

# Naproxen Induces Type X Collagen Expression in Human Bone-Marrow-Derived Mesenchymal Stem Cells Through the Upregulation of 5-Lipoxygenase

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Several studies have shown that type X collagen (COL X), a marker of late-stage chondrocyte hypertrophy, is expressed in mesenchymal stem cells (MSCs) from osteoarthritis (OA) patients. We recently found that Naproxen, but not other nonsteroidal anti-inflammatory drugs (NSAIDs) (Ibuprofen, Celebrex, Diclofenac), can induce type X collagen gene (*COL10A1*) expression in bone-marrow-derived MSCs from healthy and OA donors. In this study we determined the effect of Naproxen on COL X protein expression and investigated the intracellular signaling pathways that mediate Naproxen-induced *COL10A1* expression in normal and OA hMSCs. MSCs of OA patients were isolated from aspirates from the intramedullary canal of donors (50–80 years of age) undergoing hip replacement surgery for OA and were treated with or without Naproxen (100 µg/mL). Protein expression and phosphorylation were determined by immunoblotting using specific antibodies (COL X, p38 mitogen-activated protein kinase [p38], phosphorylated-p38, c-Jun N-terminal kinase [JNK], phosphorylated-JNK, extracellular signal-regulated kinase [ERK], and phosphorylated-ERK). Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed to determine the expression of *COL10A1* and Runt-related transcription factor 2 gene (*Runx2*). Our results show that Naproxen significantly stimulated COL X protein expression after 72 h of exposure both in normal and OA hMSCs. The basal phosphorylation of mitogen-activated protein kinases (MAPKs) (ERK, JNK, and p38) in OA hMSCs was significantly higher than in normal. Naproxen significantly increased the MAPK phosphorylation in normal and OA hMSCs. NSAID cellular effects include cyclooxygenase, 5-lipoxygenase, and p38 MAPK signaling pathways. To investigate the involvement of these pathways in the Naproxen-induced *COL10A1* expression, we incubated normal and OA hMSCs with Naproxen with and without inhibitors of ERK (U0126), JNK (BI-78D3), p38 (SB203580), and 5-lipoxygenase (MK-886). Our results showed that increased basal *COL10A1* expression in OA hMSCs was significantly suppressed in the presence of JNK and p38 inhibitors, whereas Naproxen-induced *COL10A1* expression was suppressed by 5-lipoxygenase inhibitor. This study shows that Naproxen induces COL X both at transcriptional and translational levels in normal and OA hMSCs. Elevated basal *COL10A1* expression in OA hMSCs is probably through the activation of MAPK pathway and Naproxen-induced *COL10A1* expression is through the increased 5-lipoxygenase signaling.

## Introduction

**O**STEoarthritis (OA) IS A COMMON DISORDER of articular joints characterized by slow progressive damage and loss of the articular cartilage.<sup>1-3</sup> Besides the functional and psychological impact of the disease on the affected individuals, OA is associated with a significant socio-economic burden.<sup>2</sup> All the currently approved treatments for OA, including nonsurgical and surgical approaches, are fo-

cused on relieving the symptoms of pain and stiffness as well as improving the function of the joint.<sup>4</sup> However, an effective treatment for the regeneration of the damaged articular cartilage is still lacking.

Adult mesenchymal stem cells (MSCs) are multipotent and capable of self-renewal and able to differentiate into chondrocytes, osteoblasts, and other tissues of mesenchymal origin.<sup>5</sup> Alternative treatment efforts for cartilage degeneration include tissue engineering of cartilage, using MSCs

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induced to differentiate into chondrocytes.<sup>6,7</sup> Autologous MSCs taken from OA patients are being currently considered in the biological repair of cartilage or disc lesions. However, expression of type X collagen (COL X), which is a marker of late-stage chondrocyte hypertrophic differentiation and endochondral ossification,<sup>8,9</sup> in MSCs from OA patients<sup>10–12</sup> is an undesirable degenerative phenotype. COL X is a nonfibrillar collagen, primarily a specific marker for terminally differentiated chondrocytes in the hypertrophic zone of growth plates in the growing bones.<sup>13,14</sup> Although it is well established that there is constitutive expression of COL X in chondrocytes and MSCs from OA patients,<sup>8,11</sup> the factors and intracellular mechanisms that regulate this phenomenon are not well understood.

We have recently observed among the nonsteroidal anti-inflammatory drugs (NSAIDs), commonly prescribed to OA patients, tested in our study, that specifically Naproxen but not Ibuprofen, Diclofenac, or Celebrex induces *COL10A1* gene expression as well as osteogenic marker genes, like alkaline phosphatase, osteocalcin, and bone sialoprotein, in normal human MSCs.<sup>15</sup> Similarly, several studies have revealed the negative impact of NSAIDs on articular cartilage.<sup>16,17</sup> The most important drawback of certain NSAIDs is to inhibit matrix production. NSAIDs can be classified into three categories on the basis of their different effects on chondrocyte GAG synthesis, that is, inhibitory (e.g., Indomethacin and Naproxen), stimulatory (e.g., Aceclofenac, Tenidap, and Tolmetin), or having no effect (e.g., Diclofenac, Aspirin, and Piroxicam). These differential effects may stem from their action on the function of IL-1 $\beta$  and growth factors in chondrocytes.

Since cytokines and growth factors are involved in the regulation of balance between anabolic and catabolic processes, which is disturbed in OA, it is important that the pharmacological agents should act to maintain this balance and preserve and protect the cartilage. Thus, inhibition of cytokine action and stimulation of matrix synthesis as promoted by aceclofenac is favorable for cartilage protection. It has also been shown that licofelone—a potent, competitive inhibitor of 5-LOX and COX pathways—possesses antiplatelet, analgesic, anti-inflammatory, antipyretic, and anti-bronchoconstrictory properties and also to be safe for the gastrointestinal tract at effective doses.

Preclinical studies indicated that licofenolone is effective in protecting the articular cartilage and the synovial space in degenerative joint disease and also displays antithrombotic activity.<sup>18</sup> These drugs, by inducing the expression of markers related to hypertrophy and osteogenesis, might accelerate the OA-related changes. It is necessary to identify the underlying mechanisms in order to devise steps for modulating the expression of hypertrophy- and osteogenesis-related markers in MSCs from OA patients. NSAIDs are known to exert anti-inflammatory effects mainly through cyclooxygenase inhibition, p38 MAPK, and 5-lipoxygenase pathways.<sup>19</sup> Previously it was shown that Ibuprofen, Diclofenac, and Celebrex inhibit 5-lipoxygenase,<sup>20–24</sup> while Naproxen increases 5-lipoxygenase expression.<sup>25</sup> We have previously shown that mitogen-activated protein kinases (MAPKs) regulate the expression of COL X in parathyroid-hormone-treated MSCs.<sup>26</sup> In the present study we examined whether Naproxen-induced COL X expression in normal and OA hMSCs also involves 5-lipoxygenase and/or MAPK signaling.

## Materials and Methods

### Source and isolation of MSCs

Normal human MSCs were purchased from Lonza. Osteoarthritic human mesenchymal stem cells (OA hMSCs) were isolated from bone marrow aspirates of the intramedullary canal of femur from donors (four women of age 56, 62, 72, and 79 years and three men of age 53, 59, and 78 years) undergoing total hip replacement for OA, following a protocol approved by the research ethics committee of the Jewish General Hospital (Montreal, Quebec, Canada).<sup>11,12,27</sup> Isolation of MSCs from bone marrow aspirates was carried out using previously described methods.<sup>12,28</sup>

Briefly, each aspirate was diluted 1:1 (v/v) with Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, L-glutamine, and sodium pyruvate (Wisent, Inc.) and then layered over Ficoll (Ficoll-Paque Plus; GE Healthcare Bio-Sciences). The mononuclear cell layer, after being centrifuged at 900 *g* for 30 min, was removed from the interface, washed with DMEM, and resuspended in DMEM supplemented with 10% fetal bovine serum (FBS; Hyclone), 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin. The cells were cultured in 20 mL of medium in 176-cm<sup>2</sup> culture dishes and incubated at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. After 72 h, nonattached cells were washed off and the adherent cells were thoroughly washed twice with DMEM. Cells were cultured and expanded in complete medium (DMEM high-glucose [4.5 g/L] supplemented with 10% FBS, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin) and were used within three to four passages.

