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ORIGINAL RESEARCH

Basic fibroblast growth factor induces matrix metalloproteinase-13 via ERK MAP kinase-altered phosphorylation and sumoylation of Elk-1 in human adult articular chondrocytes

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Hee-Jeong Im[1–4]
Andrew D Sharrocks[5]
Xia Lin[6]
Dongyao Yan[1]
Jaesung Kim[1]
Andre J van Wijnen[7]
Robert A Hipskind[8]

[1]Departments of Biochemistry, [2]Internal Medicine, [3]Section of Rheumatology, Orthopedic Surgery, [4]Rush University Medical Center, and Department of Bioengineering, University of Illinois at Chicago, IL USA; [5]Faculty of Life Sciences, University of Manchester, Oxford Rd, Manchester, UK; [6]Michael E DeBakey Department of Surgery, Baylor College of Medicine, Houston, Texas, USA; [7]Department of Cell Biology, University of Massachusetts Medical School, Worcester, MA, USA; [8]Institute De Genetique Moleculaire de Montpellier, France

Abstract: Degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs) and release of basic fibroblast growth factor (bFGF) are principal aspects of the pathology of osteoarthritis (OA). ECM disruption leads to bFGF release, which activates the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway and its downstream target the Ets-like transcription factor Elk-1. Previously we demonstrated that the bFGF-ERK-Elk-1 signaling axis is responsible for the potent induction of MMP-13 in human primary articular chondrocytes. Here we report that, in addition to phosphorylation of Elk-1, dynamic posttranslational modification of Elk-1 by small ubiquitin-related modifier (SUMO) serves as an important mechanism through which MMP-13 gene expression is regulated. We show that bFGF activates Elk-1 mainly through the ERK pathway and that increased phosphorylation of Elk-1 is accompanied by decreased conjugation of SUMO to Elk-1. Reporter gene assays reveal that phosphorylation renders Elk-1 competent for induction of MMP-13 gene transcription, while sumoylation has the opposite effect. Furthermore, we demonstrate that the SUMO-conjugase Ubc9 acts as a key mediator for Elk-1 sumoylation. Taken together, our results suggest that sumoylation antagonizes the phosphorylation-dependent transactivation capacity of Elk-1. This attenuates transcription of its downstream target gene MMP-13 to maintain the integrity of cartilage ECM homeostasis.

Keywords: osteoarthritis, MMP-13, bFGF, SUMO, Elk-1

Introduction

Cartilage degenerative diseases, such as osteoarthritis (OA), are both a serious cause of disability and a major source of health care costs. Accumulating evidence demonstrates that the integrated output of cellular and molecular mechanisms within entire structural joints is responsible for the impaired cartilage repair. Perturbed chondrocyte homeostasis makes a key contribution to the destruction of its own matrix through the release of destructive enzymes, including matrix metalloproteinase-13 (MMP-13).[1] Nevertheless, comparatively little is known about the role of growth factors, cytokines, and inflammatory mediators in regulating cartilage-degrading enzyme production and thus the pathogenesis of the disease.

The pathology of joint destruction is closely associated with elevated levels of basic fibroblast growth factor (bFGF) (or FGF-2).[2,3] Chondrocyte-produced bFGF is stored in the cartilage extracellular matrix (ECM) and is immediately released upon mechanical injury of cartilage. bFGF mediates an immediate response in

Correspondence: Hee-Jeong Im
Department of Biochemistry, Rush University Medical Center, Cohn Research BD 516, 1735 W Harrison, Chicago, IL 60612, USA
Tel +1 (312) 942-3091
Fax +1 (312) 942-3053
Email hee-jeong_sampen@rush.edu

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articular chondrocytes by inducing a prolonged activation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) that has striking effects on chondrocyte gene expression.[4,5] We have found that human articular chondrocytes exposed to bFGF, even at low levels, exhibit a substantially diminished response to bone morphogenetic protein-7 (BMP7).[6] As such, bFGF can severely compromise the capacity of the tissue for repair. Repeated trauma to the same joint and the resulting episodes of bFGF release may be a key component in triggering the onset of OA.

bFGF induces the transcriptional activity of the Ets-like transcription factor (Elk-1) via its phosphorylation by ERK. This stimulates the expression of Elk-1 target genes, such as c-Fos, that is critical for the transcriptional repression of ECM components in other cells.[7] Data, including our own, have demonstrated a substantial induction of MMP-13 by bFGF in human adult articular chondrocytes.[2,3,8] We previously investigated the molecular mechanisms by which the bFGF-ERK MAPK-Elk-1 signaling axis stimulates MMP-13 production in human articular cartilages.[2,3] Our findings demonstrated that bFGF binding to its receptor basic fibroblast growth factor receptor 1 (FGFR1) initiates a signaling cascade that involves Protein kinase C (PKC), which in turn activates ERK and Elk-1. The activation of Elk-1 by ERK is directly associated with the transcriptional induction of MMP-13 *via* Elk-1 binding to the proximal DNA recognition sequence in the MMP-13 promoter.[2,3]

The regulatory output of the bFGF-MAPK/ERK-Elk-1 pathway can also be altered by sumoylation, which involves posttranslational conjugation with the small ubiquitin-related modifier (SUMO). SUMO regulates diverse cellular processes through its reversible, covalent attachment to target proteins. This is mediated by the enzymes E1 (activating enzyme, Aos1-Uba2), E2 (conjugating enzyme, Ubc9) and E3 (SUMO ligase, eg, PIAS family).[9,10] Altered SUMO pathways have been linked to the onset or progression of human diseases.[11] Desumoylation of Elk-1 correlates with its activating phosphorylation by ERK upon stimulation with mitogenic factors.[9,12] In the basal state, Elk-1 is SUMO-conjugated and hence, inactive because of SUMO-mediated repression. Moreover, sumoylation strongly enhances nuclear retention of Elk-1.[13] The present study investigated whether bFGF affects the balance between sumoylation and phosphorylation of Elk-1 and thereby its ability to activate MMP-13 gene expression in human adult articular chondrocytes.

Materials and methods

Tissue acquisition, chondrocyte isolation and culture conditions

Normal human knee and ankle cartilage were obtained from tissue donors through the Gift of Hope Organ and Tissue Donor Network (Elmhurst, IL, USA) using approved institutional protocols. Each donor specimen was graded for gross degenerative changes based on a modified version of the 5-point scale of Collins.[14] Only normal or nearly normal ankle and/or knee tissues (Grade 0 or 1) were used to facilitate a comparison with osteoarthritic cartilage tissue. The donors had no known history of arthritis. OA tissue specimens that were removed during total knee arthroplasty were obtained through the Orthopedic Tissue and Implant Repository Studies (approved by IRB) at the Orthopedic Surgery Department, Rush. Samples were collected only if the patients or next-of kin of donors were informed about the use of the samples in research and signed the consent form currently approved for The Gift of Hope Organ and Tissue Donor Network or the Orthopedic Tissue and Implant Repository at Rush. Chondrocytes were isolated by enzymatic digestion of knee joint articular cartilage using pronase followed by overnight digestion with collagenase-P as described previously.[2,3,15] Isolated cells were re-suspended in a high density at 3×10^6 per mL and plated on to 12-well plates at 1 mL/well. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM/F-12) containing 10% fetal bovine serum and antibiotics (complete media) for 5 days before the experiments.

