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Basic Fibroblast Growth Factor Activates the MAPK and NFκB Pathways That Converge on Elk-1 to Control Production of Matrix Metalloproteinase-13 by Human Adult Articular Chondrocytes*

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The pathology of joint destruction is associated with elevated production of basic fibroblast growth factor (bFGF) and matrix metalloproteinase-13 (MMP-13). In osteoarthritic joint disease, expression of bFGF and MMP-13 in chondrocytes and their release into the synovial fluid are significantly increased. We have previously found that the capacity for cartilage repair in human adult articular chondrocytes is severely compromised by minimal exposure to bFGF because bFGF reduces responsiveness to bone morphogenetic protein-7 and insulin-like growth factor-1 and induces MMP-13 through protein kinase Cδ-dependent activation of multiple mitogen-activated protein kinase (MAPK) signaling pathways. Here we show using biochemical and molecular approaches that transcription factor Elk-1, a direct downstream target of MAPK, is a critical transcriptional activator of of MMP-13 by bFGF in human articular chondrocytes. We also provide evidence that Elk-1 is a direct target of NFκB and induces MMP-13 expression upon activation of the NFκB signaling pathway. Taken together, our results suggest that elevated expression of MMP-13 occurs through Elk-1 activation of both MAPK and NFκB signaling pathways, thus revealing a two-pronged biological mechanism by which bFGF controls the production of catabolic enzymes that are associated with excessive degradation of the cartilage matrix in degenerative joint diseases such as osteoarthritis.

Degenerative osteoarthritis (OA)[2] is both a serious cause of disability and a major source of health care costs. Given the physiological significance and potential clinical impact on cartilage homeostasis, comparatively little is known about the role of growth factors, cytokines, and inflammatory mediators in the pathogenesis of the disease.

Articular cartilage is a relatively uncomplicated tissue composed of chondrocytes and extracellular matrix (ECM). Despite its limited cell diversity and absence of vasculature and innervation, it is a dynamic tissue in which maintenance of the ECM depends on the delicate and physiologically regulated balance between anabolic and catabolic responses of sparsely distributed chondrocytes in normal cartilage. However, in OA, chondrocyte metabolism is unbalanced due to excessive production of matrix metalloproteinases (MMPs), aggrecanases (ADAMTS), and other proteinases by chondrocytes (1, 2). Among the MMPs, MMP-1 (collagenase-1) and MMP-13 (collagenase-3) play a significant role in the development of OA, because they are rate-limiting enzymes involved in collagen degradation (1, 3–5). MMP-1 is produced primarily by synovial cells, whereas MMP-13 is almost exclusively produced by chondrocytes that reside in the cartilage. In addition to collagen, MMP-13 also degrades the proteoglycan molecule aggrecan, suggesting a potential dual role in matrix destruction (5–7). Given the important role of MMP-13, it is vital to elucidate the key regulators and signaling pathways that control MMP-13 gene expression. Defining this mechanism is necessary to understand the molecular etiology of OA and to design new strategies for the development of therapautics to prevent the joint destruction seen in OA.

Basic FGF (or FGF-2) is a member of a large FGF family of structurally related proteins that bind heparin or heparin sulfate and modulate the growth, differentiation, migration, and survival of a wide variety of cell types (8). Basic FGF (bFGF) is expressed by chondrocytes and is stored in the ECM of adult articular cartilage (9, 10). The mitogenic capacity of bFGF in growth plate cartilage (11, 12) and adult articular cartilage (13) is well known. Nevertheless, previous studies on bFGF from a variety of species have yielded inconsistent results with regards

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[2] The abbreviations used are: OA, osteoarthritis; ECM, extracellular matrix; MMP, matrix metalloproteinase; bFGF, basic fibroblast growth factor; FGFR1, FGF receptor 1; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; IKK, IκB kinase; JNK, c-Jun N-terminal kinase; IL-1, interleukin-1; CMV, cytomegalovirus; siRNA, small interference RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PKC, protein kinase C; RT, reverse transcription; PBS, phosphate-buffered saline; DN, dominant negative; wt, wild type; PI3K, phosphatidylinositol 3-kinase.

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to production of ECM and articular cartilage homeostasis. We recently showed that bFGF has a striking antagonistic effect on the well known cartilage anabolic activities of insulin-like growth factor-1 and osteogenic protein-1, as reflected by inhibition of proteoglycan synthesis and its deposition (14). Signals generated by bFGF stimulate MMP-13 expression in several cell types, including cultured human chondrosarcoma cells (15) and osteoblasts (16), as well as human adult articular chondrocytes (17, 18), and the induction is at the transcriptional level (18). Hence, bFGF stimulation of articular chondrocytes may disturb cartilage homeostasis by increasing MMP-13 expression and ECM degradation. Despite these findings, though, the transcriptional mechanisms by which bFGF stimulates MMP-13 expression are poorly understood.

Immediate release of bFGF upon mechanical injury to cartilage leads to the rapid and sustained activation of Erk, and it occurs concomitantly with striking changes in chondrocyte gene expression (9, 10). Recently, we have reported that the activation of multiple MAPK subgroups (Erk, p38, and JNK) is required for the bFGF-mediated stimulation of MMP-13 in human adult articular chondrocytes (18). Elk-1 is a direct downstream target gene of MAPK and is a member of the Ets domain-containing ternary complex factor subfamily of transcription factors (19–23). bFGF-initiated transcriptional repression of elastin gene expression, an ECM protein produced by lung fibroblasts, has been reported to be mediated via the activation of Elk-1 (24). However, the precise physiological role of this transcription factor in chondrocytes has not yet been characterized after stimulation with bFGF. Similarly the critical role of the NFκB pathway in the stimulation of MMP-13 production by various stimuli such as IL-1β, fibronectin fragment, and hyaluranon oligonucleotide has been suggested in previous studies (25–27). Nevertheless, the precise cellular and molecular mechanism by which the NFκB pathway stimulates MMP-13 is not clearly understood.

In this study, we investigated the concept that the transcriptional activities of Elk-1 and NFκB may together control ECM homeostasis in cartilage. We identified Elk-1 as a key transcriptional regulator of MMP-13 expression by a two-pronged mechanism, because it acts both directly and indirectly. Our data show that activation of MMP-13 transcription occurs via Elk-1 directly, as well as via the NFκB pathway, in which Elk-1 plays a role as an intermediate regulatory molecule to lead to the NFκB-dependent induction of MMP-13 expression. These findings provide a mechanistic understanding of how the release of growth factors and inflammatory cytokines upon cartilage damage can result in the sustained activation of MMPs that may cause further destruction of articular cartilage.

EXPERIMENTAL PROCEDURES

Chondrocyte Isolation and Culture Conditions—Normal human ankle cartilage was obtained from tissue donors through the Gift of Hope Organ and Tissue Donor Network. Each donor specimen was graded for gross degenerative changes based on a modified version of the 5-point scale of Collins (28). Chondrocytes were isolated by enzymatic digestion of ankle articular cartilage (grade 0 or 1, which has no sign of cartilage degeneration) using Pronase followed by overnight digestion with collagenase-P as described previously (18). Isolated cells were resuspended in media at 2×10^6 per milliliter and plated onto 12-well plates at 1 ml/well. Cells were cultured in Dulbecco's modified Eagle's medium/F-12 containing 10% fetal bovine serum and antibiotics (complete media) for 5 days before the experiments.

Chondrocyte Stimulation and Immunoblotting—Cells were serum-starved by changing media to serum-free Dulbecco's modified Eagle's medium/F-12 with antibiotics for 1 day. For inhibitor studies, cells were preincubated with individual pathway-specific chemical inhibitors for 30 min before stimulation with bFGF or IL-1β. Experiments were terminated with removal of media and/or cell lysate preparation. The conditioned media was stored at 4 °C with 0.1% NaN_3 and used for the experiments within 5 days. Cell lysates were prepared using modified cell lysis radioimmune precipitation assay buffer: 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 0.25% deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM glycerol phosphate, 1 mM $NaVO_4$, with 2 mM phenylmethylsulfonyl fluoride (Sigma). Total protein concentrations of both media and cell lysates were determined by a bicinchoninic acid (BCA) protein assay (Pierce). Equal amounts of protein were resolved by 10% SDS-PAGE gels and transferred to nitrocellulose membrane for immunoblot analysis as described previously (18). Immunoreactivity was visualized using the ECL system (Amersham Biosciences) and the Signal Visual Enhancer system (Pierce), which magnifies the signal.

Plasmids Constructs—Dominant negative forms of individual MAPK expression vectors (Erk-DN, p38-DN, and JNK-DN) were kindly provided by Dr. Ralf Janknecht (Mayo Clinic, Rochester, MN). Elk-1 wild type and its positive luciferase promoter plasmid construct, c-Fos-Luc, Elk-1-associated mutant constructs (CMV-Elk-1 383A) and Gal4-Elk-1 system, including the Luciferase reporter plasmid construct (Gal4 RE-Luc), Gal4-Elk-1 wt, and Gal4-Elk1 Ser383A, were provided by Dr. A. D. Sharrocks (University of Manchester, UK) and R. A. Hipskind (Institute of Genetics, France). FGFR1-DN construct was provided by Dr. C. J. Coscia (St. Louis University). Dominant negative construct of IκBα (pCMV-IκBαM) was purchased from Clontech (Mountain View, CA). Human MMP-13 promoter construct and plasmid expression vector containing siRNA targeting Akt (Mu6pro-Akt1/2) and control siRNA plasmid construct (Mu6pro) were previously described (27, 29, 30). The p3xNFκBLuc plasmid construct, containing three copies of tandemly repeated NFκB response element sequences (5′-TTG-GGCACTCCCTG-3′) of MMP-13 promoter region was achieved by placing the NFκB response elements of MMP-13 promoter region upstream of an SV40 promoter at the SacI site of the pGL3-promoter vector, which contains a luciferase reporter gene system (Promega) as described previously (31).

Transient Transfection by Nucleofection (Electroporation)—Nucleofection methods were optimized for use with human articular chondrocytes by minor modifications of the instructions for the Nucleofector™ kit (Amaxa Biosystems) that have been described previously (18, 32). The *Renilla* vector (pRL-TK) was co-transfected as an internal control, and the luciferase activity representing promoter activity was measured using the Dual-Luciferase reporter assay system (Promega) and a lumi-

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TABLE 1
Primer sequences for semi-quantitative and real-time PCR

Genes	Primer sequences (forward/reverse) (5′-3′)	Size	Annealing temperature	Reference or accession no.
		bp	*°C*	
Semi-quantitative PCR				
MMP-13	GGCTCCGAGAAATGCAGTCTTTCTT ATCAAATGGGTAGAAGTCGCCATGC	337	64	18 NM_002427
GAPDH	CTGAGAACGGGAAGCTTGTCATCA AGTTGTCATGGATGACCTTGGCCA	318	58	18 NM_002046
Real-time PCR				
bFGF	GGCTATGAAGGAAGATGGAAGATT TGCCACATACCAACTGGTGTATTT	130	62	NM_002006
Elk-1	TTGGAGGCCTGTCTGGAGGCTGAA AGCTCTTCCGATTTCAGGTTTGGG	92	57	NM_005229
c-Fos	TAGCCTCTCTTACTACCACTCACC TGGGAATGAAGTTGGCACTGGAGA	113	53	NM_006365
GAPDH	TCGACAGTCAGCCGCATCTTCTTT GCCCAATACGACCAAATCCGTTGA	148	58	18 NM_002046

nometer (Berthold). In samples containing combinations of plasmids (*i.e.* co-transfections with Elk-1 or pCMV-IκBαM cDNA construct with MMP-13 promoter/luciferase reporter construct) we adjusted the total amount of DNA concentration to <5 μg per 100 μl of cell-Nucleofector solution complex for the entire set of experiments to minimize toxic effects observed at higher DNA concentrations. To introduce siRNA-targeting Elk-1, the validated TranSilent TF siRNAs targeting Elk-1 and GAPDH were purchased from Panomics (Fremont, CA) or SuperArray Bioscience Co. (Frederick, MD) and were introduced into human articular chondrocytes using nucleofection (as described above) by following the manufacturer's protocol. The effect of Elk-1 siRNA on bFGF-mediated MMP-13 expression was assessed by immunoblotting using anti-MMP-13 (R&D System) or anti-GAPDH antibody (Abcam, UK).