### Treatment of normal and OA hMSCs with Naproxen for determining COL X protein expression

Normal and OA hMSCs were expanded in 100  $\times$  20-mm<sup>2</sup> culture dishes in 10 mL of complete medium to 80–90% confluence. Then, the MSCs were incubated overnight in DMEM containing 1% FBS and then treated without (control) or with Naproxen sodium (Sigma-Aldrich) dissolved in DMEM containing 1% FBS for 72 h at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. Cell lysates are prepared in RIPA buffer (Sigma-Aldrich) containing 1% protease inhibitor cocktail (Sigma-Aldrich).

Since the bone marrow is well vascularized, the concentration of Naproxen in our experiments was similar to the blood-circulating levels in patients taking Naproxen. The blood concentration of Naproxen in patients is around 50–75  $\mu$ g/mL,<sup>29</sup> at 220 mg Naproxen dosage.<sup>30</sup> Naproxen has an average pharmacokinetic half-life of > 12 h when administered at high doses (500 mg b.i.d.) and it has been demonstrated that effective inhibition of COX1 can only be achieved for longer periods (up to 24 h) only when Naproxen is given at 440 mg b.i.d. dose in patients.<sup>30</sup>

Blood concentrations of Naproxen at this dosage were reported to be about 120  $\mu$ g/mL, as compared with 220 mg that achieves blood levels of  $\sim$ 75  $\mu$ g/mL. In fact, *in vitro*, Naproxen exerts near complete inhibition of both COX1 and COX2 at  $\sim$ 100  $\mu$ g/mL concentration. For these reasons, we chose to employ Naproxen at 100  $\mu$ g/mL in this study, which corresponds to intermediate blood concentration between low and high dosage of Naproxen.

#### Treatment of normal and OA hMSCs with Naproxen for examining MAPK phosphorylation

For MAPK phosphorylation studies, normal and OA hMSCs were expanded to 80–90% confluence and were serum deprived overnight and were treated without (basal phosphorylation) or with Naproxen sodium (100 µg/mL) dissolved in serum-free medium, for different time points (0.5, 1, and 6 hours at 37°C in a 5% CO<sub>2</sub> humidified atmosphere). Cell lysates are prepared in RIPA buffer containing 1% protease inhibitor and 1% phosphatase inhibitor cocktails (Sigma-Aldrich).

#### Treatment of normal and OA hMSCs with MAPK and 5-lipoxygenase inhibitors

For the studies with inhibitors, MSCs were cultured in complete DMEM to near confluence and were serum deprived for 24 h followed by treatment with MEK inhibitor (10 µM; 1, 4-diamino-2, 3-dicyano-1, 4-bis [2-aminophenylthio] butadiene, U0126; Sigma-Aldrich), c-Jun N-terminal kinase (JNK) inhibitor (1 µM, BI-78D3; Sigma-Aldrich), p38 inhibitor (20 µM, SB203580; Cell Signaling), and 5-lipoxygenase inhibitor (10 µM, MK-886; Sigma-Aldrich) for 30 min prior to stimulation with Naproxen (100 µg/mL).

#### Measurement of gene expression by real-time polymerase chain reaction

At the end of incubations total RNA from MSCs was isolated using Trizol reagent (Invitrogen). The resulting RNA pellet was washed with 75% ethanol, and then centrifuged and air-dried. Then, the pellets were suspended in 40 µL of diethylpyrocarbamate-treated (DEPC) water and RNA concentration was determined using Nanodrop 3000 fluorospectrometer. Reverse transcription was performed using the RNA (1 µg) with random primers (final concentration 0.15 µg/µL), dNTP mixture (final concentration 0.5 mM), and DEPC water in a final volume of 12 µL. After the solution was incubated at 65°C for 5 min, it was mixed with first-strand buffer, DTT, RNaseOUT, and Superscript II reverse transcriptase in a final volume of 20 µL. Then, the solution was incubated at 45°C for 50 min and then at 70°C for 15 min to inactivate the reverse transcriptase. For LightCycler real-time polymerase chain reaction (PCR), a master mix of the following reaction components was prepared with final concentrations: 10 µL SYBER PCR master mix (1×) (Qiagen), 8 µL water, 0.5 µL forward primer

(0.25 µM), and 0.5 µL reverse primer (0.25 µM) (Table 1). To this mix, 1 µL of cDNA was added as PCR template.

The reaction conditions included one cycle of 95°C for 15 min, 45 cycles of amplification and quantification (94°C for 15 s, 57°C for 30 s, and 72°C for 30 s), one cycle of melting curve (65–95°C with heating rate of 0.1°C per second with a continuous fluorescence measurement), and a final cooling step to 4°C. The crossing points (CPs) were determined by the Light Cycler software 3.3 (Roche Diagnostics) and were measured at constant fluorescence level. Every sample was run in duplicates and the average value was used in the calculation. The relative gene transcription was determined by the following equation and GAPDH was employed as the reference gene:

$$\text{Relative ratio} = \frac{2^{\Delta\text{CP target (control - sample)}}}{2^{\Delta\text{CP reference (control - sample)}}}$$

#### Protein expression

Total protein content in the cell lysates was measured by Bradford assay and equal amounts of protein from each sample were resolved on 10% acrylamide gel and, after SDS-PAGE, the separated proteins were transferred to nitrocellulose membranes by semidry electrophoretic transfer using Trans-Blot<sup>®</sup> SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). Protein expression was measured by western blot using specific antibodies directed against COL X (1:1000; Sigma-Aldrich), extracellular signal-regulated kinase (ERK), phosphorylated-ERK (Erk1/2 and pErk1/2; 1:5000), JNK, phosphorylated-JNK (JNK and pJNK; 1:1000), and p38, phosphorylated-p38 (p38, 1:1000 and p-p38, 1:500; all from Cell Signaling). GAPDH (1:10,000; Sigma-Aldrich) was used as a housekeeping gene and utilized to normalize for the expression of COL X. The phosphorylation of ERK, p38, and JNK was normalized to the corresponding nonphosphorylated total forms. Protein bands on the immunoblot were visualized using the chemiluminescent reagents (Bio-Rad Clarity<sup>™</sup> Western ECL Substrate) followed by exposure to the photographic film or by Bio-Rad VersaDoc (Bio-Rad Canada). Western blot photographic films were scanned and pixel intensity was quantified by densitometry using ImageJ (NIH) software.

