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# EP2 receptor-mediated activation of extracellular signal-regulated kinase/activator protein-1 signaling is required for the mitogenic action of prostaglandin $E_2$ in esophageal squamous-cell carcinoma

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**(a) Running Title:** EP2 receptor mediates mitogenic action of $PGE_2$

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**(c)**

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**(d) Abbreviations:** $PGE_2$, prostaglandin $E_2$; EP, E-series of prostaglandin; COX, cyclooxygenase; NSAIDs, non-steroidal anti-inflammatory drugs; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; Erk, extracellular signal-regulated kinase; JNK, c-Jun amino-terminal kinase; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; AP-1, activator protein-1; VEGF, vascular endothelium growth factor; siRNA, small interference RNA; RT-PCR, reverse transcription-polymerase chain reaction; U0126, 1,4-Diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene ethanolate; Forskolin, 7β-Acetoxy-8,13-epoxy-1α,6β,9α-trihydroxylabd-14-en-11-one; IBMX: 1-Methyl-3-isobutylxanthine; PMA, Phorbol 12-myristate 13-acetate; butaprost, (±)-15-deoxy-16S-hydroxy-

17-cyclobutyl $PGE_1$ methyl ester; sulprostone, 16-Phenoxy-ω-17,18,19,20-tetranor-prostaglandin $E_2$-methylsulfonylamide; PGE1 alcohol, 1-hydroxy Prostaglandin E1; ONO-DI-004, 17S-17,20-dimethyl-2,5,-ethano-6-oxo $PGE_1$; ONO-AE3-208, 2-(2-(2-methyl-2-naphth-1-ylacetylamino)-phenylmethyl)-benzoic acid; SC-236, 4-[5-(4-Chlorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide.

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## Abstract

The use of non-steroidal anti-inflammatory drugs is associated with a lower risk for esophageal squamous-cell carcinoma, in which overexpression of cyclooxygenase-2 (COX-2) is frequently reported. Prostaglandin $E_2$ ($PGE_2$), a COX-2-derived eicosanoid, is implicated in the promotion of cancer growth. The precise role of $PGE_2$ in the disease development of esophageal squamous-cell carcinoma, however, remains elusive. In this study, we investigated the effect of $PGE_2$ on the proliferation of cultured esophageal squamous-cell carcinoma cells (HKESC-1). Results showed that HKESC-1 cells expressed all four $PGE_2$ receptors, namely, EP1 to EP4 receptors. In this regard, $PGE_2$ and the EP2 receptor agonist butaprost markedly increased HKESC-1 cell proliferation. Moreover, the mitogenic effect of $PGE_2$ was significantly attenuated by RNA interference-mediated knockdown of EP2 receptor, indicating that this receptor mediated the mitogenic effect of $PGE_2$. In this connection, $PGE_2$ and butaprost induced phosphorylation of extracellular signal-regulated kinases-1/2 (Erk1/2), whose down-regulation by RNA interference significantly attenuated $PGE_2$-induced cell proliferation. In addition, $PGE_2$ and butaprost increased c-Fos expression and activator protein-1 (AP-1) transcriptional activity, which were abolished by the mitogen-activated protein kinase/ERK kinase (MEK) inhibitor U0126. AP-1 binding inhibitor curcumin also partially reversed the mitogenic effect of $PGE_2$. Taken together, these data demonstrate for the first time that EP2 receptor mediates the mitogenic effect of $PGE_2$ in esophageal squamous-cell carcinoma via activation Erk/AP-1 pathway. This study supports the growth-promoting action of $PGE_2$ in esophageal squamous-cell carcinoma and the potential application of EP2 receptor antagonists in the treatment of this disease.

## Introduction

Esophageal cancer is a highly aggressive malignant disease with a 5-year survival rate of 10-15% (Jemal et al., 2003). There are two major histological types of esophageal cancer, namely, squamous-cell carcinoma and adenocarcinoma, each of which has distinct etiological and pathological characteristics. Although esophageal adenocarcinomas are more prevalent in the West, esophageal squamous-cell carcinoma remains the predominant type worldwide (Souza, 2002). The etiology of esophageal squamous-cell carcinoma is multi-factorial but cigarette smoking and alcohol consumption are two of the leading risk factors (Stoner et al., 2007). Compelling epidemiological evidence also shows that regular or occasional use of aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs) is associated with a lower risk for esophageal squamous-cell carcinoma (Corley et al., 2003).

The chemoprophylactic effect of NSAIDs has been attributed to their ability to inhibit the activity of cyclooxygenase (COX), which exists in two isoforms commonly referred to as COX-1 and COX-2. COX-1 is constitutively expressed in nearly all tissues and is thought to play a ‘housekeeping’ role. COX-2, in contrast, is an immediate-early response gene product normally absent from most cells but highly inducible in response to inflammatory cytokines, growth factors, and tumor promoters (Dubios et al., 1998). In studies of human esophageal squamous-cell carcinoma, aberrant up-regulation of COX-2 expression has been reported to occur as early as at the stage of dysplasia and in over two-thirds of cases of carcinoma *in situ* and invasive carcinoma, whilst COX-2 is weakly expressed, if at all, in normal squamous esophageal epithelium (Zhi et al., 2006). Moreover, normal and cancerous esophageal tissues express similar amounts of COX-1 (Zimmermann et al., 1999). These data suggest that COX-2 but not COX-1 is

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involved in esophageal carcinogenesis. COX is the key enzyme for the conversion of arachidonic acid to prostaglandin (PG) $G_2$ and $PGH_2$. $PGH_2$ is subsequently converted to a variety of prostanoids, which include $PGE_2$, $PGD_2$, $PGF_{2\alpha}$, $PGI_2$, and thromboxane $A_2$ by each respective prostaglandin synthase. Among all prostanoids, $PGE_2$ has been found to play a crucial role in carcinogenesis. For instance, $PGE_2$ levels are elevated in various human cancers including colon, lung and esophageal squamous-cell carcinoma (Gupta and DuBois, 2000; Lau et al., 1987; Morgan, 1997). Moreover, $PGE_2$ promotes intestinal adenoma growth in $APC^{Min}$ mice (Wang, et al., 2004), and significantly enhances carcinogen-induced colon tumor incidence and multiplicity in rats (Kawamori et al., 2003). L-748706, a selective COX-2 inhibitor, has also been found to reduce tumor multiplicity in carcinogen-induced esophageal tumor in rats by reducing $PGE_2$ levels (Stoner, et al., 2005). In relation to the signaling mechanism, emerging evidence suggests that increased phosphorylation of extracellular signal-regulated kinases (Erk1/2) and Akt may be required for the stimulatory effect of $PGE_2$ on cell proliferation (Krysan et al., 2005; Cherukuri et al., 2007; Han and Wu, 2005; Leng et al., 2003).

Despite the protective effect of NSAIDs, their uses as chemoprophylactic agents have been hampered by the potential cardiovascular side-effects (Wang, et al., 2005). The COX-2 signaling pathway plays a pivotal role in the control of cell proliferation, which is fundamental to carcinogenesis. Molecules involved in the COX-2 signaling therefore become attractive targets in pathway-directed cancer therapy. To this end, there is a growing interest in the development of antagonists for $PGE_2$ receptors which are designated as EP1 to EP4 receptors. The pro-carcinogenic role of different EP receptors has been supported by studies involving the use of knock-out animals. For example, EP1- and EP4-receptor-deficient mice are resistant to carcinogen-induced aberrant crypt foci formation in the colon (Watanabe et al., 1999; Mutoh et

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al., 2002). Disruption of EP2 receptor also decreases the number and size of intestinal polyps, the intensity of angiogenesis, and vascular endothelium growth factor (VEGF) expression in *APC*$^{Min}$ mice (Sonoshita et al., 2001; Seno et al., 2002). Moreover, EP3 receptor-knockout mice develop less tumor-associated blood vessels due to the reduction of VEGF expression (Amano et al., 2003).

Characterization of EP receptors in tumor cells is only at its beginning and the precise role of each EP receptor in the pathogenesis of esophageal squamous-cell carcinoma has yet to be elucidated. In the present study, we investigated the involvement of EP receptors in the mitogenic effect of $PGE_2$ in human esophageal squamous-cell carcinoma.