Chemical Inhibitors, Growth Factors, and Cytokines—The PKC inhibitor bisindolylmaleimide I (10 μM), PKCδ inhibitor (Rottlerin, 4 μM), PKCε inhibitor, and PKCα/β inhibitor (GÖ6976, 5 nM); NFκB inhibitor SN50 (peptide inhibitor, 10 μM), IKK inhibitor II (wedelolactone, 10 μM), FGFR1 inhibitor (SU5402, 5 μM), LY294002 (50 nM), rapamycin (20 nM), and wortmanin (10 μM); Ras inhibitor (manumycin A, 3 μM), Raf inhibitor (Raf1 kinase inhibitor 1, 20 μM), MEK inhibitor (PD098059, 20 μM), p38 inhibitor (SB203580, 10 μM), and JNK inhibitor (SP600125, 20 μM) were purchased from Calbiochem. Human recombinant bFGF and IL-1β were provided by NCI (National Institutes of Health) and Amgen, respectively. Optimal doses were evaluated by dose-dependent experiments for the use of chemical and peptide inhibitors to ensure specific effects on signaling pathways operative in human adult articular chondrocytes.

Reverse Transcription and PCR—Reverse transcription (RT) was carried out with 500 ng of total cellular RNA using either real-time or the One-Step RT-PCR System (Invitrogen) following the instructions provided by the manufacturer. For all experiments, optimal conditions were determined by initially generating cycle number-dependent expression curves and the linear range for the PCR amplification. The same amounts of total RNA or genomic DNA were subjected to One-Step RT-PCR simultaneously to minimize experimental variation due to differences in amplification efficiency. The assessment for GAPDH was performed in parallel (18, 25). The One-Step RT-PCR was performed using 30 cycles of 95 °C for 30 s, 62 °C for 1 min, and 72 °C for 40 s in the presence of 50 pmol of sense and antisense primers. The primer sequences and the conditions for their use are summarized in Table 1. The resulting PCR products were resolved in 1.5% agarose gels and visualized by staining with ethidium bromide and UV transillumination. Integrated density values for testing genes were normalized relative to GAPDH values to yield a semi-quantitative assessment. For real-time PCR, the primer sets specific to human genes and optimized conditions were used to generate <200-bp coding regions, spanning at least one exon and an intron as we performed previously (18).

Preparation of Nuclear Extracts—The nuclear extracts were prepared by using the nuclear extraction kit (Panomics) according to the manufacturer's protocol. Following stimulation, culture media was removed, and the cells were then lysed with 1 ml buffer A, incubated on ice for 10 min, and centrifuged at 15,000 × *g* at 4 °C for 3 min. The supernatants were discarded, and the pellets were resuspended in 150 μl of buffer B. The samples were incubated on ice for 2 h and centrifuged, and the supernatants were aliquoted and stored at −80 °C.

Gel Shift Assays (Electrophoretic Mobility Shift Assays)—Gel shift assays of Elk-1 and NFκB were performed by using the commercially available kits (Panomics). Following stimulation, the nuclear extracts (prepared as mentioned above) were incubated with the biotin-labeled double-stranded transcription factor (Elk-1 and NFκB) either consensus sequence or the sequence present in MMP-13 gene. The samples were then resolved on a 6% non-denaturing polyacrylamide gel and transferred to Pall Biodyne B membrane, blocked, and incubated with Streptavidin-horseradish conjugate. The bands were visualized with a chemiluminescence-imaging system. Unlabeled, double-stranded wild-type oligonucleotides were used to determine the binding specificity of the assay.

Nuclear Translocalization of Elk-1—Chondrocytes plated at a density of 100,000 in 4-well chamber slides were cultured for 24 h in complete media containing 10% fetal bovine serum. The medium was then changed to serum-free medium for 18 h. Chemical inhibitors were incubated for 1 h prior to treatment with bFGF (100 ng/ml). The treatment was then aborted by washing the cells with 1× PBS three times followed by fixation in 4% paraformaldehyde for 15 min at room temperature. The cells were then washed with 1× PBS and incubated with 0.2% Triton X-100 prepared in PBS for 10 min at room temperature.

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After permeabilizing the cells with Triton X-100, the cells were washed and blocked with 10% normal goat serum in PBS for 60 min at room temperature. After washing the cells with PBS, the primary antibody prepared in 1% bovine serum albumin made in PBS (1:200 dilution) was then added, and the mixture was incubated overnight at 4 °C. The cells were then washed and incubated with green fluorescent-labeled anti-mouse secondary antibody (prepared in 1% bovine serum albumin-PBS) for 1 h at 4 °C in the dark. The cells were then washed, the slides were prepared using the mounting media containing 4′,6-diamidino-2-phenylindole (Molecular Probes), which can counterstain the nucleus, and visualized using the fluorescent microscope (Nikon Eclipse E600 microscope) connected to a PC running MetaView Imaging Series 6.1. The nuclear image was visualized using a UV filter, whereas the cytoplasmic fluorescence staining was visualized by using a blue filter. Both images were overlaid using MetaView imaging software.

Histology—Safranin Orange staining was performed using full thickness cartilage slices prepared from OA knee joint tissues and a 4-mm diameter puncher. These explants were cultured in 1.0 ml of Dulbecco's modified Eagle's medium/F-12 containing 10% fetal bovine serum. Following a 2-day recovery period, the cartilage explants were treated with or without bFGF (100 ng/ml) under serum-free conditions (mini-ITS). Following 14 days of incubation, the slices were fixed with 4% paraformaldehyde for overnight and embedded in paraffin, and 8 μM sections were prepared. The paraffin sections were deparaffinized and stained with Safranin Orange staining to assess matrix proteoglycan loss.

Type II Collagen Digestion Assay—75 ml of culture media from each sample to be tested was activated with 1 μM 4-aminophenylmercuric acetate (Sigma, catalog no. A9563) for 1 h at 37 °C in the dark. After activation, 75 ml of 0.5 mg/ml chick sternal type II collagen (Sigma, catalog no. C9301) was added to the activated culture media, and the mixture was vortexed thoroughly followed by placing the mixture of culture media and type II collagen (150 μl) in a heating block for 24 h at 37 °C. The reaction was terminated by the addition of 150 μl of 2× reducing Laemmli sample buffer (Bio-Rad, catalog no. 161-0737), and the samples were then resolved on 8% SDS-PAGE followed by staining with Coomassie Brilliant Blue R-250 for 2 h. The gel was destained with water containing 10% methanol and 10% acetate acid.

Statistical Analyses—The statistical significance of results was determined by analysis of variance, using StatView 5.0 software (SAS Institute, Cary, NC). Data interpretation was performed by statistical normalization as assessed with histograms, and a 0.05 significance level was used for all statistical tests.

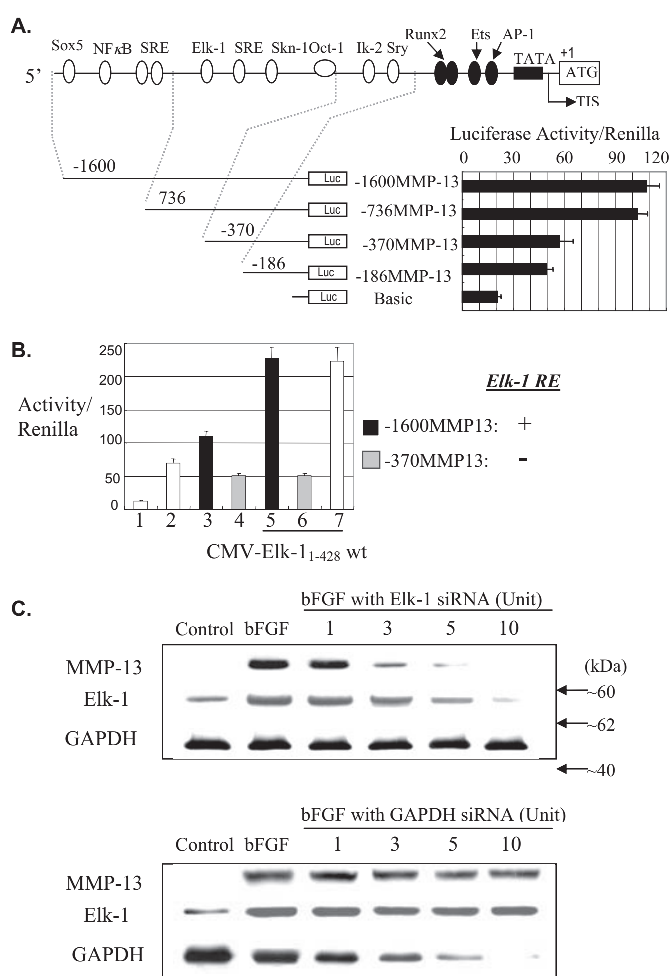

FIGURE 1. **Elk-1 motif is critical for MMP-13 expression.** *A*, MMP-13 promoter-reporter deletion constructs were transiently transfected into human adult articular chondrocytes by nucleofection. 24 h after post-transfection, the luciferase activity representing the MMP-13 promoter activity was measured. A *Renilla* vector was co-transfected as an internal control for normalization. The length of the promoter fragments tested is indicated. The *numbers* correspond to the relative positions with respect to the transcription start site ATG. Cells transfected with empty vector were used as a control. The data represent three different donors measured in triplicate for each experiment. *B*, human articular chondrocytes were transiently co-transfected with −1600 (with Elk-1 motif "+") or −370 MMP-13 (without Elk-1 motif "−") promoter luciferase reporter constructs with wild-type Elk-1 cDNA construct (CMV-Elk-1_{1-428}). 24 h after post-transfection, the cells were harvested and subjected for luciferase activity. The c-Fos-Luc promoter reporter plasmid was used as a positive response control to Elk-1. *Lane* identification: *1*, empty CMV vector only; *2*, c-Fos-Luc; *3*, −1600MMP-13; *4*, −370MMP-13; *5*, −1600MMP-13 with CMV-Elk-1_{1-428}wt; *6*, −370MMP-13 with CMV-Elk-1_{1-428} wt; and *7*, c-Fos-Luc with CMV-Elk-1_{1-428} wt. The data represent three different donors measured in triplicate for each experiment. *C*, validated human specific siRNAs, targeting Elk-1 and GAPDH (Panomics), were introduced into human adult articular chondoryctes with different doses (units) as indicated. The transfected cells were cultured in the presence or absence of bFGF for an additional 24 h. The conditioned media were analyzed for MMP-13. The effect of siRNA was evaluated by using anti-Elk-1 or anti-GAPDH antibody.