Chondrocyte stimulation, chemical inhibitors and immunoblotting

Cells were serum-starved by changing media to serum-free DMEM/F-12 with antibiotics for one day. For pharmacological inhibitor studies, cells were pre-incubated with individual pathway-specific chemical inhibitors for 1 hour before stimulation with FGF at the concentration of 100 ng/mL (NCI). Chemical inhibitors used in the study included SU5402 for FGFR (1 μM), Raf inhibitor (Raf1 kinase inhibitor 1, 20 μM), PD98059 (20 μM) for MAPK and mitogen-activated protein kinase, SB203580 (2 μM) for p38, and SP600125 (20 μM) for c-Jun N-terminal kinases (JNK). These concentrations have been used for our previous studies to minimize non-specific inhibitory effects.[2,3,15] Experiments were terminated with removal of media and/or cell lysate preparation. The conditioned media was stored at 4 °C with 0.1% sodium azide and used for the experiments within 5 days. Cell lysates were prepared using

modified cell lysis RIPA buffer: 20 mM Tris (pH 7.5), 150 mM sodium chloride, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 0.25% deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM glycerol phosphate, 1 mM sodium vanadate (VII) oxide, 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 amount of protein was resolved by 10% SDS-PAGE gels and was transferred to nitrocellulose membrane for immunoblot analysis as described previously.[2] Immunoreactivity was visualized using the ECL system (Amersham) and the Signal Visual Enhancer system (Pierce) which magnifies the signal.

Plasmids constructs

Elk-1 wild type (wt) and Elk-1-associated mutant constructs, containing cytomegalovirus (CMV) promoter such as CMV-Elk-1 S383A (unphosphorylatable), CMV-Elk-1 K230R/K249R (unsumoylatable), and GAL4-Elk-1 system including Luciferase reporter plasmid construct (GAL4 RE-Luc), GAL4-Elk-1wt, GAL4-Elk1 S383A, GAL4-Elk-1 K230R/K249R, CMV-DN-Ubc9 and Ubc9 wt cDNA constructs and Human MMP-13 promoter construct (–1600 from ATG) were previously described.[9,16]

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.[2,17] 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 luminometer (Berthold). In samples containing combinations of plasmids (ie, co-transfections with GAL4-Elk-1 or mutant cDNA constructs with GAL4 RE-Luc 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.

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 minutes, and centrifuged at 15,000 g at 4 °C for 3 minutes. The supernatants were discarded and the pellets resuspended in 150 µL of buffer B. The samples were incubated on ice for 2 hours, centrifuged and supernatants were aliquoted and stored at −80 °C.

Electrophoretic-mobility shift assay

Gel shift assays of Elk-1 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 consensus sequence (Elk-1). The samples were then resolved on a 6% non-denaturing polyacrylamide gel and transferred to Pall Biodyne B membrane, blocked, incubated with Streptavidin-HRP 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.

Immunoprecipitation (IP)

To detect SUMO-conjugation of endogenous Elk-1 and SUMO-1, cells (3×10^6/well on a 12-well plate) with and without bFGF stimulation were subjected to lysis in a buffer supplemented with 5% SDS and 20 mM isopeptidase inhibitor N-ethylmaleimide. Cell lysates then were diluted with the same buffer without SDS to achieve an SDS concentration of 0.5%. IP of Elk-1 was performed by using anti-Elk-1 antibody and by following manufacturer's protocol (Seize X Protein A IP kit; Pierce Biotechnology). Briefly, the supernatants (500 µg protein) were transferred to new tubes containing the antibody immobilized to the protein A agarose gel using the cross-linker disuccinimidyl suberate (DSS) 12.5 mL for antibody crosslinking. The samples were mixed end-over-end overnight at 4 °C followed by washing three times with binding/washing buffer (0.14 M sodium chloride, 0.008 M sodium phosphate, 0.002 M potassium phosphate and 0.01 M potassium chloride, pH 7.4). The samples were then eluted using elution buffer and subjected to SDS-PAGE. SUMO-conjugation and/or de-conjugation of Elk-1 were detected by following immunoblotting with anti-SUMO-1 antibody.

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.

Results

bFGF-activation of Elk-1 is preferentially regulated by ERK MAP kinase, and its trans-activity is controlled by the SUMO pathways in human articular chondrocytes.

Previously, we reported that the endogenous levels of bFGF and MMP-13 are highly upregulated in arthritic knee joint such as OA and RA patients compared to those levels in normal.[2] Comparison of normal and OA human cartilage revealed a dramatic elevation of basal ERK activity in OA chondrocytes that correlates with high basal transcriptional potential and DNA binding activity of Elk-1, which is a known to be a direct phosphorylation substrate for MAP kinases.[3] Because Elk-1 is critical for MMP-13 stimulation,[3] we examined whether ERK MAP kinase is indeed a major upstream modulator of Elk-1-MMP-13 transactivation. Serum-starved human articular chondrocytes cultured in monolayear were pre-incubated with pathway-specific inhibitors for MAP kinase subgroups (p38i, ERK inhibitor, and JNKi) for 30 minutes prior to the stimulation of the cells with bFGF for 5 minutes. Inhibitors for basic fibroblast growth factor receptor1initiated FGFR1i[3] and Raf (Rafi), an upstream kinase of MEK-ERK MAP kinase were included in parallel for comparison. The modulation of Elk-1 activity was detected using a phosphospecific anti-Elk-1 antibody (Ser383). The results show that although pharmacological inhibitors of all three MAP kinases reduce the activity of Elk-1, ERK inhibitor (ERKi) is most potent in blocking Elk-1 phosphorylation, and thus the intrinsic activities of Elk-1, as reflected by reduced levels of the Serine 383 phospho-epitope (Figure 1A). These results were further validated by examining the Elk-1 protein binding activity to its cognate DNA response elements by gel shift assays, using nuclear extract from human articular chondrocytes treated with or without pathway-specific inhibitors in the presence of bFGF (Figure 1B). Consistent with the reduced Elk-1 activation reflected by the reduced detection of Ser383 phospho-epitope, the inhibitors of each MAP kinase subgroup significantly reduce Elk-1 protein-DNA interaction with ERKi being the most potent (Figure 1B, *lane 3*).