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## Materials and methods

### *Chemicals and Drugs*

Butaprost (EP2 receptor agonist), sulprostone (EP3/EP1 receptor agonist), $PGE_1$ alcohol (EP4/EP3 receptor agonist) and antibodies to EP1-EP4 receptors and COX-2 were purchased from Cayman Chemical Co. Inc. (Ann Arbor, MI). Erk1- and Erk2-siRNA and antibody to COX-1 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). EP2 receptor-siRNA and control siRNA were from Qiagen (Hilden, Germany). All other primary antibodies were purchased from Cell Signaling Technology (Beverley, MA). ONO-DI-004 (EP1 receptor agonist), ONO-AE3-240 (EP3 receptor antagonist), and ONO-AE3-208 (EP4 receptor antagonist) were kindly provided by Ono Pharmaceutical Co., Ltd. (Osaka, Japan). Reagents for electrophoresis were from Bio-Rad (Hercules, CA). All the other chemicals and reagents were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise specified.

### *Cell culture*

Human esophageal squamous cell lines, HKESC-1, HKESC-2 and HKESC-3, were kindly provided by Prof. G. Srivastava (Department of Pathology, The University of Hong Kong). HKESC-1 and HKESC-2 were established from moderately-differentiated human esophageal squamous-cell carcinoma (Hu et al., 2000; Hu et al., 2002). HKESC-3 was established from a well-differentiated human esophageal squamous-cell carcinoma (Hu et al., 2002). Another two cell lines KYSE150 and EC109 were established from poorly-differentiated human esophageal squamous-cell carcinoma. KYSE150 was purchased from the Japanese Collection of Research Biosources (JCRB). EC109 was provided by Cancer Institute Chinese Academy of Medical Sciences. HKESC-1, HKESC-2 and HKESC-3 were maintained in MEM medium, KYSE150

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was maintained in Ham F-12 medium, and EC109 was maintained in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37$^{o}$C in a humidified atmosphere of 5% $CO_2$ and 95% air.

*[$^{3}$H]thymidine incorporation assay*

Cell proliferation was assessed as the amount of DNA synthesis by measuring the incorporation of [$^{3}$H]thymidine into DNA. Briefly, cells were seeded into 24-well plates overnight for attachment, then serum deprived for 24 h and stimulated with $PGE_2$ or selective EP receptor agonists for another 24 h. To study the effects of antagonists or inhibitors, cells were pretreated with specific antagonists or inhibitors for 1 h prior to treatment with $PGE_2$. In the next step, 0.5 µCi/ml [$^{3}$H]thymidine (Amersham, Arlington Heights, IL) was added to each well, and the cells were further incubated for another 4 h. The amount of DNA synthesized was measured by liquid scintillation spectrometry with a beta-counter (Beckman Instruments, Fullerton, CA). The final concentration of vehicle did not exceed 0.2% (v/v) in cell culture medium, which showed no effects on cell proliferation.

*Conventional and Real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)*

Total RNA was isolated from esophageal squamous-cell carcinoma cells using TRIzol reagent (Invitrogen, Carlsbad, CA). Two micrograms of total RNA was used to generate the first strand of cDNA by reverse transcription using the ThermoScript$^{TM}$ RT-PCR system (Invitrogen) in accordance with the manufacture's instructions. PCR was then performed using the following primer pairs: EP1 receptor, 5'-CCAATGCTGGTGTTGGTGGC-3' (forward) and 5'-AGGGTGGGCTGGCTTAGTCG-3' (reverse); EP2 receptor, 5'-CCACCTCATTCTCCTGGCTA-3' (forward) and 5'-CGACAACAGAGGACTGAACG-3'

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(reverse); EP3 receptor, 5'-CTTCGCATAACTGGGGCAAC-3' (forward) and 5'-TCTCCGTGTGTGTCTTGCAG-3' (reverse); EP4 receptor, 5'-AGACGACCTTCTACACGC-3' (forward) and 5'-GACGAATACTCGCACCAC-3' (reverse) and beta-actin, 5'-AACACCCCAGCCATGTACG-3' (forward) and 5'-CGCTCAGGAGGAGCAATGA-3' (reverse). Conditions for PCR were 95$^{o}$C for 5 min, 35 cycles of 94°C for 30 sec, 55-60°C (see below) for 30 sec and 72°C for 1 min. The final extension step was at 72$^{o}$C for 10 min. The annealing temperature was 58$^{o}$C for EP1 receptor, 55$^{o}$C for EP4 receptor, 60$^{o}$C for EP2, EP3 receptors and β-actin. A negative control which was the PCR reaction without prior reverse transcription was included to exclude PCR amplification of genomic DNA. The PCR products were electrophoresed on 1.2% (W/V) agarose gels containing 0.5 μg/ml ethidium bromide. Gel photographs were then analyzed in a multianalyzer (Bio-Rad, Hercules, CA). The expected size of the amplified fragment was 314 bp for EP1 receptor, 216 bp for EP2 receptor, 300 bp for EP3 receptor, 731 bp for EP4 receptor and 623 bp for beta-actin. For quantitation of mRNA expression, real-time PCR was performed with an iQ$^{TM}$ Multicolor Real-Time PCR Detection System (Bio-Rad) using the SYBR GreenER$^{TM}$ qPCR Supermix (Invitrogen) as recommended by the manufacturer. Real-time PCR was performed using the following primer pairs: c-Fos, 5'-AGGGCTGGCGTTGTGA-3' (forward) and 5'-CGGTTGCGGCATTTGG-3' (reverse); FosB, 5'-CCAGCGGAACTACCAGT-3' (forward) and 5'-CTGCTGCTAGTTTATTTCGT-3' (reverse); Fra-1, 5'-GCATGTTCCGAGACTTCG-3' (forward) and 5'-ATGAGGCTGTACCATCCACT-3' (reverse); Fra-2, 5'-CCAAGACCTGGCGTGA-3' (forward) and 5'-CGGATGCGACGCTTCT-3' (reverse); c-Jun, 5'-CTGCGTCTTAGGCTTCTCC-3' (forward) and 5'-TCGCCCAAGTTCAACAA-3' (reverse); JunB, 5'-GTACCCGACGACCACCATC-3' (forward) and 5'-CGGTCTGCGGTTCCTCCTT-3' (reverse); JunD, 5'-CTTCGCTGCCGAACCTGTG-3'

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(forward) and 5'-CGTCTGTGGCTCGTCCTTGA-3' (reverse) and β-actin, 5'-AGCACTGTGTTGGCGTACAG-3' (forward) and 5'-CTCTTCCAGCCTTCCTTCCT-3' (reverse). PCR conditions were 95 $^{o}$C for 30 s, 55 $^{o}$C for 30 s and 72 $^{o}$C for 30 s for 45 cycles. The mRNA expression was calculated using the comparative threshold cycle ($C_T$) method and normalized against expression of β-actin.

*Western blot*

Cells were lysed in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L sodium chloride, 0.5 % α-cholate acid, 0.1 % SDS, 2 mmol/L EDTA, 1% Triton X-100 and 10% glycerol], containing protease and phosphatase inhibitors. After sonication for 30 s on ice and centrifugation for 15 min at 14,000 g at 4$^{o}$C, the supernatant was collected and protein concentration was determined by assay kit (Bio-Rad). Equal amount of protein (50 μg/lane) were resolved with SDS-polyacrylamide gel electrophoresis, and transferred to Hybond C nitrocellulose membranes (Amersham). The membranes were probed with primary antibodies overnight at 4$^{o}$C and incubated for 1 h with secondary peroxidase-conjugated antibody at room temperature. The signals on the membrane were visualized by enhanced chemiluminescence (Amersham) and exposed to X-ray film (FUJI Photo Film, Tokyo, Japan).

*RNA interference*

Cells were transiently transfected with small interference RNA (siRNA) oligonucletides by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For each transfection, 20 pmol target-specific siRNA (Erk1, Erk2, EP2) or control siRNA were added to each well and incubated at 37 $^{o}$C for 6 h. The transfected cells were then growth arrested for

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another 18 h before treatments.