RESULTS

Identification of an Elk-1 Response Element in the Distal Promoter Region of the MMP-13 Gene That Regulates MMP-13 Expression in Human Adult Articular Chondrocytes—Our previous studies suggest that bFGF-mediated up-regulation of MMP-13 occurs via a transcriptional stimulation (18). To define elements controlling MMP-13 transcription, MMP-13 promoter-luciferase reporter constructs were transiently transfected into primary human articular chondrocytes. Deletion from −1600 (−1600MMP-13) to −736 (−736MMP-13) upstream of the ATG of the MMP-13 promoter region shows no significant change in the basal promoter activity (Fig. 1*A*), consistent with our previous report with an immortalized chondrocyte cell line (30, 32). Further deletion from −736 to −370 of the MMP-13 promoter (−370MMP-13) significantly reduces basal promoter activity (up to ∼40% reduction). This

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decreased level of promoter activity is similar to the activity observed for the −186MMP-13 promoter that contains minimal core transcription factor response elements, including motifs for Runx2, ETS, and AP-1. These results suggest that the distal −736 to −370 promoter region of the MMP-13 gene encompasses key elements that regulate MMP-13 promoter activity in human adult articular chondrocytes.

Bioinformatic analysis reveals the presence of an Elk-1 recognition motif (5′-CAGGAC) between positions −636 and −630 of the MMP-13 promoter region adjacent to putative serum response elements. Because bFGF activates the MAPK-AP-1 pathway to stimulate MMP-13 expression in human adult articular chondrocytes (18), and because Elk-1 is an MAPK-responsive factor in other biological contexts (19–24), we examined the role of Elk-1 in MMP-13 expression. Cells were transiently co-transfected with the −1600MMP-13 promoter-reporter construct, which contains the putative Elk-1 motif, and the −370MMP-13 construct, which lacks the Elk-1 binding site, in the presence or absence of a wild type Elk-1 expression vector. Forced expression of Elk-1 to mimic the activation of bFGF-signaling cascades significantly enhances the activity of the −1600 promoter by 2-fold but has no appreciable effect on the −370 promoter (Fig. 1*B*). Cells transfected with the empty expression vector were analyzed as negative controls (Fig. 1*B*, *lane 1*), whereas a c-*fos*-responsive promoter-reporter construct (c-*fos*-Luc) shows significant enhancement of luciferase activity (Fig. 1*B*, *lane 7*), which is consistent with the stimulation of c-*fos* expression by Elk-1 (24, 34–36).

To examine whether Elk-1 levels are critical for the control of MMP-13 expression, we performed siRNA experiments using a commercially available Elk-1 siRNA (siElk-1, Panomics). Human primary articular chondrocytes transfected with siElk-1 were incubated in the absence or presence of bFGF. Cells with bFGF stimulation increase both MMP-13 and Elk-1 protein production. The introduction of siElk-1 significantly blocked these up-regulations in a dose-dependent manner while the level of internal control GAPDH remained unchanged (Fig. 1*C*, *upper panel*). Introduction of siRNA-targeting GAPDH was performed in parallel as a control in which siRNA-targeting GAPDH had no effect on bFGF stimulation of MMP-13 and Elk-1, whereas the GAPDH protein level was decreased dose dependently (*lower panel*). Our results suggest that Elk-1 plays a critical role as a primary transcriptional regulator of MMP-13 transactivation after stimulation with bFGF in human adult articular chondrocytes.

bFGF-mediated Phosphorylation of Elk-1 at Serine 383 Is Linked to Biological Activation of Elk-1 in Human Adult Articular Chondrocytes—Elk-1 was rapidly activated by bFGF (within 5 min), and the activation was sustained beyond 60 min as reflected by phosphorylation of Elk-1 at the amino acid residue serine 383 (Fig. 2*A*). We examined the transcriptional activating ability of Elk-1 using a GAL4-responsive reporter gene in which multimerized GAL4 sites drive luciferase, and chimeric Elk-1 proteins fuse to the GAL4 DNA binding domain (GAL4 BD) (Fig. 2*B*). Human primary chondrocytes co-transfected with the GAL4-responsive promoter construct (GAL4 RE-Luc), and expression vectors for the GAL4-Elk-1 fusion protein (GAL4-Elk-1_{1-428} wt) did not exhibit activation of the reporter

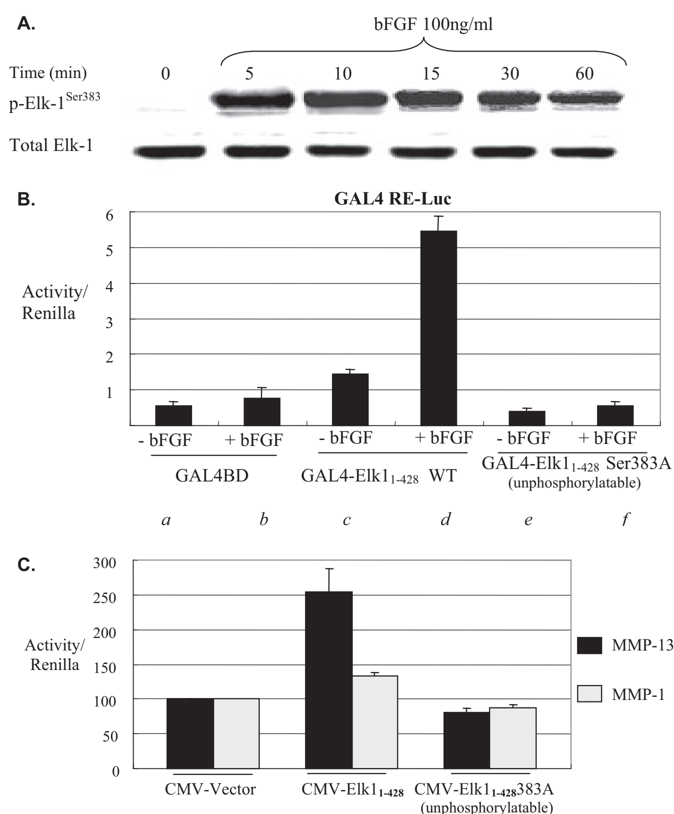

FIGURE 2. **Phosphorylation of Elk-1 at Ser^{383} is essential for the MMP-13 stimulation by bFGF in human articular chondrocytes.** *A*, serum-starved cells in monolayer were treated with bFGF for the indicated periods of time. Cell lysates were then prepared and analyzed by immunoblotting with phospho-specific anti-Elk-1 (Ser^{383}) antibody. Total Elk-1 was used as a control for normalization. *B*, cells were transiently co-transfected with a reporter construct, containing GAL4 element immediately upstream of the luciferase gene (GAL4RE-Luc) with the GAL4-Elk-1 fusion proteins (GAL4-Elk-1_{1-428}wt or unphosphorylatable mutant, GAL4-Elk-1_{1-428}S383A). 24 h after post-transfection, cells were treated with or without bFGF for an additional 24 h at which point the cell lysates were harvested and analyzed for the luciferase activity. Cells co-transfected with GAL4BD, the backbone of Elk-1 expression vectors, were used as controls. The data represent three different donors measured in triplicate for each experiment. *C*, cells were transiently co-transfected with the −1600MMP-13 or 562MMP-1 promoter construct and wild-type (CMV-$Elk1_{1-428}$) or mutant (CMV-$Elk1_{1-428}$Ser383A) Elk-1 cDNA constructs. 24 h after post-transfection, cells were harvested and used for the analysis of luciferase activity. Co-transfection with empty CMV-Vector was used as a control. The data represent three different donors measured in triplicate for each experiment.

activity (Fig. 2, *B* and *C*) until stimulation of cells with bFGF (Fig. 2, *B* and *D*). In the presence of bFGF, the luciferase activity was significantly increased, reflected by an ~4-fold induction of GAL4 promoter activity, suggesting the stimulation of intrinsic transcriptional activity of Elk-1 via the biological action of bFGF. Co-expression of a vector containing only GAL4 BD with GAL4 RE-Luc was used as a negative control in the experiments (Fig. 2*B*, *panels a* and *b*).

The phosphoryaltion of Elk-1 at the serine 383 residue appeared to be essential for the transcriptional activating ability of Elk-1 in human adult articular chondrocytes. We observed that cells co-transfected with GAL4-Elk-1_{1-428} S383A, in which the serine 383 residue was replaced with a non-phosphorylatable alanine, had no stimulatory effect on the reporter activity either in the absence (Fig. 2*B*, *panel e*) or presence of bFGF (Fig. 2*B*, *panel f*). These results suggest that bFGF exerts

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its biological effect by phosphorylating Elk-1 at serine 383, thereby promoting its transcriptional potential in enhancing expression of target genes such as MMP-13.

We further examined whether phosphorylation of Elk-1 at serine 383 by bFGF is functionally linked to transactivation of MMP-13 in human adult articular chondrocytes. Cells were transiently co-transfected with the unphosphorylatable mutant Elk-1 expression vector (CMV-Elk-1_{1-428} S383A) and the MMP-13 promoter-reporter construct that contains an Elk-1 response element (Fig. 2*C*). The MMP-13 promoter-driven luciferase activity of cells expressing wild type Elk-1_{1-428} increased greater than 2-fold, but cells expressing the Elk-1_{1-428} S383A mutant failed to stimulate MMP-13 transactivation. Unlike MMP-13, co-expression of Elk-1 with MMP-1 promoter (−562 upstream of ATG)-luciferase reporter construct (30) did not show a significant transactivation. These results suggest that phosphorylation of Elk-1 at the serine 383 residue is essential for the biological activity of Elk-1 and specific transactivation of MMP-13 in human adult articular chondrocytes.

Activation of Elk-1 by bFGF Is Specific to the Pathways Associated with MMP-13 Transactivation—Previously, we reported that bFGF activates PKCδ, which, in turn, transduces its signal to activate multiple MAPK subgroups (Erk, p38, and JNK) to regulate MMP-13 production (18). Because Elk-1 is a direct downstream target of the MAPK pathway in non-chondrocytic cells (19–23), and because our data show that Elk-1 is critical for MMP-13 expression (see Figs. 1 and 2), we examined whether Elk-1 is a major effector of bFGF/PKCδ/MAPK-dependent pathways that control MMP-13 expression in human adult articular chondrocytes. Serum-starved chondrocytes cultured in monolayer were incubated with pathway-specific individual inhibitors for MAPK subgroups (Erki, p38i, and JNKi) and PKC isoforms (PKCδi, PKCα/βi, and PKCϵ) 1 h prior to stimulation with bFGF for 5 min. We included a broad-spectrum PKC inhibitor (bisindolylmaleimide I), which can block signals generated from various PKC isoforms, including PKCδ, as a control (37). The modulation of Elk-1 activity was detected using phospho-specific anti-Elk-1 antibody (Ser-383). The blots were stripped and re-probed with total anti-Elk-1 antibody to normalize for equal loading of protein in each lane. The results showed that blocking MAPK subgroups (Erk, p38, and JNK) or the PKCδ signaling pathways prevented phosphorylation of Elk-1 (Fig. 3*A*). Importantly, inhibition of PKCα/β and PKCϵ signaling pathways that are modulated by bFGF, but not associated with MMP-13 expression (18), failed to block Elk-1 activation.

