# Src Kinase Regulates Nitric Oxide-induced Dedifferentiation and Cyclooxygenase-2 Expression in Articular Chondrocytes via p38 Kinase-dependent Pathway

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**ABSTRACT**

**Background:** Nitric oxide (NO) in articular chondrocytes regulates dedifferentiation and inflammatory responses by modulating MAP kinases. In this study, we investigated whether the Src kinase in chondrocytes regulates NO-induced dedifferentiation and cyclooxygenase-2 (COX-2) expression. **Methods:** Primary chondrocytes were treated with various concentrations of SNP for 24 h. The COX-2 and type II collagen expression levels were determined by immunoblot analysis, and prostaglandin $E_2$ ($PGE_2$) was determined by using a $PGE_2$ assay kit. Expression and distribution of p-Caveolin and COX-2 in rabbit articular chondrocytes and cartilage explants were determined by immunohistochemical staining and immunocytochemical staining, respectively. **Results:** SNP treatment stimulated Src kinase activation in a dose-dependent manner in articular chondrocytes. The Src kinase inhibitors PP2 [4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo(3,4-d)pyrimidine], a significantly blocked SNP-induced p38 kinase and caveolin-1 activation in a dose-dependent manner. Therefore, to determine whether Src kinase activation is associated with dedifferentiation and/or COX-2 expression and $PGE_2$ production. As expected, PP2 potentiated SNP-stimulated dedifferentiation, but completely blocked both COX-2 expression and $PGE_2$ production. And also, levels of p-Caveolin and COX-2 protein expression were increased in SNP-treated primary chondrocytes and osteoarthritic and rheumatoid arthritic cartilage, suggesting that p-Caveolin may play a role in the inflammatory responses of arthritic cartilage. **Conclusion:** Our previously studies indicated that NO caused dedifferentiation and COX-2 expression is regulated by p38 kinase through caveolin-1 (1). Therefore, our results collectively suggest that Src kinase regulates NO-induced dedifferentiation and COX-2 expression in chondrocytes via p38 kinase in association with caveolin-1. **(Immune Network 2006;6(4):204-210)**

**Key Words:** Src kinase, Caveolin-1, Dedifferentiation, COX-2, p38 kinase

## Introduction

Chondrocytes in cartilage are differentiated from mesenchymal cells during embryonic development. Differentiated chondrocytes, which are the only cell type found in normal mature cartilage, synthesize sufficient amounts of cartilage-specific extracellular matrix (ECM) to maintain matrix integrity (1). This homeostasis is destroyed in degenerative diseases, such as osteoarthritis and rheumatoid arthritis. Arthritis is characterized by structural and biochemical changes in chondrocytes and cartilage, including degradation of cartilage matrix, insufficient synthesis of ECM because of loss of chondrocyte phenotype. However, the differentiated phenotype is unstable both *in vivo* and *in vitro* and thus lost by a process designated "dedifferentiation" upon exposure of cells to interleukin-1β (1,2), nitric oxide (NO) (3), or retinoic acid (4,5) and during serial monolayer culture (6,7). NO mediates the regulation of chondrocyte

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phenotype and survival by inducing dedifferentiation and apoptosis (3,8,9). We showed previously that direct production of NO by treatment of primary culture articular chondrocytes with a NO donor, sodium nitroprusside (SNP), led to apoptosis, dedifferentiation, and cyclooxygenase (COX)-2 expression via a complex protein kinase signaling cascade involving mitogen-activated protein (MAP) kinase and protein kinase C (PKC) (10-13). For example, NO-induced activation of extracellular signal-regulated protein kinase (ERK) promotes dedifferentiation, COX-2 expression, and inhibition of apoptosis, whereas NO-induced p38 kinase activati2-3dpon triggers apoptosis, COX-2 expression, and maintains differentiated phenotypes (10,11). And also, we recently observed that p38 kinase regulates dedifferentiation in association with caveolin-1 phosphorylation (14).

Caveolin, a family of 21～24 kDa integral membrane proteins are a principal component of caveolae membranes (15). Although expression of caveolae structure and expression of caveolin family, caveolin-1, -2 and -3, was known in chondrocytes, the exact functional role of caveolae and caveolins in chondrocytes remain unclear (16).