*Luciferase reporter gene transactivation assay*

Transient transfectation with pAP-1 (PMA)-TA-Luc luciferase reporter plasmid (Clontech Laboratories, Mountain View, CA) was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. In brief, HKESC-1 cells were transfected with a 10:1 ratio of the pAP-1 (PMA)-luc plasmid and pRL-TK plasmid (Promega, Madison, WI). Cell lysates were then subjected to dual-luciferase reporter assay system, and luciferase activities were measured with a Lumat LB9501 luminometer (Berthold Company, Wildbad, Germany). Firefly luciferase activities were normalized to *Renilla* luciferase activity for transfection efficiency.

*$PGE_2$ assay*

The measurement of $PGE_2$ in the cell culture medium was carried out using the Correlate-EIA Prostaglandin $E_2$ Enzyme Immunoassay kit from Assay Design Inc. (Ann Arbor, MI) according to the manufacturer's instructions. In brief, 4 x $10^4$ cells/well were plated in 24-well plate in the presence of 10% serum. At confluence, fresh culture medium with 1% serum was added and incubated for another 24 h, after which supernatants were collected for $PGE_2$ measurement.

*Cyclic AMP assay*

Intracellular cyclic AMP assay was performed according to the manufacturer's instructions (Amersham Corporation). Briefly, $1\times10^6$ cells were treated with $PGE_2$, butaprost, forskolin for 10 minutes in the presence of phosphodiesterase inhibitor IBMX (100 μM) to prevent the breakdown of cAMP. The cAMP level was then measured using a non-acetylation EIA procedure. The cAMP level was expressed as picomoles per milligram of protein.

*Statistical analysis*

Results were expressed as the mean ± SEM for at least three independent experiments. Statistical analysis was performed with an analysis of variance (ANOVA) followed by the Turkey's t-test. *P* values less than 0.05 were considered statistically significant.

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## Results

*HKESC-1 cells expressed all four EP receptor subtypes, COX-1 and COX-2 and actively secreted $PGE_2$*

We determined the expression of EP1 to EP4 receptors expression in a panel of human esophageal squamous-cell carcinoma cell lines (HKESC-1, HKESC-2, HKESC-3, EC109, and KYSE150). Results from RT-PCR showed that the tested five esophageal squamous-cell carcinoma cell lines expressed mRNAs for all EP receptor subtypes (Fig. 1A). Furthermore, in the tested cell lines, the protein expression of EP receptors was confirmed by Western blot analysis in which specific EP receptors were detected at the anticipated molecular weight using EP receptor-specific antibodies (Fig. 1B). The protein expression of COX-1 and COX-2 was also detected in these cell lines (Fig. 1B). HKESC-1 cells were thereafter elected as the working cell line for further analysis. In HKESC-1 cells, aside from the expression of EP receptors, we also investigate whether HKESC-1 cells could actively secrete $PGE_2$. In this regard, the basal release of $PGE_2$ was determined to be 2.27±0.02 ng / mg of total protein over 24 h (data not shown).

*$PGE_2$ or EP2 agonist butaprost increased HKESC-1 cell proliferation*

To study the effect of $PGE_2$ on proliferation of esophageal squamous-cell carcinoma cells, HKESC-1 cells were treated with $PGE_2$ at concentrations ranging from 0.1 to 10 μmol/L. Results showed that $PGE_2$ at these concentrations significantly increased HKESC-1 cell proliferation in a concentration-dependent manner (Fig. 2A). In the next step, we determined which EP receptor mediated the mitogenic effect of $PGE_2$ using selective EP receptor agonists or antagonists. Results showed that EP2 receptor agonist butaprost at the concentration of 25 μmol/L substantially increased HKESC-1 cell proliferation to an extent similar to that of 10 μmol/L $PGE_2$

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while EP1 receptor agonist ONO-DI-004 and EP3/EP1 receptor agonist sulprostone at all concentrations tested only minimally stimulated HKESC-1 cell proliferation (Fig. 2B). These data indicated that EP2 receptor, and to a lesser extent EP1 receptor, were involved in mediating the stimulatory of $PGE_2$. The involvement of EP3 and EP4 receptors was further excluded based on the finding that EP4/EP3 receptor agonist $PGE_1$ alcohol exhibited no effect on cell proliferation (Fig. 2B) whilst EP3 receptor antagonist ONO-AE3-240 and EP4 receptor antagonist ONO-AE3-208 failed to attenuate $PGE_2$-induced cell proliferation as shown in Fig. 2C.

*Knockdown of EP2 receptor attenuated the mitogenic effect of $PGE_2$*

As EP2 receptor agonist butaprost strongly increased HKESC-1 cell proliferation as compared with other agonists, the role of the EP2 receptor in $PGE_2$-induced cell proliferation was further investigated by RNA interference experiment. Using specific siRNA, down-regulation of EP2 receptor significantly attenuated $PGE_2$-induced proliferation in HKESC-1 cells (Fig. 2D top). The efficacy of the EP2 receptor depletion was further verified by Western blot analysis in which the results showed that EP2 receptor-siRNA successfully down-regulated EP2 receptor protein levels 24-h post-transfection (Fig. 2D bottom).

*$PGE_2$ or butaprost increased Erk1/2 phosphorylation*

As phosphorylation of Akt and Erk1/2 has been suggested to mediate the growth-promoting effect of $PGE_2$ in other cancer cell types (Krysan et al., 2005; Cherukuri et al., 2007; Han and Wu, 2005; Leng et al., 2003), we examined the direct effects with $PGE_2$ on the phosphorylation of these proteins. As shown in Fig. 3A, treatment of $PGE_2$ from 10 min to 30 min significantly stimulated the phosphorylation of Erk1/2, whereas it exerted no influence on the phosphorylation of Akt. Moreover, Western blot analysis revealed that the phosphorylation of p38 or JNK,

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members of the mitogen-activated protein kinase (MAPK) family in which Erk1/2 belongs, was not affected by $PGE_2$ treatment. To further examine whether Erk1/2 are involved in mediating the stimulatory effect of $PGE_2$ on cell proliferation, Erk1-siRNA and Erk2-siRNA were used to silence their expressions. It was demonstrated that knockdown of Erk1 or Erk2 protein expression significantly attenuated $PGE_2$-induced HKESC-1 cell proliferation (Fig. 3B and 3C). Since EP2 receptor appeared to mediate the mitogenic effect of $PGE_2$, we also examined the effect of the EP2 receptor agonist butaprost on Erk1/2 phosphorylation. It was shown that butaprost at 25 μmol/L also markedly increased Erk1/2 phosphorylation after 10 min treatment (Fig. 3D).

*$PGE_2$ or butaprost up-regulated the mRNA expression of Fos and Jun family members*

The data presented so far indicated that Erk1/2 phosphorylation participated, at least in part, in the mitogenic effect of $PGE_2$ on HKESC-1 cells. In this connection, the transcription factor activator protein-1 (AP-1), which consists of different members from the Fos and Jun families, has been reported to be induced upon Erk1/2 phosphorylation to mediate the effect on cell proliferation (Karin, 1995; Shaulian and Karin, 2002). We therefore measured the mRNA expression levels of these AP-1 components in HKESC-1 cells treated with or without $PGE_2$. As shown in Fig. 4A and 4B, $PGE_2$ significantly increased the mRNA levels of c-Fos, FosB, Fra-1, c-Jun, and JunB, whereas it did not alter mRNA levels of Fra-2 or JunD. To this end, stimulating the cells with $PGE_2$ for 30 min resulted in a marked change in the expression of c-Fos, up to 18-fold increase compared with untreated cells. In parallel, EP2 receptor agonist butaprost significantly increased the mRNA levels of c-Fos, FosB, Fra-1, c-Jun, JunB, and JunD, whereas it showed no effects on Fra-2 mRNA level (Fig. 3C and 3D). Similar to $PGE_2$ treatment, the change in c-Fos expression was the most prominent among the up-regulated genes, up to 10-fold increase compared with control. We also observed that the time-course changes in FosB, Fra-1 and c-Jun

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mRNA levels between $PGE_2$ and butaprost treated cells were not exactly the same. The difference may be due to the fact that butaprost is a highly selective EP2 receptor agonist whilst $PGE_2$ can activate all four EP receptor subtypes, EP1, EP3 and EP4 receptors may also involve in regulating the expression of these genes.