We further verified the biological activation of Elk-1 in the presence of bFGF by using GAL4 binding assay system.[9] The GAL4 binding assay system components include a luciferase reporter plasmid, containing multiple GAL4 response elements (GAL4 RE-Luc) that responds to the expression of Elk-1 proteins fused to GAL4 DNA-binding domain (BD) (GAL4-Elk-1 wt). These plasmids were co-transfected into human primary articular chondrocytes by nucleofection followed by measurement of luciferase reporter activity, which reflects the inherent transactivation potential of 11 (Figure 1C). Consistent with other data (Figures 1A and B), bFGF significantly induces the transactivation of Elk-1, and this bFGF-dependent stimulation is reduced by FGFR1

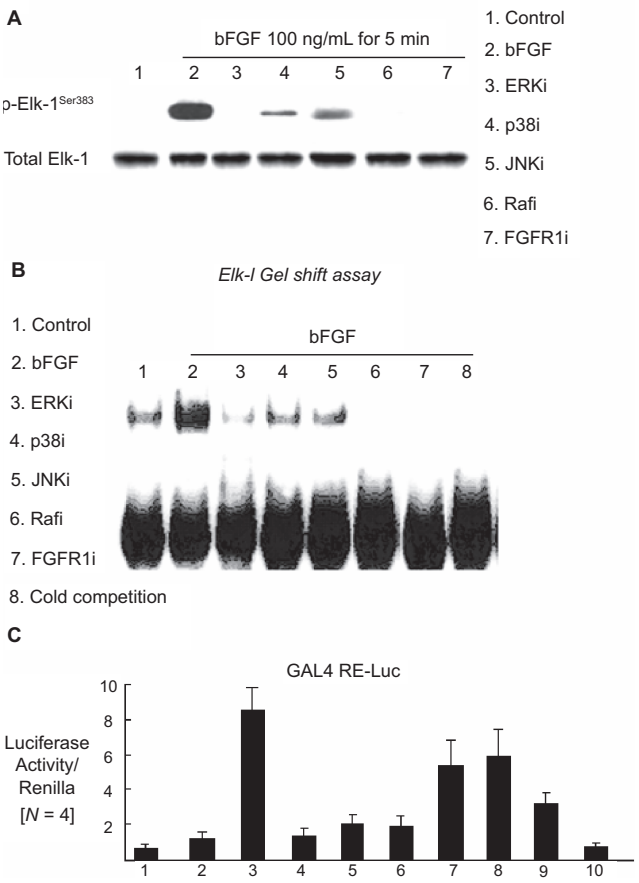

Figure 1 Elk-1 is activated by the ERK MAP kinase and the SUMO-1 pathways. Serum-starved cells in monolayer for 24 hours were treated with individual pharmacological pathway-specific inhibitors of MAP kinase subgroups 1 hour prior to the treatment with bFGF (100 ng/mL) for 5 minutes. **A**) The cell lysates were prepared and immunoblotted with anti-phosphospecific Elk-1^{Ser383} antibody. The membrane was stripped and re-probed with total Elk-1 antibody for normalization. **B**) Elk-1 protein-DNA interaction was examined by gel shift using biotin labeled double-stranded Elk-1 DNA consensus motif. The oligo probes were incubated with nuclear extracts derived from untreated cells (Control) or cells treated with bFGF for 45 minutes in the presence of inhibitors specific for FGFR1, Raf and each MAP kinase subgroup. Nuclear extract incubated with excess amount of unlabeled probe (cold competition) was used for specificity control (*lane 6*). Lane identification: 1 Control, 2. bFGF, 3. bFGF with ERK inhibitor (ERKi, PD098059, 20 μM), 4. bFGF with p38 inhibitor (p38i, SB203580, 10 μM), 5. JNK inhibitor (JNKi, SP600125, 20 μM). **C**) 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). Twenty-four hours after post-transfection, the cells were serum-starved, then, were treated with individual pharmacological pathway-specific inhibitors of MAP kinase subgroups 1 hour prior to the treatment with bFGF for an additional 24 hours 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 (*lane* 1). For the association of the SUMO pathway with the transactivation capability of Elk-1, SUMO-1 cDNA construct was co-transfected (*lanes 9,10*). The luciferase activity representing the transactivation capability of Elk-1 was measured. A renilla vector was co-transfected as an internal control for normalization. Lane identification: 1 GAL4 BD vector; 2 GAL4-Elk-1 wt (untreated); 3. GAL4-Elk-1 wt with bFGF 100 ng/mL; 4. GAL4-Elk-1 wt with bFGF 100 ng/mL and FGFR1i; 5. GAL4-Elk-1 wt with bFGF 100 ng/mL and Rafi; 6. GAL4-Elk-1 wt with bFGF 100 ng/mL and ERKi; 7. GAL4-Elk-1 wt with bFGF 100 ng/mL and p38i; 8. GAL4-Elk-1 wt with bFGF 100 ng/mL and JNKi; 9. GAL4-Elk-1 wt with bFGF 100 ng/mL and SUMO-1; 10. GAL4-Elk-1 wt with SUMO-1 (untreated). The data represent four different donors measured in triplicate for each experiment.

Abbreviations: Elk-1, Ets-like transcription factor; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein kinase.

and the individual MAP kinase subgroup-specific inhibitors. Among the inhibitors of MAP kinase subgroups, ERKi has the strongest impact on Elk-1 activation, suggesting that Elk-1 is preferentially controlled by the ERK MAP kinase pathways in human articular chondrocytes. Interestingly, co-expression of SUMO-1 also demonstrates significantly inhibited bFGF-initiated Elk-1 activation, suggesting that transactivation of Elk-1 by bFGF may be regulated through the SUMO pathways in human primary articular chondrocytes.

Basic FGF-dependent phosphorylation of Elk-1 by ERK/MAPK signaling decreases SUMO-conjugation of Elk-1 in human primary adult articular chondrocytes.

Our results suggest that Elk-1 is preferentially regulated by the ERK MAP kinase (Figure 1A and B), and that trans-activation capability of Elk-1 may be regulated by SUMO-1 conjugation in human primary chondrocytes (Figure 1C). Therefore, we assessed whether endogenous conjugation of SUMO-1 to Elk-1 is altered by activation of ERK MAP kinase upon bFGF stimulation. Human articular chondrocytes in monolayer were stimulated with bFGF during an extended time course (0 minutes, 2 minutes, 1 hour, 6 hours, and 24 hours) followed by immunoprecipitation (IP) and western blot analysis by using antibodies for Elk-1 and SUMO-1. Within 2 minutes, it appears that the SUMO conjugation to Elk-1 is reduced by bFGF thus suggesting that ERK MAP kinase signaling promotes Elk-1 desumoylation (Figure 2, *lane 1* versus *2*). The SUMO-Elk-1 conjugation is undetectable after 1 hour of stimulation with bFGF (*lane 3*), and to our surprise, this desumoylation of Elk-1 is sustained even after 24 hours of bFGF stimulation (*lane 5*). Importantly, we observed significantly reduced SUMO conjugation of Elk-1 in OA chondrocytes (*lane 7*) where we consistently detect hyper-activation of ERK-Elk-1,[3] suggesting the pathophysiological links of desumoylation of Elk-1 to OA condition. Another key point is that activation of Elk-1 (represented by phosphorylation at Ser 383), is reciprocal with SUMO-conjugation of Elk-1 (*lanes 1–4* and *7*). One hour pre-incubation of cells with a specific ERK MAPK inhibitor (ERKi) prior to the stimulation with bFGF reverses the bFGF-mediated desumoylation of Elk-1 (*lane 6*). Our results suggest that the persistent activation of ERK by bFGF may deregulate the activity of Elk-1 by perturbing the equilibrium between conjugation and de-conjugation of SUMO to Elk-1. Further, our results suggest that these interrupted SUMO pathways may be associated with the hyper-activation of the ERK-Elk-1 pathways that are pathologically associated with OA.[3]

