

Celecoxib suppresses fibroblast proliferation and collagen expression by inhibiting ERK1/2 and SMAD2/3 phosphorylation

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Abstract. This study aimed to investigate whether celecoxib suppresses fibroblast proliferation and collagen expression by inhibiting extracellular signal-regulated kinase 1/2 (ERK1/2) and SMAD2/3 phosphorylation. Celecoxib was added to NIH/3T3 fibroblasts stimulated by fibroblast growth factor-2 (FGF-2) or transforming growth factor- β 1 (TGF- β 1). NIH/3T3 fibroblast proliferation and viability were assessed by MTT assays; ERK1/2 expression and SMAD2/3 expression were assessed by quantitative RT-PCR and Western blot analysis. The results indicated that celecoxib suppressed cell proliferation (IC_{50} FGF⁺ group, $75 \pm 1.9 \mu\text{mol/l}$) stimulated by FGF-2, and also inhibited cell viability (IC_{50} FGF⁻ group, $252 \pm 2.3 \mu\text{mol/l}$) by inhibiting ERK1/2 phosphorylation but not ERK1/2 expression. In addition, celecoxib treatment led to the apoptosis of NIH/3T3 fibroblasts (IC_{50} FGF⁻ group, $35 \pm 1.4 \mu\text{mol/l}$). Celecoxib also suppressed collagen expression (0.35-fold COL3 and 0.43-fold COL1 with $320 \mu\text{mol/l}$ celecoxib relative to the untreated group following stimulation for 3 h, $p < 0.01$) when stimulated by TGF- β 1, by inhibiting SMAD2/3 phosphorylation but not SMAD2/3 expression. Celecoxib is capable of inhibiting ERK1/2 and SMAD2/3 phosphorylation, which is responsible for NIH/3T3 fibroblast proliferation and collagen expression.

Introduction

Adhesions are a serious complication following trauma or surgery. Injury to the joint or tendon often causes restrictive fibrosis or adhesions, which severely restricts functional recovery (1,2). Adhesions occur following abdominal and pelvic surgery, often causing the serious complications of intestinal obstruction, chronic pain, and infertility in women (3).

Certain selective and nonselective cyclooxygenase-2 enzyme (COX-2) inhibitors have been shown to inhibit adhesion formation, but have rarely been applied clinically due to their limited effect (4-10). Celecoxib, a selective COX-2 inhibitor, has been shown to produce a maximal reduction in intra-abdominal adhesion formation in comparison to rofecoxib and nonselective COX-2 inhibitors, and therefore provides a promising therapy for all types of adhesions (11,12). Celecoxib has been demonstrated to inhibit of intra-abdominal adhesions through an antiangiogenic mechanism involving COX-2.

However, celecoxib is unique among the coxibs and traditional NSAIDs. Certain COX-2-independent mechanisms are thought to be involved in celecoxib's pharmacological effect. It is known that transforming growth factor- β 1 (TGF- β 1) and fibroblast growth factor-2 (FGF-2) play very significant roles in fibroblast proliferation and collagen expression. These processes lead to the formation of adhesions via extracellular signal-regulated kinase 1/2 (ERK1/2) and SMAD2/3 phosphorylation (13-16). It is not known whether celecoxib can suppress the proliferation and collagen expression of NIH/3T3 cells by inhibiting the phosphorylation of ERK1/2 and SMAD2/3 or by inhibiting ERK1/2 and SMAD2/3 expression. In the present study, we sought to investigate the above-described issues, and to delineate the mechanism by which celecoxib is implicated in preventing the formation of adhesions.

Materials and methods

Cell culture and stimulation. NIH/3T3 cells, which were purchased from Sigma-Aldrich (St. Louis, MO, USA), were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% calf serum (Gibco), 2 mM L-glutamine, penicillin, and streptomycin. The cells were seeded onto 90-mm tissue culture dishes and grown to 80% confluence, then used for ERK1/2 (Cell Signaling, USA), SMAD2/3 (Cell Signaling), collagen (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and cell proliferation assays.

Measurement of cell proliferation and viability by methylthiazolotetrazolium (MTT) assay. NIH/3T3 cells ($5 \times 10^4/\text{ml}$) were seeded in 96-well culture plates. NIH/3T3 cells, with and without stimulation by FGF-2, were treated with increasing

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Table I. PCR primers of target genes.