*$PGE_2$ induced c-Fos protein expression which was abolished by MEK inhibitor U0126*

To further confirm the stimulatory effect of $PGE_2$ on c-Fos expression, we verified the up-regulation of c-Fos protein levels by Western blot analysis. Results showed that the expression level of c-Fos protein at basal condition was almost undetectable whilst it was dramatically elevated in response to $PGE_2$ treatment, reaching its peak level at 1 h post-treatment (Fig. 5A). In addition, MAPK/ERK kinase (MEK) inhibitor U0126 at the concentration of 1 μmol/L completely abolished $PGE_2$-induced c-Fos expression (Fig. 5B). Similarly, EP2 receptor agonist butaprost but not EP1 receptor agonist ONO-DI-004, EP3/EP1 receptor agonist sulprostone, or EP4/EP3 receptor agonist PGE1 alcohol (Fig. 5C) markedly elevated c-Fos protein expression.

*$PGE_2$ or butaprost enhanced the transcriptional activity of AP-1 which was abolished by MEK inhibitor U0126*

As changes in the expression of AP-1 components might not exactly mirror the transcriptional activity of AP-1, we next determined AP-1 transcriptional activity in response to $PGE_2$ and butaprost treatment by dual-luciferase reporter assay. As shown in Fig. 6A, $PGE_2$ or butaprost significantly increased AP-1 transcriptional activity. In this experiment, phorbol 12-myristate 13-acetate (PMA) was used as a positive control for AP-1 activity. In this respect, butaprost enhanced AP-1 transcriptional activity to an extent similar to that of $PGE_2$. MEK inhibitor U0126 also completely prevented the increase in AP-1 transcriptional activity induced by $PGE_2$. To

further examine whether up-regulation of AP-1 transcriptional activity was required for the mitogenic effect of $PGE_2$, the AP-1 binding inhibitor curcumin (Guo et al., 2001) was used. To this end, curcumin significantly attenuated cell proliferation induced by $PGE_2$ (Fig. 6B).

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**Discussion**

Over-expression of COX-2 and the subsequent elevation of $PGE_2$ levels have been implicated in the pathogenesis of human esophageal squamous-cell carcinoma (Zhi et al., 2006; Zimmermann et al., 1999; Morgan, 1997). Here we demonstrate that both endogenous and exogenous $PGE_2$ stimulate the proliferation of a human esophageal squamous-cell carcinoma cell line HKESC-1 (Fig. 2A), in which the endogenous production of $PGE_2$ and cell proliferation can be suppressed by the COX-2-selective inhibitor SC-236 (data not shown). $PGE_2$ also shows mitogenic effects on other four esophageal squamous-cell carcinoma cell lines (HKESC-2, HKESC-3, EC109 and KYSE150) with different extents (data not shown). In line with this finding, previous work reported by Zimmermann *et al.* also demonstrates that treating OSC-2 cells, another human esophageal squamous-cell carcinoma cell line, with the COX-2-selective inhibitor suppresses $PGE_2$ synthesis and cell proliferation (Zimmermann et al., 1999). These observations indicate that $PGE_2$ exerts its pro-carcinogenic effect in esophageal squamous-cell carcinoma, at least in part, through direct stimulation of cell proliferation. In this connection, EP receptors have been reported to mediate the mitogenic effects of $PGE_2$ in different cell types (Fulton et al., 2006). In the present study, we show for the first time that all four EP receptor subtypes, namely, EP1 to EP4 receptors, are expressed in a panel of human esophageal squamous-cell carcinoma cell lines (Fig. 1). Further characterization by pharmacological and RNA interference approaches reveals that EP2 receptor mediates the mitogenic effect of $PGE_2$ in HKESC-1 cells, in which the EP2 receptor agonist butaprost mimics the mitogenic effect of $PGE_2$ whilst knockdown of EP2 receptor attenuates the $PGE_2$-induced proliferative response (Fig. 2B and 2D). Upregulation of EP receptors has been reported in rat Barrett's metaplasia, a pre-malignant lesion of esophageal adenocarcinoma, induced by duodenal contents reflux (Jang et al., 2004). Here we provide direct

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evidence that EP2 receptor plays a predominant role in the mediation of the stimulatory effect of $PGE_2$ in esophageal squamous-cell carcinoma. Indeed, the importance of EP2 receptor in $PGE_2$-induced cell proliferation has been documented in a variety of cancers such as colon cancer, epidermoid carcinoma, and lung carcinoma (Castellone et al., 2005; Donnini et al., 2007; Han and Roman, 2004).

MAPK cascades (Erk1/2, p38, and JNK) and the phosphatidylinositol 3-kinase (PI3k)/Akt pathway are key signaling molecules involved in the regulation of cell proliferation, survival and differentiation. It therefore comes as no surprise that deregulation of these signaling pathways frequently occurs in human cancer, including esophageal squamous-cell carcinoma (Chattopadhyay et al., 2007; Li et al., 2007). Our results demonstrate that $PGE_2$ markedly increased the phosphorylation of Erk1/2, but not JNK or p38, in cultured esophageal squamous-cell carcinoma cells (Fig. 3A). RNA interference-mediated down-regulation of Erk1 or Erk2 also attenuates the stimulatory effect of $PGE_2$ on cell proliferation (Fig. 3B and 3C), suggesting that phosphorylation of Erk1/2 but not the other two members of MAPK cascades is required for the mitogenic effect of $PGE_2$. Intriguingly, activation of Erk1/2 has also been shown to up-regulate the activity of COX-2 (Chun et al., 2003), which has been observed aberrantly up-regulated in esophageal squamous-cell carcinoma (Zhi et al., 2006; Zimmermann et al., 1999). It is therefore possible that COX-2-derived $PGE_2$ may enhance a positive feedback loop to stimulate cell proliferation in esophageal squamous-cell carcinoma cells. Regulation of Erk1/2 activity by cAMP has been observed in some cell lines (Gerits et al., 2008). As EP2 receptor is a Gs protein coupled receptor, it may regulate Erk1/2 activity via cAMP pathway. In this respect, we investigated effects of cAMP on activation of Erk1/2 in HKESC-1 cells. Although forskolin, an adenylate cyclase activator, increased intracellular cAMP level more potently than EP2 agonist butaprost and $PGE_2$ (Supplementary Fig. 1), it does not influence Erk1/2 phosphorylation and

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cell proliferation (Supplementary Fig. 2A and 2B). Thus, mechanism other than cAMP pathway, may be involved in EP2 receptor mediated HKESC-1 cell proliferation. This needs further exploration. Apart from the MAPK cascade, Akt has been implicated in $PGE_2$-induced cholangiocarcinoma and hepatocellular carcinoma cell proliferation (Han and Wu, 2005; Leng et al., 2003). Akt becomes activated as a result of phosphorylation of Thr308 within the T loop of the catalytic domain and Ser473 located in a C-terminal, noncatalytic region of the enzyme, termed the "hydrophobic motif". In this regard, our results show that treating HKESC-1 cells with $PGE_2$ did not alter the expression of total Akt or its phosphorylation at Ser473. Furthermore, phosphorylated Akt at Thr308 was undetectable irrespective of the presence of $PGE_2$ (Fig. 3A), suggesting that Akt may not be involved in $PGE_2$-induced cell proliferation in esophageal squamous-cell carcinoma cells.