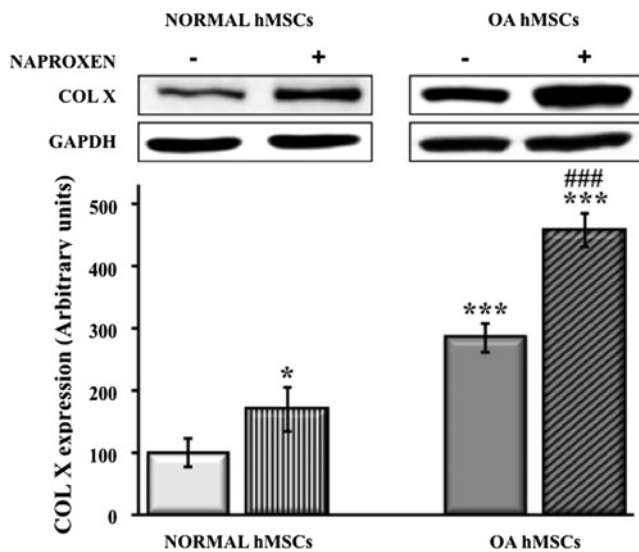
#### Statistical analysis

Experiments were carried out using the cells from three donors for normal MSCs and from four to seven donors for

TABLE 1. PRIMER SEQUENCES

Gene	Sequence	Product size (bp)
<i>ALOX5</i>	Forward: TGG AGA GCA AAG AAG ACA TCC Reverse: GCC GTA CAC GTA GAC ATC GTT	123
<i>RUNX2</i>	Forward: CAG ACC AGC AGC ACT CCA TA Reverse: CAG CGT CAA CAC CAT CAT TC	178
<i>COL10A1</i>	Forward: AAT GCC TGT GTC TGC TTT TAC Reverse: ACA AGT AAA GAT TCC AGT CCT	130
<i>GAPDH</i>	Forward: TGA AGG TCG GAG TCA ACG GAT Reverse: TTC TCA GCC TTG ACG GTG CCA	181





**FIG. 1.** Effect of Naproxen on type X collagen (COL X) expression in normal and osteoarthritis (OA) hMSCs. Normal hMSCs (Lonza) and the MSCs isolated from bone marrow aspirates of OA human donors undergoing total hip arthroplasty were cultured in DMEM (1% FBS and 1% PS) without or with Naproxen (100  $\mu\text{g}/\text{mL}$ ) for up to 72 h. COL X protein expression was determined by immunoblotting. GAPDH was used as a housekeeping gene and utilized to normalize the results. Values represent the mean  $\pm$  SE of four experiments (\* $p < 0.05$ ; \*\*\* $p < 0.001$  compared with untreated normal hMSCs; ### $p < 0.001$  compared with untreated OA hMSCs). DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; hMSCs, human mesenchymal stem cells.

OA hMSCs. Statistical analysis was performed using analysis of variance followed by Fisher's protected least significant difference *post-hoc* test using Prism statistical program. Results are presented as the mean  $\pm$  SE. Differences were considered statistically significant with  $p < 0.05$ .

## Results

### Effect of Naproxen on COL X protein expression in normal and OA hMSCs

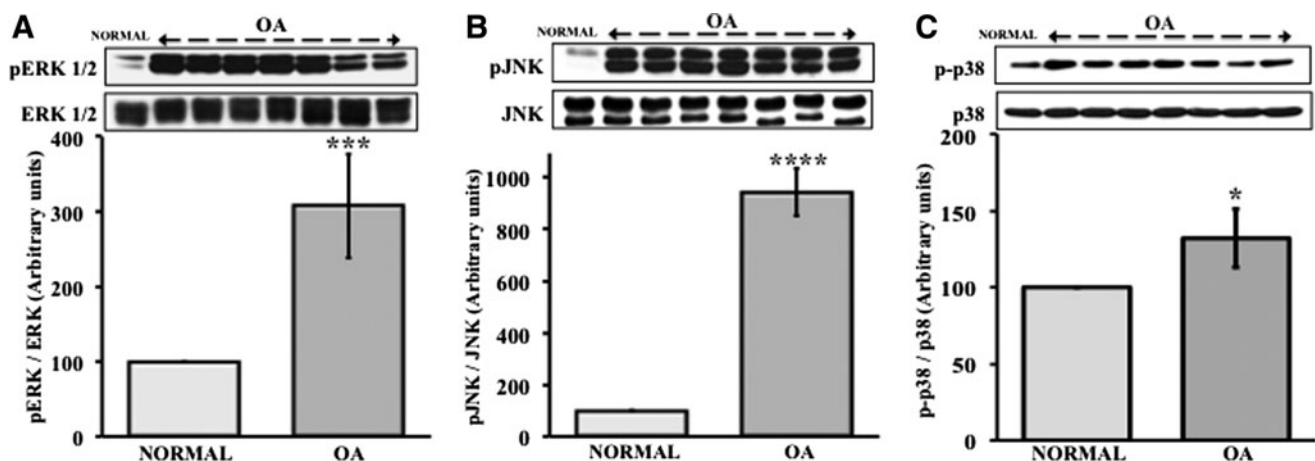
Treatment of normal MSCs with Naproxen (100  $\mu\text{g}/\text{mL}$ ) for up to 72 h significantly increased COL X protein expression (170%  $\pm$  20%). MSCs isolated from OA patients showed elevated basal COL X expression as compared with normal MSCs (285%  $\pm$  14%) and the expression was further augmented significantly by treatment with Naproxen for 72 h (459%  $\pm$  16%) (Fig. 1).

### Basal phosphorylation levels of MAPKs in normal versus OA hMSCs

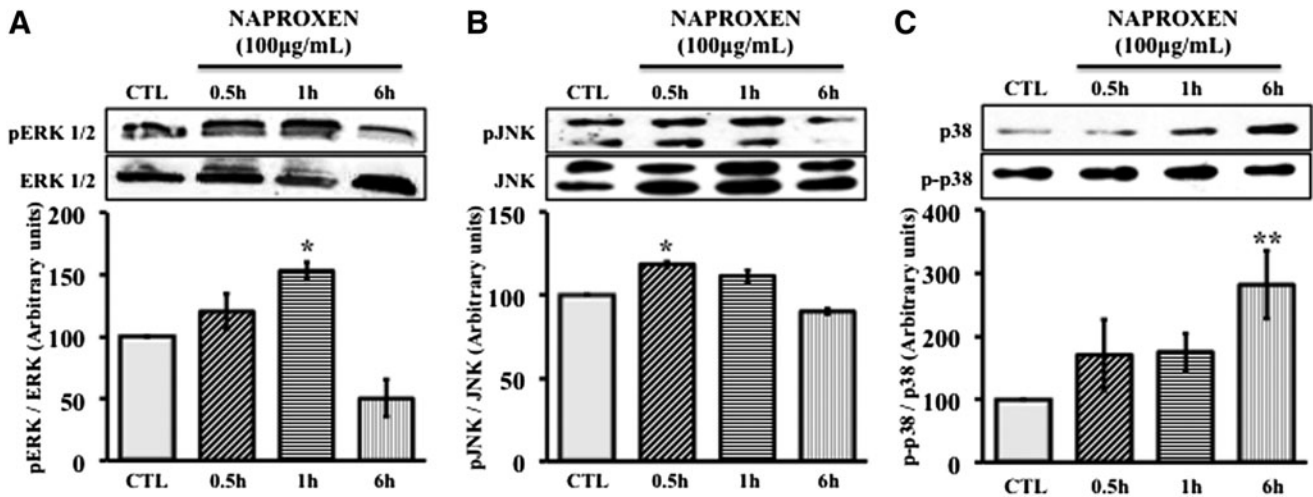
MAPKs are important mediators of inflammation and modulate intracellular signal transductions involved in expression of osteogenesis markers like Runt-related transcription factor 2 (*Runx2*), alkaline phosphatase (ALP), and osteocalcin (OC).<sup>31</sup> *Runx2* is known to regulate transcription of *COL10A1*.<sup>32</sup> Since MSCs from OA patients show elevated *COL10A1* expression, we first examined whether there is any relationship between MAPK signaling and the expression of COL X in MSCs. Basal phosphorylation levels of ERK, JNK, and p38 in MSCs from both normal and OA patients were measured and the results showed that the phosphorylation of all these three MAPKs in MSCs from OA patients was significantly higher (pERK1/2 [307%  $\pm$  69%] [Fig. 2A]; pJNK [943%  $\pm$  92%] [Fig. 2B]; and p-p38 [132%  $\pm$  19%] [Fig. 2C]) than in normal MSCs.