Target genes	PCR primers
ERK2	Sense: 5'-GGCGGGCCCCGAGATGGTC-3' Antisense: 5'-TGAAGCGCAGTAGGATTTTAT-3'
COL3	Sense: 5'-GGCGCCCCAGGAGAAAAG-3' Antisense: 5'-CAGGAGGACCAGGGCGACCAC-3'
COL1	Sense: 5'-GACCTCCGGCTCCTGCTCCTCTTA-3' Antisense: 5'-ACAGCACTCGCCCTCCCGTTTTTG-3'
β -actin	Sense: 5'-AGAGGGAAATCGTGCGTGAC-3' Antisense: 5'-CCATACCCAGGAAGGAAGGCT-3'

concentrations of celecoxib. After 48 h, these cells were harvested, washed and incubated with 10 μ l MTT solution for 4 h. Subsequently, 100 μ l dimethyl sulfoxide (DMSO) were added to dissolve the crystals. The plates were incubated at room temperature for 10 min prior to measurement of the absorbance at 570 nm. Each sample was assayed in triplicate and each experiment was repeated three times.

Quantitative RT-PCR. Total RNA was isolated from the NIH/3T3 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Isolated RNA was converted into cDNA using M-MLV reverse transcriptase (Takara, Japan). Quantitative RT-PCR was performed using the QuantiTect SYBR-Green RT-PCR kit (Qiagen, Germany) and a detection system (Cepheid, Sunnyvale, CA, USA). Quantitative RT-PCR was performed in a total reaction volume of 25 μ l, containing 12.5 μ l of QuantiTect SYBR-Green RT-PCR master mix, 3 μ l of the cDNA template and 0.2 μ M of each target-specific primer designed to amplify a section of each gene. The specific sense and antisense primers that were used are shown in Table I. Following PCR, a melting curve analysis was performed to demonstrate the specificity of each PCR product as a single peak. A control reaction containing all the components except for the template was included in all of the experiments. COL3 and COL1 gene expression levels were normalized to the corresponding gene expression levels of mouse β -actin.

Western blot analysis. NIH/3T3 cells were harvested and lysed with lysis buffer. The cell lysates were centrifuged at 13000 \times g for 15 min at 4°C, and the supernatants were collected for Western blot analysis. Equal amounts of protein were separated by SDS-PAGE using a 12% gel and electro-transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). Following blocking with 5% non-fat milk, the

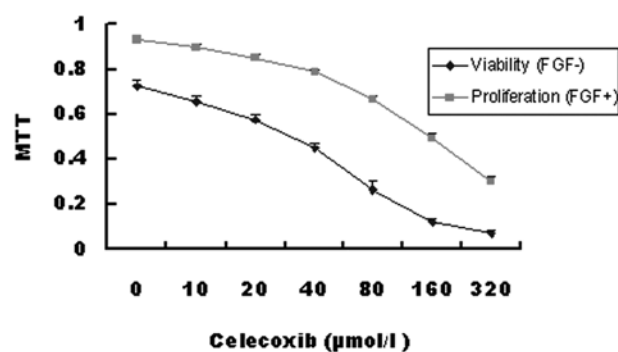


Figure 1. Cell proliferation was assessed by MTT assays following 48 h at various concentrations of celecoxib (0–320 μ mol/l) with (FGF⁺ group) or without (FGF⁻ group) FGF-2 stimulation. The FGF⁺ group was treated daily with 5 ng/ml FGF-2. The results were obtained from three independent experiments and the MTT results are expressed as the means \pm SD.

membranes were incubated with antibodies against COL3, COL1 (Santa Cruz), ERK1/2 and SMAD2/3 (Cell Signaling) for 1 h at room temperature. A monoclonal anti-GAPDH antibody (Cell Signaling) was used as the control. Following washing, the membranes were incubated with horseradish peroxidase-conjugated IgG (Acris Antibodies, Germany) for 1 h, and immunoreactive bands were detected by chemiluminescence (Amersham).