Elevated AP-1 activity, which is associated with increased proliferation, has been frequently documented in various types of human cancer and is related to multi-stage development of tumors (Young et al., 2003; Liu et al., 2002). In mammalian cells, the AP-1 transcription factor is a heterodimeric complex that mainly comprises members of the Jun and Fos protein families, most of which belong to the category of immediate-early response genes and are promptly induced following growth factor stimulation (Karin, 1995). AP-1 activity is predominantly governed by the MAPK cascade whose activation status is in turn influenced by extracellular stimuli such as growth factors, pro-inflammatory cytokines and UV radiation. In the context of cell proliferation, the most important mediator of growth factor is believed to be Erk1/2 whose phosphorylation causes induction of c-Fos which subsequently heterodimerizes with Jun proteins to form stable AP-1 dimer (Shaulian and Karin, 2002). Concordantly, our study reveals that $PGE_2$ dramatically increased c-Fos expression and AP-1 transcriptional activity (Fig. 5A and 6A), both

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which can be abolished by the MEK inhibitor U0126 (Fig. 5B and 6A), suggesting that Erk1/2 phosphorylation is required for $PGE_2$-induced c-Fos expression and AP-1 activation. Above all, AP-1 binding inhibitor curcumin significantly attenuated $PGE_2$-induced cell proliferation (Fig. 6B), revealing that AP-1 activation is required for $PGE_2$-induced cell proliferation in esophageal squamous-cell carcinoma. In parallel, the EP2 receptor agonist butaprost induces Erk1/2 phosphorylation (Fig. 3D), c-Fos expression (Fig. 4C and 5C), and AP-1 activity to a similar magnitude as $PGE_2$ exposure (Fig. 6A), indicating that EP2 receptor mediates the effects of $PGE_2$ on these parameters. This conclusion is substantiated by the fact that EP1 receptor agonist, EP3/EP1 receptor agonist, or EP4/EP3 receptor agonist shows minimal or no effect on c-Fos protein expression (Fig. 5C). To our knowledge, this is the first study to demonstrate the participation of Erk/AP-1 pathway in $PGE_2$-induced cell proliferation through EP2 receptor in human esophageal squamous-cell carcinoma.

In conclusion, we demonstrate that $PGE_2$ promotes human esophageal squamous-cell carcinoma cell proliferation mainly through EP2 receptor. Moreover, the phosphorylation of Erk1/2 and the subsequent AP-1 activation are required for the mitogenic effect of $PGE_2$. Given the recent concerns regarding the safety of conventional COX-2 inhibitors (Vanchieri, 2004), our findings suggest that, by blocking only $PGE_2$ signaling instead of global prostaglandin synthesis, targeting only at EP2 receptor may represent a promising therapeutic strategy for the treatment of esophageal squamous-cell carcinoma that deserves further clinical investigation.

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**Footnotes:**

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**Legends for figures:**

**Figure 1**. Expression of EP receptors in human esophageal squamous-cell carcinoma cells. (A) Results from RT-PCR revealed that the transcripts of all four EP receptor subtypes, EP1 to EP4 receptors, were present in a panel of esophageal squamous-cell carcinoma cell lines (HKESC-1, HKESC-2, HKESC-3, EC109 and KYSE150). Direct PCR amplifications of mRNA without prior reverse transcription were used as negative control. (B) The protein expression of all four EP receptor subtypes in these cell lines was further verified by Western blot. The protein expression of COX-1 and COX-2 was also verified by Western blot.

**Figure 2**. Involvement of EP receptors in $PGE_2$-induced HKESC-1 cell proliferation. Cells were deprived of serum for 24 h and thereafter treated for another 24 h with (A) $PGE_2$, (B) EP1 receptor agonist ONO-DI-004, EP2 receptor agonist butaprost, EP3/EP1 receptor agonist sulprostone or EP4/EP3 receptor agonist $PGE_1$ alcohol at the indicated concentrations. In a parallel set of experiments, serum-deprived HKESC-1 cells were pre-treated for 1 h with specific (C) EP3 receptor antagonist ONO-AE3-240 or EP4 receptor antagonist ONO-AE3-208 prior to treatment with 10 μmol/L $PGE_2$ for another 24 h. Cell proliferation was then determined as the amount of DNA synthesized by [$^3$H]thymidine incorporation assay. (D) After transfection with EP2 receptor-siRNA, cells were treated with 10 μmol/L $PGE_2$ for 24 h and examined for proliferation by [$^3$H]thymidine incorporation assay. The efficacy of the EP2 receptor depletion by EP2 receptor-siRNA was verified by Western blot analysis. Scrambler siRNA was used as a control. beta-actin was used to evaluate protein loading. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate. **, $p<0.01$, ***, $p<0.001$ versus respective control group; †, $p<0.001$versus $PGE_2$-treated group.

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**Figure 3**. Stimulatory effect of $PGE_2$ and EP2 receptor agonist butaprost on phosphorylation of Erk1/2 in HKESC-1 cells. (A) Serum-deprived cells were lysed after 10 μmol/L $PGE_2$ stimulation, and lysates were probed with phospho-Erk1/2, Erk1/2, phospho-p38, p-38, phospho-JNK, phospho-Akt (Thr308), phospho-Akt (Ser473), Akt antibodies, as indicated. Data shown are representative of three independent experiments. (B) After transfection with Erk1- or Erk2-siRNA, cells were treated with 10 μmol/L $PGE_2$ for 24 h and examined for proliferation by [$^3$H]thymidine incorporation assay. Scrambler siRNA was used as a control. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate. **, $p<0.01$, ***, $p<0.001$ versus respective control group; †, $p<0.001$ versus $PGE_2$-treated group. (C) The expressions of Erk1 and Erk2 after respective siRNA transfection were evaluated by Western blot analysis. Scrambler siRNA was used as a control. beta-actin was used to evaluate protein loading. Data shown are representative of three independent experiments. (D) Serum-deprived cells were lysed after 25 μmol/L butaprost stimulation for 10 min, and lysates were probed with phospho-Erk1/2 and Erk1/2 antibodies. Data shown are representative of three independent experiments.

**Figure 4**. Effects of $PGE_2$ and EP2 receptor agonist butaprost on the mRNA expression of members of Fos and Jun families in HKESC-1 cells as determined by quantitative real-time PCR. The mRNA expression of members of (A) Fos and (B) Jun families (expressed as % of control) was up-regulated in response to treatment with 10 μmol/L $PGE_2$ for 30 min and 60 min. The mRNA expression of members of the (C) Fos and (D) Jun families (expressed as % of control) showed similar increases in response to treatment with EP2 receptor agonist butaprost (25 μmol/L) for 30 min and 60 min. beta-actin was used as an internal control for normalization. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate. **,

*p*<0.01, ***, *p*<0.001 versus respective control group.

**Figure 5**. Involvement of EP2 receptor and Erk1/2 phosphorylation in $PGE_2$ induced c-Fos protein expression. (A) Serum-deprived HKESC-1 cells were exposed to 10 μmol/L $PGE_2$ and collected at 0, 1, 2, 3, 6, 12 and 24 h for the determination of c-Fos protein expression by Western blot analysis. (B) Cells were pre-treated with MEK inhibitor U0126 for 1 h prior to treatment with 10 μmol/L $PGE_2$ for another 1 h. Cells were thereafter collected for determination of c-Fos protein level by Western blot analysis. Protein expression of c-Fos in response to (C) EP1 receptor agonist ONO-DI-004, EP2 receptor agonist butaprost, EP3/EP1 receptor agonist sulprostone or EP4/EP3 receptor agonist $PGE_1$ alcohol treatment was determined by Western blot analysis. Serum-deprived cells were collected after 1 h treatment with respective EP receptor agonists. beta-actin was used to evaluate protein loading. Data shown are representative of three independent experiments.

**Figure 6**. Involvement of AP-1 activation in $PGE_2$-induced cell proliferation in HKESC-1 cells. (A) Cells were transfected with a ratio 10:1 of the pAP-1 (PMA)-luc plasmid and pRL-TK plasmid. After exposure to 10 μmol/L $PGE_2$ or 25 μmol/L butaprost for 6 h, cells were collected for determination of AP-1 activity. For investigating the role of Erk1/2 phosphorylation in $PGE_2$-induced AP-1 activation, cells were pretreated with 1 μmol/L MEK inhibitor U0126 for 1 h prior to treatment with 10 μmol/L $PGE_2$ for another 6 h. pAP-1 (PMA)-luc luciferase activities were normalized by pRL-TK luciferase activities for transfection efficiency. PMA at 100 nmol/L was used as a positive control. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate. ***, *p*<0.001 versus respective control group, †, *p*<0.001 versus $PGE_2$-treated group. (B) Serum-deprived cells were pre-treated with 10 μmol/L curcumin

for 1 h prior to treatment with 10 μmol/L $PGE_2$ for another 24 h. Cell proliferation was then examined by [$^3$H]thymidine incorporation assay. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate. *, $p<0.05$, ***, $p<0.001$ versus respective control group; †, $p<0.001$ versus $PGE_2$-treated group.

