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## EP<sub>4</sub> receptor regulates collagen type-I, MMP-1, and MMP-3 gene expression in human tendon fibroblasts in response to IL-1 $\beta$ treatment

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### Abstract

Tendinopathy is accompanied by inflammation, tendon matrix degradation, or both. Inflammatory cytokine IL-1 $\beta$ , which is a potent inflammatory mediator, is likely present within the tendon. The purpose of this study was to determine the biological impact of IL-1 $\beta$  on tendon fibroblasts by assessing the expression of cPLA<sub>2</sub>, COX-2, PGE<sub>2</sub> and its receptors (EP), collagen type-I, and MMPs. We also studied the role of the p38 MAPK pathway in IL-1 $\beta$ -induced catabolic effects. We found that IL-1 $\beta$  increased the intracellular levels of cPLA<sub>2</sub> and COX-2, and also increased secretion of PGE<sub>2</sub>. Induction of MMPs, such as MMP-1 and MMP-3 at the mRNA level, was also observed after stimulation with IL-1 $\beta$ . Furthermore, the presence of IL-1 $\beta$  significantly decreased the level of collagen type-I mRNA in tendon fibroblasts. These effects were found to be mediated by selective upregulation of EP<sub>4</sub> receptor, which is a member of G-protein-coupled receptor that transduces the PGE<sub>2</sub> signal. Blocking EP<sub>4</sub> receptor by a specific chemical inhibitor abolished IL-1 $\beta$ -induced catabolic effects. These results suggest that IL-1 $\beta$ -induced catabolic action on tendon fibroblasts occurs via the upregulation of two key inflammatory mediators, cPLA<sub>2</sub> and COX-2, which are responsible for the synthesis of PGE<sub>2</sub>. IL-1 $\beta$  further stimulates the expression of EP<sub>4</sub> receptor, suggesting positive feedback regulation which may lead to accelerated catabolic processes in tendon fibroblasts. Studies using pathway-specific chemical inhibitors suggest that the p38 MAPK pathway is the key signaling cascade transducing IL-1 $\beta$ -mediated catabolic effects. Collectively, our findings suggest that the EP<sub>4</sub> receptor mediates the IL-1 $\beta$ -induced catabolic metabolism via the p38 MAPK pathway in human tendon fibroblasts and may play a major role in the tendon's degenerative changes often seen in the later stages of tendinopathy.

### Keywords

tendon fibroblasts; interleukin-1 $\beta$ ; prostaglandin E<sub>2</sub>; collagen; MMPs; EP receptors

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## 1. Introduction

Tendinopathy is a collective term that refers to a group of tendon disorders that commonly occur in both occupational and athletic settings (Almekinders and Temple, 1998). It is clinically associated with manifestation of pain and inflammation, greatly debilitating the workforce. Although the etiology of tendinopathy is considered multi-factorial, repetitive mechanical loading that causes tendon injuries is one of the major causative factors (Almekinders, 1998). Tendinopathy is pathologically characterized by tendon inflammation and/or degeneration (Riley, 2004). The anti-inflammatory drugs that have been prescribed over the years treat only the symptoms, not the root cause of the disease (Wang et al., 2006). Moreover, various causative factors for tendon degeneration that have not been fully characterized may precede tendon rupture (Riley, 2004). Therefore, it is essential to understand the cellular and molecular mechanisms of tendon degeneration during its early stages for the effective treatment of tendinopathy.

Tendon matrix is rich in collagens, such as collagen type-I (Wang, 2005). It is known that matrix remodeling is enabled by the balance of matrix metalloproteinases (MMPs) and their inhibitors, such as tissue inhibitors of metalloproteinases (TIMPs) (Kjaer, 2004). Imbalance in the expression of MMPs and their inhibitors can contribute to tendon matrix degradation, as collagen degradation is initiated by MMPs. It is proposed that prior to tendon matrix degradation, subtle changes involving the release of inflammatory cytokines such as IL-1 by infiltrating macrophages/monocytes may occur (Tsuzaki et al., 2003a). In addition, tendons *in vivo* are subjected to repetitive motion which may cause microinjuries, which is sufficient to induce endogenous IL-1 $\beta$  (Tsuzaki et al., 2003b). IL-1 $\beta$ , a potent inflammatory cytokine, has been reported to be present in the synovium of inflamed rotator cuffs, which may enhance inflammatory intensity at the site of injury (Gotoh et al., 2000; Gotoh et al., 2002). IL-1 $\beta$  acts through its specific receptor, which is shown to be present in tendon fibroblasts, the occupation of which can activate numerous signaling pathways, including MAPKs (Bankers-Fulbright et al., 1996; Tsuzaki et al., 2003b).

As in other connective tissue injuries, tendon injuries are likely accompanied by upregulation of IL-1 $\beta$ . *In vitro* studies have shown that IL-1 $\beta$  can induce inflammatory mediators such as COX-2, PGE<sub>2</sub>, and the matrix-degrading enzymes MMPs, which are all involved in tendon matrix degradation (Archambault et al., 2002; Tsuzaki et al., 2003b). Although some of these effects are suggested to be modulated through MAPK pathways in other fibroblasts (Brauchle et al., 2000; Kida et al., 2005; Saklatvala et al., 1999), the extent to which MAPKs are involved in inflammatory pathway regulation in tendon fibroblasts is not well understood. The three well-characterized subfamilies of MAPKs (ERK1/2, JNKs, and p38 MAPKs) are, however, implicated in the expression and activation of pro-inflammatory molecules such as COX-2 (Kyriakis and Avruch, 2001). Therefore, the intracellular signaling mechanisms triggered by IL-1 $\beta$ , as well as this pro-inflammatory cytokine-generated signaling pathway responsible for PGE<sub>2</sub> production, need to be explored in tendon fibroblasts.