Statistical analysis. Data are shown as the means \pm standard deviation (SD). Statistical analysis was carried out by one-way analysis of variance (ANOVA) with values of $p < 0.05$ and $p < 0.01$ representing significance. Dose-response curves were fitted using nonlinear regression analysis and the IC_{50} of the inhibitors was calculated. Values of $p < 0.05$ were considered statistically significant. These statistical analyses were done using SigmaPlot and Graphic Pad software.

Results

Celecoxib inhibits NIH/3T3 fibroblast proliferation and viability. As shown in Fig. 1, the MTT assay revealed that FGF-2 significantly promoted NIH/3T3 fibroblast proliferation (1.27-fold, $p < 0.05$), but this proliferation was markedly inhibited by celecoxib treatment (IC_{50} FGF⁺ group, 75 ± 1.9 μ mol/l). Notably, celecoxib treatment also significantly inhibited NIH/3T3 fibroblast viability (IC_{50} FGF⁻ group, 252 ± 2.3 μ mol/l). The effects of celecoxib were dose-dependent, with greater inhibition at higher doses.

Celecoxib inhibits up-regulated p-ERK1/2 stimulated by FGF-2 in NIH/3T3 fibroblasts. As shown in Fig. 2, FGF-2 significantly increased p-ERK1/2 in NIH/3T3 fibroblasts (2.9-fold and 4.9-fold after 15 and 30 min, respectively, $p < 0.01$), but this activation was markedly inhibited by celecoxib treatment (0.3-fold with 320 μ mol/l celecoxib and 0.57-fold with 40 μ mol/l celecoxib relative to the untreated group after TGF- β 1 stimulation for 30 min, $p < 0.01$). Notably, the basal p-ERK1/2 level was not affected by celecoxib. In addition, neither FGF-2 nor celecoxib affected ERK1/2 expression.