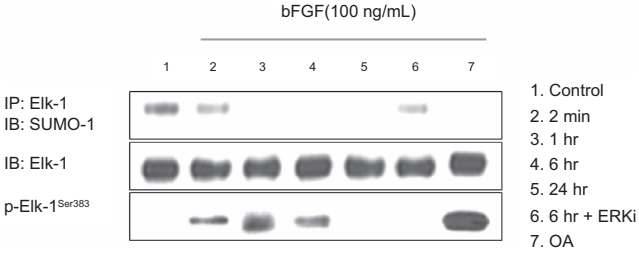

Figure 2 Endogenous sumoylation and phosphorylation of Elk-1 by bFGF are reciprocal. Human articular chondrocytes isolated from normal or OA tissues in monolayer were stimulated with bFGF (100 ng/mL) in a time-course as indicated in the presence or absence of ERK MAP kinase-specific inhibitor (ERKi). Elk-1 protein was immunoprecipitated using A agarose gel and anti-Elk-1 antibody. Alteration of the endogenous SUMO-conjugation to Elk-1 by bFGF was determined by following immunoblotting with anti-SUMO-1 antibody. The same immunoblot was stripped and then re-blotted with phosphospecific anti-Elk-1 (Ser383) antibody.
Abbreviations: bFGF, basic fibroblast growth factor; ERK, extracellular signal-regulated kinase; ERKi, ERK inhibitor; MAP, mitogen-activated protein kinase; OA, osteoarthritis; SUMO, small ubiquitin related modifier.

Elk-1 phosphorylation and sumoylation exert opposite biological effects on MMP-13 expression in human adult articular chondrocytes.

Our results suggest that bFGF-mediated activation of Elk-1 is regulated by the SUMO pathway (Figure 1C), and the phosphorylation of Elk-1 at serine 383 is linked to the functional trans-activating capability of Elk-1 on MMP-13 in human adult articular chondrocytes.[3] SUMO conjugation at the R motif [SUMO modification site ($\psi K \times E$)] of Elk-1 appears to be critical for the suppression of the transcriptional activity of Elk-1.[9] We therefore examined whether the Elk-1 SUMO modification sites in Elk-1 counterbalance phosphorylation of Elk-1, thereby inhibiting MMP-13 transcription in human articular chondrocytes.

In previous report, the mutation of these two SUMO modification sites has been shown to prevent conjugation to SUMO molecules in other types of tissue/cell systems.[9] First, we verified the functional significance of the two SUMO modification sites using a double mutation plasmid construct of K230R/K249R in the full-length Elk-1 cDNA construct (GAL4-Elk-1K230R/K249R) using human primary articular chondrocytes. A chimeric protein in which Elk-1 is fused to the GAL4 DNA binding domain (Elk-1K230R/K249R) was co-expressed by nucleofection with GAL4-responsive reporter plasmids (GAL4 RE-Luc), containing multiple GAL4 response elements that drive luciferase production. The transcriptional activating ability of Elk-1 was evaluated in parallel with Elk-1 mutants that are either unsumoylatable (GAL4-Elk-1K230R/K249R) or unphosphorylatable (GAL4-Elk-1 S383A: Ser 383 residue was replaced with a non-phosphorylatable alanine) as schematically shown in Figure 3A. Mutation of the lysine SUMO modification sites