PGE<sub>2</sub> has been identified as a central lipid mediator in inflammation and pain in many tissues, including tendons (Almekinders and Temple, 1998; Rocha et al., 2003). It is an arachidonic acid (AA) metabolite released from membrane phospholipids via activation of cPLA<sub>2</sub> and COX-2. Both *in vivo* and *in vitro* experimental models of tendinopathy have provided evidence suggesting that production of PGE<sub>2</sub> may be an important step in the development of tendinopathy. For example, in rabbit tendons, repeated exposure to PGE<sub>2</sub> results in localized tendon degeneration (Khan et al., 2005); in human tendon fibroblasts, repetitive mechanical loading elevates the production of PGE<sub>2</sub> (Khan et al., 2005; Li et al., 2004; Wang et al., 2003b; Wang et al., 2004). Furthermore, the major catabolic functions of PGE<sub>2</sub> in connective tissues are inhibition of collagen type-I synthesis and induction of MMPs (Fall et al., 1994;

Kim et al., 2005; Ruwanpura et al., 2004; Varga et al., 1987). In tendon fibroblasts, for instance, exogenous PGE<sub>2</sub> decreases collagen type-I production and upregulates MMP-1 and -3 gene and protein expression (Cilli et al., 2004; Thampatty et al., 2006).

PGE<sub>2</sub> acts through G-protein-coupled receptors called EP receptors, which are classified into four subtypes, EP<sub>1</sub> through EP<sub>4</sub> (Narumiya et al., 1999). Each of these receptors has unique signal transduction mechanisms as a result of coupling to different G proteins. EP<sub>1</sub> receptor stimulates intracellular calcium while EP<sub>2</sub> and EP<sub>4</sub> receptors activate adenylate cyclase which results in elevation of intracellular cAMP levels. EP<sub>3</sub> receptor has variants that mediate multiple signaling pathways (Negishi et al., 1995). PGE<sub>2</sub>-induced catabolic effects in fibroblasts are shown to be mediated via upregulation of one of its receptors mentioned previously. For instance, activation of EP<sub>2</sub> receptor has been shown to reduce collagen type-I mRNA expression in lung fibroblasts (Choung et al., 1998). Also, PGE<sub>2</sub> is unable to suppress proliferation and collagen synthesis in lung fibroblasts from EP<sub>2</sub>-deficient mice despite the expression of other EP receptors (Moore et al., 2005). Furthermore, IL-1 $\beta$ -induced MMP-3 production is upregulated via EP<sub>1</sub> receptors in cells from periodontally-diseased tissue (Ruwanpura et al., 2004). However, little is presently known about the expression and regulation of EP receptors in human tendon fibroblasts.

The aims of the current study were to show that 1) exogenous IL-1 $\beta$  induces inflammatory mediators such as cPLA<sub>2</sub>, COX-2, and PGE<sub>2</sub> via its specific functional receptor; 2) IL-1 $\beta$ -induced PGE<sub>2</sub> (i.e. endogenous PGE<sub>2</sub>) mediates catabolic effects by modulating the target gene expression of the PGE<sub>2</sub>-mediated signaling pathway such as downregulation of collagen type-I and upregulation of MMPs; and 3) this catabolic effect of IL-1 $\beta$  is mediated via the p38 MAPK signaling cascade as a key mechanistic molecule in human tendon fibroblasts. To test these hypotheses, a novel cell culture model was used. In this culture model, human tendon fibroblasts were grown in microgrooved culture surfaces, as opposed to the smooth culture surface, to allow the fibroblasts to mimic the alignment and shape of these cells *in vivo* (Wang et al., 2003a). We report here that IL-1 $\beta$  increased the protein levels of cPLA<sub>2</sub>, COX-2, and PGE<sub>2</sub> expressed by human tendon fibroblasts. The accelerated production of PGE<sub>2</sub> appeared to occur via its functional receptor EP<sub>4</sub>, which is selectively upregulated in the presence of IL-1 $\beta$  playing a role as a positive feedback regulator. These upregulation of inflammatory mediators by IL-1 $\beta$  were, at least in part, triggered by the activation of the p38 MAPK signaling pathway. We also found significant induction of MMPs and reduction of collagen type-I mRNA after stimulation with IL-1 $\beta$  in human tendon fibroblasts.

## 2. Materials and methods

### 2.1. Fibroblast culture

Human patellar tendon fibroblasts (HPTFs) were isolated and maintained from tendon samples of two healthy male donors as previously described (Thampatty et al., 2006; Yang et al., 2005). The protocol for obtaining tendon samples was approved by the Institutional Review Board of the University of Pittsburgh Medical Center (IRB #0407060). Briefly, washed and minced tendon samples were transferred to 100 mm polystyrene Petri dishes and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 50U/mL penicillin, and 50U/mL streptomycin (P/S; Life Technologies, Rockville, MD). The cultures were maintained in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C and subcultured up to six times without change in cell morphology and doubling time.

### 2.2. Culture experiments

HPTFs were plated on culture surfaces in custom-made silicone dishes (culture area 3 cm x 6 cm) which were transparent, elastic, and nontoxic to cultured cells (Wang and Grood, 2000).

A special feature of the dish was that the culture surface was fabricated with microgrooves of width 10  $\mu\text{m}$  and depth 3  $\mu\text{m}$ , with 10  $\mu\text{m}$  wide ridges between the grooves (Fig. 1). To promote cell attachment, the microgrooved surfaces of the dishes were pre-coated with 10  $\mu\text{g/ml}$  ProNectin-F (Sigma, St Louis, MO).

Next,  $2 \times 10^5$  HPTFs were plated in each silicone dish and grown in DMEM supplemented with 10% FBS and 1% P/S. After 24 hrs, the growth medium was replaced by DMEM supplemented with 1% serum. The fibroblasts were then treated with human recombinant IL-1 $\beta$  (Chemicon International Inc., Temecula, CA) at 0.01, 0.1, and 1 ng/ml doses. Cells without IL-1 $\beta$  treatment served as controls. Human recombinant IL-1 receptor antagonist (IL-1ra) (R&D Systems, Minneapolis, MN) at concentrations of 10 and 100 ng/ml, p38 MAPK inhibitor, SB203580 (Calbiochem, La Jolla, CA) at concentrations of 1 and 10  $\mu\text{M}$ , and EP<sub>4</sub> receptor antagonist AH23848 (Sigma, St. Louis, MO) at concentrations of 0.1, 1 and 10  $\mu\text{M}$  were added wherever appropriate 30 min prior to treating cells with IL-1 $\beta$ . The culture media were sampled after incubation for 24 hrs to measure PGE<sub>2</sub> levels, and cell lysates were collected to obtain total cellular protein for Western blot analysis and total RNA for RT-PCR.