Basic FGF mediates activation of the PI3K/Akt pathway in human adult articular chondrocytes as reflected by rapid phosphorylation of Akt at amino acid serine 473 within 5 min (Fig. 3*B*). This phosphorylation event renders Akt functionally active as a kinase, and consequently results in phosphorylation of its downstream targets, p70S6K and GSK3β. However, blocking the PI3K/Akt pathway did not affect the bFGF-dependent stimulation of MMP-13 at both protein and mRNA levels (Fig. 3*C*). Inhibition of the MAPK pathway had no significant impact on bFGF activation of PI3K/Akt signaling (Fig. 3*D*) and *vice versa* (data not shown), suggesting that there was no discernable molecular cross-talk between the individual MAPK subgroups and the PI3K/Akt pathway. The MAPK pathway-dependent activation of Elk-1 was further supported by gel shift assay, using double-stranded Elk-1 sequences of MMP-13 and nuclear extracts prepared from primary chondrocytes that were preincubated with pharmacological inhibitors 1 h prior to stimulation with bFGF for 45 min. Consistent with our other results, we observed inhibition of Elk-1 protein-DNA interactions by pathway-specific inhibitors that are linked to MMP-13 expression, which includes MAPKs and PKCδ, after stimulation with bFGF (Fig. 3*E*). These results were further confirmed by using an Elk-1 activation-mediated GAL4 binding system in the presence of chemical inhibitors or co-expression of siRNA (Akt-siRNA) or dominant negative forms of MAPK subgroups (Erk-DN, p38-DN, and JNK-DN) (Fig. 3*F*). Again, consistent with our other data, blocking the Erk pathway either by pharmacological inhibitor or by overexpression of Erk-DN expression vector abolishes the bFGF-mediated activation of Elk-1, which is similar to the inhibitory level seen by blocking the FGF receptor 1 (either chemical inhibitor or overexpression of FGFR1-DN). Inhibition of the p38 and JNK pathways partially, but significantly, reduced bFGF-induced Elk-1 activation by both chemical inhibitors as well as the overexpression of dominant negative constructs. On the other hand, interfering with the PI3K/Akt pathway via either chemical inhibitor or introduction of Akt-siRNA vector (Mu6pro-Akt1/2) shows no influence on Elk-1 activation by bFGF. Control siRNA vector (Mu6pro) and CMV-vector (backbone of DNs) were used as a negative control for the experiment. Collectively, these data demonstrate that bFGF induction of MMP-13 production occurs via bFGF-responsive signaling pathways that mediate the activation of MAPK (preferentially Erk MAPK)-Elk-1 in human adult articular chondrocytes.

Basic FGF Activation of the NFκB Pathway Regulates MMP-13 Expression in Human Adult Articular Chondrocytes—Our results show that stimulation of the cells with bFGF activates components of IκB kinase (IKK) ($IKK\alpha^{Ser180}/IKK\beta^{Ser181}$ and IKKγ (also known as NEMO)), which are important kinases for NFκB activation (Fig. 4*A*). Phosphorylation of IKK, representing activation, is rapid (within 5 min), and the activation is sustained for >1 h after stimulation with bFGF.

To assess the role for NFκB activation in bFGF stimulation of MMP-13 expression, serum-starved cells were incubated with NFκB pathway-specific inhibitors 1 h prior to stimulation with bFGF. We used two different pharmacological inhibitors: peptide inhibitor SN50, which blocks the nuclear translocalization of activated NFκB, and wedelolactone, a cell-permeable, selective inhibitor of IKKα/β kinase activity ($IC_{50} < 10$ μM, Calbiochem). Conditioned media from cells treated with each of these inhibitors were analyzed for MMP-13 by immunoblotting. Pretreatment of chondrocytes with each NFκB inhibitor significantly decreased the bFGF-mediated MMP-13 expression at both protein and mRNA levels (Fig. 4*B*, *lanes 3* and *5*). Because inhibition of the NFκB pathway can cause apoptosis in chondrocytes (38), we performed DNA fragmentation analysis to ensure that the reduced MMP-13 production was not due to cell death. We found that cells incubated with chemical or peptide inhibitors of NFκB showed no signs of apoptosis (data not shown). For comparison, experiments were performed in par-

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allel with IL-1β, an inflammatory cytokine known to stimulate the NFκB pathway (25), and we obtained similar results to those observed after stimulation with bFGF.

The pharmacological inhibitor studies were further validated by transient transfection studies. Human adult articular chondrocytes were transiently co-transfected with a construct (pCMV-IκBαM), expressing a dominant negative form of IκBα, and the MMP-13 promoter/luciferase reporter plasmid. Expression of IκBαM blocks endogenous NFκB signaling by competitively binding to NFκB and preventing the release of activated NFκB (18, 30, 32). Our results showed that exogenous expression of IκBαM dramatically inhibited the bFGF-mediated stimulation of MMP-13 promoter activity (Fig. 4*C*, *lane 4*), which is consistent with our pharmacological inhibitor studies. Collectively, these results demonstrate the critical role of the NFκB pathway in the stimulation of MMP-13 expression by bFGF in human adult articular chondrocytes.

NFκB Pathway Regulation of MMP-13 Is Independent of NFκB Protein-DNA Interaction—The promoter region of the MMP-13 gene from −1600 to −736 (relative to the ATG start site) contains a single putative NFκB response element (Fig. 1*A*) (31). We analyzed the functional significance of this NFκB motif using the −1600 and −736 MMP-13 promoter deletion constructs, which differ in the presence of the NFκB response element (Fig. 5*A*). Cells transfected with the −1600 MMP-13 and −736 MMP-13 constructs did not exhibit a significant difference in stimulation of MMP-13 promoter activity by bFGF. We further verified the functional significance of this NFκB motif present in the MMP-13 promoter by transiently transfecting a p3xNFκBLuc construct that contains three copies of tandemly repeated NFκB response element sequences of the MMP-13 promoter region into human adult