Src family kinases (SFKs) are one of the most studied groups of tyrosine kinases and can mediate a variety of signaling pathways. c-Src signaling played a major role in transducing MSU-induced NO production and MMP-3 expression via p38 activation. Thus, c-Src as novel sites for potential therapeutic intervention in cartilage degradation (17). In addition to, the specific activity of pp60c-Src kinase regulate matrix mineralization in hypertrophic chondrocyte culture (18). However, little is known about the expression of SFKs in articular chondrocytes.

In the studies described herein we examined the role of Src kinase using a PP2, a potent and selective inhibitor of the Src family tyrosine kinases (13) on SNP-induced dedifferentiation and inflammatory responses. We found that dedifferentiation, COX-2 expression, and $PGE_2$ production by NO was regulated by Src kinase signaling pathway via activation of p38 kinase and p-Caveolin.

## Materials and Methods

*Isolation and monolayer culture of rabbit articular chondrocytes and experimental culture condition.* Articular chondrocytes were isolated from cartilage slices of 2-week-old New Zealand white rabbits by enzymatic digestion as described previously (19). Cartilage slices were dissociated enzymatically for 6 h in 0.2% collagenase type II (381 U/mg solid, Sigma) in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Gaithersburg, MD). Individual cells were suspended in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine-calf serum, 50μg/ml streptomycin, and 50 units/ml penicillin, after which and they were then plated on culture dishes at a density of $5\times10^4$ cells/$cm^2$. The medium was changed every 2 days after seeding, and cells reached confluence in approximately 5 days. Differentiation status of articular chondrocytes was determined by examining the accumulation of sulfated glycosaminoglycan with Alcian Blue staining or expression of type II collagen expression was detected using antibodies purchased from Chemicon (Temecula, CA) by immunoblot analysis as described previously (19).

*Immunoblot analysis.* Whole cell lysates were prepared by extracting proteins using a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% sodium dodecylsulfate, supplemented with protease inhibitors [10μg/ml leupeptin, 10μ g/ml pepstatin A, 10μg/ml aprotinin and 1 mM of 4-(2-aminoethyl) benzenesulfonyl fluoride] and phosphatase inhibitors (1 mM NaF and 1 mM $Na_3VO_4$). The proteins were size-fractionated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The nitrocellulose sheet was then blocked with 3% non-fat dry milk in Tris-buffered saline. COX-2 was detected using antibody purchased from Cayman Chemical (Ann Arbor, MI), and p-Caveolin and pSrc were detected using antibodies purchased from Santa Cruz Biotech. (Santa Cruz, CA). pp38 was purchased from Chemicon (Temecula, CA). Blotss were developed using a peroxidase-conjugated secondary antibody and visualized with an ECL system.

*Immunohistochemistry and imunofluorescence microscopy.* Rabbit joint cartilage explants or arthritic cartilage were in 4% paraformaldehyde in PBS for 24 h at 4°C, washed with PBS, dehydrated with graded etanol, embedded in paraffin, and sectioned at 4μm thickness. The sections were stained by standard procedures using Alcian blue or antibody against p-Caveolin or COX-2 and visualized by developing with a kit purchased from DAKO (Carpinteria, CA), following the procedure recommended by the manufacturer. Expression and distribution of type II collagen and COX-2 in rabbit articular chondrocytes were determined by indirect immunofluorescence microscopy, as described previously (19). Briefly, chondrocytes were fixed with 3.5% paraformaldehyde in PBS for 10 min at room temperature. The cells were permeabilized and blocked with 0.1% Triton X-100 and 5% fetal calf serum in PBS for 30 min. The fixed cells were washed and incubated for 1 h with antibody (10μg/ml) against type II collagen or COX-2. The cells were washed, incubated with rhodamine-conjugated secondary antibodies for 30 min, and observed under a fluorescence microscope.

*$PGE_2$ Assay.* $PGE_2$ production was determined by measuring the levels of cellular and secreted $PGE_2$ using an assay kit (Amersham Pharmacia Biotech, NJ, UK). Briefly, chondrocytes were seeded in standard 96-well microtiter plates at $2\times10^4$ cells/well. Following addition of the indicated pharmacological reagents, total cell lysate was used to quantify the amount of $PGE_2$, according to the manufacturer's protocol. $PGE_2$ levels were calculated against a standard curve of $PGE_2$ and normalized against the amount of genomic DNA.