### 2.3. Western blot analysis

Standard Western blotting technique was used to determine cPLA<sub>2</sub> and COX-2 protein expression levels as described previously (Yang et al., 2004). Briefly, equal amounts of the denatured cellular proteins were fractionated by electrophoresis on 10% SDS-polyacrylamide gels, and the separated proteins were then transferred to nitrocellulose membranes. After blocking for non-specific binding, the blots were incubated for 1 hr with monoclonal antibody of anti-cPLA<sub>2</sub> (Cell Signaling, Beverly, Massachusetts) followed by 1 hr incubation with goat anti-rabbit IgG (HRP-linked Antibody, Cell Signaling, Beverly, Massachusetts). After washing, the proteins on the membranes were then detected with the ECL Plus detection system (Amersham Pharmacia Biotech, Piscataway, New Jersey) according to the manufacturer's protocol. The membranes were stripped and probed with antibody of anti-COX-2 (Cayman Chemical, Ann Arbor, Michigan) for 1 hr followed by incubation with goat anti-mouse IgG (Jackson Immunoresearch Lab, Inc., West Grove, PA) for 1 hr and the proteins were detected as described earlier. The membranes were probed with GAPDH antibody (Biogenesis Inc, Kingston, New Hampshire) for an internal control of equal protein loading.

### 2.4. PGE<sub>2</sub> Assay

PGE<sub>2</sub> levels in the media were assayed using a commercially available ELISA kit (Assay Designs, Ann Arbor, Michigan) following the manufacturer's protocol. This assay is based on the competitive binding technique in which PGE<sub>2</sub> present in the sample competes with a fixed amount of alkaline phosphatase-labeled PGE<sub>2</sub> for sites on a mouse monoclonal antibody. The amount of PGE<sub>2</sub> in each sample was determined using a standard curve generated from known amounts of PGE<sub>2</sub> ranging from 39–2500 pg/ml. The sensitivity of the assay was 13.4 pg/ml.

### 2.5. RT-PCR

Reverse transcription (RT) was carried out with 1  $\mu\text{g}$  of total cellular RNA using the ThermoScript RT-PCR System (Invitrogen) for first strand cDNA synthesis in 20  $\mu\text{L}$  of reaction volume. The primer sequences and optimized annealing temperature for each primer set are provided in Table 1. For all experiments, the conditions were determined to be in the linear range for the PCR amplification. Briefly, all samples were subjected to RT, and subsequent amplification of the cDNA samples was performed by PCR at the same time. The cDNA samples were then assessed for GAPDH expression. Genomic DNA was included for the PCR to ensure that there was no genomic DNA contamination in the total RNA samples. The cDNA was amplified by PCR using 28 cycles at 95°C for 30 seconds, 55–60°C for 30 seconds, and 72°C for 30 seconds in the presence of *Taq* polymerase (Invitrogen) and 50 pmol

of sense and antisense primers. PCR products were resolved on 1.5% agarose gels by electrophoresis and visualized by staining with ethidium bromide and UV transillumination.

### 3. Results

#### 3.1. IL-1 $\beta$ induced cPLA<sub>2</sub> and COX-2 protein expression

The effect of IL-1 $\beta$  at 0.01, 0.1, and 1 ng/ml on cPLA<sub>2</sub> and COX-2 protein expression was determined (Fig. 2). The control shows basal expression of cPLA<sub>2</sub> suggesting that tendon cells constitutively express cPLA<sub>2</sub>. At 0.01 ng/ml concentration of IL-1 $\beta$ , cPLA<sub>2</sub> expression markedly increased, and higher dosages (0.1 and 1 ng/ml) further augmented the intracellular protein expression. This augmentation of cPLA<sub>2</sub> by IL-1 $\beta$  was significantly reduced by co-incubation with IL-1ra, although IL-1ra alone did not affect cPLA<sub>2</sub> protein expression (Fig. 2).

Unlike expression of cPLA<sub>2</sub>, basal protein level of COX-2 in tendon cells was not detectable. Stimulation of cells with IL-1 $\beta$  increased COX-2 protein level in a dose-dependent manner. Specifically, IL-1 $\beta$  at 0.01 ng/ml slightly increased COX-2 protein expression, but both 0.1 ng/ml and 1 ng/ml IL-1 $\beta$  markedly increased it (Fig. 2). Again, co-incubation with IL-1ra completely inhibited the IL-1 $\beta$ -mediated stimulation of COX-2 production. Collectively, these data suggest an IL-1 $\beta$ -specific cellular response to regulate the expression of inflammatory mediators such as COX-2 in human tendon fibroblasts.

#### 3.2. IL-1 $\beta$ induced PGE<sub>2</sub> production

Activation cPLA<sub>2</sub> and COX-2 can elevate PGE<sub>2</sub> production (Funk, 2001). Therefore, we examined the effect of IL-1 $\beta$  on PGE<sub>2</sub> production in tendon fibroblasts. Induction of cPLA<sub>2</sub> and COX-2 protein expression was accompanied by an increase in PGE<sub>2</sub> release. For example, treatment of tendon fibroblasts with 0.1 ng/ml IL-1 $\beta$  for 24 hrs resulted in only a slight induction of PGE<sub>2</sub>, but 1 ng/ml IL-1 $\beta$  induced a phenomenal increase in PGE<sub>2</sub> production (Fig. 3). IL-1ra at 100 ng/ml completely inhibited IL-1 $\beta$ -induced PGE<sub>2</sub> production, again suggesting IL-1 $\beta$ -specific cellular response to regulate PGE<sub>2</sub> expression in human tendon fibroblasts.

#### 3.3. p38 MAPK inhibitor abolished IL-1 $\beta$ -induced cPLA<sub>2</sub> and COX-2 protein expression and PGE<sub>2</sub> production

Stimulation of cells with IL-1 $\beta$  significantly increased cPLA<sub>2</sub> protein level (Fig. 4), and this upregulation was abrogated in the presence of a pathway-specific chemical inhibitor of p38 MAPK, SB203580 (Fig. 4). SB203580 with concentration of 1  $\mu$ M was sufficient to reduce the induction of cPLA<sub>2</sub> by IL-1 $\beta$ . Similarly, IL-1 $\beta$ -stimulation of COX-2 protein was inhibited by SB203580 (Fig. 4). In addition, PGE<sub>2</sub> production induced by IL-1 $\beta$  at 0.1 and 1 ng/ml was also inhibited by 10  $\mu$ M SB203580 (Fig. 5). These results suggest the involvement of the p38 MAPK signaling pathway in IL-1 $\beta$ -induced cPLA<sub>2</sub> and COX-2 protein expression and PGE<sub>2</sub> production.