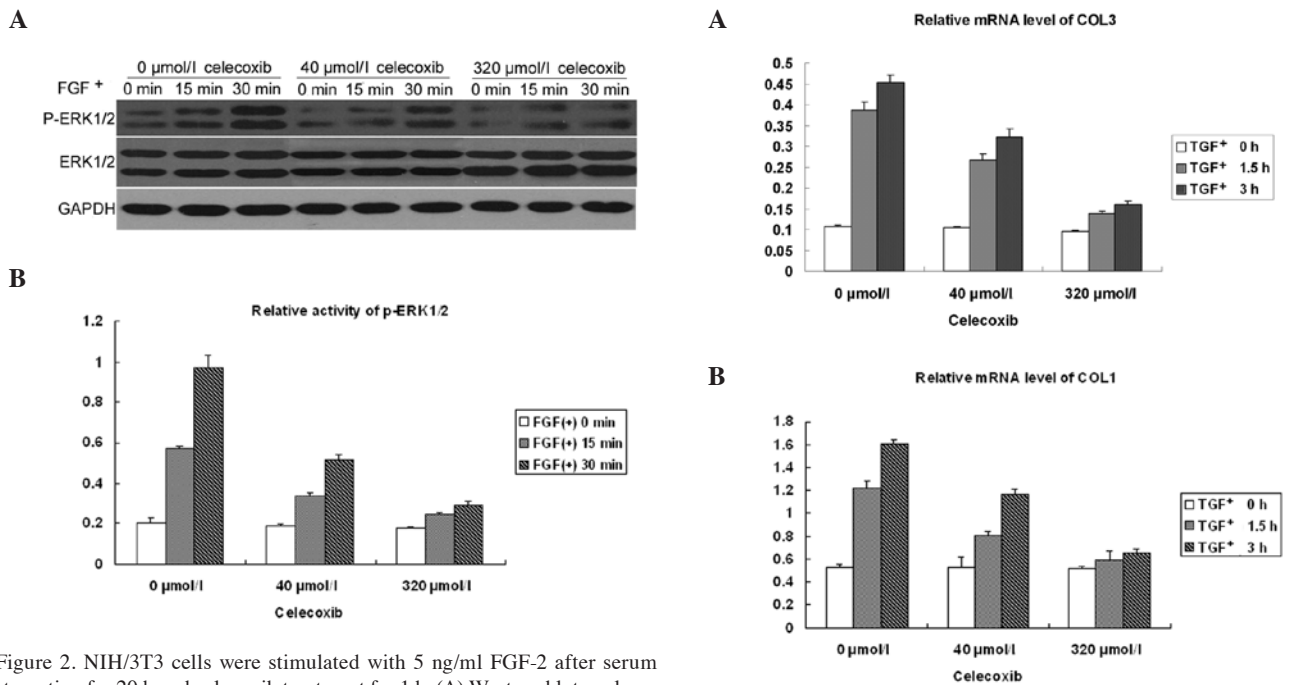


Figure 2. NIH/3T3 cells were stimulated with 5 ng/ml FGF-2 after serum starvation for 20 h and celecoxib treatment for 1 h. (A) Western blot analyses were performed to show the effects of various concentrations of celecoxib. The results were obtained from three independent experiments. (B) The relative activity of p-ERK1/2 is expressed as the mean \pm SD.

Celecoxib inhibits collagen expression of NIH/3T3 fibroblasts stimulated by TGF- β 1. As shown in Fig. 3, TGF- β 1 significantly increased COL3 and COL1 expression in NIH/3T3 fibroblasts (3.55-fold COL3 and 4.18-fold COL1 after 1.5 h and 3 h, respectively, $p < 0.01$; 1.2-fold COL1 and 3.08-fold COL3 after 1.5 h and 3 h, respectively, $p < 0.01$). However, this promotion was markedly inhibited by celecoxib treatment (0.35-fold COL3 with 320 $\mu\text{mol/l}$ celecoxib and 0.72-fold COL1 with 40 $\mu\text{mol/l}$ celecoxib relative to the untreated group after TGF- β 1 stimulation for 3 h, $p < 0.01$; 0.43-fold COL1 with 320 $\mu\text{mol/l}$ celecoxib and 0.74-fold COL1 with 40 $\mu\text{mol/l}$ celecoxib relative to the untreated group after TGF- β 1 stimulation for 30 min, $p < 0.01$). Celecoxib treatment did not inhibit basal COL3 and COL1 expression.

Celecoxib inhibits up-regulated p-SMAD2/3 stimulated by TGF- β 1 in NIH/3T3 fibroblasts. As shown in Fig. 4, TGF- β 1 significantly increased p-SMAD2/3 in NIH/3T3 fibroblasts (1.93-fold and 3-fold after 15 and 30 min, respectively, $p < 0.01$). However, this activation was markedly inhibited by celecoxib treatment (0.42-fold with 320 $\mu\text{mol/l}$ celecoxib and 0.63-fold with 40 $\mu\text{mol/l}$ celecoxib relative to the untreated group after TGF- β 1 stimulation for 30 min, $p < 0.01$). Notably, SMAD2/3 expression of the NIH/3T3 fibroblasts was not affected by TGF- β 1 and celecoxib treatment. In addition, basal p-SMAD2/3 levels were not affected by celecoxib.

Discussion

Formation of all types of adhesions are dependent on angiogenesis (17,18). Fibroblasts from these adhesions express the COX-2 enzyme while fibroblasts in non-adhesion-bearing areas do not express this enzyme (19). In addition, there is