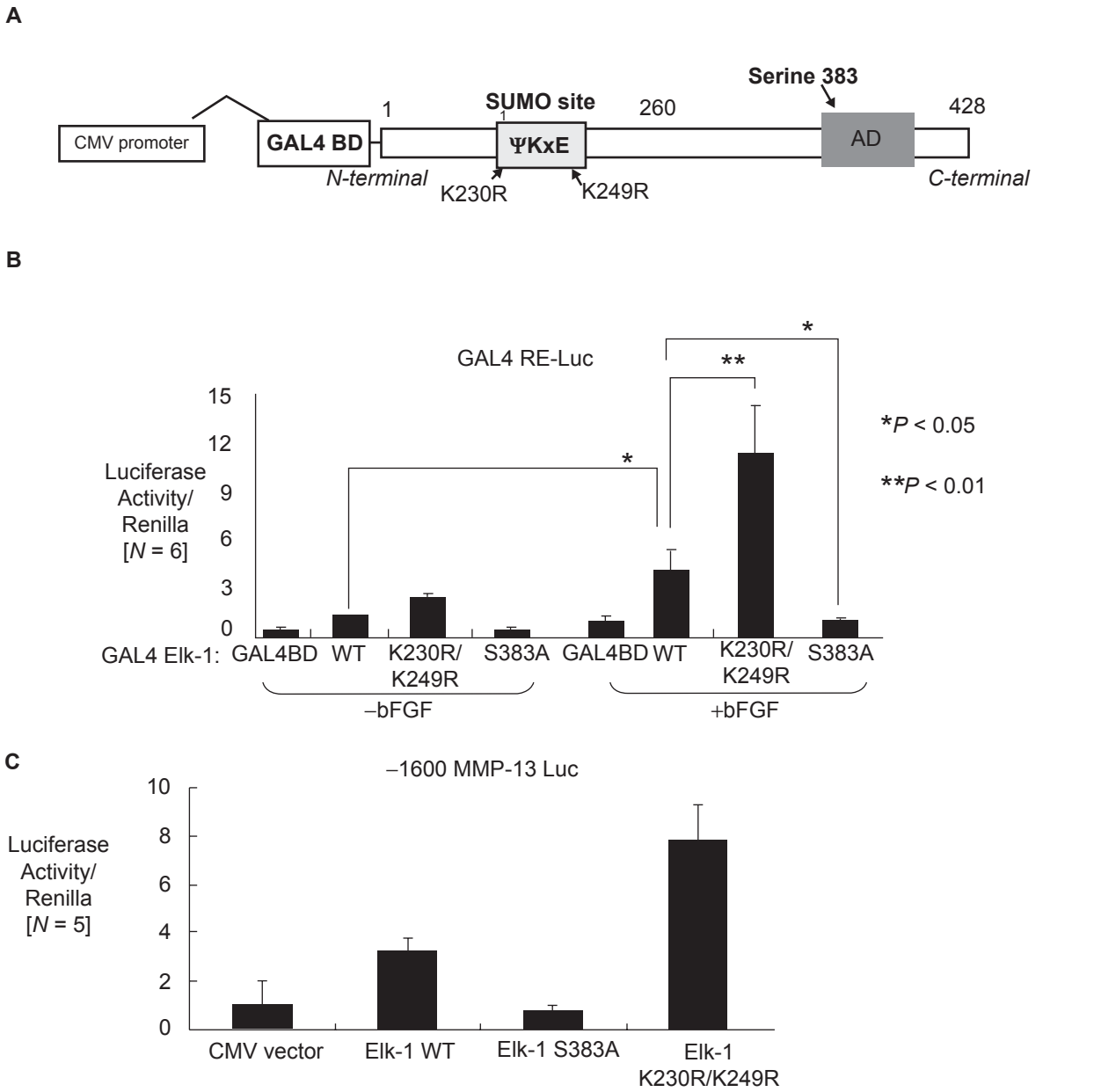

Figure 3 Elk-1-regulation of MMP-13 is modified by the SUMO pathway. **A**) Schematics of GAL4-Elk-1 fusion protein structure with mutations of SUMO-conjugation site (K230R and K249R within the SUMO site, ψK × E) and activation domain (AD) site of Elk-1 (Ser383) (Modified from Yang et al, 2003[9]); **B**) Cells were transiently co-transfected with a reporter construct containing GAL4 element immediately upstream of the luciferase gene (GAL4RE-Luc) with (1) the GAL4-Elk-1 fusion proteins (GAL4 Elk-1wt) containing GAL4BD and the Elk-1 protein, (2) unsumoylatable Elk-1 mutant (GAL4 Elk-1 K230R/K249R), or (3) unphosphorylatable Elk-1 mutant (GAL4 Elk-1 S383A). Twenty-four hours after post-transfection, cells were treated with bFGF (100 ng/mL) for an additional 24 hrs at which point the cell lysates were prepared and analyzed for the luciferase activity. **C**) MMP-13 promoter-reporter construct (–1600 MMP-13 Luc) was transiently co-transfected with (1) CMV empty vector as a control, (2) Elk-1 wt, (3) unphosphorylatable Elk-1 mutant (Elk-1 S383A) or (4) unsumoylatable Elk-1 mutant (Elk-1 K230R/K249R). Twenty-four hours after post-transfection, cells were treated with bFGF (100 ng/mL) for an additional 24 hours at which point the cell lysates were prepared and analyzed for the luciferase activity that represents MMP-13 promoter activity. A renilla vector was co-transfected as an internal control for normalization. The data represent 3 (Ubc9) or 4 (DN-Ubc9) different donors, respectively, measured in duplicates for each experiment.

Abbreviations: bFGF, basic fibroblast growth factor; CMV, cytomegalovirus; Elk-1, Ets-like transcription factor; ERK, extracellular signal-regulated kinase; ERKi, ERK inhibitor; MAP, mitogen-activated protein kinase; MMP, matrix metalloproteinase; OA, osteoarthritis; SUMO, small ubiquitin related modifier.

on Elk-1 strongly enhances the ability of the fusion protein to activate the GAL4 luciferase reporter gene (GAL4 RE-Luc) upon bFGF stimulation in human adult articular chondrocytes. Basic FGF activates Elk-1 represented by phosphorylation at Ser 383 (Figure 2). Consistently, the presence of bFGF stimulation increases in GAL4RE-Luc reporter gene activity. In contrast, over expression of the unphosphorylatable Elk-1 mutant (GAL4-Elk-1 S383A) shows no induction of luciferase activity that represents the activity of Elk-1 in the presence of bFGF stimulation (Figure 3B).

Next, we examined the inverse functional linkage between phosphorylation and sumoylation of Elk-1 on the activation of MMP-13 gene transcription in human primary adult articular chondrocytes in the presence of bFGF stimulation (100 ng/mL). Wild type Elk-1 cDNA vector (Elk-1wt) as well as the Elk-1 mutant that are either unsumoylatable (Elk-1K230R/K249) or unphosphorylatable mutant (Elk-1 S383A) was co-transfected with a –1600MMP-13Luc promoter reporter plasmid construct. After 48 hours, the cell lysates were analyzed for luciferase activity driven by the MMP-13 promoter. Expression of Elk-1 S383A significantly reduces MMP-13 transcription compared to expression of wild type Elk-1 (Elk-1wt) (Figure 3C). However, expression of Elk-1K230R/K249 significantly enhances MMP-13 promoter activity compared to the expression of Elk-1wt. Collectively our results suggest that phosphorylation and sumoylation of Elk-1 control, respectively, activation and de-activation of the transactivation capability of Elk-1. Thus, these two post-translational modifications exert opposite biological actions on the expression of downstream target genes, such as MMP-13 in human articular chondrocytes.

The SUMO-conjugating enzyme (E2) Ubc9 controls the transactivation potential of Elk-1 in human adult articular chondrocytes.

We investigated whether an alteration of a key SUMO pathway affects the biological activation of Elk-1 to control MMP-13 gene transcription in human adult articular chondrocytes after stimulation with bFGF. The core SUMO consensus motif ψKxE is recognized by the E2-conugating enzyme Ubc9, which makes key interactions with this motif,[18] and transfers SUMO onto the lysine (K) residue. *In vitro* studies revealed that an introduction of Ubc9 is sufficient to promote substrate sumoylation, suggesting that Ubc9 is a key mediator of the SUMO pathways.[19,20] We used expression vectors for wild type Ubc9 and a dominant-negative mutant (DN-Ubc9) to enhance or block SUMO modification. DN-Ubc9 is an enzymatically inactive protein in which SUMO-conjugating activity is specifically disabled by mutation of a cysteine (C93S) in its catalytic site, and hence, is expected to occlude binding of wild-type Ubc9 to substrates.[21,22] First, we tested if alteration of the Ubc9 pathway plays a role in the activity of Elk-1 in human primary articular chondrocytes by performing co-transfection studies with either GAL4-Elk-1wt or the unsumoylatable mutant of Elk-1 expression vector (GAL4-Elk-1 K230R/K249R). Alterations in Elk-1 activity were assessed by luciferase activity driven by GAL4-Luc reporter plasmid. Results obtained by measuring GAL4-driven luciferase activity demonstrate that expression of DN-Ubc9 significantly increases bFGF-induced transactivation by wild type Elk-1 (Figure 4A). In contrast, the expression of Ubc9 does not enhance Elk-1 activity (Figure 4B). In confirmation of these results, the activity of the unsumoylatable mutant of Elk-1 (GAL4-Elk-1K230R/K249R) was not altered by co-expression of either WT- or DN-Ubc9 (Figures 4A and B) in human articular chondrocytes.