*Data analyses and statistics.* The results are expressed as the means±S.E. values calculated from the specified number of determinations. A student's test was used to compare individual treatments with their respective control values. A probability of $p<0.05$ was taken as denoting a significant difference.

## Results

*SNP treatment induces dedifferentiation of articular chondrocytes.* To examine the effects of NO generator SNP on articular cartilage chondrocytes, cartilage explant cultures and primary culture chondrocytes were treated with 1 mM SNP for 24 h, and expression of cartilage-

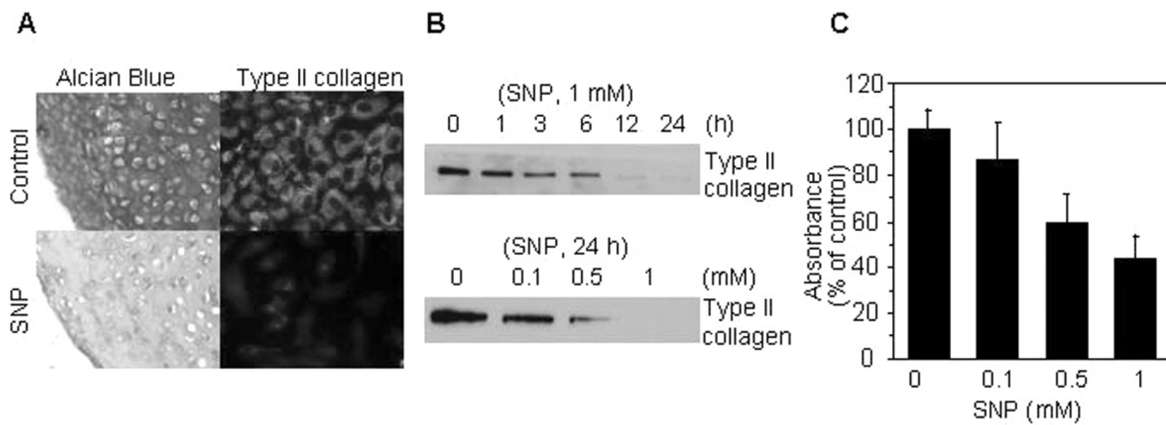


**Figure. 1.** NO causes dedifferentiation of chondrocytes. Cartilage explants (left panel) and primary clulture chondrocytes (right panel) were untreated or treated with 1 mM SNP for 24 h. Proteoglycan and type II collagen were detected by Alcian blue staining and immunocytochemical staining and, respectively (A). Chondrocytes were treated with 1 mM SNP for various time periods (upper panel) or with the indicated concentrations of SNP for 24 h (lower panel). The expression of type II collagen was determined immunoblot analysis (B). Accumulation of sulfated glycosaminoglycan in cells treated with the indicated concentrations of SNP for 24 h was quantified by Alcian Blue staining (C). The data represent the results of a typical experiment conducted at least three times with similar results.