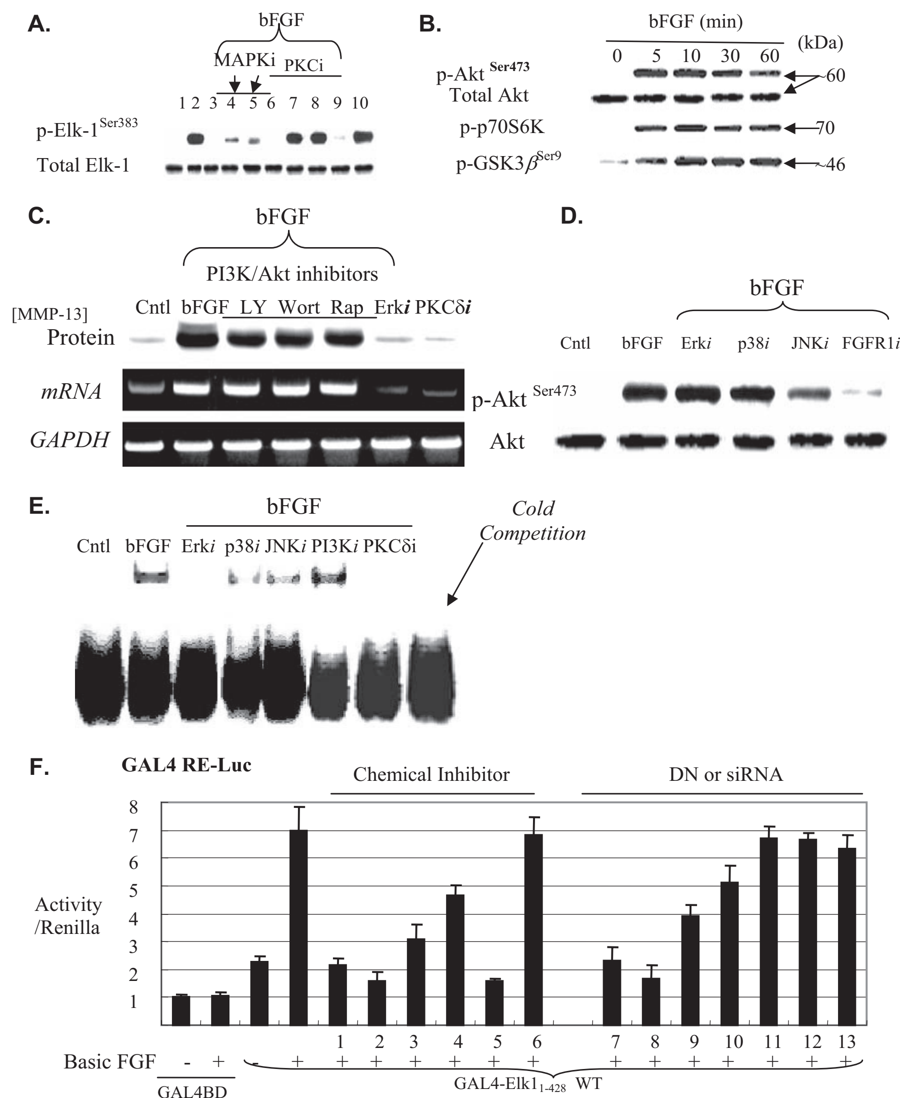

FIGURE 3. *A*, Elk-1 activation by bFGF via multiple MAPK subgroups and the PKCδ pathway. Serum-starved cells in monolayers were preincubated with individual pathway-specific chemical inhibitors 1 h prior to the treatment with bFGF (100 ng/ml) for 10 min. The cell lysates were prepared and immunoblotted with anti-phosphospecific Elk-1^{Ser383} antibody. The membrane was stripped and reprobed with total Elk-1 antibody for normalization. *Lane* identification: *1*, control; *2*, bFGF; *3*, bFGF with Erk inhibitor (PD098059, 20 μM); *4*, bFGF with p38 inhibitor (SB203580, 10 μM); *5*, JNK inhibitor (SP600125, 20 mM); *6*, PKCδ inhibitor (rottlerin, 4 mM); *7*, PKCα/β inhibitor (GÖ6976, 5 nM); *8*, PKCε peptide inhibitor (1 μM); *9*, bisindolylmaleimide I (10 μM); and *10*, PI3K/Akt inhibitor (LY294002, 50 nM). *B*, serum-starved cells were treated with bFGF (100 ng/ml) for different time points as indicated. The cell lysates were then immunoblotted with anti-phosphospecific Akt^{Ser473}, and its downstream target molecules, p70S6K or GSK3β^{Ser9}. The membranes were striped and reprobed with total Akt for normalization. *C*, serum-starved cells were preincubated with various inhibitors that can selectively block the PI3K/Akt pathway (LY294002 (50 nM), wortmannin (10 μM), rapamycin (20 nM)), Erk (PD098059, 20 μM), or PKCδ (rottlerin, 4 μM) 1 h prior to the treatment with bFGF (100 ng/ml) for 24 h. The conditioned media was then analyzed for MMP-13 production by immunoblot, whereas the cells were harvested for analysis of MMP-13 expression at mRNA level by RT-PCR. GAPDH mRNA was analyzed for normalization. *D*, cells were preincubated with inhibitors of Erk (PD98059), p38 (SB203580), or JNK (SP600125) or FGFR1 inhibitor (SU5402, 5 μM) 1 h prior to the treatment with bFGF for 10 min. The cell lysates were analyzed for the activation of Akt by immunoblotting using phosphospecific anti-Akt (Ser^{473}) antibody. The membranes were stripped and reprobed with total Akt antibody for normalization. *E*, Elk-1 protein-DNA interaction was examined by gel shift using biotin-labeled double-stranded Elk-1 DNA motif present in the MMP-13 promoter. The oligonucleotide probes were incubated with nuclear extracts derived from untreated cells (control) or cells treated with bFGF for 45 min in the presence of inhibitors specific for Erk (PD098059), p38 (SB203580), JNK (SP600125), PI3K/Akt (LY294002), or PKCδ (rottlerin). Nuclear extract incubated with excess amount of unlabeled probe (cold competition) was used for specificity control. *F*, cells were transiently co-transfected with a reporter construct containing GAL4 element immediately upstream of the luciferase gene (GAL4RE-Luc) and with the GAL4-Elk-1 fusion proteins (GAL4-Elk-1_{1-428}wt) containing GAL4BD and the Elk-1 protein. 24 h after post-transfection, cells were treated with bFGF (100 ng/ml) for an additional 24 h at which point the cell lysates were prepared and analyzed for the luciferase activity. Cells co-transfected with GAL4BD (no Elk-1) were used as a negative control. The data represent three different donors measured in triplicate for each experiment. *Lane* identification: for co-transfected with GAL4-Elk1_{1-428}wt and stimulated with bFGF in the presence of specific inhibitors: *1*, FGFR1; *2*, Erk; *3*, p38; *4*, JNK; *5*, PKCδ; *6*, PI3K/Akt (LY294002); for dominant negative plasmids: *7*, FGFR1-DN; *8*, Erk-DN; *9*, p38-DN; *10*, JNK-DN; or for siRNA plasmids: *11*, Akt (Mu6pro-Akt1/2); *12*, control siRNA plasmid (Mu6pro), and *13*, CMV vector were used for control.

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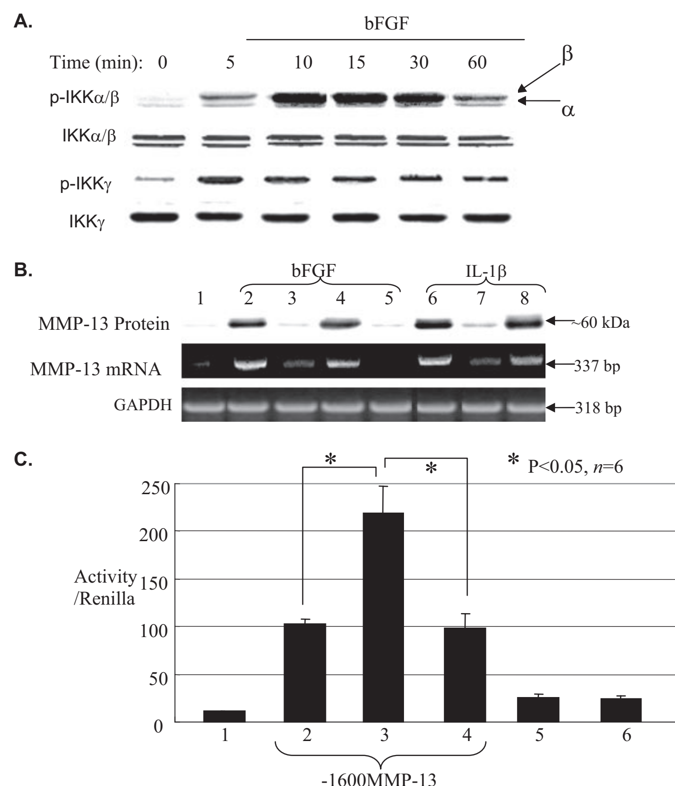

FIGURE 4. **Basic FGF-mediated MMP-13 stimulation occurs via the NFκB pathway in human adult articular chondrocytes.** *A*, serum-starved cells were stimulated with bFGF for the indicated periods of time. Cell lysates were resolved on SDS-PAGE, followed by immunoblotting with the antibody specific to the phosphorylation sites of IKKα (Ser[180])/IKKβ (Ser[181]) or IKKγ (Ser[376]). The membranes were striped and reprobed with total IKKα/β or IKKγ for normalization. *B*, cells were cultured with or without bFGF in the presence or absence or NFκB peptide inhibitor (SN50) or control peptide for 24 h. For comparison, cells were treated with IL-1β (10 ng/ml) in the presence of SN50. Conditioned media was immunoblotted with anti-MMP-13 antibody. Total RNA extracted from the cells was used for analyzing MMP-13 mRNA levels. GAPDH mRNA was used as control for normalization. *C*, promoter construct, −1600MMP-13 was transiently co-transfected with IκBαM, a mutant IκB cDNA construct, which can block the endogenous NFκB-signaling pathway. The transfected cells were treated with bFGF for 24 h followed by analysis for the relative luciferase activity after normalization of the transfection efficiency by *Renilla*. *Lane* identification: *1*, pCMV-IκBαM only; *2*, −1600MMP-13 promoter construct only; *3*, −1600MMP-13 treated with bFGF 100 ng/ml; *4*, pCMV-IκBαM with bFGF 100 ng/ml; *5*, pGL2E only (backbone of MMP-13 promoter-reporter construct); and *6*, CMV-vector only (backbone of the IκBαM expression vector).

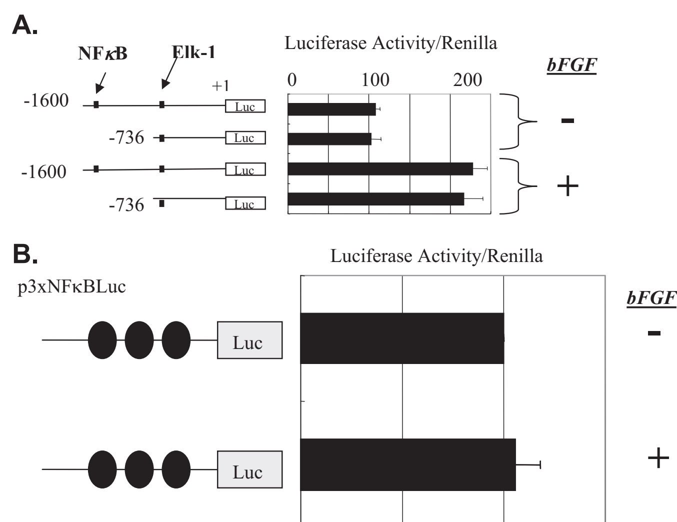

FIGURE 5. *A*, −1600 or −736 MMP-13 promoter-reporter constructs (and *Renilla* vector) were transiently transfected into human adult articular chondrocytes. 48 h after posttransfection, cells were treated with or without bFGF for 24 h. Cell lysates were then prepared and used for dual luciferase assay system. *B*, cells were transiently transfected with p3xNFκBLuc plasmid construct which contains three copies of tandemly repeated NFκB response element sequences of MMP-13 promoter region. After 48 h of incubation, cells were stimulated with bFGF with additional incubation for 24 h. The relative luiferase units were calculated by normalizing the *Renilla* activity that was co-transfected. The data represent three different donors measured in triplicate for each experiment.

articular chondrocytes. Similar to those results obtained from the promoter deletion analysis, the transfected cells showed no response to the stimulation with bFGF (Fig. 5*B*). Our results suggest that the NFκB response element present in the MMP-13 promoter region may not be functional and, thus, has no impact on bFGF stimulation of the MMP-13 promoter.

Elk-1 Is a Key Regulator That Mediates NFκB Pathway-dependent Control of MMP-13 Expression upon bFGF Stimulation—It is important to determine how bFGF-mediated activation of the NFκB pathway can regulate MMP-13 expression independent of direct binding of NFκB to its response element. Our real-time PCR results showed that stimulation of cells with bFGF rapidly increased *elk-1* mRNA levels within 1 h (>2-fold), and this induction was abolished in the presence of a peptide inhibitor of the NFκB pathway, which blocks the translocation of activated NFκB (Fig. 6*A*). These results suggest that bFGF activation of NFκB may regulate Elk-1 at the transcriptional level to control MMP-13 expression in human adult articular chondrocytes. Thus, we determined whether the NFκB pathway is able to modulate the activation of Elk-1 protein after stimulation with bFGF. Serum-starved cells were treated with bFGF in a time course in the presence or absence of NFκB inhibitor, and the cell lysates were analyzed for the phosphorylation of Elk-1 by immunoblot, using phospho-specific anti-Elk-1 antibody (Ser[383]). Pretreatment of the cells with the NFκB inhibitor significantly decreased the bFGF-mediated activation of Elk-1 as represented by phosphorylation of Elk-1 at serine 383 (Fig. 6*B*).

NFκB-dependent activation of Elk-1 after stimulation with bFGF was further supported by Elk-1 protein-DNA interaction in the presence of NFκB inhibitors, which can block a specific regulatory step to activate the NFκB pathway. Nuclear extracts were prepared from human adult articular chondrocytes treated with or without bFGF. Cells treated with bFGF showed a significant increase in Elk-1 protein-DNA-binding activity (Fig. 6*C*, *lane 2*) compared with untreated control chondrocytes (*lane 1*). Importantly, the presence of NFκB inhibitors that can block either NFκB unclear translocalization or IKKα/β activity (*lanes 3* and *4*, respectively) significantly reduced the bFGF-induced Elk-1 protein-DNA interaction. In the absence of bFGF, these inhibitors decreased the basal level of Elk-1 protein-DNA interaction (data not shown). Specificity of binding was confirmed by incubating nuclear extracts with an excess of unlabeled Elk-1 oligonucleotides (*lane 5*).