#### 3.4. IL-1 $\beta$ modulated the expression of collagen type-I, MMP-1, and MMP-3 at the level of mRNA via specific upregulation of EP<sub>4</sub> receptor expression

We determined the effect of treating tendon fibroblasts with 1 ng/ml IL-1 $\beta$  for 24 hrs on collagen type-I at the mRNA level. There was substantial downregulation of collagen type-I expression after 24 hrs of treatment (Fig. 6). On the other hand, collagen type-III, which is a minor component of tendon matrix, did not show any change in its expression after IL-1 $\beta$  treatment (data not shown), suggesting that the IL-1 $\beta$ -mediated pathway mainly decreases the expression of collagen type-I as a target gene in human tendon fibroblasts.



We also determined the effect of IL-1 $\beta$  on MMP-1 and -3 at the mRNA levels. IL-1 $\beta$  at 1 ng/ml significantly increased the gene expression of both MMP-1 and MMP-3 after 24 hrs of treatment (Fig. 6). Collectively, our data suggest that IL-1 $\beta$  exerts catabolic effects in tendon fibroblasts via upregulating MMP-1 and -3 and downregulating collagen type-I mRNA expression.

In order to determine whether IL-1 $\beta$  regulates EP receptor expression, we examined the expression patterns of EP receptors after IL-1 $\beta$  treatment. Initially, we tested if human tendon fibroblasts express EP receptors and if so, whether the presence of IL-1 $\beta$  affects the expression of these EP receptors (EP<sub>1</sub> to EP<sub>4</sub>). The expression of EP<sub>3</sub> was not detectable in human tendon fibroblasts, whereas the expression of EP<sub>1</sub>, EP<sub>2</sub>, and EP<sub>4</sub> was observed (data not shown). The most notable change in terms of differential expression among EP receptors was EP<sub>4</sub> receptor. After 24 hrs of treatment with IL-1 $\beta$  at 1 ng/ml, EP<sub>4</sub> receptor mRNA level was considerably higher in treated cells than in control cells (Fig. 6). This stimulatory effect by IL-1 $\beta$  was not observed in other EP receptors, EP<sub>1</sub> and EP<sub>2</sub>, suggesting a specific upregulation of EP<sub>4</sub> receptor by IL-1 $\beta$  in human tendon fibroblasts (data not shown).

Since we observed specific upregulation of EP<sub>4</sub> receptor expression by IL-1 $\beta$  treatment, we determined the effect of blocking EP<sub>4</sub> receptor using a specific EP<sub>4</sub> receptor antagonist, AH23848 (Coleman et al., 1994). The effect of various concentrations of the antagonist on the expression of collagen type-I, EP<sub>4</sub>, MMP-1, and MMP-3 at the mRNA level was tested with and without IL-1 $\beta$  treatment. The stimulatory or suppressive biological effects induced by IL-1 $\beta$  were inhibited by pretreatment with AH23848 in a dose-dependent manner (Fig. 6, lanes 6–8). These results suggest that the antagonist could effectively block the catabolic action induced by IL-1 $\beta$ .

#### 4. Discussion

A novel culture model was used to investigate the inflammatory responses of HPTFs to IL-1 $\beta$  treatment. Tendon fibroblasts in this culture model aligned parallel to each other and had an elongated shape similar to that of the cells *in vivo*. Moreover, treatment of the tendon fibroblasts with IL-1 $\beta$  did not change their morphology and did not cause cell death, suggesting that IL-1 $\beta$  at the concentrations used in this study likely did not elicit toxic effects. Since cell organization and shape influence cell function (Chen et al., 1998; Wang et al., 2003a), this culture model system may more accurately reflect the inflammatory responses of human tendon fibroblasts to IL-1 $\beta$ , which is the potent inflammatory cytokine produced due to tendon microinjuries resulting from repetitive mechanical loading of tendons *in vivo*.

With such a novel culture model, this study showed that IL-1 $\beta$  induced significant, dose-dependent upregulation of cPLA<sub>2</sub> protein expression. IL-1 $\beta$  also increased intracellular production levels of COX-2 and PGE<sub>2</sub> relative to untreated cells. These IL-1 $\beta$ -mediated cellular responses were inhibited by co-incubation with IL-1ra, suggesting the specificity of IL-1 $\beta$  in its inflammatory actions mediated via its biologically functional receptor. Therefore, IL-1ra may offer protection of tendon cells against IL-1 $\beta$ -mediated inflammatory insult. Recently, we and others have shown that IL-1 $\beta$  induces dose-dependent increase in COX-2 expression at the mRNA level in human tendon fibroblasts (Tsuzaki et al., 2003b; Yang et al., 2005). Current results further confirm our hypothesis that IL-1 $\beta$  induces the inflammatory signaling pathway in tendon fibroblasts by upregulation of inflammatory modulators, including cPLA<sub>2</sub>, COX-2, and PGE<sub>2</sub> at both mRNA and protein levels via specific functional IL-1 $\beta$  receptor. Previously, IL-1 $\beta$  has been shown to induce cPLA<sub>2</sub>, COX-2, and PGE<sub>2</sub> in various other cell types such as gingival fibroblasts, neuroblastoma, and neuroglioma cells, suggesting an IL-1 $\beta$ -mediated inflammatory process (Fiebich et al., 2000; Kida et al., 2005; Moolwaney and Igwe, 2005). Our studies using human tendon fibroblasts show results consistent with these

previous reports using other tissue/cell types. IL-1 $\beta$  has also been shown to enhance the expression of prostaglandin E synthase (mPGES), which catalyzes the final step in the conversion of PGH<sub>2</sub> to PGE<sub>2</sub> in rheumatoid synovial fibroblasts (Kojima et al., 2003).

cPLA<sub>2</sub> is a key enzyme that plays an important role in the inflammation process, supplying AA for cellular production of inflammatory eicosanoids such as prostaglandins and leukotrienes. Furthermore, cPLA<sub>2</sub> itself participates in tissue inflammation. For example, intratracheal, intradermal, and intra-articular injection of cPLA<sub>2</sub> in rabbits induces profound inflammatory lesions (Gonzalez-Buritica et al., 1989). Current study in human tendon cells also shows that concomitant with the elevated expression of cPLA<sub>2</sub>, its downstream inflammatory mediators, COX-2 and PGE<sub>2</sub> are increased in their levels. COX-2 has been recognized as playing an important role in tissue inflammation. For example, in a collagen-induced arthritis model, mice deficient in COX-2 exhibited a significant reduction in synovial inflammation and joint destruction (Loftin et al., 2002; Myers et al., 2000).