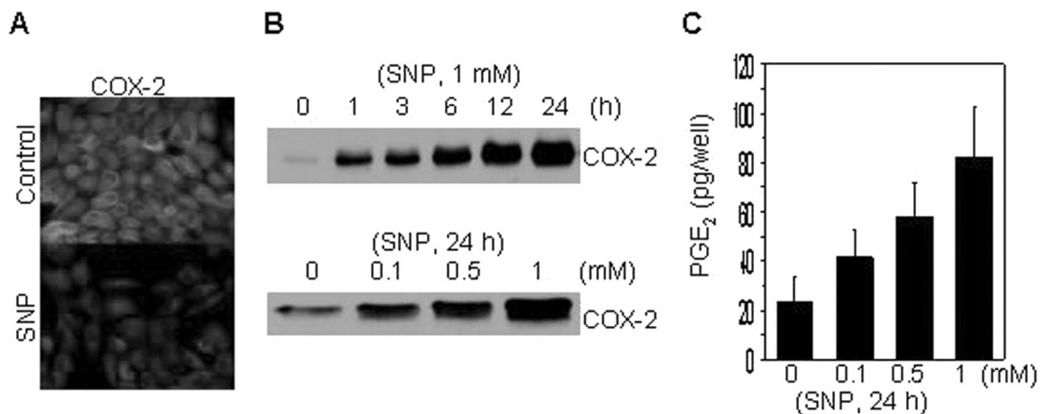


**Figure. 2.** NO causes COX-2 expression and $PGE_2$ production in articular chondrocytes. Primary clulture chondrocytes were untreated or treated with 1 mM SNP for 24 h. COX-2 expression was detected by immunocytochemical staining (A). Chondrocytes were treated with 1 mM SNP for various time periods (upper panel) or with the indicated concentrations of SNP for 24 h (lower panel). The expression of COX-2 was determined immunoblot analysis (B). Levels of cellular and secreted $PGE_2$ were determined by using a $PGE_2$ assay kit (C). The data in A represent the results of a typical experiment conducted four times, and B signifies the average values with standard deviation (n=4).

specific matrix molecules were determined. NO caused a dramatic loss of type II collagen and sulfated proteoglycan as determined by immunostaining and Alcian blue staining, respectively (Fig. 1A). NO also inhibited type II collagen expression in articular chondrocytes in a time- and dose-dependent manner (Fig. 1B). Similarly, SNP treatment of articular chondrocytes inhibited the accumulation of sulfated proteoglycan in a dose- dependent manner (Fig. 1C). These results indicate that NO induces dedifferentiation in both cartilage explants and primary culture cells. *SNP treatment induces COX-2 expression and $PGE_2$ production in articular chondrocytes.* The effect of NO on COX-2 expression and $PGE_2$ production was investigated in articular chondrocytes. In primary culture articular chondrocytes, NO stimulated COX-2 expression as determined by immunocytochemical staining (Fig. 2A). And also, NO induce COX-2 expression in a time- and dose-dependent manner as determined by immunoblot analysis (Fig. 2B). Consistent with the induction of COX-2 expression, NO stimulated $PGE_2$ production (Fig. 2C). These data indicate that NO stimulates COX-2 expression and $PGE_2$ production in articular chondrocytes.

*Src kinase stimulates dedifferentiation and COX-2 expression.* To investigate the signaling pathway stimulating dedifferentiation and COX-2 expression of SNP-treated chondrocytes, we examined changes in the activity of Src and p38 kinase, and activation of caveolin-1. As shown in Fig. 3A, Src kinase activity was increased in a time-dependent manner as determined by phosphorylation status of the protein. Consistent with our previous observation, levels of p38 kinase phosphorylation began to increase at 3 h, reached peak levels at 12 h, and decreased thereafter. Similar to the pattern of Src activation, caveolin-1 activation was also increased in a time-dependent manner (Fig. 3A). To determine the association between Src kinase and p38 kinase, chondrocytes were treated with SNP in the presence and absence of PP2, and expression of p38 activation as determined by immunoblot analysis. The addition of PP2 to SNP-treated chondrocytes blocked the increased phosphorylation of p38 kinase in a dose-dependent manner (Fig. 3B, middle panel). And, caveolin-1 tyrosine phosphorylation is also blocked by inhibition of Src kinase (Fig. 3B, lower

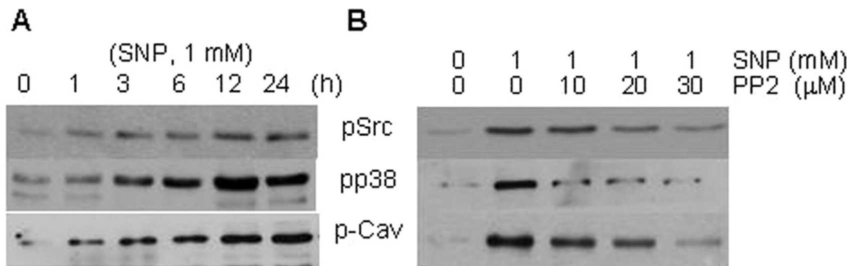


**Figure. 3.** Activation of Src kinase, p38 kinase and caveolin-1 in SNP-treated chondrocytes. Chondrocytes were treated with 1 mM SNP for various time periods (A). Chondrocyte cells were treated with 1 mM SNP for 24 h in the presence of indicated concentrations of PP2 (B). pSrc, pp38 and p-Caveolin-1 (p-Cav) were detected by immunoblot analyses. The data represent the results of a typical experiment conducted at least three times with similar results.