### Effect of Naproxen on MAPK phosphorylation in normal and OA hMSCs

We investigated whether Naproxen has any effect on MAPK signaling in normal MSCs and OA hMSCs. Normal and OA hMSCs were incubated with Naproxen (100  $\mu\text{g}/\text{mL}$ ) for 0.5 to 6 h. In normal MSCs, phosphorylation of ERK significantly increased by 1 h (153%  $\pm$  12%) following



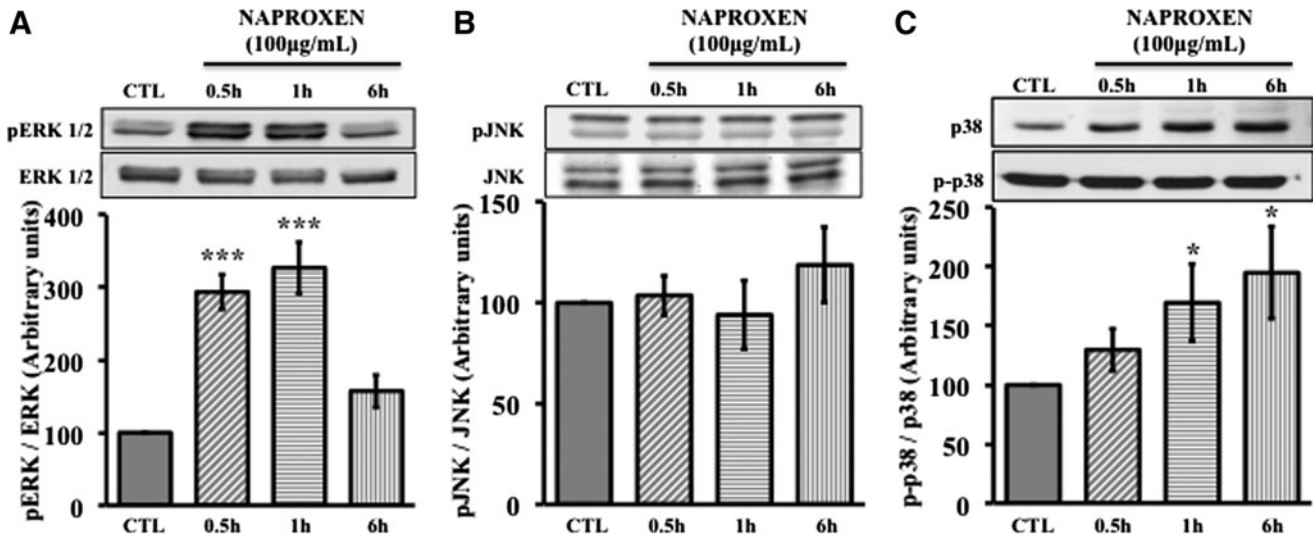
**FIG. 2.** Increased mitogen-activated protein kinase (MAPK) activation in OA hMSCs as compared with normal hMSCs. MSCs isolated from bone marrow aspirates of OA human donors undergoing total hip arthroplasty and normal human MSCs (Lonza) were cultured in complete DMEM to near confluence. Then, the cells were serum starved for 24 h. The protein expression and phosphorylation of MAPKs (ERK, JNK, and p38) in MSC lysates were determined by immunoblotting. Values represent the mean  $\pm$  SE of seven donors (A: pERK1/2, 307  $\pm$  69, \*\*\* $p < 0.001$ ; B: pJNK, 943  $\pm$  92, \*\*\*\* $p < 0.0001$ ; and C: p-p38, 132  $\pm$  19, \* $p < 0.05$ ) as percentage of normal human MSCs (100%). ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.



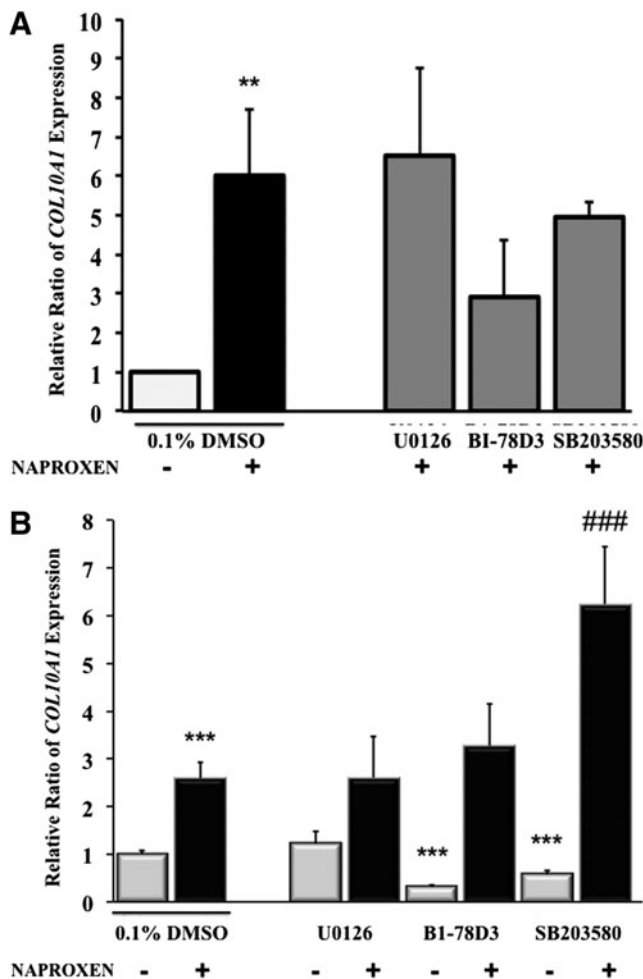
**FIG. 3.** Effect of Naproxen on phosphorylation of MAPKs in normal hMSCs. Normal hMSCs were cultured to near confluence in complete DMEM. Then, the cells were serum starved for 24 h followed by treatment with Naproxen (100  $\mu$ g/mL) or without (CTL) for up to 6 h. Protein expression and phosphorylation of ERK 1/2, JNK, and p38 were determined by immunoblotting and protein bands were quantified using Image J (NIH) software. Total ERK 1/2, JNK, and p38 were used to normalize corresponding phosphorylated forms. Values represent the mean  $\pm$  SE of three experiments. (A) Effect of Naproxen on phosphorylation of extracellular signal-regulated kinase (ERK). Naproxen significantly increased the phosphorylation of ERK at 1 h ( $153 \pm 12$ ,  $*p < 0.05$ ), which reached to below control levels after 6 h ( $50 \pm 26$ ). (B) Effects of Naproxen on phosphorylation of c-Jun N-terminal kinase (JNK). Naproxen significantly increased the phosphorylation of JNK in 0.5 h ( $119 \pm 3$ ,  $*p < 0.05$ ), which reached to control levels after 6 h. (C) Effects of Naproxen on phosphorylation of p38. The phosphorylation of p38 increased by 30 min and this increase was statistically significant at 6 h ( $282 \pm 106$ ,  $**p < 0.01$ ).

