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E Series of Prostaglandin Receptor 2-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[S]

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ABSTRACT

The use of nonsteroidal 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. However, the precise role of PGE_2 in the disease development of esophageal squamous cell carcinoma 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 series of prostaglandin (EP) receptors, namely, EP1 to EP4 receptors. In this regard, PGE_2 and the EP2 receptor agonist (±)-15-deoxy-16*S*-hydroxy-17-cyclobutyl PGE_1 methyl ester (butaprost) markedly increased HKESC-1 cell proliferation. Moreover, the mitogenic effect of PGE_2 was significantly attenuated by RNA interference-mediated knockdown of the 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 inhibitor 1,4-diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)-butadiene ethanolate (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 the EP2 receptor mediates the mitogenic effect of PGE_2 in esophageal squamous cell carcinoma via activation of the 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.

Esophageal cancer is a highly aggressive malignant disease with a 5-year survival rate of 10 to 15% (Jemal et al., 2003). There are two major histological types of esophageal cancer, 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 multifactorial, but cigarette smoking and alcohol consumption are

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ABBREVIATIONS: NSAIDs, nonsteroidal anti-inflammatory drugs; COX, cyclooxygenase; PG, prostaglandin; Erk, extracellular signal-regulated kinase; Akt, protein kinase B; EP, E series of prostaglandin; butaprost, (±)-15-deoxy-16*S*-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; siRNA, small interference RNA; ONO-DI-004, 17*S*-17,20-dimethyl-2,5,-ethano-6-oxo PGE_1; ONO-AE3-208, 2-(2-(2-methyl-2-naphth-1-ylacetylamino)-phenylmethyl)-benzoic acid; RT-PCR, reverse transcription-polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate; IBMX, 1-methyl-3-isobutylxanthine; JNK, c-Jun amino-terminal kinase; MAPK, mitogen-activated protein kinase; AP-1, activator protein 1; U0126, 1,4-diamino-2,3-dicyano-1,4-bis-(o-aminophenylmercapto)butadiene ethanolate; MEK, MAPK/Erk kinase; SC-236, 4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]-benzenesulfonamide; L-748706, 3-(3,4-difluorophenyl)-4-(4-methylsulfonyl)phenyl-2-(5*H*)-furanone; forskolin, 7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxylabd-14-en-11-one; ONO-AE3-240, 2-[2-{[4-methyl-2-(1-naphthyl)pentanoyl]amino}-4-(1*H*-pyrazol-1-ylmethyl)benzyl]benzoic.

two of the leading risk factors (Stoner et al., 2007). Compelling epidemiological evidence also shows that regular or occasional use of aspirin or other nonsteroidal 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, whereas 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 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 (Lau et al., 1987; Morgan, 1997; Gupta and DuBois, 2000). Moreover, PGE_2 promotes intestinal adenoma growth in APC^{Min} mice (Wang et al., 2004), and it 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 (Erk) 1/2 and protein kinase B (Akt) may be required for the stimulatory effect of PGE_2 on cell proliferation (Leng et al., 2003; Han and Wu, 2005; Krysan et al., 2005; Cherukuri et al., 2007).

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. Therefore, molecules involved in the COX-2 signaling become attractive targets in pathway-directed cancer therapy. To this end, there is a growing interest in the development of antagonists for E series of prostaglandin (EP) receptors, which are designated as EP1 to EP4 receptors. The procarcinogenic role of different EP receptors has been supported by studies involving the use of knockout 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 al., 2002). Disruption of the EP2 receptor also decreases the number and size of intestinal polyps, the intensity of angiogenesis, and vascular endothelium growth factor 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 vascular endothelium growth factor 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.

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 to EP4 receptors and COX-2 were purchased from Cayman Chemical (Ann Arbor, MI). Erk1- and Erk2-small interference RNA (siRNA) and antibody to COX-1 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). EP2 receptor siRNA and control siRNA were obtained from QIAGEN GmbH (Hilden, Germany). All other primary antibodies were purchased from Cell Signaling Technology Inc. (Danvers, 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 obtained from Bio-Rad (Hercules, CA). All of 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, Hong Kong, China). 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 (Osaka, Japan). EC109 was provided by the Cancer Institute Chinese Academy of Medical Sciences (Beijing, China). HKESC-1, HKESC-2, and HKESC-3 were maintained in minimal essential medium; KYSE150 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°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. In brief, 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 before treatment with PGE_2. In the next step, 0.5 μCi/ml [^{3}H]thymidine (GE Healthcare, 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 Coulter, 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. 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 reverse transcription-polymerase chain reaction (RT-PCR)