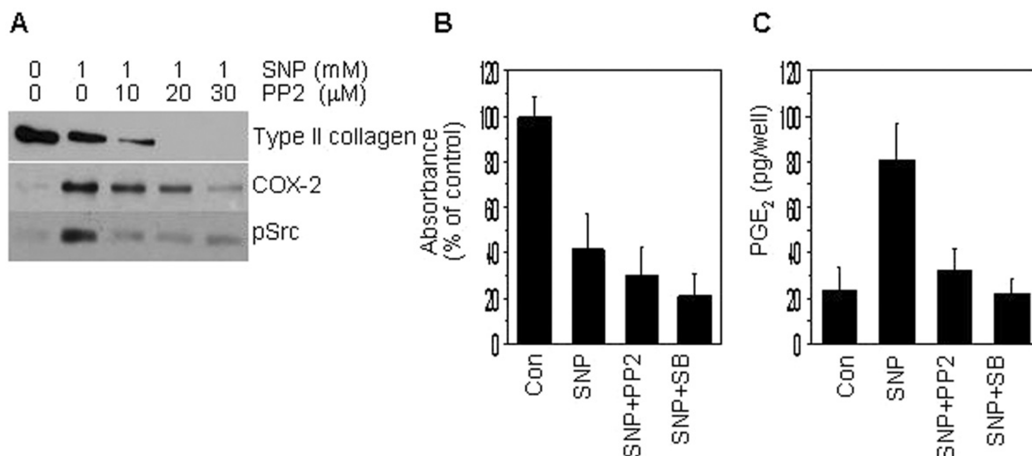


**Figure. 4.** Src kinase induces dedifferentiation and COX-2 expression. Articular chondrocyte cells were treated with 1 mM SNP for 24 h in the presence of indicated concentrations of PP2. Type II collagen, COX-2 and pSrc were detected by immunoblot analyses (A). Chondrocytes were treated with 1 mM SNP for 24 h in the absence or presence of indicated concentrations of 20μM PP2 or 20μM SB203580. Accumulation of sulfated glycosaminoglycan was quantified by Alcian blue staining (B). Levels of cellular and secreted $PGE_2$ were determined by using a $PGE_2$ assay kit (C). The data represent the average values with standard deviation (n=4).

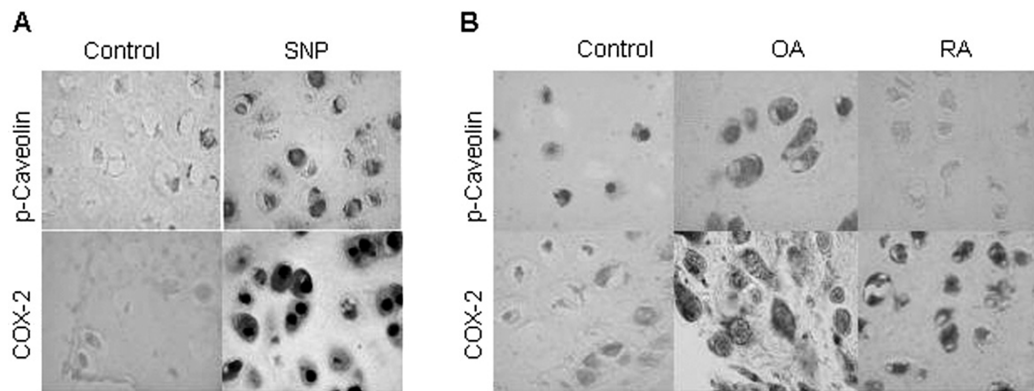


**Figure. 5.** Increased levels of p-Caveolin and COX-2 in arthritic cartilage. Cartilage were untreated or treated with 1 mM SNP for 24 h. Expression levels of p-Caveolin and COX-2 was detected by immunohistochemical staining (A). Expression of p-Caveolin and COX-2 proteins was detected from normal (Control) and osteoarthritis-affected (OA) and rheumatoid-affected human joint cartilage (RA) (B). Tissue sections were counter stained with hematoxylin. The data represent the results of a typical experiment conducted at least three times.

panel).