In addition to the inflammatory role of cPLA<sub>2</sub> and COX-2, PGE<sub>2</sub> is involved in many inflammatory conditions. Basal levels of PGE<sub>2</sub> may be maintained by constitutively-expressed COX-1 for normal cellular function; however, elevated levels of PGE<sub>2</sub> by IL-1 $\beta$  treatment can participate in the development of certain inflammatory diseases such as in neurodegenerative diseases (Angel et al., 1994; Sun et al., 2004; Xu et al., 2003) and in the formation of inflammatory lesions in gingival tissue (Yucel-Lindberg et al., 1995). Moreover, PGE<sub>2</sub> decreases cell proliferation and collagen synthesis in lung fibroblasts (Fine and Goldstein, 1987; Fine et al., 1989; Kawamoto et al., 1995#61). Our recent studies have shown that treatment of HPTFs with exogenous PGE<sub>2</sub> decreases cell proliferation and collagen synthesis, which may eventually lead to tendon degeneration, a hallmark of tendinopathy at later stages (Cilli et al., 2004). Thus, the expression of the inflammatory mediators, cPLA<sub>2</sub> and COX-2 and a high level of PGE<sub>2</sub> production by HPTFs in response to exposure of IL-1 $\beta$  *in vivo* may contribute to tendon inflammation and/or degeneration often seen in repetitive motion-induced tendon injuries.

Besides the upregulation of inflammatory mediators, we observed that IL-1 $\beta$  significantly downregulates the expression of collagen type-I at the mRNA level in human tendon fibroblasts. Collagen type-I is the major component of tendon matrix. Thus, IL-1 $\beta$ -mediated suppression of collagen type-I gene expression may lead to the reduced deposition of ECM. Consequently, it might affect normal tissue remodeling and perhaps lead to development of tendinopathy. There is experimental evidence that the downregulation of collagen type-I by IL-1 $\beta$  is mediated via endogenous PGE<sub>2</sub> generated by IL-1 $\beta$ . For example, it has been shown that the inhibitory effect of IL-1 $\beta$  on lung fibroblast collagen production is partially due to the effects of newly synthesized PGE<sub>2</sub> (Diaz et al., 1993). These results indicate that inflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$  inhibit the expression of  $\alpha$ 1(I) procollagen gene at the transcriptional level by a PGE<sub>2</sub>-dependent pathway as well as through the effect of endogenous PGE<sub>2</sub> released under the stimulus of inflammatory cytokines. Thus, it is reasonable to speculate that IL-1 $\beta$  disturbs human tendon homeostasis in part through the induction of PGE<sub>2</sub>, which initiates inhibition of collagen production.

In this study, we also showed that IL-1 $\beta$  upregulates both MMP-1 and MMP-3 at the level of mRNA in human tendon fibroblasts, which may accelerate tendon matrix degradation. Our results are consistent with previous observations in tendon cells (Tsuzaki et al., 2003b; Yang et al., 2005) and are also in agreement with the observation in cardiac fibroblasts that IL-1 $\beta$  decreases collagen synthesis and increases MMP activity (Siwik et al., 2000). Furthermore, collagen degradation by IL-1 $\beta$  has been accompanied by release and activation of multiple MMPs such as MMP-1, -8, and -13 in gingival fibroblasts (Cox et al., 2006). Collectively, these results suggest that IL-1 $\beta$  contributes to abnormal collagen matrix turn over in tendons.

The elevated expression of MMPs induced by IL-1 $\beta$  is also thought to be mediated via endogenous PGE<sub>2</sub> in fibroblasts. For example, it was shown that IL-1 $\beta$ -induced MMP-3 production in human gingival fibroblasts was regulated by PGE<sub>2</sub> (Ruwanpura et al., 2004). In addition, we have recently shown that exogenous PGE<sub>2</sub> upregulates MMP-1 and -3 at the levels of mRNA and protein in tendon fibroblasts (Thampatty et al., 2006). Additional evidence for the potential role of endogenous PGE<sub>2</sub> in mediating catabolic effects in tendon fibroblasts is provided by the current observation that IL-1 $\beta$  specifically upregulates the expression of EP<sub>4</sub> receptor among other EP receptors. Blocking the EP<sub>4</sub> receptor signaling pathway by using selective EP<sub>4</sub> receptor antagonist switches the catabolic effects in favor of more anabolic effects. For example, we observed that IL-1 $\beta$ -mediated suppression of collagen type-I or stimulation of MMPs is reversed to the control level using human tendon fibroblasts. It is well established that PGE<sub>2</sub> exerts its effects via its EP receptors (EP<sub>1</sub>–EP<sub>4</sub>). The diversity and selectivity of PGE<sub>2</sub> effects are dependent on the expression of these four different EP subtypes of PGE<sub>2</sub> receptors (Negishi et al., 1995). Previous observations in other fibroblasts (lung, gingival) provide evidence for the specific upregulation of EP receptor subtypes such as EP<sub>1</sub> and EP<sub>2</sub> in the matrix degradation process (Choung et al., 1998; Moore et al., 2005; Ruwanpura et al., 2004). Selective upregulation of EP<sub>4</sub> receptor expression by IL-1 $\beta$  has been reported in PGE<sub>2</sub>-mediated cervical ripening process (Schmitz et al., 2003). Interestingly, IL-1 $\beta$  also selectively stimulates the expression of EP<sub>4</sub> receptor in human tendon fibroblasts. Potentially, the specific upregulation of EP<sub>4</sub> receptors in tendon fibroblasts by inflammatory cytokine may trigger inflammatory signaling pathways ultimately leading to tendon matrix degradation and thus tendinopathy.