Next, we assessed whether interruption of SUMO pathways by DN-Ubc9 promotes transcriptional activation of the MMP-13 gene through activation of Elk-1. Conversely, we also tested whether co-expression of wild type of Ubc9 cDNA expression vector can inhibit MMP-13 transcription. The MMP-13 promoter luciferase reporter construct (–1600MMP-13Luc) was co-transfected with expression vectors for either wild type or DN-mutant forms of Ubc9 followed by incubation for 48 hours in the presence or absence of bFGF stimulation (100 ng/mL). The resulting cell lysates were analyzed for the MMP-13 gene transcription represented by luciferase activity. Significant induction of transcriptional activity of MMP-13 ($P < 0.01$) is observed by co-expression of DN-Ubc9 (Figure 4C) either with or without bFGF stimulation compared to controls (ie, co-transfection of MMP-13Luc reporter plasmid with CMV empty vector). Although the effect of Ubc9 on the basal MMP-13 gene transcription remains unclear, the inhibitory effect by Ubc9 on MMP-13 after stimulation with bFGF is significant ($P < 0.05$). Our results collectively suggest that the SUMO-conjugating E2 enzyme Ubc9 supports sumoylation of Elk-1 to regulate transactivation of MMP-13 gene expression by Elk-1 in human primary adult articular chondrocytes.

Discussion

Although the altered SUMO pathways have been reported to be associated with various human diseases,[11] it has been unclear whether the SUMO pathways affect cartilage homeostasis. Our results suggest that ERK MAPK-dependent phosphorylation of Elk-1 promotes desumoylation and enhances the functional activity of Elk-1 in human primary articular chondrocytes, similar to previous findings in other biological contexts.[9,23] Furthermore, the current studies suggest that bFGF-activation of Elk-1 is preferentially regulated by ERK MAP kinase, and the MMP-13 gene transcription may be controlled by the dynamic balances between phosphorylation and desumoylation of Elk-1, suggesting the critical role of the SUMO pathways in transactivity of Elk-1. We propose that the SUMO pathways are critical regulators of the transactivation potential of Elk-1 in human primary

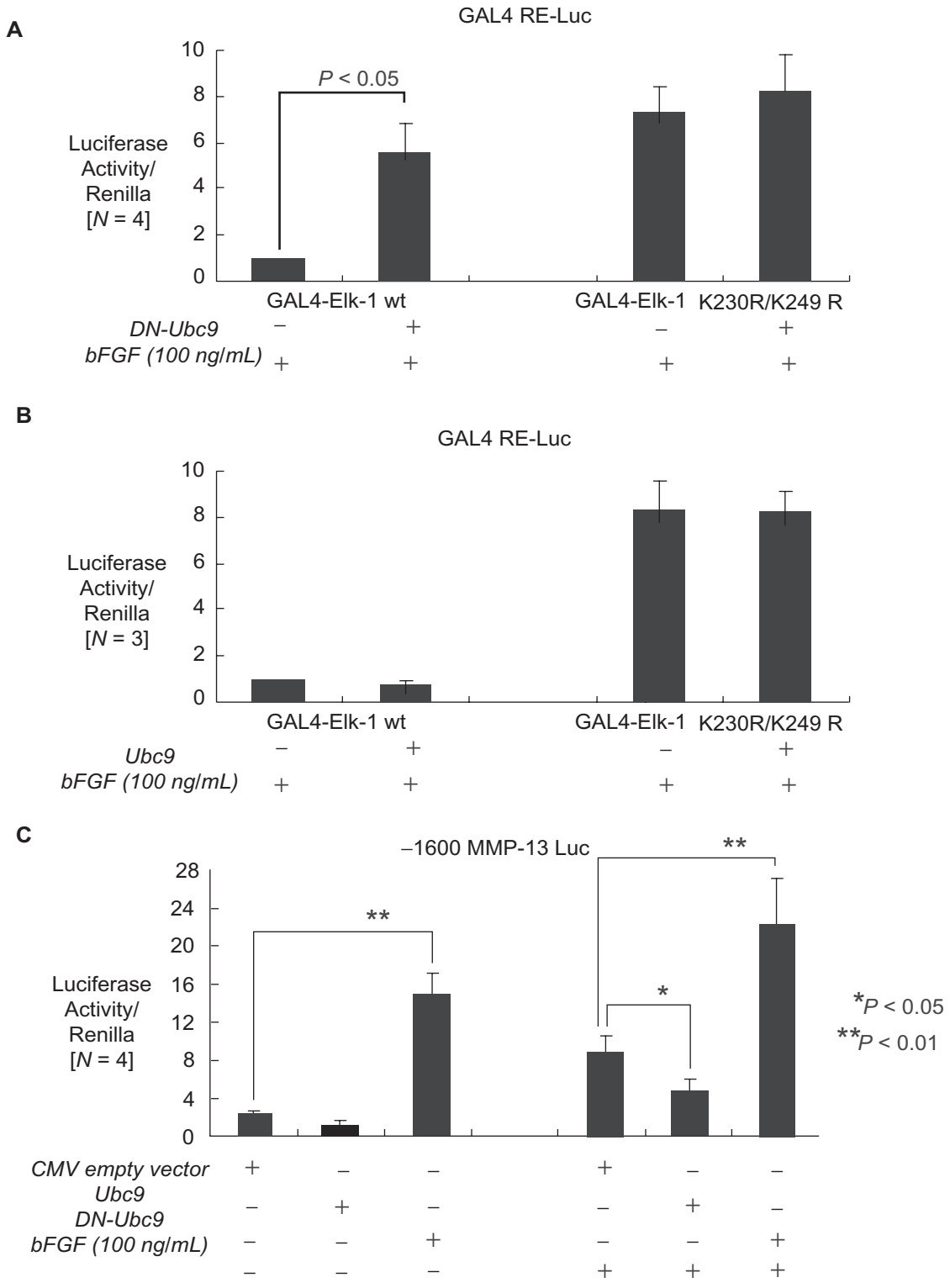

Figure 4 Interference with the SUMO pathways by DN-Ubc9 alters Elk-1-driven transcription of MMP-13. Cells were transiently co-transfected with a reporter construct GAL4RE-Luc with **A**) the GAL4-Elk-1 fusion proteins (GAL4 Elk-1wt) containing GAL4BD and the Elk-1 protein, or **B**) unsumoylatable Elk-1 mutant (GAL4 Elk-1 K230R/K249R) in the presence or absence of DN-Ubc9 or Ubc9 cDNA construct. Twenty-four hours after post-transfection, cells were treated with bFGF (100 ng/mL) for an additional 24 hours at which point the cell lysates were prepared and analyzed for the luciferase activity that represents transactivating capability of Elk-1. **C**) MMP-13 promoter-reporter construct (–1600 MMP-13 Luc) was transiently co-transfected either with Ubc9 or DN-Ubc9. CMV empty vector was used as a control. Twenty-four hours after post-transfection, cells were treated with/without bFGF (100 ng/mL) for an additional 24 hours at which point the cell lysates were prepared and analyzed for the luciferase activity that represents MMP-13 promoter activity. A renilla vector was co-transfected as an internal control for normalization. These data represent 4 different donors measured in triplicate for each experiment.