Naproxen treatment and by 6 h it reached below control levels ( $50\% \pm 26\%$ ) (Fig. 3A). Similarly Naproxen significantly increased the phosphorylation of JNK by 0.5 h ( $119\% \pm 3\%$ ), which also decreased to below control levels by 6 h (Fig. 3B). Naproxen treatment increased phosphory-

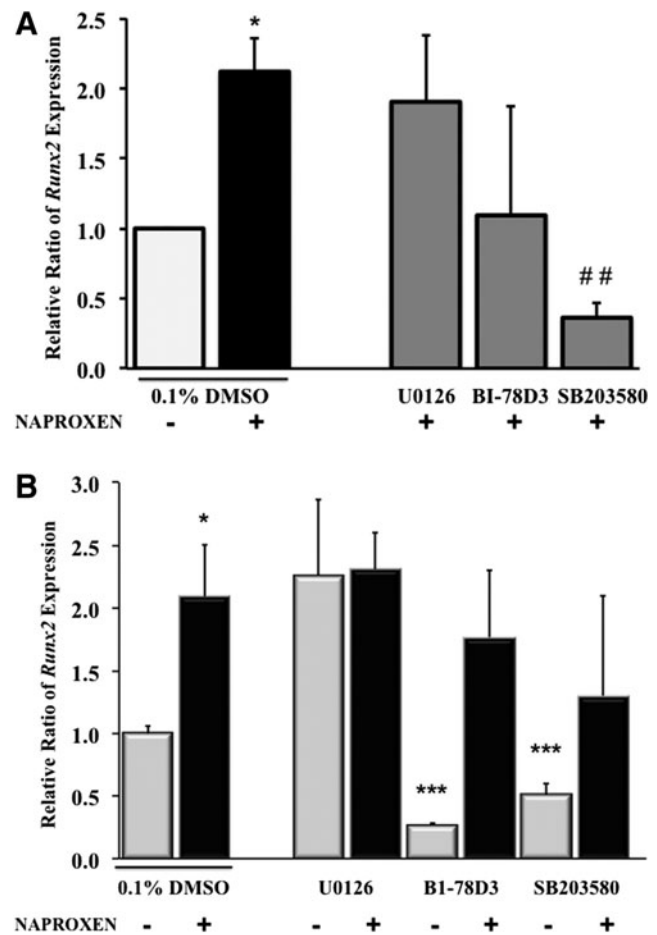
lation of p38 by 0.5 h and reached significant levels by 6 h ( $282\% \pm 106\%$ ) (Fig. 3C). In OA hMSCs, phosphorylation of ERK significantly increased by 0.5 h ( $294 \pm 24$ ) following Naproxen treatment and after 6 h reached to nonsignificant levels (Fig. 4A). Naproxen treatment had no significant



**FIG. 4.** Effect of Naproxen on phosphorylation of MAPKs in OA hMSCs. OA hMSCs were cultured to near confluence in complete DMEM. Then, the cells were serum starved for 24 h and were treated without (CTL) or with Naproxen (100  $\mu$ g/mL) for up to 6 h. Protein expression and phosphorylation of ERK 1/2, JNK, and p38 were determined by immunoblotting and protein bands were quantified using Image J (NIH) software. Total ERK 1/2, JNK, and p38 were used to normalize corresponding phosphorylated forms. Values represent the mean  $\pm$  SE of four donors. (A) Effect of Naproxen on phosphorylation of ERK. Naproxen had significantly increased the phosphorylation of ERK by 0.5 h ( $294 \pm 24$ ,  $***p < 0.001$ ) and reached to nonsignificant levels only after 6 h. (B) Effects of Naproxen on phosphorylation of JNK. Naproxen did not change the phosphorylation of JNK as compared with control. (C) Effects of Naproxen on phosphorylation of p38. The phosphorylation of p38 increased by 0.5 h and this increase was statistically significant at 1 h ( $170 \pm 32$ ,  $*p < 0.05$ ) and 6 h ( $195 \pm 39$ ,  $*p < 0.05$ ).



**FIG. 5.** (A) Effect of MAPK inhibitors on Naproxen-induced *COL10A1* expression in normal MSCs. Normal MSCs (Lonza) were cultured in complete DMEM to near confluence. Then, the cells were serum deprived for 24 h and were treated with or without Naproxen (100  $\mu$ g/mL) in the absence or presence of MAPK inhibitors (ERK inhibitor: U0126 [10  $\mu$ M], JNK inhibitor: BI-78D3 [1  $\mu$ M], and p38 inhibitor: SB203580 [20  $\mu$ M]). Gene expression was measured by real-time RT-PCR. GAPDH was used as the housekeeping gene to normalize the results. Values represent the mean  $\pm$  SE of three experiments (\*\* $p$  < 0.01; compared with untreated control [0.1% DMSO]). (B) Effect of MAPK inhibitors on Naproxen-induced *COL10A1* expression in OA hMSCs. MSCs isolated from bone marrow aspirates of OA human donors undergoing total hip arthroplasty were cultured in complete DMEM to near confluence. Then, the cells were serum deprived for 24 h followed by treatment without or with Naproxen (100  $\mu$ g/mL) in presence or absence of MAPK inhibitors (ERK inhibitor: U0126 [10  $\mu$ M], JNK inhibitor: BI-78D3 [1  $\mu$ M], and p38 inhibitor: SB203580 [20  $\mu$ M]). Gene expression was measured after 24 h of treatment by real-time RT-PCR. GAPDH was used as the housekeeping gene to normalize the results. Values represent the mean  $\pm$  SE of six donors (\*\* $p$  < 0.001, compared with untreated control [0.1% DMSO]; ### $p$  < 0.001, compared with Naproxen [100  $\mu$ g/mL] and 0.1% DMSO treated).



**FIG. 6.** (A) Effect of MAPK inhibitors on Naproxen-induced Runx2 expression in normal MSCs. Normal MSCs (Lonza) were cultured in complete DMEM to near confluence. Then, the cells were serum deprived for 24 h followed by treatment with or without Naproxen (100  $\mu$ g/mL) in absence or presence of MAPK inhibitors (ERK inhibitor: U0126 [10  $\mu$ M], JNK inhibitor: BI-78D3 [1  $\mu$ M], and p38 inhibitor: SB203580 [20  $\mu$ M]). Gene expression was measured by real-time RT-PCR. GAPDH was used as the housekeeping gene to normalize the results. Values represent the mean  $\pm$  SE of three experiments (\* $p$  < 0.05, compared with untreated control [0.1% DMSO]; ## $p$  < 0.01, compared with Naproxen [100  $\mu$ g/mL] and 0.1% DMSO treated). (B) Effect of MAPK inhibitors on Naproxen-induced Runx2 expression in OA hMSCs. MSCs isolated from bone marrow aspirates of OA human donors undergoing total hip arthroplasty were cultured in complete DMEM to near confluence and were serum deprived for 24 h. Then, the cells were treated without or with Naproxen (100  $\mu$ g/mL) in presence or absence of MAPK inhibitors (ERK inhibitor: U0126 [10  $\mu$ M], JNK inhibitor: BI-78D3 [1  $\mu$ M], and p38 inhibitor: SB203580 [20  $\mu$ M]). Gene expression was measured after 24 h of treatment by real-time RT-PCR. GAPDH was used as the housekeeping gene to normalize the results. Values represent the mean  $\pm$  SE of six donors (\* $p$  < 0.05; \*\*\* $p$  < 0.001, compared with untreated control [0.1% DMSO]).



effect on the phosphorylation of JNK (Fig. 4B) but increased the phosphorylation of p38 by 0.5 h and this increase was statistically significant at 1 h ( $170 \pm 32$ ) and continued to be significant even after 6 h ( $195 \pm 39$ ) (Fig. 4C).

#### Effect of MAPK inhibitors on Naproxen-induced COL10A1 expression in normal and OA hMSCs

To investigate whether the MAPKs are implicated in the Naproxen-induced COL10A1 expression, normal and OA hMSCs were treated with Naproxen (100  $\mu\text{g}/\text{mL}$ ) in the presence of MAPK inhibitors. In normal MSCs, MAPK inhibitors did not show any significant effect on Naproxen-induced COL10A1 expression (Fig. 5A) even though COL10A1 expression seems to show a downward trend in presence of JNK (BI-78D3) and p38 (SB203580) inhibitors. In OA hMSCs, basal COL10A1 expression, which was significantly higher than in normal MSCs, was attenuated by JNK inhibitor (BI-78D3) and p38 inhibitor (SB203580) by 0.69-fold and 0.43-fold, respectively, while ERK inhibitor (U0126) had no effect. Naproxen-induced COL10A1 expression in OA hMSCs was not altered by ERK and JNK inhibitors; on the other hand, p38 inhibitors appeared to increase its expression further by approximately threefold over Naproxen alone (Fig. 5B).