The use of the GAL4 DNA binding system further validated our hypothesis that bFGF-mediated activation of Elk-1 is NFκB-dependent (Fig. 6*D*). Chondrocytes co-transfected

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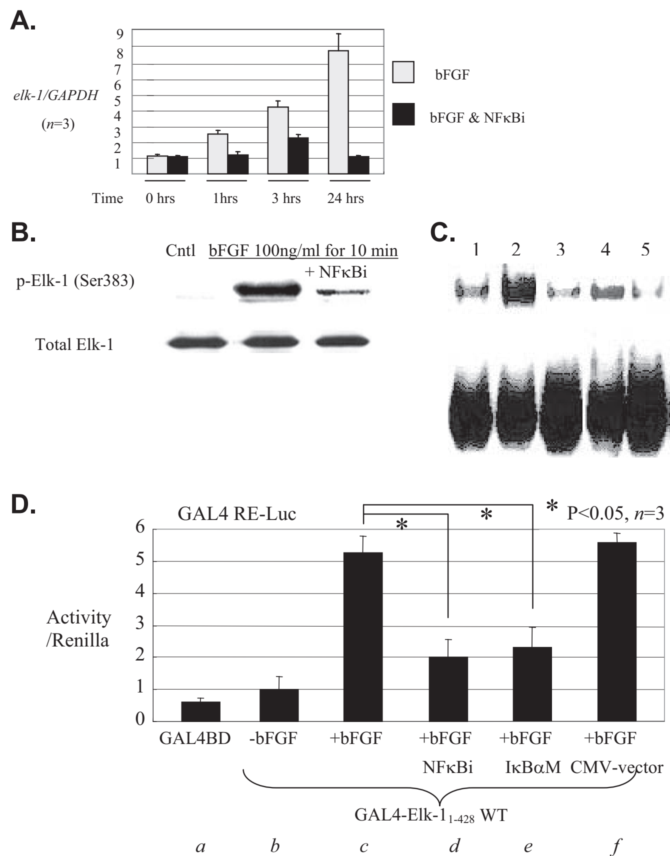

FIGURE 6. **Regulation of Elk-1 by NFκB in human articular chondrocytes.** *A*, cells were pre-treated with the NFκB pathway inhibitor (wedelolactone, 20 μM) for 1 h prior to bFGF treatment for the indicated period of time. The cDNA prepared from total RNA was analyzed by real-time PCR for the expression of *elk-1*. The level of GAPDH mRNA was used for normalization. The gel picture represents three different donors measured in triplicate for each experiment. *B*, serum-starved cells were treated with bFGF for 10 min in the presence or absence of the NFκB pathway inhibitor (wedelolactone, 20 μM). Cell lysates were analyzed by immunoblot with phospho-Elk-1^{Ser383} and total Elk-1 antibodies. *C*, Elk-1 gel-shift analysis was performed using nuclear extracts derived from untreated chondrocytes (*lane 1*) or cells treated with bFGF for 45 min (*lane 2*) in the absence or presence of SN50, a blocker of translocalization of NFκB (*lane 3*) or wedelolactone, an inhibitor of IKKα/β (*lane 4*). *Lane 5* represents the nuclear extracts incubated with excess amount of cold (unlabeled) probe for specificity test. *D*, cells were transiently co-transfected with a reporter construct GAL4RE-Luc, and GAL4-Elk-1 fusion protein, GAL4-Elk-1_{1-428}wt, 24 h after post-transfection, cells were treated with bFGF for 24 h in the presence or absence of the NFκB pathway inhibitor (NFκBi and wedelolactone) or overexpression of IκBαM. Then the cell lysates were used for the analysis of luciferase activity. Cells transfected with GAL4BD or CMV-vector alone was used as a control, and *Renilla* vector was used for normalization. The data represent three different donors measured in triplicate for each experiment.

with the GAL4 RE-Luc and GAL4-Elk-1_{1-428} wt exhibit dramatic increases in promoter activity in response to bFGF (Fig. 6*D*, *c*). In the presence of an inhibitor of IKKα/β (*d*) or overexpression of IκBαM (*e*), which can prevent the release of activated NFκB, this induction of Elk-1 activity was significantly reduced, whereas the overexpression of CMV-vector (backbone of IκBαM) had no inhibitory effect on the bFGF-mediated Elk-1 activation (*f*). Collectively, our results suggest that bFGF-mediated activation of the NFκB pathway modulates the biological function of Elk-1 at transcriptional and post-translational levels, because Elk-1 acts as a downstream target regulatory molecule. Thus, we conclude that, after stimulation with bFGF in human adult articular chondrocytes, Elk-1 functions as a key intermediate regulatory molecule in the NFκB pathway that controls MMP-13 expression.

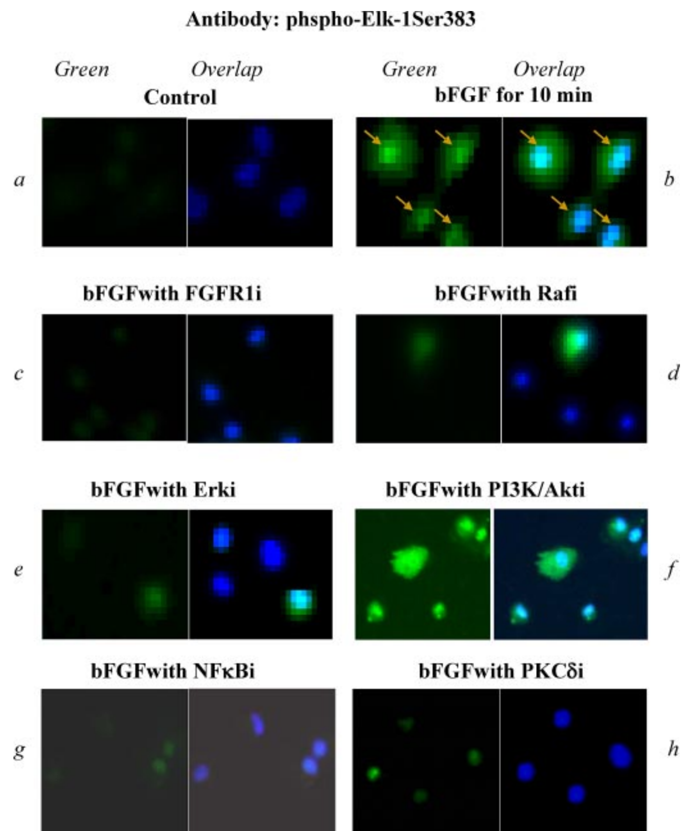

FIGURE 7. **Basic FGF promotes the nuclear translocalization of Elk-1 in human articular chondrocytes.** Cells plated in 4-well chamber slides at 100,000 cells/well were serum-starved for 24 h followed by treatment with or without bFGF in the presence or absence of inhibitors of FGFR1 (SU5402), Raf (Raf1 kinase inhibitor), Erk (PD098059), PI3K/Akt (LY294002), NFκB (wedelolactone) or PKCδ (rottlerin) for 10 min. The cells were then fixed with 2% paraformaldehyde, treated with 0.2% Triton X-100, and blocked with 10% normal goat serum followed by overnight incubation with phospho-Elk-1^{Ser383} antibody. On the next day the cells were washed, incubated with green flourescent-labeled anti-rabbit secondary antibody for 45 min, mounted using 4′,6-diamidino-2-phenylindole, which stains the nucleus, and visualized under the fluorescent microscope.

Nuclear Localization of Activated Elk-1 May Be Required for Biological Effect of bFGF in Human Adult Articular Chondrocytes—Nuclear localization of activated transcription factors has been shown to be vital for fulfillment of many of their biological activities regulating target genes. Thus, we further elucidated the corresponding relationship between bFGF-mediated regulation of nucleocytoplasmic trafficking of activated Elk-1 through phosphorylation of serine 383 and the stimulation of MMP-13 expression. The association of Elk-1 nuclear localization with the inhibitors that specifically block the bFGF-mediated signaling pathways leading to MMP-13 production was examined. In our time-course translocalization studies using anti-phospho-specific Elk-1^{Ser383} antibody, we observed that the nuclear localization occurs within 5 min and reached the maximum nuclear localization level between 10 and 20 min after stimulation with bFGF (Fig. 7*b*). This level of localization was gradually decreased over 1 h (data not shown).

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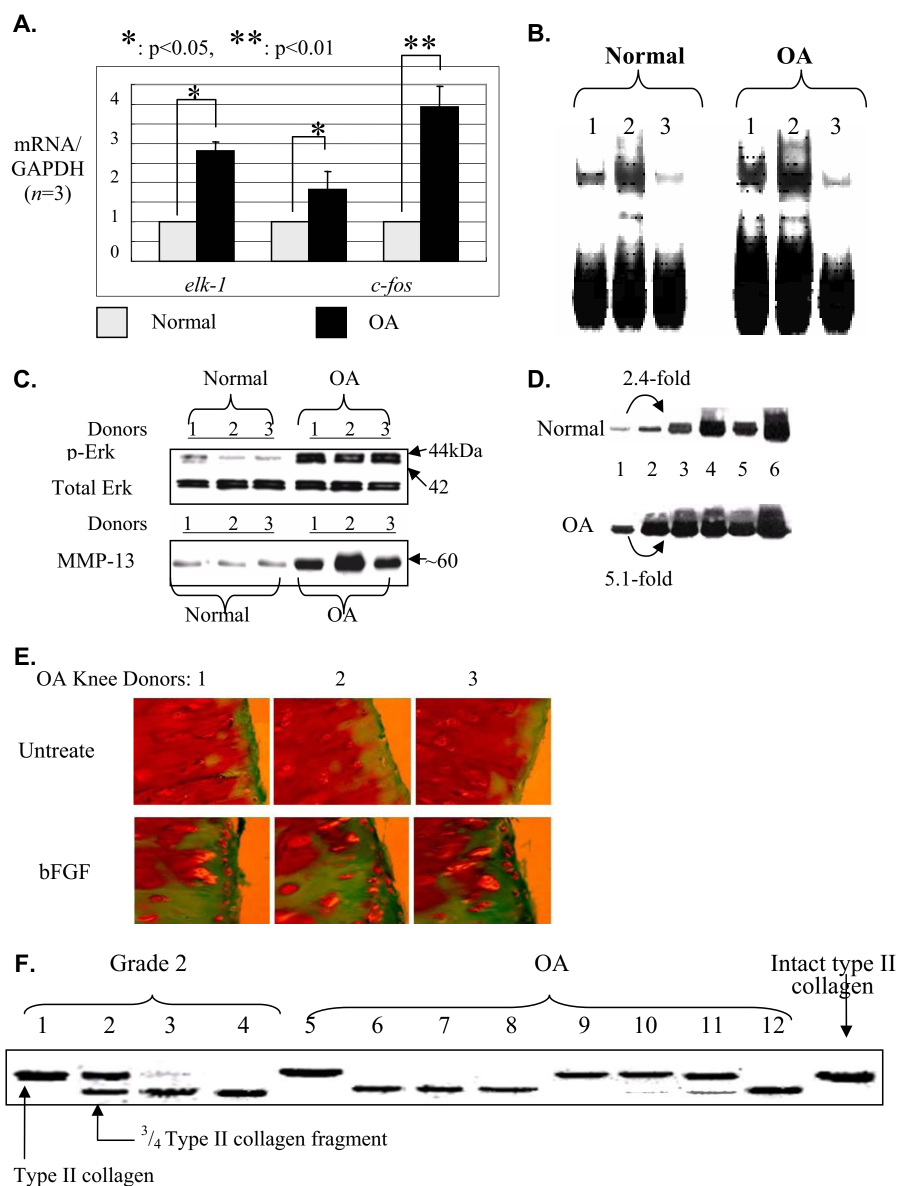