Abbreviations: bFGF, basic fibroblast growth factor; CMV, cytomegalovirus; DN-Ubc9, dominant-negative Ubc9; Elk-1, Ets-like transcription factor; ERK, extracellular signal-regulated kinase; ERKi, ERK inhibitor; MAP, mitogen-activated protein kinase; MMP, matrix metalloproteinase; OA, osteoarthritis; SUMO, small ubiquitin related modifier.

adult articular chondrocytes and thus influence expression of downstream target genes that control normal cartilage homeostasis.

Our studies show significantly increased endogenous desumoylation of Elk-1 in human OA chondrocytes, suggesting that these altered SUMO pathways are associated with pathological condition of OA. Importantly, these current findings are consistent with our previous results in which we observed that the activation of Elk-1 by ERK MAP kinase is critical for transcriptional stimulation of MMP-13, and that the hyper-activation of ERK MAP kinase and Elk-1 is persistent in OA cartilage tissues compared to normal articular cartilage.[3] One major finding of this study is the dynamic and reciprocal relationship between sumoylation and activation of Elk-1 (represented by phosphorylation at Ser 383). Our time course studies reveal that Elk-1 maintains desumoylation at 24 hours even when phosphorylation of Elk-1 is below the level of detection (see Figure 2, *lane 5*). We predict that Elk-1 may nonetheless be phosphorylated at this time-point (albeit at undetectable levels), because we and others have observed that ERK1/2 phosphorylation, a key upstream activator of Elk-1, is sustained for >72 hours in both human and/or bovine articular chondrocytes in response to bFGF (unpublished data Im H.J. et al 2009). It remains to be investigated for a conceptual or technical reason for the lack of detectable Ser383 phosphorylation of Elk-1 and its relationship with sustained desumoylation of Elk-1 after 24 hours.

Our prior studies demonstrated that the bFGF-mediated stimulation of MMP-13 transcription occurs through the activation of MAP kinase and NFκB that both converge on Elk-1.[3] Notably, the NFκB pathway is also a target of the SUMO pathway,[23,24] thus potentially clarifying the molecular and cellular basis for constitutive upregulation of NFκB activity in OA compared to normal knee joint cartilage tissues. Our current findings that show increased desumoylation of Elk-1 in OA chondrocytes and the findings in our previous studies in which we observed the persistent activation of the ERK-Elk-1 pathways in OA may together reflect coordinated deregulation of the MAPK/ERK-Elk-1 and NFκB-Elk-1 pathways due to pathological alterations in sumoylation levels.[3] Thus, alterations in the homeostatic SUMO pathways is closely associated with the impaired cartilage regeneration and increased production of cartilage degrading enzyme production.

The current study provides experimental evidence for the concept that Elk-1-dependent stimulation of MMP-13 expression is modulated by Ubc9-dependent sumoylation of Elk-1. The significant induction of the transactivation potential of Elk-1 and concomitant increase in MMP-13 gene transcription was observed upon expression of DN-Ubc9 enzyme in the primary human articular chondrocytes regardless of stimulation by bFGF. This finding is consistent with the critical role of Ubc9 in mediating SUMO conjugation observed in other cell types and tissues.[19,20] We demonstrate that (i) an inhibition of E2 SUMO conjugating enzyme (by over expression of DN-Ubc9) stimulates catabolic enzyme expression (eg, MMP-13). On the other hand, (ii) the increase of E2 SUMO conjugating enzyme (by overexpression of Ubc9) downregulates MMP-13 transcription. The potential therapeutic applications of our observations are depicted in Figure 5. For example, we suggest that activation of E2

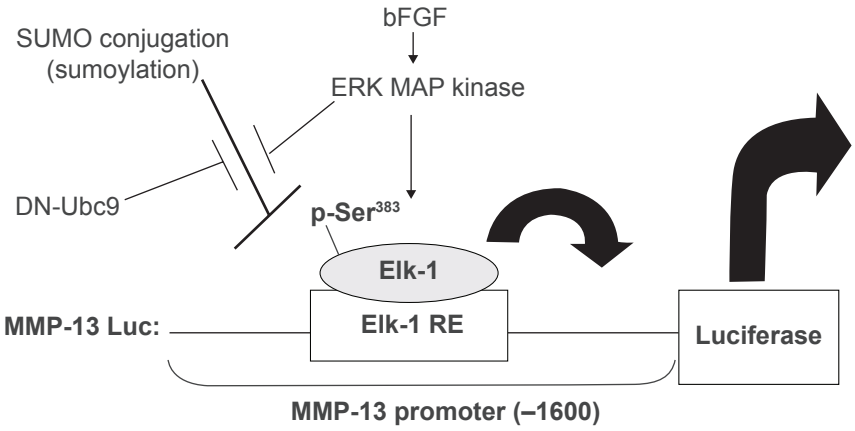

Figure 5 Schematic presentation of fine balances between phosphorylation and sumoylation of Elk-1 to regulate MMP-13 transcription. Basic FGF-activation of the ERK MAP kinase pathways phosphorylate Elk-1 (Ser383) to stimulate transcription of MMP-13 which can be modified by the SUMO pathways, such as Ubc9, SUMO-conjugating enzyme (E2) in human adult articular chondrocytes.

Abbreviations: bFGF, basic fibroblast growth factor; CMV, cytomegalovirus; DN-Ubc9, dominant-negative Ubc9; Elk-1, Ets-like transcription factor; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein kinase; MMP, matrix metalloproteinase; SUMO, small ubiquitin related modifier.

SUMO conjugating enzymes may be an effective strategy to block the potentially catabolic downstream effects of Elk-1 upon bFGF induction.

Apart from effects on substrate recognition, our results suggest that Elk-1 sumoylation may be regulated by the levels of enzymes that are associated with the SUMO pathways. For example, it has been shown that recombinant protein inhibitor of activated Signal Transducers and Activator of Transcription (STAT)xα protein inhibitors of actwated STAT (PIASxα) that functions as E3 SUMO-1 protein ligases enhances Ubc9-mediated sumoylation of the target proteins *in vitro*.[25,27] Furthermore, Yang and Sharrocks discovered that PIASxα promotes desumoylation upon ERK activation, but preserves sumoylation when p38 is activated.[28] Thus, it is possible that PIASxα may also control Elk-1 activity, and whether PIASxα acts as an Elk-1 activator or suppressor in human articular chondrocytes still remains to be examined.