#### Effect of MAPK inhibitors on Naproxen-induced Runx2 expression in normal and OA hMSCs

In normal MSCs, ERK inhibitor (U0126) did not show any effect on Naproxen-induced Runx2 expression while it was suppressed by JNK inhibitor (BI-78D3) ( $\sim 1$ -fold) and by p38 inhibitor (SB203580) (1.7-fold), which was statistically significant (Fig. 6A). In OA hMSCs, similar to COL10A1, Runx2 expression was significantly attenuated by

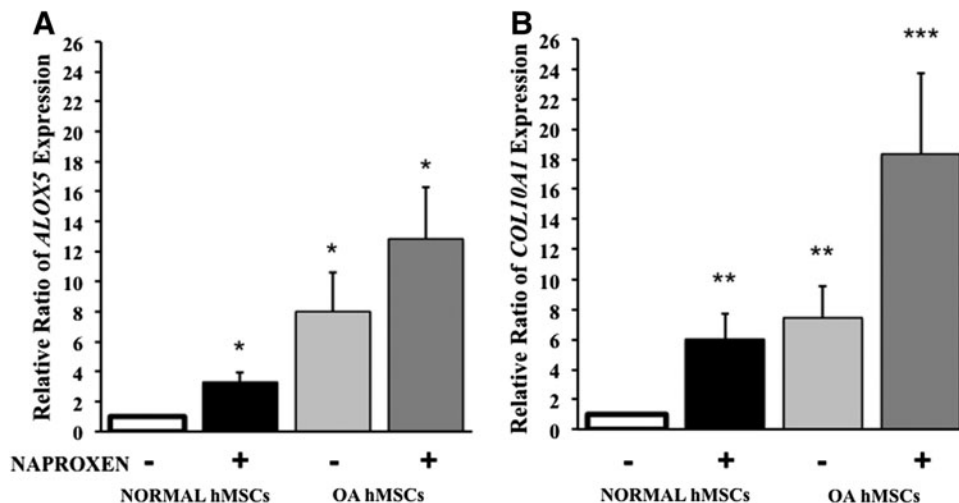
JNK inhibitor (BI-78D3) and p38 inhibitor (SB203580) by 0.7- and 0.5-fold, respectively, while ERK inhibitor (U0126) had no effect. Naproxen-induced Runx2 expression in OA hMSCs was not altered significantly by any of the MAPK inhibitors (Fig. 6B), but similar to COL10A1, there was a downward trend in expression with JNK and p38 inhibitors.

#### Effect of Naproxen on 5-lipoxygenase (ALOX5) and COL10A1 gene expression in normal and OA hMSCs

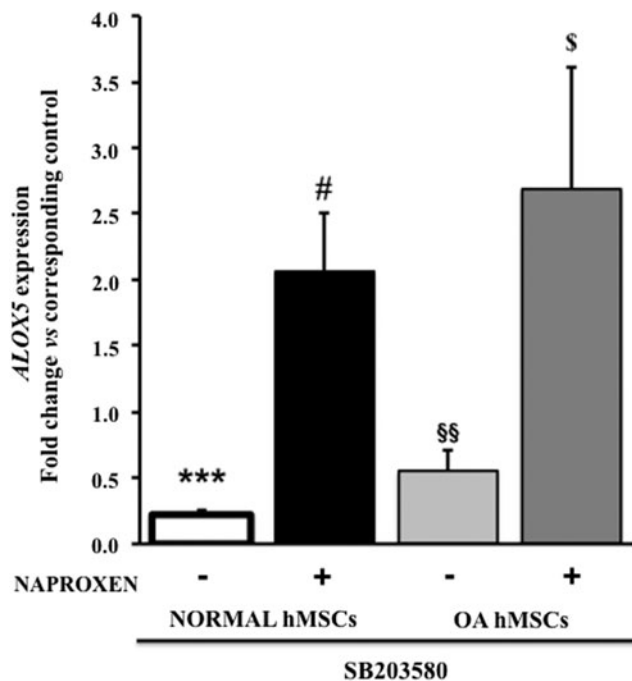
Naproxen treatment for 24 h significantly increased ALOX5 gene expression (3.29-fold) in normal MSCs. Basal ALOX5 gene expression on OA hMSCs is significantly higher (8-fold) compared with normal hMSCs. OA hMSCs treated with Naproxen had significantly higher ALOX5 gene expression (13-fold over untreated normal hMSCs and 5-fold over untreated OA hMSCs) (Fig. 7A). Similarly Naproxen treatment augmented the COL10A1 expression in both normal (6-fold) and OA hMSCs (18-fold). Basal COL10A1 expression in OA hMSCs was significantly higher (seven-fold) as compared with normal hMSCs (Fig. 7B). These results indicate that there was a clear correlation between the expression of ALOX5 and COL10A1.

#### Effect of p38 inhibitor on 5-lipoxygenase (ALOX5) gene expression in normal and OA hMSCs

Treatment with p38 inhibitor (SB203580) alone significantly suppressed ALOX5 gene expression in normal (0.78-fold) and OA hMSCs (0.45-fold) while it further upregulated ALOX5 gene expression in the presence of Naproxen both in normal (2.06-fold) and OA hMSCs (2.69-fold) when compared with corresponding p38-inhibitor untreated controls (Fig. 8).



**FIG. 7.** Effect of Naproxen on 5-lipoxygenase (ALOX5) and type X collagen (COL10A1) gene expression in normal and OA hMSCs. Normal hMSCs (Lonza) and the MSCs isolated from bone marrow aspirates of OA human donors undergoing total hip arthroplasty were cultured in complete DMEM to near confluence and were serum deprived for 24 h followed by treatment without or with Naproxen (100  $\mu\text{g}/\text{mL}$ ). ALOX5 (A) and COL10A1 (B) gene expression was measured after 24 h of treatment by real-time RT-PCR. GAPDH was used as the housekeeping gene to normalize the results. Normal hMSC values represent the mean  $\pm$  SE of three experiments (\* $p < 0.05$ ; \*\* $p < 0.01$ ; compared with untreated normal hMSC control). OA hMSC values represent the mean  $\pm$  SE of six donors (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; compared with untreated normal hMSC control).



**FIG. 8.** Effect of p38 inhibitor on 5-lipoxygenase (*ALOX5*) gene expression in normal and OA hMSCs with and without Naproxen treatment. Normal hMSCs (Lonza) and the MSCs isolated from bone marrow aspirates of OA human donors undergoing total hip arthroplasty were cultured in complete DMEM to near confluence. Then, the cells were serum deprived for 24h followed by treatment without or with p38 inhibitor (SB203580, 20  $\mu$ M) and Naproxen (100  $\mu$ g/mL). *ALOX5* gene expression was measured after 24 h of treatment by real-time RT-PCR. GAPDH was used as the housekeeping gene to normalize the results. Normal hMSC values represent the mean  $\pm$  SE of three experiments (\*\*\* $p$  < 0.001, compared with untreated normal hMSC control; # $p$  < 0.05, compared with Naproxen-treated normal hMSCs without p38 inhibitor). OA hMSC values represent the mean  $\pm$  SE of six donors (§§ $p$  < 0.01, compared with untreated OA hMSC control; § $p$  < 0.05, compared with Naproxen-treated OA hMSCs without p38 inhibitor).