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**Fig.1**

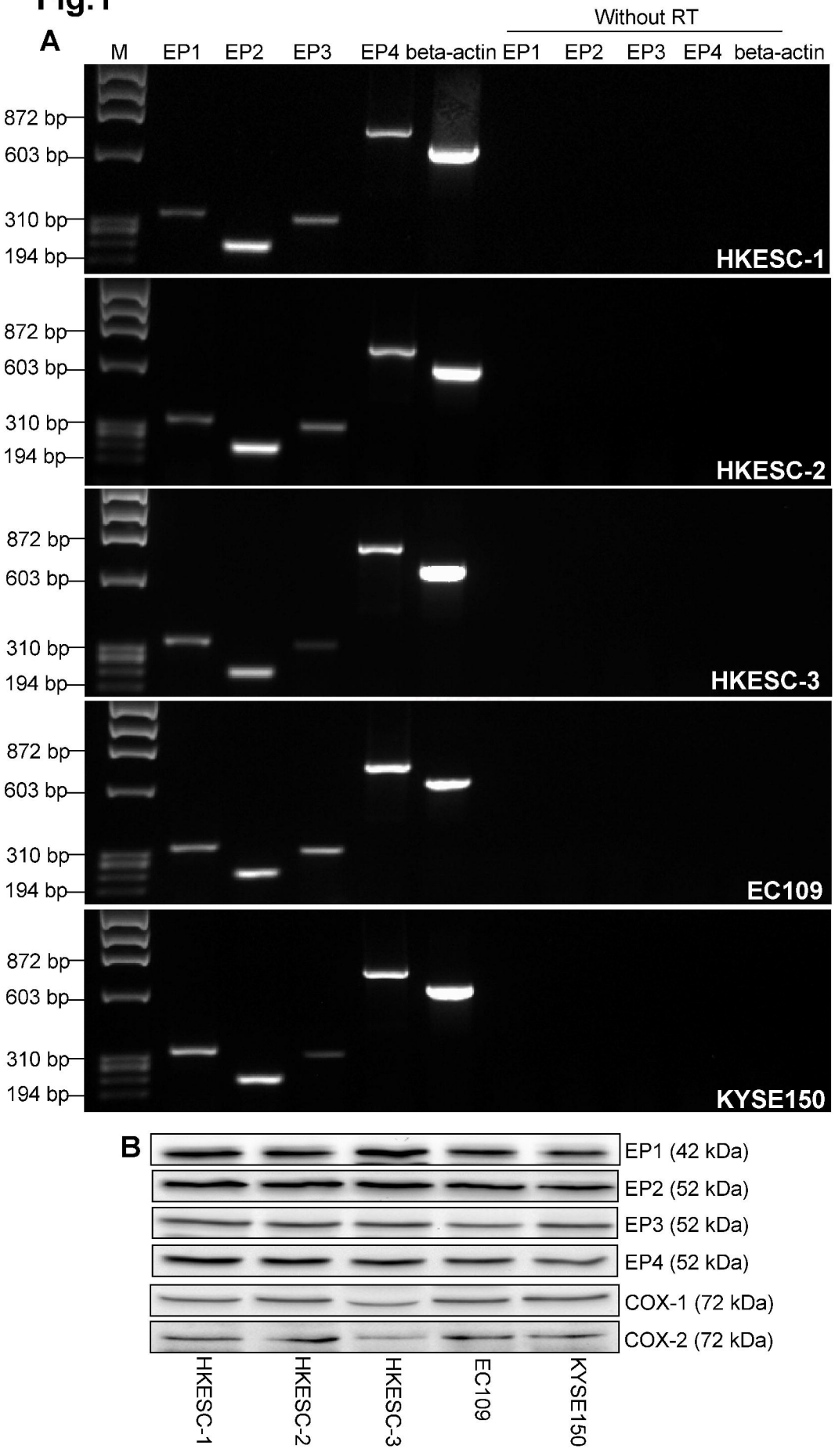

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**Fig. 2**

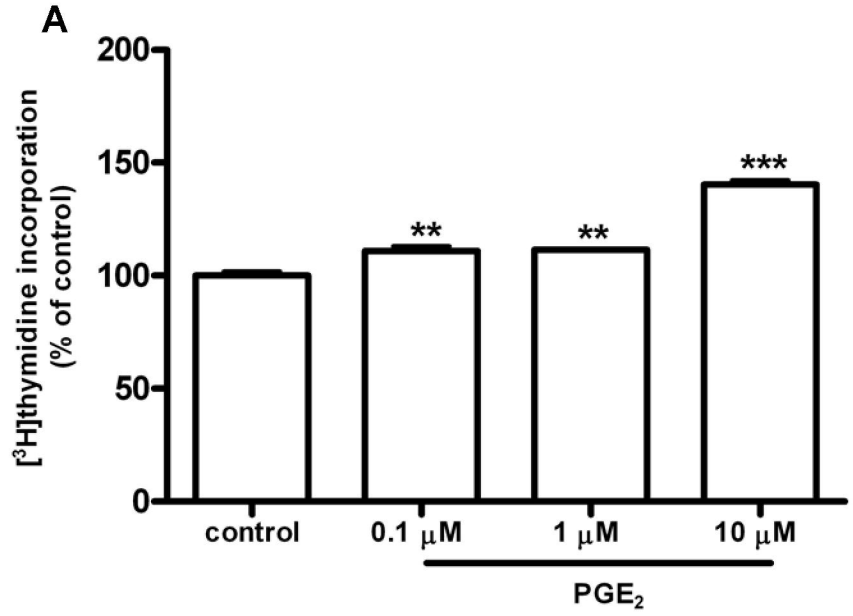

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**Fig. 2**