To determine whether Src kinase stimulated dedifferentiation and COX-2 expression, chondrocytes were treated with 1 mM SNP in the presence and absence of PP2. As shown in Fig. 4A, type II collagen expression, a differentiation marker of articular chondrocytes (17), was completely blocked (upper panel), and COX-2 expression was blocked in a dose-dependent manner (middle panel). To elucidate the above data, we next examined sulfated proteoglycan accumulation and $PGE_2$ production as determined by Alcian blue staining and using a kit assay, respectively. Similarly, SNP treatment of primary culture cells blocked the accumulation of sulfated proteoglycan (Fig. 4B) and $PGE_2$ production by PP2 (Fig. 4C). The inhibition of sulfated proteoglycan accumulation and $PGE_2$ production by inhibition of p38 kinase with SB203580 is consistent with our previous data (14,20). Taken together, these results indicate that activity of Src kinase is required for COX-2 expression, and subsequent $PGE_2$ production induced by NO.

*Increased levels of caveolin-1 and COX-2 in cartilage explants culture.* To examine the in vivo significance of p-Caveolin and COX-2 expression in cartilage, explants of rabbit joint cartilage were cultured in the absence or presence of SNP. SNP treatment of cartilage explants caused increased p-Caveolin protein levels with a concomitant increase in COX-2 expression (Fig. 5A). We also examined the expression levels of p-Caveolin and COX-2 in human osteoarthritic and rheumatoid arthritic cartilage obtained from patients undergoing total knee arthroplasty. Relatively normal parts of the joint (*i.e.*, undamaged parts of the osteoarthritic joint) showed undetectable expression of p-Caveolin and COX-2 (Fig. 5B). However, expression of p-Caveolin and COX-2 was significantly increased in osteoarthritis-affected part of cartilage and rheumatoid arthritic cartilage. Taken together, these results suggest that the increased expression levels of p-Caveolin and COX-2 may contribute to the cartilage destruction during arthritic disease.

## Discussion

Our previous studies in primary articular chondrocytes indicated that NO-induced apoptosis and dedifferentiation of articular chondrocytes were regulated by opposite functions of mitogen-activated protein (MAP) kinase subtypes, extracellular signal-regulated protein kinase (ERK), and p38 kinase (10). NO-induced activation of ERK-1/-2 induces dedifferentiation with the inhibitory effects on apoptosis, whereas activation of p38 kinase induces apoptosis and is responsible for the maintenance of differentiated phenotype. In addition to MAP kinase signaling, NO production stimulates expression of inflammatory molecules such as COX-2 and $PGE_2$ (10,11). We also previously demonstrated that activation of caveolin-1 is necessary for NO-induced dedifferentiation, COX-2 expression, and $PGE_2$ production through p38 kinase activation (14). Volonte et al. (21), demonstrated that tyrosine phosphorylation of caveolin-1 on tyrosine 14 was mediated by the activation of p38 kinase and Src tyrosine kinase.

We characterized signaling pathways involved in NO-induced dedifferentiation and COX-2 expression in articular chondrocytes, focusing on the functional relationship between Src kinase and p38 kinase signaling. Here, we show that pSrc and p38 kinase activated by NO in a similar kinetic pattern. The addition of PP2 to SNP-treated chondrocytes inhibited the increase phosphorylation of p38 kinase and

p-Caveolin and slowed the inhibition of both type II collagen expression and proteoglycan synthesis. Similar to the pattern of type II collagen, COX-2 expression and $PGE_2$ production were also blocked in SNP-treated cells in the presence of PP2.

Several inflammatory mediators, including nitric oxide and PG, have been implicated in the disease process of osteo- and rheumatoid arthritis. PGs are produced by cytokine-stimulated chondrocytes in arthritis-affected cartilage via induction of COX-2 expression (8,22-25). Our current observation of increased expression of COX-2 in arthritis-affected chondrocytes is consistent with the reports by Amin et al. (8) and Jacques et al. (26), showing increased expression and activity of COX-2 in osteoarthritic cartilage. And also, we first demonstrated increased expression levels of p-Caveolin in arthritis-affected chondrocytes. Based on the observation that high levels of p-Caveolin in arthritic cartilage might be associated with increased COX-2 expression.

These data suggest that Src kinase activation is necessary to NO-induced dedifferentiation and COX-2 expression in articular chondrocytes through p38 kinase-dependent pathway.

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