#### Effect of 5-lipoxygenase inhibitor (MK-886) on COL10A1 gene expression in normal and OA hMSCs

Naproxen-induced *COL10A1* expression was significantly attenuated by 5-lipoxygenase inhibitor in normal (threefold) and OA hMSCs (twofold) (Fig. 9).

#### Effect of 5-lipoxygenase inhibitor (MK-886) on Runx2 gene expression in normal and OA hMSCs

Naproxen-induced *Runx2* expression was suppressed by the 5-lipoxygenase inhibitor in normal and OA hMSCs and was statistically significant in OA hMSCs (1.7-fold) (Fig. 10).

## Discussion

The purpose of this study was to investigate the mechanism underlying the induction of *COL10A1* by Naproxen in human MSCs and the signal transduction pathways involved. Human MSCs from OA patients, which are used as

a potential cell source for cartilage tissue engineering, have elevated expression of COL X, a marker of late-stage chondrocyte hypertrophy and associated with endochondral ossification.<sup>15</sup> Other osteogenesis-related markers are also present in increased levels in MSCs from OA patients.<sup>8,10-12</sup> We have recently investigated the effect of commonly prescribed drugs to OA patients, including NSAIDs and acetaminophen, on the expression of markers of chondrocyte hypertrophy and osteogenesis in normal and OA hMSCs and our results showed that Naproxen, an NSAID and a nonspecific inhibitor of cyclooxygenase, can significantly increase the expression levels of *COL10A1* in human MSCs from normal and OA subjects.<sup>15</sup>

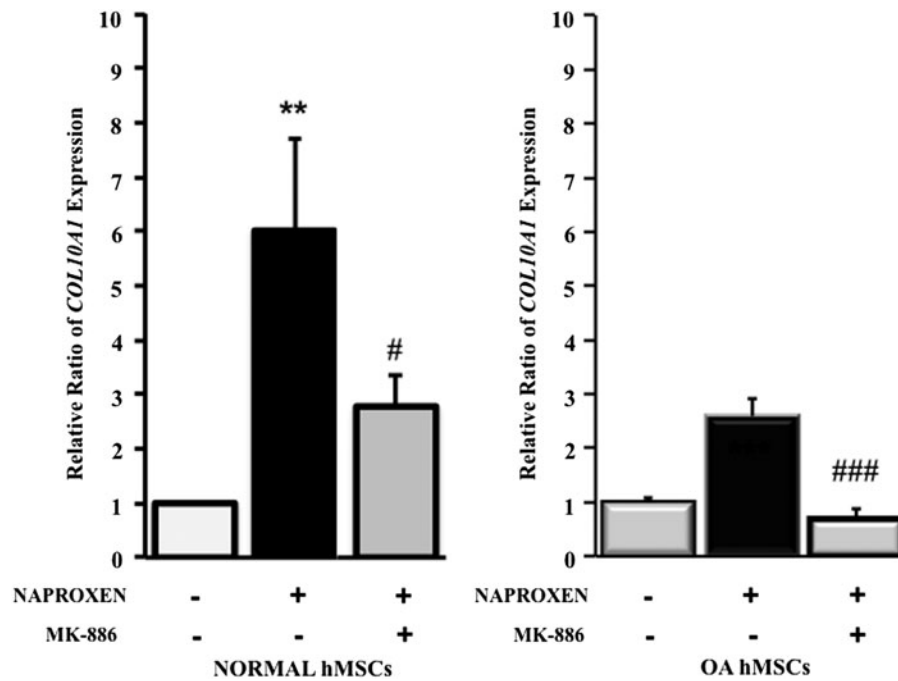
In the present study, using normal and OA hMSCs, we further show that the effect of Naproxen on COL X expression is not only at the transcriptional level, but also at the protein level. Similar detrimental effects of Naproxen on matrix synthesis both in normal and OA articular cartilage were reported earlier.<sup>17</sup> The nonselective NSAIDs like Naproxen, by inhibiting cyclooxygenases 1 and 2, can reduce production of prostaglandins and downstream activation of p38 but this can lead to accumulation of arachidonic acid and its diversion to the formation of leukotrienes via lipoxygenase pathway. Leukotrienes are known to enhance the phosphorylation/activation of p38 MAPK, which in turn stabilizes the cyclooxygenase-2 mRNA.<sup>33,34</sup> The *COL10A1* transcription factor *Runx2* is known to be activated/phosphorylated by p-p38 and thus increases the expression of *COL10A1*.<sup>31,32</sup>

Since NSAIDs can alter MAPK signaling, in particular, p38 MAPK, in this study we investigated whether altered MAPK signaling pathways play a role in Naproxen-induced increase in the expression of *COL10A1* in human MSCs. Our results indicate MAPK involvement in the elevated *COL10A1* expression in OA hMSCs as the increased basal *COL10A1* was attenuated with p38 and JNK inhibitors. On the other hand, Naproxen-induced *COL10A1* expression in both normal and OA hMSCs was unchanged by MAPK inhibitors, indicating involvement of a different signaling mechanism.

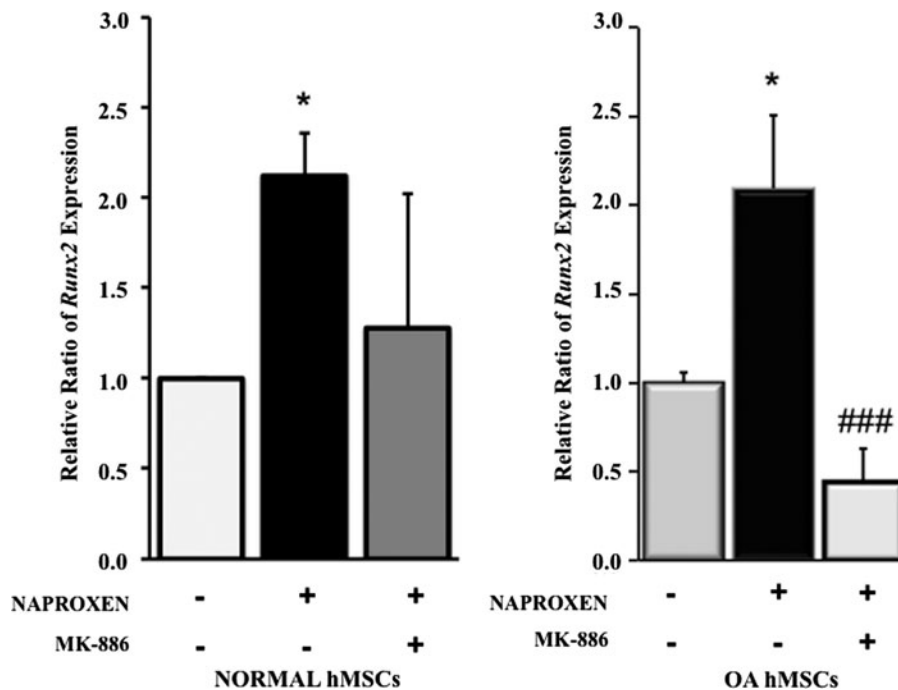
Previously it was shown in human OA chondrocytes that Naproxen increased 5-lipoxygenase mRNA by 330% after 24 h of incubation.<sup>25</sup> In our current study we observed a significant increase in 5-lipoxygenase mRNA levels with Naproxen treatment for 24 h, by 3.29-fold in normal MSCs and by 13-fold in OA hMSCs as compared with untreated normal MSCs, indicating a possible diversion of accumulated arachidonic acid toward lipoxygenase pathway. 5-Lipoxygenase catalyzes oxidation of arachidonic acid to leukotrienes (LTB<sub>4</sub>) through several intermediary steps.<sup>35</sup> These leukotrienes are known to activate MAPKs (p38 and ERK),<sup>36,37</sup> which are instrumental in activation/phosphorylation of *COL10A1* transcription factor *Runx2* and thus increasing its expression.<sup>31</sup> In the present study, we found that Naproxen-induced *COL10A1* and *Runx2* expression was significantly reduced both in normal and OA hMSCs by 5-lipoxygenase inhibitor.