FIGURE 8. **Comparison studies between normal and OA cartilage.** *A*, total RNA extracted from the cartilage tissue (normal and OA knees) were subjected to real-time PCR analysis for *elk-1*, *bFGF*, and c-*fos* gene expression. GAPDH mRNA was analyzed as a control for normalization purpose. *B*, Elk-1 protein-DNA interaction was tested using biotin-labeled probes incubated with nuclear extracts derived from untreated chondrocytes (*lanes 1*) or cells treated with bFGF for 45 min (*lanes 2*) of both normal and OA cells. Nuclear extracts incubated with excess amount of cold (unlabeled) probe were used for specificity purpose (*lanes 3*). *C*, total tissue protein extracted directly (without cell culture) from normal and OA knee cartilage (from three different donors) were resolved by 10% SDS-PAGE and immunoblotted with anti- phopshospecific Erk1/2 or MMP-13 antibodies. Total Erk was blotted as a control for loading equal amount of protein into each lane. *D*, cells isolated from normal and OA were serum-starved for 24 h followed by treatment with bFGF in different doses as indicated for 24 h. The conditioned media were then analyzed for MMP-13 production by immunoblot. For comparison purposes, cells were also treated with different doses of IL-1β. Lanes: *1*, control; *2*, bFGF (10 ng/ml); *3*, bFGF (50 ng/ml); *4*, bFGF (100 ng/ml); *5*, IL-1β (1 ng/ml); and *6*, IL-1β (10 ng/ml). *E*, Safranin Orange staining was performed using full thickness cartilage explants prepared from OA knee joint tissues, and the explants were cultured in the presence or absence of bFGF (100 ng/ml) in mini-ITS. Following 14 days of incubation, the explants were fixed with 4% paraformaldehyde for overnight and embedded in paraffin, and 8 μM sections were prepared for Safranin Orange staining to assess matrix proteoglycan loss. Representative results from three different donors are shown ($n = 6$). *F*, type II collagen digestion assay was performed by using human knee chondorcytes (grade 2 and OA) cultured in different condition as indicated. The culture media was activated with 1 μM 4-aminophenylmercuric acetate for 1 h at 37 °C, and then incubated with chick sternal type II collagen. The samples were then resolved on 8% SDS-PAGE followed by staining with Coomassie Brilliant Blue R-250 for 2 h. The gel was destained with water, containing 10% methanol and acetate acid to determine collagen type II degradation (3/4 type II collagen fragment). Intact type II collagen was included as a control. *Lanes*: *1*, Grade 2 control; *2*, bFGF (100 ng/ml); *3*, IL-1β (10 ng/ml); *4*, tumor necrosis factor-α (10 ng/ml); *5*, OA control; *6*, bFGF (100 ng/ml); *7*, IL-1β (10 ng/ml); *8*, tumor necrosis factor-α (10 ng/ml); *9*, OA control; *10*, bFGF plus Erki; *11*, bFGF plus NFκBi; and *12*, bFGF plus PI3K/Akti.

Thus, we selected 10 min for bFGF stimulation of cells for further studies. The translocalization of phosphorylated Elk-1^{Ser383} to the nuclear region was significantly reduced by pharmacological inhibitors of FGF receptor (*c*), as well as the Raf (*d*), Erk MAPK (*e*), NFκB (*g*), and PKCδ (*h*) pathways. With less extent, we observed decreased Elk-1 nuclear localization in the presence of p38 and JNK inhibitors (data not shown). However, inhibition of the PI3K/Akt pathway (*f*), PKCα/β and PKCϵ (data not shown), which have no influence on the activation of Elk-1 nor on MMP-13 expression (Fig. 3), failed to modulate nuclear translocalization of activated Elk-1 after stimulation with bFGF. Our results suggest that the close association of biological activation of Elk-1 with the ability of Elk-1 to upregulate MMP-13 is concomitantly related to the nuclear translocalization of Elk-1 after stimulation with bFGF.

Normal and OA Comparison Studies on the Erk MAPK-Elk-1-MMP-13 Pathway—We examined the potential links between Elk-1 activity and cartilage-degenerative diseases by comparing the protein activity and expression of Elk-1 in normal and OA cells. Human adult articular cartilage tissue from normal knee (asymptomatic, grade 0 or 1) and degenerative cartilage obtained after knee replacement surgery were used for this study. Without culturing cells in monolayer, we directly isolated total RNA from the cartilage tissues and performed real-time PCR using human-specific primer sets. Compared with normal cells, the elevated basal level of *elk-1* mRNA expression in OA cells was evident. Expression of both *bfgf* and c-*fos*, which are downstream target genes of Elk-1, was also significantly increased in OA compared with normal cells (Fig. 8*A*). Next, we examined whether Elk-1 DNA-binding activity, which represents the function of this transcription factor, is up-regulated in OA compared with normal cells. Serum-

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starved cells in monolayers were incubated in the presence or absence of bFGF for 45 min and prepared for nuclear extract followed by gel-shift assay. A markedly increased basal level of Elk-1-DNA-binding activity was observed in OA compared with normal cells (Fig. 8*B*, *Normal versus OA*, *lane 1*). Treatment with bFGF (100 ng/ml) further elevated Elk-1 protein-DNA interactions in both normal and OA cells (Normal *versus* OA, *lane 2*). This elevated Elk-1 protein-DNA interaction in OA cells corresponded to the elevated basal activity of Erk MAPK, a direct upstream regulator of Elk-1 (Fig. 8*C*, *upper panel*). Again, the enhanced basal activity of Erk was closely linked to stimulated production of MMP-13 at a basal level in OA compared with normal cells (Fig. 8*C*, *lower panel*). Furthermore, basal DNA binding activities of AP-1, which is a downstream target of Elk-1, were also significantly increased in OA cells compared with normal human articular chondrocytes (data not shown). These highly up-regulated abnormal basal activities of critical transcription factors in OA cells could explain why basal production of MMP-13 is higher in OA and why OA cells are hypersensitized to stimulation with bFGF (Fig. 8*D*).

We further examined the functional correlation between bFGF and articular cartilage degeneration by performing Safranin Orange staining, a marker to assess proteoglycan loss, using OA cartilage explant culture after incubation with or without bFGF for 14 days (Fig. 8*E*). Our results show accelerated Safranin Orange staining in the presence of bFGF in OA knee tissues (*lower panel*) compared with tissues not treated with bFGF (*upper panel*).

Using conditioned medium collected from bFGF-stimulated early-stage OA cells (grade 2) and normal cells, we also observed bFGF and its signaling pathway-mediated degradation of collagen type II. Incubation of cells with bFGF generated the ¾ fragment of type II collagen (Fig. 8*F*, *lanes 2* and *6*), suggesting the potential links between bFGF and collagen digestion. Similar results were observed in the presence of well known catabolic pro-inflammatory cytokines (IL-1β and tumor necrosis factor-α), which we included in our experiments in parallel for comparison (*lanes 3*, *4*, *7*, and *8*). Importantly, the bFGF-induced collagen digestion was completely abolished by co-incubation with pathway-specific inhibitors of Erk (*lane 10*) and NFκB pathway (*lane 11*), both upstream regulators of Elk-1 (Figs. 3, 6, and 7). Consistent with our other data, in the current study, blocking the PI3K/Akt pathway, which had no effect on Elk-1 activity and MMP-13 production (Figs. 3 and 7), showed no impact on the type II collagen degradation by bFGF (*lane 12*). Collectively, our results suggest that the bFGF/Erk/NFκB/Elk-1 pathways co-operatively deregulate in OA and may represent an important pathophysiological link in cartilage degeneration.

DISCUSSION

This study focuses on the molecular mechanisms that mediate the biological impact of bFGF on human adult articular cartilage homeostasis and complements previous studies from our group (14, 18). In human adult articular chondrocytes, binding of bFGF to its cognate receptor, FGFR1, activates multiple signaling cascades (18, 38), including the MAPK pathway, PKCδ, NFκB, and PI3K/Akt (Ref. 18 and current study). We have previously shown that NFκB is one of the most critical activators of MMP-13 expression in response to fibronectin fragment (38). The present studies show that NFκB pathway is also the ultimate target of bFGF signaling in human articular chondrocytes and is associated with the bFGF-mediated MMP-13 stimulation. The MMP-13 promoter region contains a putative NFκB recognition motif (-1005 to -1014 upstream of ATG), and this element is not responsive to stimulation with bFGF. Accordingly, deletion of this motif did not affect basal or bFGF-stimulated levels of MMP-13 promoter activity. Similarly, Fujioka and colleagues (39) have demonstrated that vascular endothelial growth factor gene expression is regulated by the NFκB signaling pathway, even though the promoter of vascular endothelial growth factor lacks the NFκB binding motif. They proposed an indirect mechanism by which vascular endothelial growth factor stimulates Elk-1 expression which, in turn, controls the AP-1 component c-*fos*. Our current data suggest that NFκB-mediated stimulation of MMP-13 by bFGF is mediated by NFκB activation of the key ETS-domain-containing transcription factor Elk-1. Using human primary adult articular chondrocytes, we found that blocking the activation of the NFκB pathway not only significantly reduces the bFGF-mediated phosphorylation and biological activation of Elk-1 but also *elk-1* mRNA accumulation in response to bFGF. We propose that NFκB activation, which may be under control of MAPK (JNK, Erk, and p38), may have a dual role as an upstream regulator of both Elk-1 and, thereby, c-Fos, resulting in the control of MMP-13 expression. Our results collectively indicate that multiple molecular networks converge to activate Elk-1, leading to the up-regulation of MMP-13 expression.

Blocking the biological activity of bFGF by inhibiting FGFR1, MAPKs, or NFκB is associated with reduced Elk-1 activation, nuclear translocalization, and Elk-1 protein-DNA-binding activity, which may result in the down-regulation of MMP-13 expression. These findings suggest a close link between Elk-1 function and MMP-13 expression in human adult articular chondrocytes. Interestingly, our data suggest that the bFGF-mediated activation of PI3K/Akt signaling cascades is completely independent from other known signaling events associated with MMP-13 stimulation. Investigation of the potential role of the PI3K/Akt signaling pathway after stimulation with bFGF may provide insight into the precise biological role of bFGF in human adult articular cartilage homeostasis (Fig. 9).

Using chimeric GAL4/Elk-1 proteins in transcriptional assays, we verified that bFGF biologically activates the inherent transactivation potential of Elk-1 in both a quantitative and sensitive manner. This bFGF-dependent stimulation was reduced by blocking the pathway controlled by FGFR1, the individual MAPK subgroups and NFκB. The activation of multiple MAPK subgroups and NFκB appeared to be required and essential to stimulate MMP-13 expression in a coordinated and integrated manner. Among the MAPK subgroups, Erk exhibited the most potent impact on Elk-1 activation, suggesting that Erk-Elk-1 is the preferential regulatory pathway utilized by bFGF in human articular chondrocytes. The bFGF-mediated activation of Elk-1 was completely abolished in the presence of an inhibitor of MEK1/2 (direct upstream regulator of Erk),

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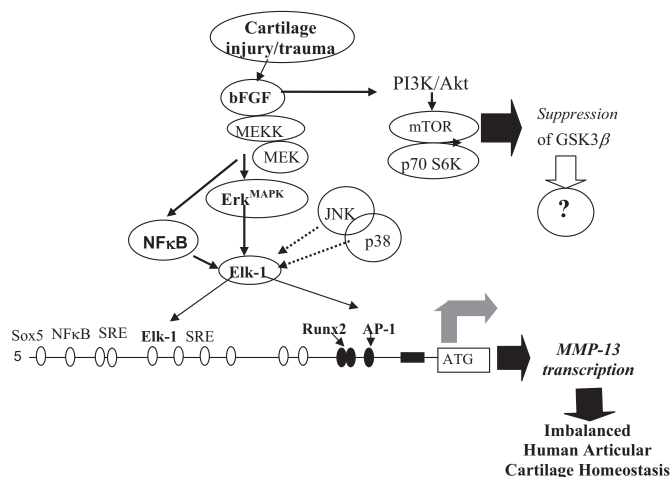

FIGURE 9. **Schematic presentation of the transcriptional regulation of MMP-13 gene expression.** Basic FGF activates the MAPK and NFκB pathways, which converge to activate a key transcription regulatory molecule Elk-1 for MMP-13 gene transcription in human adult articular chondrocytes.

whereas blocking the JNK, p38, and NFκB pathways partially inhibited bFGF-mediated Elk-1 activity, suggesting that partial reduction of Elk-1 is sufficient enough to down-regulate MMP-13 expression.