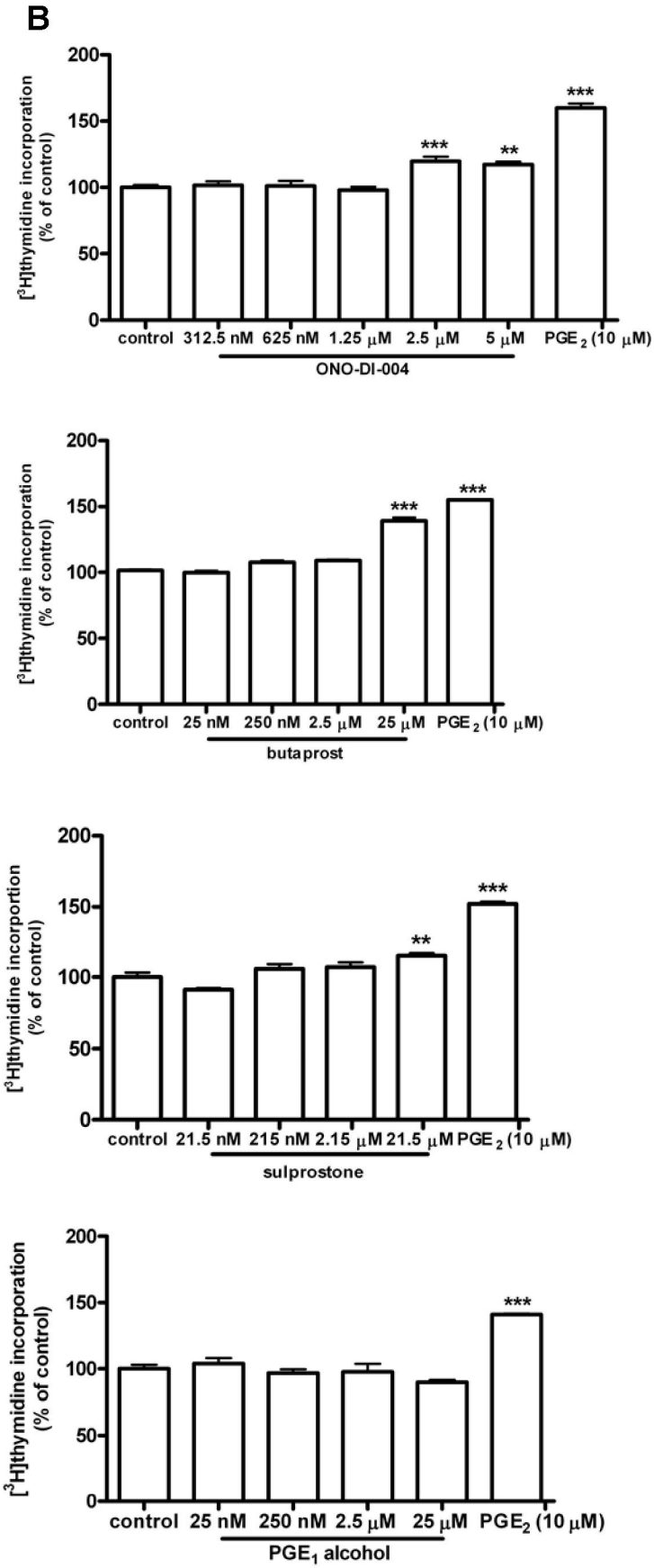

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**Fig. 2**

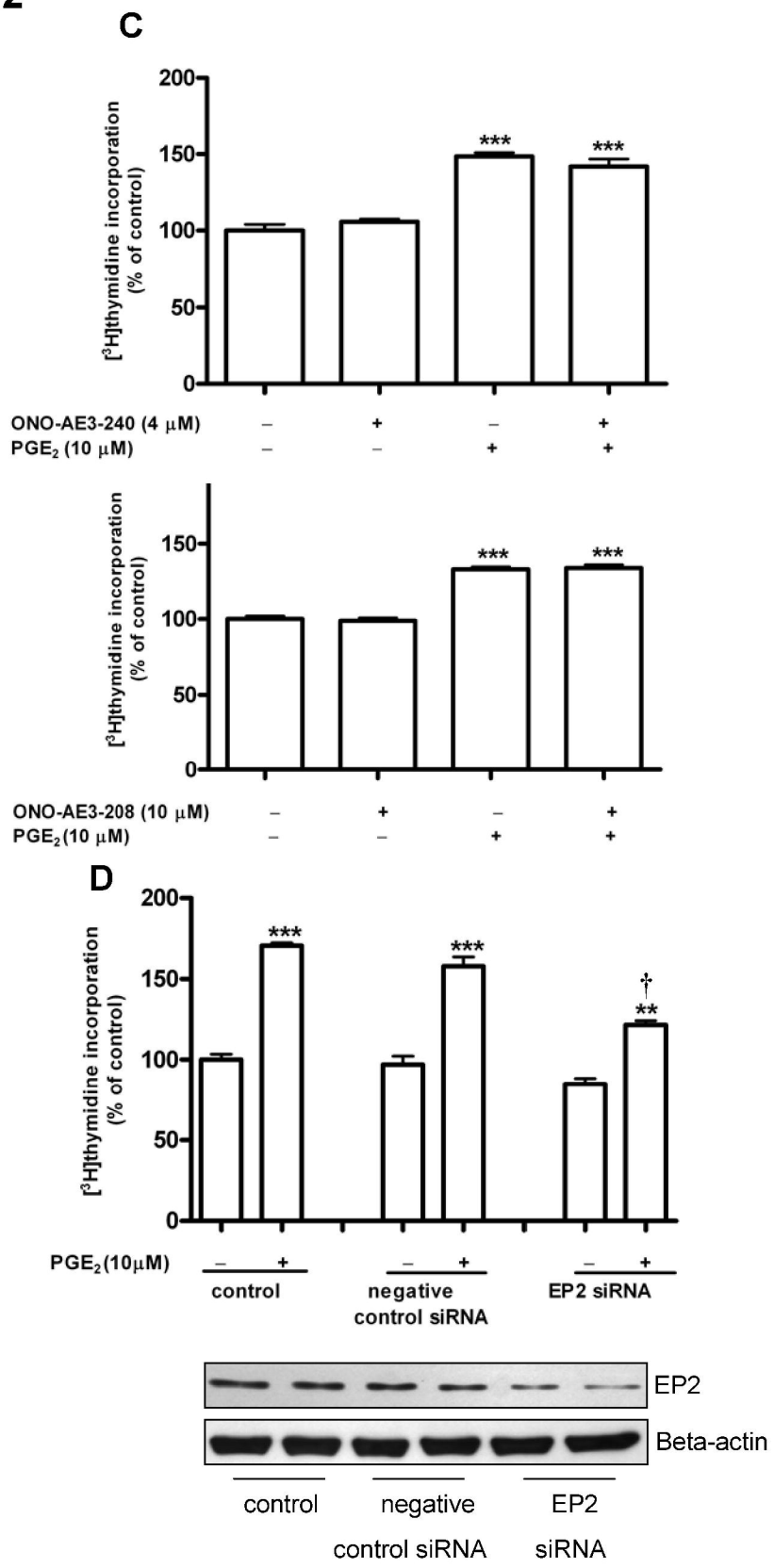

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**Fig.3**

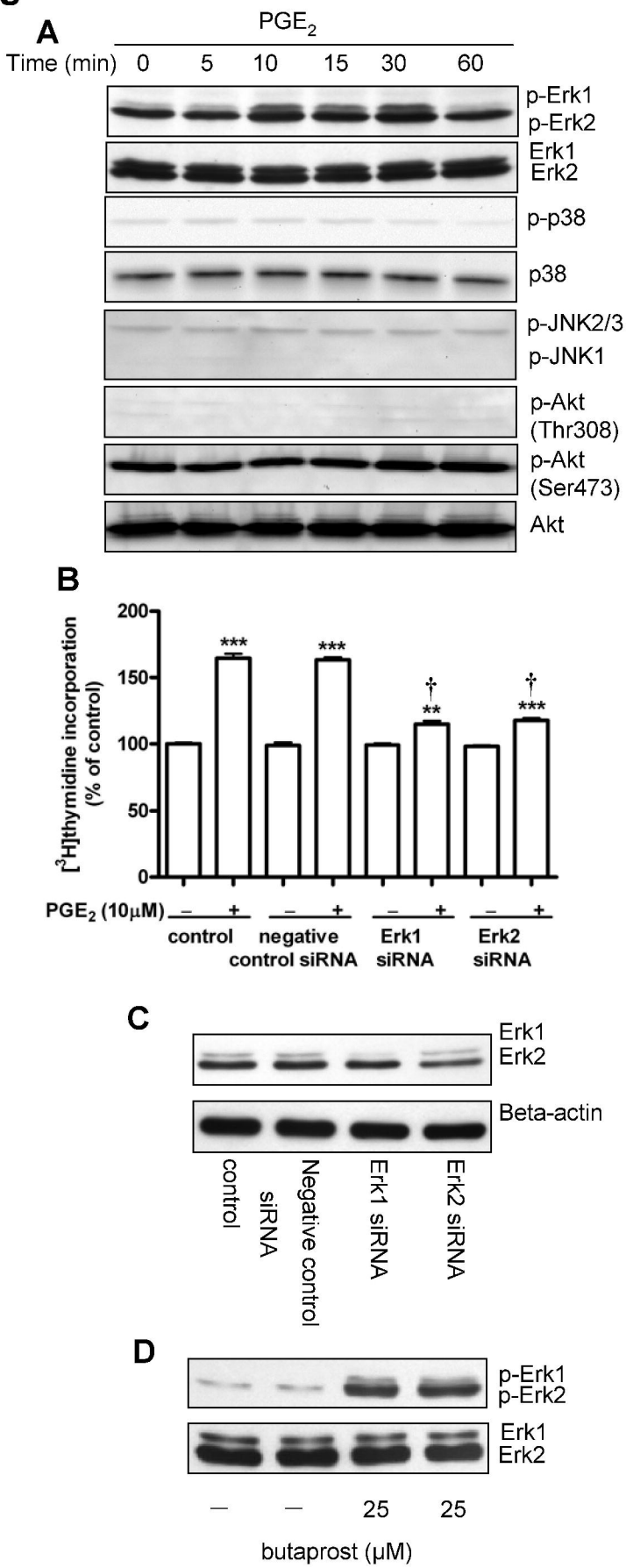