We noted that all four different SUMO isoforms (SUMO-1 to SUMO-4) are ubiquitously expressed regardless of tissue types and metabolic conditions (eg, knee, ankle, normal and OA chondrocytes) at both mRNA and protein levels (data not shown). Nevertheless, co-expression of SUMO-1 significantly inhibits bFGF-initiated Elk-1 activation, suggesting that not only sumoylation-associated enzymes, such as Ubc9, but also SUMO isoforms could attenuate Elk-1.

In conclusion, our data indicate that bFGF activates Elk-1 *via* induction of ERK MAP kinases, and that Elk-1 activity appears to be negatively regulated by Ubc9-dependent sumoylation. We propose that sumoylation and phosphorylation are two apparently antagonistic post-translational modifications that can function as a sophisticated molecular switch to control the bFGF-dependent expression of MMP-13 and other ECM degrading enzymes at the transcriptional level in human primary adult articular chondrocytes.

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Disclosures

The authors declare no conflicts of interest.

References

1. Shlopov BV, Gumanovskaya ML, Hasty KA. Autocrine regulation of collagenase 3 (matrix metalloproteinase 13) during osteoarthritis. *Arthritis Rheum*. 2000;43:195–205.
2. Im HJ, Muddasani P, Natarajan V, et al. Basic fibroblast growth factor stimulates matrix metalloproteinase-13 via the molecular cross-talk between the mitogen-activated protein kinases and protein kinase Cdelta pathways in human adult articular chondrocytes. *J Biol Chem*. 2007;282:11110–11121.
3. Muddasani P, Norman JC, Ellman M, van Wijnen AJ, Im HJ. Basic fibroblast growth factor activates the MAPK and NFkappaB pathways that converge on Elk-1 to control production of matrix metalloproteinase-13 by human adult articular chondrocytes. *J Biol Chem*. 2007;282:31409–31421.
4. Vincent T, Hermansson M, Bolton M, Wait R, Saklatvala J. Basic FGF mediates an immediate response of articular cartilage to mechanical injury. *Proc Natl Acad Sci U S A*. 2002;99:8259–8264.
5. Vincent TL, Hermansson MA, Hansen UN, Amis AA, Saklatvala J. Basic fibroblast growth factor mediates transduction of mechanical signals when articular cartilage is loaded. *Arthritis Rheum*. 2004;50:526–533.
6. Loeser RF, Chubinskaya S, Pacione C, Im HJ. Basic fibroblast growth factor inhibits the anabolic activity of insulin-like growth factor 1 and osteogenic protein 1 in adult human articular chondrocytes. *Arthritis Rheum*. 2005;52:3910–3917.
7. Carreras I, Rich CB, Jaworski JA, et al. Functional components of basic fibroblast growth factor signaling that inhibit lung elastin gene expression. *Am J Physiol Lung Cell Mol Physiol*. 2001;281:L766–L775.
8. Wang X, Manner PA, Horner A, Shum L, Tuan RS, Nuckolls GH. Regulation of MMP-13 expression by RUNX2 and FGF2 in osteoarthritic cartilage. *Osteoarthritis Cartilage*. 2004;12:963–973.
9. Yang SH, Jaffray E, Hay RT, Sharrocks AD. Dynamic interplay of the SUMO and ERK pathways in regulating Elk-1 transcriptional activity. *Mol Cell*. 2003;12:63–74.
10. Matunis MJ, Pickart CM. Beginning at the end with SUMO. *Nat Struct Mol Biol*. 2005;12:565–566.
11. Gill G. SUMO and ubiquitin in the nucleus: different functions, similar mechanisms? *Genes Dev*. 2004;18:2046–2059.
12. Yang SH, Sharrocks AD. PIASx acts as an Elk-1 coactivator by facilitating derepression. *EMBO J*. 2005;24:2161–2171.
13. Salinas S, Briancon-Marjollet A, Bossis G, et al. SUMOylation regulates nucleo-cytoplasmic shuttling of Elk-1. *J Cell Biol*. 2004;165:767–773.
14. Muehleman C, Bareither D, Huch K, Cole AA, Kuettner KE. Prevalence of degenerative morphological changes in the joints of the lower extremity. *Osteoarthritis Cartilage*. 1997;5:23–37.
15. Im HJ, Li X, Muddasani P, et al. Basic fibroblast growth factor accelerates matrix degradation via a neuro-endocrine pathway in human adult articular chondrocytes. *J Cell Physiol*. 2008;215:452–463.
16. Lin X, Liang M, Liang YY, Brunicardi FC, Feng XH. SUMO-1/Ubc9 promotes nuclear accumulation and metabolic stability of tumor suppressor Smad4. *J Biol Chem*. 2003;278:31043–31048.
17. Pulai JI, Chen H, Im HJ, et al. NF-kappa B mediates the stimulation of cytokine and chemokine expression by human articular chondrocytes in response to fibronectin fragments. *J Immunol*. 2005;174:5781–5788.
18. Reverter D, Lima CD. Insights into E3 ligase activity revealed by a SUMO-RanGAP1-Ubc9-Nup358 complex. *Nature*. 2005;435:687–692.

19. Yang SH, Sharrocks AD. Interplay of the SUMO and MAP kinase pathways. *Ernst Schering Res Found Workshop*. 2006:193–209.
20. Hay RT. SUMO: a history of modification. *Mol Cell*. 2005;18:1–12.
21. Desterro JM, Rodriguez MS, Kemp GD, Hay RT. Identification of the enzyme required for activation of the small ubiquitin-like protein SUMO-1. *J Biol Chem*. 1999;274:10618–10624.
22. Girdwood D, Bumpass D, Vaughan OA, et al. P300 transcriptional repression is mediated by SUMO modification. *Mol Cell*. 2003;11:1043–1054.
23. Desterro JM, Rodriguez MS, Hay RT. SUMO-1 modification of IkappaBalpha inhibits NF-kappaB activation. *Mol Cell*. 1998;2:233–239.
24. Huang TT, Wuerzberger-Davis SM, Wu ZH, Miyamoto S. Sequential modification of NEMO/IKKgamma by SUMO-1 and ubiquitin mediates NF-kappaB activation by genotoxic stress. *Cell*. 2003;115:565–576.
25. Bossis G, Melchior F. SUMO: regulating the regulator. *Cell Div*. 2006;29:1–13.
26. Guo B, Yang SH, Witty J, Sharrocks AD. Signalling pathways and the regulation of SUMO modification. *Biochem Soc Trans*. 2007;35:1414–1418.
27. Kotaja N, Karvonen U, Janne OA, Palvimo JJ. PIAS proteins modulate transcription factors by functioning as SUMO-1 ligases. *Mol Cell Biol*. 2002;22:5222–5234.
28. Yang SH, Sharrocks AD. PIASxalpha differentially regulates the amplitudes of transcriptional responses following activation of the ERK and p38 MAPK pathways. *Mol Cell*. 2006;22:477–487.

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