This study has certain limitations. We did not determine the time course of IL-1 $\beta$  effect on cPLA<sub>2</sub> and COX-2 protein expression and PGE<sub>2</sub> production. Also, we did not investigate the possible involvement of other MAPK pathways such as ERK1/2 or JNK in IL-1 $\beta$ -mediated effects in tendon fibroblasts. These pathways have also been implicated in IL-1 $\beta$ -mediated inflammatory effects (Kida et al., 2005; Laporte et al., 1999). It is also important to investigate the potential anabolic role of EP<sub>4</sub> receptor antagonist in models of tendinopathy *in vitro* and *in vivo*. Since repetitive mechanical loading is considered as one of the causative factors for development of tendinopathy, it would be interesting to investigate how EP<sub>4</sub> receptor antagonist may regulate the inflammatory mediator release under mechanical loading conditions. Previously, we have shown that a small magnitude mechanical loading of tendon fibroblasts decreases COX-2 and MMP-1 gene expression and PGE<sub>2</sub> production that were stimulated by IL-1 $\beta$  (Yang et al., 2005). EP<sub>4</sub> receptor antagonist together with small magnitude loading may further augment this effect, favoring tendon inflammation reduction. Additionally, we are in the process of developing “exercise animal models” of tendinopathy to induce tendon microinjuries in mice running on treadmills with various loading intensities. In this model, administration of EP<sub>4</sub> receptor antagonist to reduce tendon inflammation and degeneration may be a suitable clinical approach toward effective treatment of tendinopathy.

In conclusion, this study shows that treatment of HPTFs with the inflammatory cytokine IL-1 $\beta$  increases cPLA<sub>2</sub> and COX-2 protein expression and PGE<sub>2</sub> production. Furthermore, IL-1 $\beta$  induces catabolic effects in tendon matrix by downregulating collagen gene expression and upregulating MMP gene expression. Importantly, we found that the biological impact of IL-1 $\beta$  appeared to be mediated via EP<sub>4</sub> receptor, which is specifically upregulated by IL-1 $\beta$  in human tendon fibroblasts. These effects are at least in part mediated via the p38 MAPK signaling pathway. IL-1 $\beta$  is the inflammatory cytokine produced when tendon microinjuries occur resulting from repetitive mechanical loading of tendons *in vivo*. Our study using a novel cell culture system that preserves tendon fibroblastic phenotype *in vivo* shows that the exposure of tendon fibroblasts to IL-1 $\beta$  induces the expression and production of inflammatory mediators and matrix degradation, which may contribute to tendon inflammation and thus participate in



the pathogenesis of tendinopathy. The use of selective EP<sub>4</sub> receptor antagonist may be an attractive therapeutic option for the treatment of tendinopathy at early stages.

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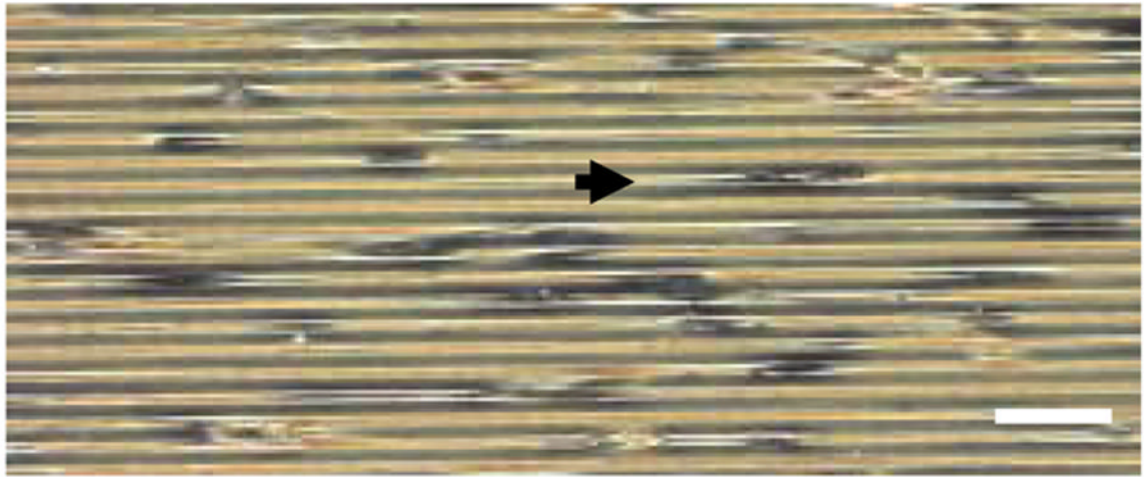
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## Abbreviations

AA

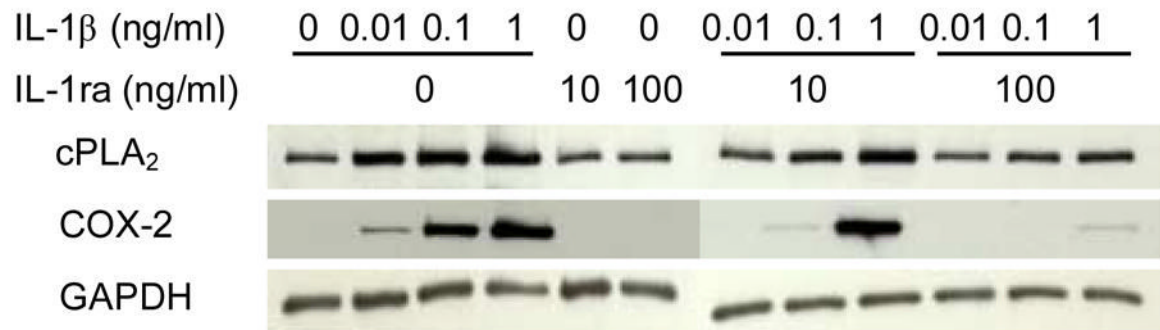
Arachidonic acid

<b>cAMP</b>	Cyclic adenosine monophosphate
<b>COX-2</b>	Cyclooxygenase-2
<b>DMEM</b>	Dulbecco's Modified Eagle Medium
<b>EP</b>	Prostaglandin E receptor
<b>ELISA</b>	Enzyme-linked immunosorbent assay
<b>ERK</b>	Extracellular regulated kinase
<b>FBS</b>	Fetal bovine serum
<b>GAPDH</b>	Glyceraldehyde phosphate dehydrogenase
<b>HHTF</b>	Human patellar tendon fibroblast
<b>IL-1<math>\beta</math></b>	Interleukin-1 $\beta$
<b>IL-1ra</b>	Interleukin-1 receptor antagonist
<b>JNK</b>	c-Jun N-terminal kinase
<b>MAPK</b>	Mitogen activated protein kinase
<b>MMP</b>	Matrix metalloproteinase
<b>PGE<sub>2</sub></b>	Prostaglandin E <sub>2</sub>
<b>cPLA<sub>2</sub></b>	Cytosolic phospholipase A <sub>2</sub>
<b>RT-PCR</b>	Reverse transcription-polymerase chain reaction
<b>TIMP</b>	Tissue inhibitors of metalloproteinases