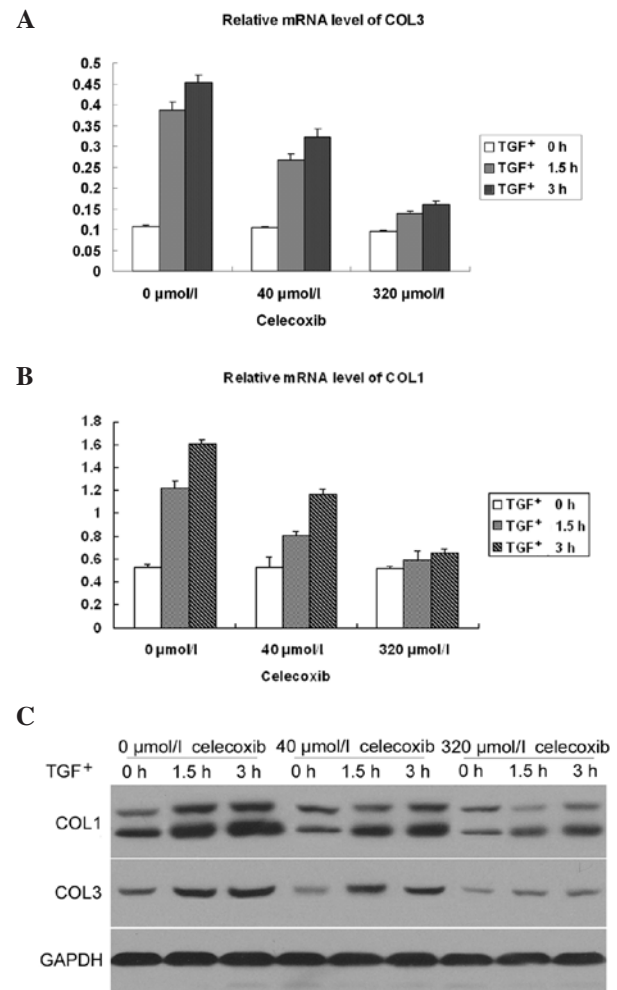


Figure 3. NIH/3T3 cells were stimulated with 1 ng/ml TGF- β 1 after serum starvation for 20 h and celecoxib treatment for 1 h. (A,B) Quantitative RT-PCR and (C) Western blot analyses were performed to show the effects of the various concentrations of celecoxib. The results were obtained from three independent experiments, and the relative COL3 and COL1 mRNA levels are expressed as the means \pm SD.

experimental evidence that COX-2-produced prostaglandins may modulate FGF and vascular endothelial growth factor-induced angiogenesis (20). Thus, the COX-2 inhibitor, celecoxib, may selectively inhibit angiogenesis associated with newly-forming adhesions through a COX-2 mechanism (11). However, previous studies have suggested that, although COX-2 may play an active role in one or more biological responses during cutaneous wound healing, these enzymes are not essential, possibly due to the presence of other additional pathways with compensatory mechanisms (21). In view of the fact that an adhesion is understood to be an aberrant form of the normal tissue healing process (17), specific additional pathways with compensatory mechanisms could also exist in adhesion formation. It is possible that, apart from its effect on COX-2, celecoxib may inhibit adhesion formation through other mechanisms.

Certain studies have shown that TGF- β 1 and FGF-2 play very crucial roles in adhesion formation (3,4). TGF- β 1 and FGF-2 promote collagen expression and fibroblast proliferation through the activation of SMAD2/3 and ERK1/2, leading to adhesion formation. Thus SMAD2/3 and ERK1/2 represent

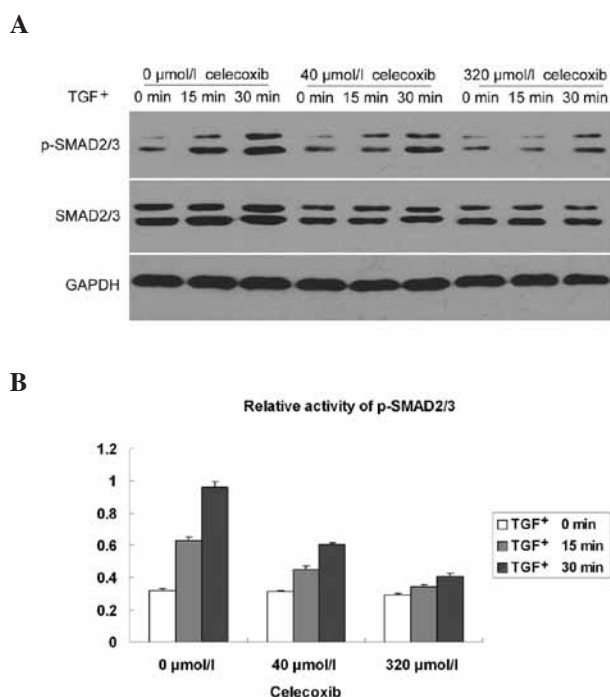


Figure 4. NIH/3T3 cells were stimulated with 1 ng/ml TGF- β 1 after serum starvation for 20 h and celecoxib treatment for 1 h. (A) Western blot analyses were performed to show the effects of various concentrations of celecoxib. The results were obtained from three independent experiments. (B) The relative activity of p-SMAD2/3 is expressed as the mean \pm SD.