Our results also show that overexpression of Elk-1 is selective for MMP-13, because we did not observe a significant induction of MMP-1. Because bFGF and inflammatory cytokines are present at elevated levels in the synovial fluids of patients with osteoarthritis, the possibility arises that both Erk-Elk-1 and NFκB pathways are concomitantly activated in chondrocytes under pathological conditions, and NFκB-directed modulation of Elk-1 may cooperate to activate shared target genes of these two rapidly inducible transcription factors.

The basal gene expression levels of *bfgf*, *elk-1*, and c-*fos* were increased in OA cartilage in comparison to that of normal articular cartilage. In addition, the basal activity of Erk and Elk-1 was highly up-regulated in OA compared with that of normal articular chondrocytes. Moreover, the increased basal activity levels of Erk-Elk-1 were correlated with increased basal levels of MMP-13 in OA cartilage as well as synovial fluids. In our separate protein-DNA interaction experiments, followed by individual gel shift assays, we observed dramatically elevated basal activity of NFκB and AP-1 in OA cells (data not shown). The elevated basal activities of well known inflammatory pathway-associated transcription factors perhaps can explain why the biological response to bFGF was accelerated in OA cells. Collectively, our results demonstrate pathophysiological links between abnormally high basal levels of activated Erk-Elk-1 and increased MMP-13 expression found in OA cartilage.

From our previous data and current studies, the anti-anabolic and catabolic effect on cartilage by bFGF is apparent in human adult articular cartilage (14, 18). The negative biological action of bFGF on articular cartilage is significantly magnified when cartilage tissues are already in the process of degeneration (from the stage of early degeneration to OA condition). These findings suggest that naturally increased bFGF expression and its cognate receptor FGFR1 in OA cells may participate in the detrimental inhibition of cartilage repair resulting in tissue degeneration. Previously, we reported that bFGF stimulates MMP-13 expression together with up-regulation of various pro-inflammatory cytokines (18) and potent inhibition of proteoglycan synthesis in human adult articular cartilage (14). In the current study, we observed bFGF-mediated proteoglycan loss (by Safranin Orange staining) and type II collagen cleavage in an *in vitro* assay, which was rescued in the presence of inhibitors of Erk MAPK and NFκB, further supporting our previous results. Inhibitors of the PI3K/Akt pathway, however, failed to rescue bFGF-mediated type II collagen degradation. This result is consistent with our other data in this study and confirms that bFGF-mediated stimulation of collagenase production occurs as the PI3K/Akt-independent pathway.

In conclusion, we have provided compelling evidence for the critical role of the Elk-1 transcription factor in the transcriptional stimulation of MMP-13 (collagenase-3), but not MMP-1 (collagenase-1), in response to bFGF in human adult articular chondrocytes. We further provide evidence for an indirect regulatory role of NFκB on MMP-13 expression through its activation of Elk-1 as a critical intermediate transcriptional regulator. MAPK signaling (especially Erk) modulates Elk-1 activity, Elk-1 regulates AP-1, and AP-1 is one of the core transcription factors critical for MMP-13 gene expression. Furthermore, MAPK also controls RUNX2 activity, which is a key regulator of MMP-13 gene expression (40). Thus, bFGF may control MMP-13 by the coordinated molecular regulation of multiple regulatory factors and their cognate motifs present in the MMP-13 promoter: Elk-1, NFκB, and the core factors, AP-1, ETS, and RUNX2.

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REFERENCES

1. Pelletier, J. P., Martel-Pelletier, J., and Howell, D. S. (2001) *Arthrits and Allied Conditions: A Textbook of Rheumatologyi*, Lippincott Williams & Wilkins, Baltimore, 14th Ed., pp. 2195–2245
2. Martel-Pelletier, J., Welsch, D. J., and Pelletier, J. P. (2001) *Best Pract. Res. Clin. Rheumatol.* **15,** 805–829
3. Reboul, P., Pelletier, J. P., Tardif, G., Cloutier, J. M., and Martel-Pelletier, J. (1996) *J. Clin. Invest.* **97,** 2011–2019
4. Fernandes, J. C., Martel-Pelletier, J., Lascau-Coman, V., Moldovan, F., Jovanovic, D., Raynauld, J. P., and Pelletier, J. P. (1998) *J. Rheumatol.* **25,** 1585–1594
5. Burrage, P. S., Mix, K. S., and Brinckerhoff, C. E. (2006) *Front. Biosci.* **11,** 529–543
6. Miwa, H. E., Gerken, T. A., Hyunh, T. D., Flory, D. M., and Hering, T. M. (2006) *Biochim. Biophys. Acta* **1760,** 472–486
7. Fosang, A. J., Last, K., and Maciewicz, R. A. (1996) *J. Clin. Invest.* **98,** 2292–2299
8. Bikfalvi, A., Klein, S., Pintucci, G., and Rifkin, D. B. (1997) *Endocr. Rev.* **18,** 26–45
9. Vincent, T., Hermansson, M., Bolton, M., Wait, R., and Saklatvala, J.

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(2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 8259–8264
10. Vincent, T. L., Hermansson, M. A., Hansen, U. N., Amis, A. A., and Saklatvala, J. (2004) *Arthritis Rheum.* **50,** 526–533
11. O'Keefe, R. J., Crabb, I. D., Puzas, J. E., and Rosier, R. N. (1994) *J. Orthop. Res.* **12,** 299–310
12. Rosselot, G., Vasilatos-Younken, R., and Leach, R. M. (1994) *J. Bone. Miner. Res.* **9,** 431–439
13. Osborn, K. D., Trippel, S. B., and Mankin, H. J. (1989) *J. Orthop. Res.* **7,** 35–42
14. Loeser, R. F., Chubinskaya, S., Pacione, C., and Im, H. J., (2005) *Arthritis Rheum.* **52,** 3910–3917
15. Uria, J. A., Balbin, M., Lopez, J. M., Alvarez, J., Vizoso, F., Takigawa, M., and Lopez-Otin, C. (1998) *Am. J. Pathol.* **153,** 91–101
16. Varghese, S., Rydziel, S., and Canalis, E. (2000) *Endocrinology* **141,** 2185–2191
17. Wang, X., Manner, P. A., Horner, A., Shum, L., Tuan, R. S., and Nuckolls, G. H. (2004) *Osteoarthritis Cartilage* **12,** 963–973
18. Im, H. J., Muddasani, P., Natarajan, V., Schmid, T. M., Block, J. A., Davis, F., van Wijnen, A. J., and Loeser, R. F. (2007) *J. Biol. Chem.* **282,** 11110–11121
19. Gille, H., Kortenjann, M., Thomae, O., Moomaw, C., Slaughter, C., Cobb, M. H., and Shaw, P. E. (1995) *EMBO J.* **14,** 951–962
20. Gille, H., Strahl, T., and Shaw, P. E. (1995) *Curr. Biol.* **5,** 1191–1200
21. Janknecht, R., and Hunter, T. (1997) *EMBO J.* **16,** 1620–1627
22. Whitmarsh, A. J., Shore, P., Sharrocks, A. D., and Davis, R. J. (1995) *Science* **269,** 403–407
23. Cahill, M. A., Janknecht, R., and Nordheim, A. (1996) *Curr. Biol.* **6,** 16–19
24. Carreras, I., Rich, C. B., Jaworski, J. A., Dicamillo, S. J., Panchenko, M. P., Goldstein, R., and Foster, J. A. (2001) *Am. J. Physiol.* **281,** L766–L775
25. Mengshol, J. A., Vincenti, M. P., Coon, C. I., Barchowsky, A., and Brinckerhoff, C. E. (2000) *Arthritis Rheum.* **43,** 801–811
26. Forsyth, C. B., Pulai, J., and Loeser, R. F. (2002) *Arthritis Rheum.* **46,** 2368–2376
27. Ohno, S., Im, H. J., Knudson, C. B., and Knudson, W. (2006) *J. Biol. Chem.* **281,** 17952–17960
28. Muehleman, C., Bareither, D., Huch, K., Cole, A. A., and Kuettner, K. E. (1997) *Osteoarthritis Cartilage* **5,** 23–37
29. Roberts, M. S., Woods, A. J., Dale, T. C., van der Sluijs, P., and Norman, J. C. (2004) *Mol. Cell. Biol.* **24,** 1505–1515
30. Im, H. J., Pacione, C., Chubinskaya, S., Van Wijnen, A. J., Sun, Y., and Loeser, R. F. (2003) *J. Biol. Chem.* **278,** 25386–25394
31. Im, H. J., Kang, S. W., and Loh, H. H. (1999) *Brain Res.* **829,** 174–179
32. Pulai, J. I., Chen, H., Im, H.-J., Kumar, S., Hanning, C., Hegde, P. S., and Loeser, F. R. (2005) *J. Immunol.* **174,** 5781
33. Cavigelli, M., Dolfi, F., Claret, F. X., and Karin, M. (1995) *EMBO J.* **14,** 5957–5964
34. Gutzman, J. H., Nikolai, S. E., Rugowski, D. E., Watters, J. J., and Schuler, L. A. (2005) *Mol. Endocrinol.* **19,** 1765–1778
35. Yang, S. H., Jaffray, E., Hay, R. T., and Sharrocks, A. D. (2003) *Mol. Cell.* **12,** 63–74
36. Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, P., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F., Duhamell, L., Charon, D., and Kirilovsky, J. (1991) *J. Biol. Chem.* **266,** 15771–15781
37. Kuhn, K., and Lotz, M. (2001) *Arthritis Rheum.* **44,** 1644–1653
38. Loeser, R. F., Forsyth, C. B., Mamarel, A. M., and Im, H. J. (2003) *J. Biol. Chem.* **278,** 24577–24585
39. Fujioka, S., Niu, J., Schmidt, C., Sclabas, G. M., Peng, B., Uwagawa, T., Li, Z., Evans, D. B., Abbruzzese, J. L., and Chiao, P. J. (2004) *Mol. Cell. Biol.* **24,** 7806–7819
40. Papachristou, D. J., Pirttiniemi, P., Kantomaa, T., Papavassiliou, A. G., and Basdra, E. K. (2005) *Histochem. Cell Biol.* **124,** 215–223

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