Identifying the signaling mechanisms that regulate the expression of markers of chondrocyte hypertrophy in human MSCs is necessary in order to help preventing hypertrophic differentiation and ossification during cartilage tissue engineering.



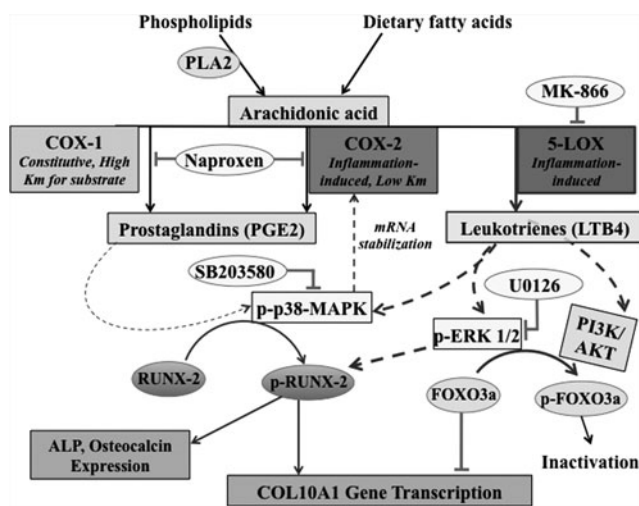


**FIG. 9.** Effect of 5-lipoxygenase inhibitor (MK-886) on Naproxen-induced type X collagen (*COL10A1*) gene expression in normal and OA hMSCs. Normal hMSCs (Lonza) and the MSCs isolated from bone marrow aspirates of OA human donors undergoing total hip arthroplasty were cultured in complete DMEM to near confluence and were serum deprived for 24 h followed by treatment without or with 5-LOX inhibitor (MK-886, 10  $\mu$ M) and Naproxen (100  $\mu$ g/mL). *COL10A1* gene expression was measured after 24 h of treatment by real-time RT-PCR. GAPDH was used as the housekeeping gene to normalize the results. Normal hMSC values represent the mean  $\pm$  SE of three experiments (\*\* $p < 0.01$ , compared with untreated normal hMSC control; # $p < 0.05$ , compared with Naproxen-treated normal MSCs without 5-LOX inhibitor). OA hMSC values represent the mean  $\pm$  SE of six donors (### $p < 0.001$ , compared with Naproxen-treated OA hMSCs without 5-LOX inhibitor).



**FIG. 10.** Effect of 5-lipoxygenase inhibitor (MK-886) on Naproxen-induced RUNX2 gene expression in normal and OA hMSCs. Normal hMSCs (Lonza) and the hMSCs isolated from bone marrow aspirates of OA human donors undergoing total hip arthroplasty were cultured in complete DMEM to near confluence and were serum deprived for 24 h followed by treatment without or with 5-LOX inhibitor (MK-886, 10  $\mu$ M) and Naproxen (100  $\mu$ g/mL). RUNX2 gene expression was measured after 24 h of treatment by real-time RT-PCR. GAPDH was used as the housekeeping gene to normalize the results. Normal hMSC values represent the mean  $\pm$  SE of three experiments (\* $p < 0.05$ , compared with Naproxen-treated normal hMSCs without 5-LOX inhibitor). OA hMSC values represent the mean  $\pm$  SE of six donors (### $p < 0.001$ , compared with Naproxen-treated OA hMSCs without 5-LOX inhibitor).

MAPK signaling has been proposed to be involved in the different stages of chondrogenic differentiation.<sup>38</sup> There is growing evidence that MAPK signaling pathways probably play a critical role in OA-related changes of chondrocytes.<sup>39–42</sup> Further, it has been shown that inhibition of one or more MAP kinases in chondrocytes both *in vitro* and *in vivo* results in slowing down of the progression of OA-related changes.<sup>41,43,44</sup> The possible involvement of MAPK in the upregulation of markers of chondrocyte hypertrophy and ossification became evident as all the MAPKs (p38, JNK, and ERK) are activated even under basal conditions in MSCs from OA patients as compared with normal MSCs. Thus, the elevated expression of *COL10A1* seen in MSCs from OA patients in earlier studies<sup>15</sup> and in the present studies is associated with MAPK signaling. Besides



**FIG. 11.** Arachidonic acid is liberated from the cell membrane phospholipids by cytoplasmic phospholipase A2 (PLA2). Free arachidonic acid can be metabolized through cyclooxygenase (COX) and the lipoxygenase (LOX) pathways. In the COX pathway, arachidonic acid is enzymatically converted to biological mediators called prostanoids, including prostaglandins (PGs) by specific prostaglandin synthases through several intermediary steps. COX-1 is the constitutive cyclooxygenase and maintains basal levels of prostaglandins, whereas COX-2 is inducible and produces prostaglandins under inflammatory conditions. In the 5-LOX pathway, arachidonic acid is converted into biologically active metabolites, such as leukotrienes (LTB4), which also trigger inflammatory response. Prostaglandin-mediated activation of p38 leads to the stabilization of COX-2 mRNA, which further enhances the prostaglandin production and inflammation through COX-2 pathway. Activated p38-MAPK phosphorylates and activates Runx-2 transcription factor, which triggers the expression of *COL10A1* gene. Leukotrienes (LTB4) derived from 5-LOX pathway also activate p38-MAPK as well as ERK1/2 by phosphorylation. Phospho-ERK1/2 can also lead to the activation of Runx-2. Besides activating Runx-2, phospho-ERK1/2 is also known to inactivate and alleviate the inhibitory action of FOXO3a, thus further increasing the *COL10A1* gene expression. Naproxen, by inhibiting both the COX-1 and COX-2, causes diversion of arachidonic acid to 5-LOX-pathway-mediated leukotriene production. These leukotrienes (LTB4) in turn upregulate *COL10A1* gene expression.

p38 MAPK, ERK1/2 activation may also contribute to the upregulation of *COL10A1* gene expression by inactivating the inhibitory transcription factor Foxo3a (Fig. 11). NSAIDs that increase the flux through 5-lipoxygenase pathways and/or increase the expression of 5-lipoxygenase can potentially lead to further elevation in the expression of COL X and aggravate the pathogenesis of OA.

Considering the importance of NSAIDs as the most prescribed antipain medication for patients with OA, the results from the present study point toward caution that needs to be exercised, while suggesting the use of these drugs. This is particularly true for drugs like Naproxen that target only COX and those with similar molecular action. One can speculate that drugs such as licofelone that show dual inhibition of COX and LOX pathways are more likely to have better beneficial effects in preventing inflammatory responses than the COX inhibitors.<sup>45</sup> In fact, it has recently been reported in a 2-year clinical trial with 161 knee OA patients that the total knee replacements were much higher in patients receiving Naproxen than in patients given licofelone,<sup>46,47</sup> indicating the beneficial significance of choosing a COX/LOX inhibitor over COX-only inhibitor in OA patients. Interestingly, licofelone also possesses anticancer effects and is currently at advanced stage of clinical development status with completed phase III trials.<sup>48</sup>

In conclusion, the findings of our study contribute to advancing our understanding of the molecular mechanisms underlying *COL10A1* expression in human MSCs, implicating MAPK signaling pathways in the chondrocyte hypertrophic differentiation of OA hMSCs. Our study also explains how NSAIDs like Naproxen aggravate the pathogenesis of OA by increasing COL X expression via 5-lipoxygenase. Further research is required to identify and target other signaling pathways involved in this process, which may provide a basis for finding therapy directed toward reverting the hypertrophic changes associated with OA.

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#### Disclosure Statement

The authors have no conflicts of interest to declare.

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