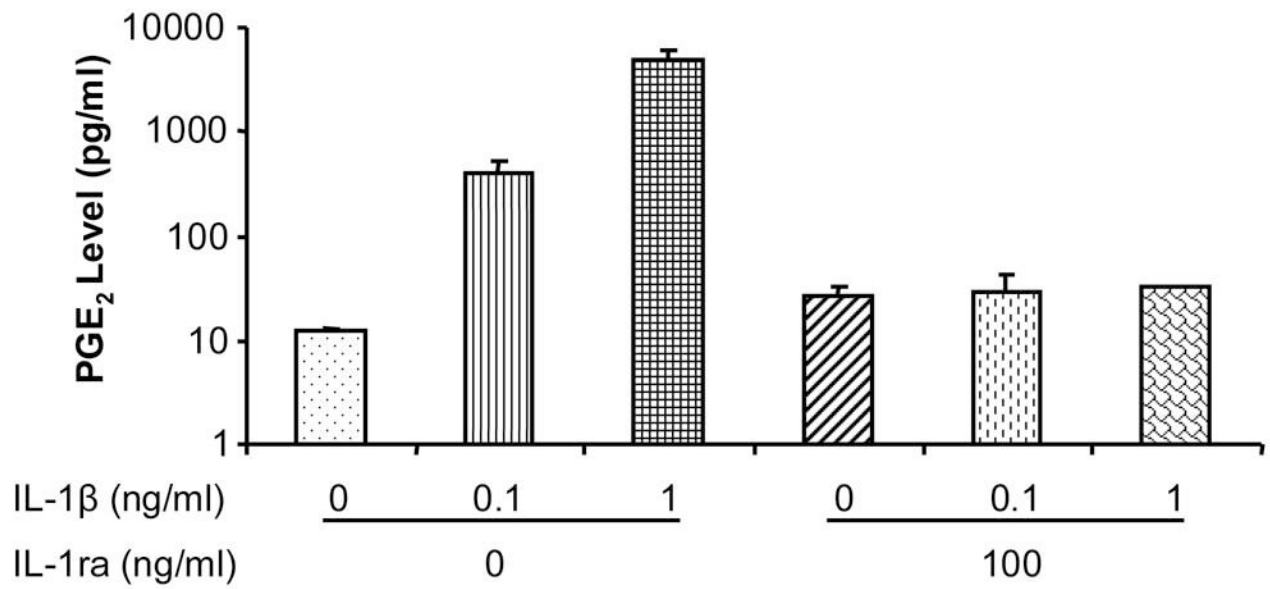


**Fig 1.** Human tendon fibroblasts were grown on microgrooved culture surfaces. The cells remained aligned and the shape during cell culture experiments. The cell alignment and shape mimic the situation *in vivo*. (The arrow points to a fibroblast on the microgrooved substrate. Bar: 50  $\mu\text{m}$ ).