possible therapeutic targets for preventing adhesion formation. However, regretfully, there is no SMAD2/3 or ERK1/2 inhibitor approved for clinical use. The results of the present study support the hypothesis that, apart from being a selective COX-2 inhibitor, celecoxib is also an inhibitor of ERK1/2 and SMAD2/3 phosphorylation, suppressing fibroblast proliferation and collagen expression. Celecoxib has various roles in ERK1/2 activation in various cells. For example, in human non-small cell lung cancer cells, treatment with celecoxib alone had no effect (22). However, in human hepatic stellate cells (HSCs), celecoxib significantly attenuated ERK1/2 activation. In addition, celecoxib up-regulated p-ERK1/2 in COX-2-negative human ovarian cancer cells, but not in COX-2-positive human ovarian cancer cells (23). In the present study, celecoxib inhibited up-regulated p-ERK1/2 stimulated by FGF-2 in NIH/3T3 fibroblasts. It is known that celecoxib can inhibit the proliferation of most types of cells, including endometrial stromal cells, retinal pigment epithelial cells and oral squamous cancer cells (24-26). However, COX-2 inhibition is neither necessary nor sufficient for celecoxib to suppress tumor cell proliferation and focus formation *in vitro* (27). The present study also shows the growth inhibition of NIH/3T3 fibroblasts treated with celecoxib. In view of the key role of ERK1/2 in mitogen-activated cell proliferation, celecoxib was considered to reduce NIH/3T3 fibroblast proliferation by inhibiting the up-regulation of p-ERK1/2. Celecoxib also exerted a different effect on collagen expression in various types of cells. For example, celecoxib inhibited COL1 mRNA and protein production in HSCs (28), but the expression of COL1 and COL3 remained unchanged in tendon cells treated

with celecoxib (29). As in HSCs, celecoxib also inhibited COL1 and COL3 protein production in NIH/3T3 fibroblasts in the present study. Notably, this study also demonstrates that celecoxib can inhibit up-regulated p-SMAD2/3 stimulated by TGF- β 1 in NIH/3T3 fibroblasts, which contributes to collagen expression. Thus, celecoxib inhibits COL1 and COL3 protein production in NIH/3T3 fibroblasts by inhibiting the up-regulation of p-SMAD2/3. However, celecoxib had little effect on the basal ERK1/2, p-ERK1/2, SMAD2/3 and p-SMAD2/3 expression, and so had no influence on normal cells, which would account, at least in part, for its low cytotoxicity. Certain studies have indicated that ERK1/2 plays a synergistic role in SMAD2/3-mediated collagen synthesis initiated by TGF- β 1 in mesangial cells (16). Previously, we also demonstrated that siRNA-mediated ablation of one of the ERK1/2 isoforms, ERK2, could markedly inhibit the COL1 and COL3 synthesis stimulated by TGF- β 1 in NIH/3T3 cells (15). Thus, in the present study, inhibition of stimulated collagen synthesis was also partly a result of the effect of celecoxib in inhibiting p-ERK1/2 stimulated by TGF- β 1.

Collagen content is known to be a major factor in determining the mechanical properties of the tissue in the early stage of tissue repair (30,31). In this study, COL3 and COL1 were used as an indicator of fibrogenic activity as they, particularly COL1, are the main collagens in the formation of adhesions. However, the extent of adhesions was thought to be a consequence of fibroblast proliferation and angiogenicity. Celecoxib may inhibit adhesion formation by these three processes, and thus has a significant pharmacological effect in reducing formation of adhesions. These pharmacological properties of celecoxib were confirmed in the reduction of scar tissue formation, which is an abnormal tissue healing process, along with fibroblast proliferation and collagen deposition (32). In addition, celecoxib may also delay chronic gastric ulcer and esophageal ulcer healing, and delay ileal anastomosis (33-35). Traditional views consider that COX-2 inhibition by celecoxib plays a significant role in these processes. In fact, inhibition of ERK and SMAD by celecoxib is also implicated in its pharmacological effect. However, a selective COX-2 inhibitor, SC-791, did not retard normal wound healing without disrupting re-epithelialization or decreasing collagen deposition and tensile strength (21). A possible reason is that SC-791 is not an ERK and SMAD inhibitor, and that the inhibitory effect on COX-2 was compromised by compensated ERK and SMAD activation. Future studies are required in order to further elucidate the mechanisms of action of celecoxib on these various processes.

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