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**Fig.4**

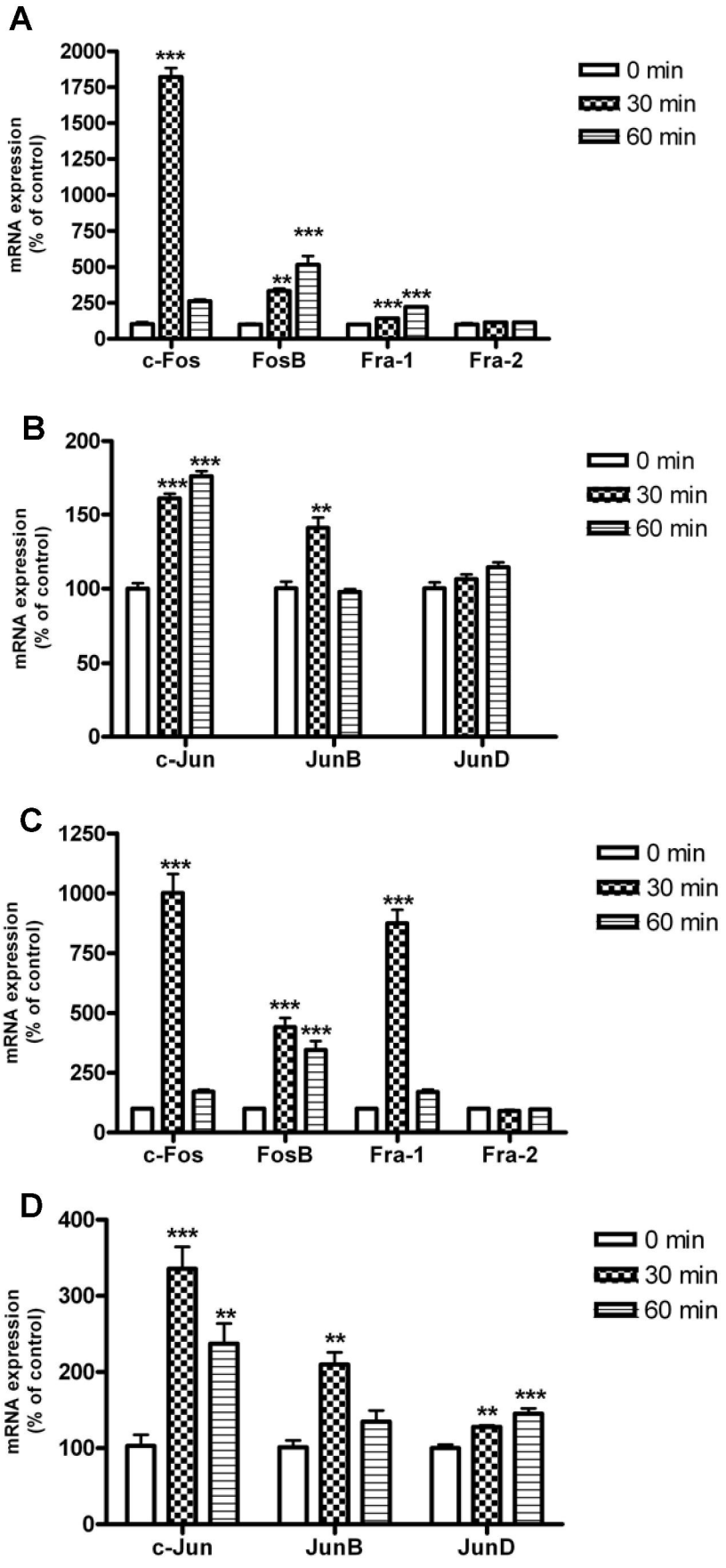

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**Fig. 5**

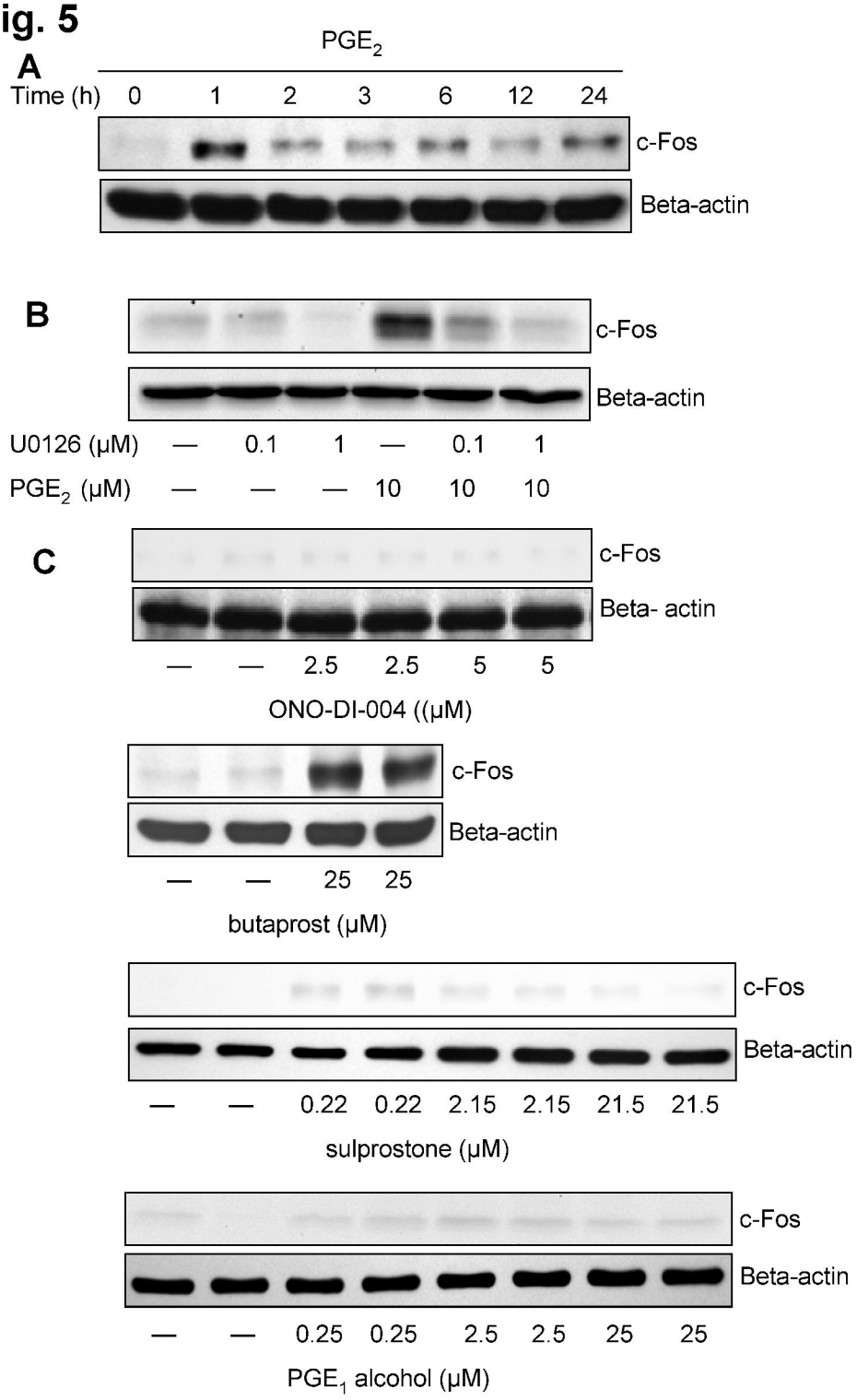

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**Fig. 6**