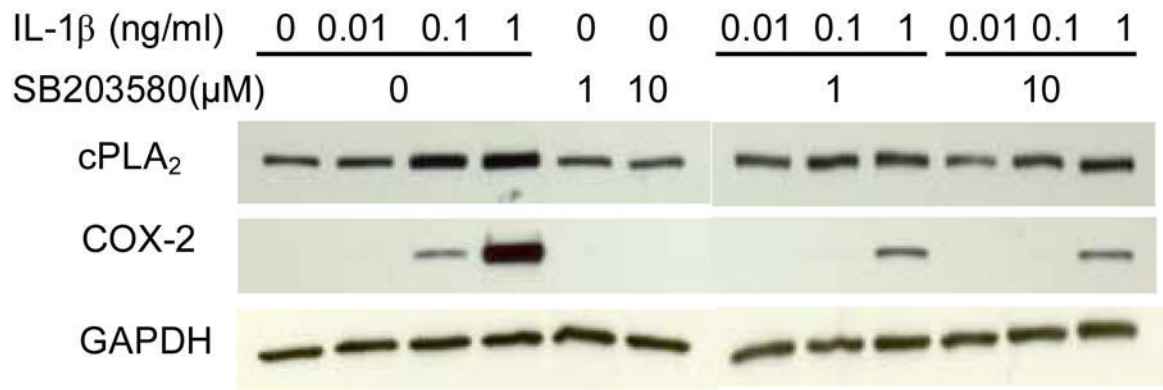




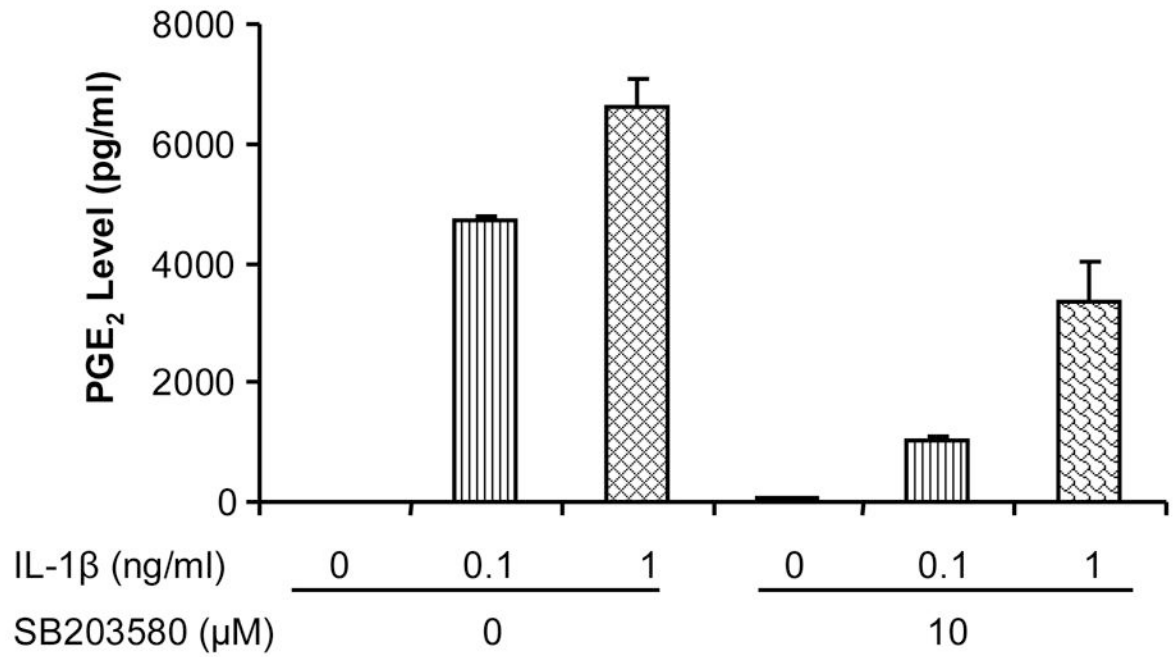
**Fig 2.** Effects of IL-1 $\beta$  and IL-1ra on cPLA<sub>2</sub> and COX-2 protein expression. A representative Western blot result of three independent experiments is shown. The data show that IL-1 $\beta$  increased cPLA<sub>2</sub> and COX-2 protein expressions in dose-dependent manner and co-incubation with IL-1ra reduced the augmentations of protein expressions. IL-1ra by itself did not have any effect on both protein expressions.



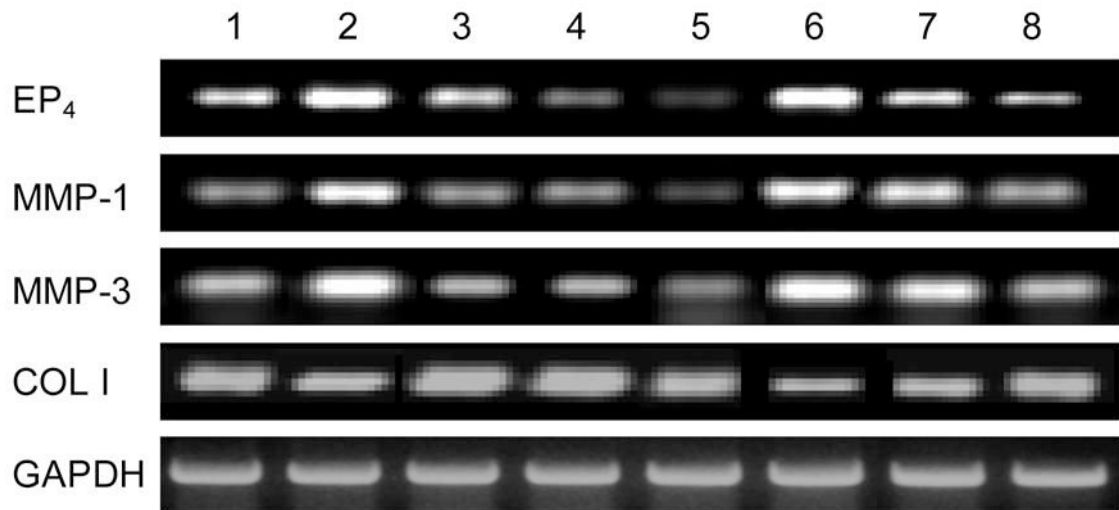
**Fig 3.** Effects of IL-1 $\beta$  and IL-1ra on PGE<sub>2</sub> production. IL-1 $\beta$  increased PGE<sub>2</sub> release in a dose-dependent manner and IL-1ra markedly inhibited the PGE<sub>2</sub> production. IL-1ra by itself did not have any effect on PGE<sub>2</sub> production. Two independent experiments were performed with a sample size of 6.

**Fig. 4.**

Effects of IL-1 $\beta$  and SB203580 on cPLA<sub>2</sub> and COX-2 protein expression. A representative experimental result from three independent experiments is shown. The data show that IL-1 $\beta$  increased cPLA<sub>2</sub> and COX-2 protein expressions in a dose-dependent manner. SB203580 reduced both protein expressions, but by itself did not affect the protein expressions.



**Fig. 5.** Effects of IL-1 $\beta$  and SB203580 on PGE<sub>2</sub> production. IL-1 $\beta$  increased PGE<sub>2</sub> levels in a dose-dependent manner and SB203580 decreased them. Two independent experiments were performed with a sample size of 6.



**Fig. 6.** Effects of IL-1 $\beta$  (1 ng/ml) and AH23848 (0.1, 1, and 10  $\mu$ M) on the expressions of collagen-I, MMP-1, MMP-3, and EP<sub>4</sub> at the level of mRNA. IL-1 $\beta$  decreased collagen type-I whereas it increased MMP-1, MMP-3, and EP<sub>4</sub> gene expression (lane 2) compared to the control (lane 1). A pretreatment with AH23848, a specific chemical inhibitor of EP<sub>4</sub>, inhibited the biological impact of IL-1 $\beta$  in a dose-dependent manner (lanes 6–8). Lanes 3–5 represent AH23848 treatment alone.



**Table 1**

Primer sequences for RT-PCR

Gene Annealing temperature	Primer Sequence Forward/Reverse	PCR Product Size (bp)
Collagen-I 58°C	5'-GGT TAC TAC TGG ATT GAC C-3' 5'-TTG CCA GTC TCC TCA TCC-3'	328
MMP-1 58°C	5'-CAACT CTGGAGTAAT GTCACA -3' 5'-T ACATCAAAGC CCCGATATCA -3'	295
MMP-3 55°C	5'-TTT TGG CCA TCT CTT CCT TCA-3' 5'-TGT GGA TGC CTC TTG GGT ATC-3'	138
EP-4 55°C	5'-TTTCCAGACTGAGCAGGACAAGGT-3' 5'-ATAGGCATGGTTGATGGCCAGGTA-3'	525
GAPDH 58°C	5'-AAATTCCATGGCACCGTCAAGGCT-3' 5'-CTCATGGTTCACACCCATGACGAA-3'	295