system (Invitrogen) in accordance with the manufacturer'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′ (reverse); EP3 receptor, 5′-CTTCGCATAACTGGGGCAAC-3′ (forward) and 5′-TCTCCGTGTGTGTCTTGCAG-3′ (reverse); EP4 receptor, 5′-AGACGACCTTCTACACGC-3′ (forward) and 5′-GACGAATACTCGCACCAC-3′ (reverse); and β-actin, 5′-AACACCCCAGCCATGTACG-3′ (forward) and 5′-CGCTCAGGAGGAGCAATGA-3′ (reverse). Conditions for PCR were 95°C for 5 min, 35 cycles of 94°C for 30 s, 55 to 60°C (see below) for 30 s, and 72°C for 1 min. The final extension step was at 72°C for 10 min. The annealing temperature was 58°C for the EP1 receptor, 55°C for the EP4 receptor, and 60°C for the EP2 and 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 the EP1 receptor, 216 bp for the EP2 receptor, 300 bp for the EP3 receptor, 731 bp for the EP4 receptor, and 623 bp for β-actin. For quantitation of mRNA expression, real-time PCR was performed with an iQ Multicolor Real-Time PCR Detection System (Bio-Rad) by using the SYBR GreenER 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′ (forward) and 5′-CGTCTGTGGCTCGTCCTTGA-3′ (reverse); and β-actin, 5′-AGCACTGTGTTGGCGTACAG-3′ (forward) and 5′-CTCTTCCAGCCTTCCTTCCT-3′ (reverse). PCR conditions were 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s for 45 cycles. The mRNA expression was calculated using the comparative threshold cycle method and normalized against expression of β-actin.

Western Blot. Cells were lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 0.5% α-cholate acid, 0.1% SDS, 2 mM 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°C, the supernatant was collected, and protein concentration was determined by assay kit (Bio-Rad). Equal amounts of protein (50 μg/lane) were resolved with SDS-polyacrylamide gel electrophoresis and transferred to Hybond C nitrocellulose membranes (GE Healthcare). The membranes were probed with primary antibodies overnight at 4°C and incubated for 1 h with secondary peroxidase-conjugated antibody at room temperature. The signals on the membrane were visualized by enhanced chemiluminescence (GE Healthcare) and exposed to X-ray film (Fuji Photo Film, Tokyo, Japan).

RNA Interference. Cells were transiently transfected with siRNA oligonucleotides 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°C for 6 h. The transfected cells were then growth-arrested for another 18 h before treatments.

Luciferase Reporter Gene Transactivation Assay. Transient transfection with pAP-1 (phorbol 12-myristate 13-acetate; PMA)-TA-Luc luciferase reporter plasmid (Clontech, 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 a dual-luciferase reporter assay system, and luciferase activities were measured with a Lumat LB9501 luminometer (Berthold Technologies, Bad 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 by using the Correlate-EIA Prostaglandin E_2 Enzyme Immunoassay kit from Assay Designs (Ann Arbor, MI) according to the manufacturer's instructions. In brief, 4×10^4 cells/well were plated in a 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.

cAMP Assay. Intracellular cAMP assay was performed according to the manufacturer's instructions (GE Healthcare). In brief, 1×10^6 cells were treated with PGE_2, butaprost, and forskolin for 10 min in the presence of phosphodiesterase inhibitor IBMX (100 μM) to prevent the breakdown of cAMP. The cAMP level was then measured using a nonacetylation enzyme immunoassay procedure. The cAMP level was expressed as picomoles per milligram of protein.

Statistical Analysis. Results were expressed as the mean ± S.E.M. for at least three independent experiments. Statistical analysis was performed with an analysis of variance followed by the Turkey's *t* test. *p* values less than 0.05 were considered statistically significant.

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 five tested 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 investigated 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 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 μM. 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 25 μM concentration substantially increased HKESC-1 cell proliferation to an extent similar to that of 10 μM PGE_2, whereas the EP1 receptor agonist ONO-DI-004 and EP3/EP1 receptor agonist sulprostone at all concentrations tested only minimally stimulated HKESC-1 cell prolif-

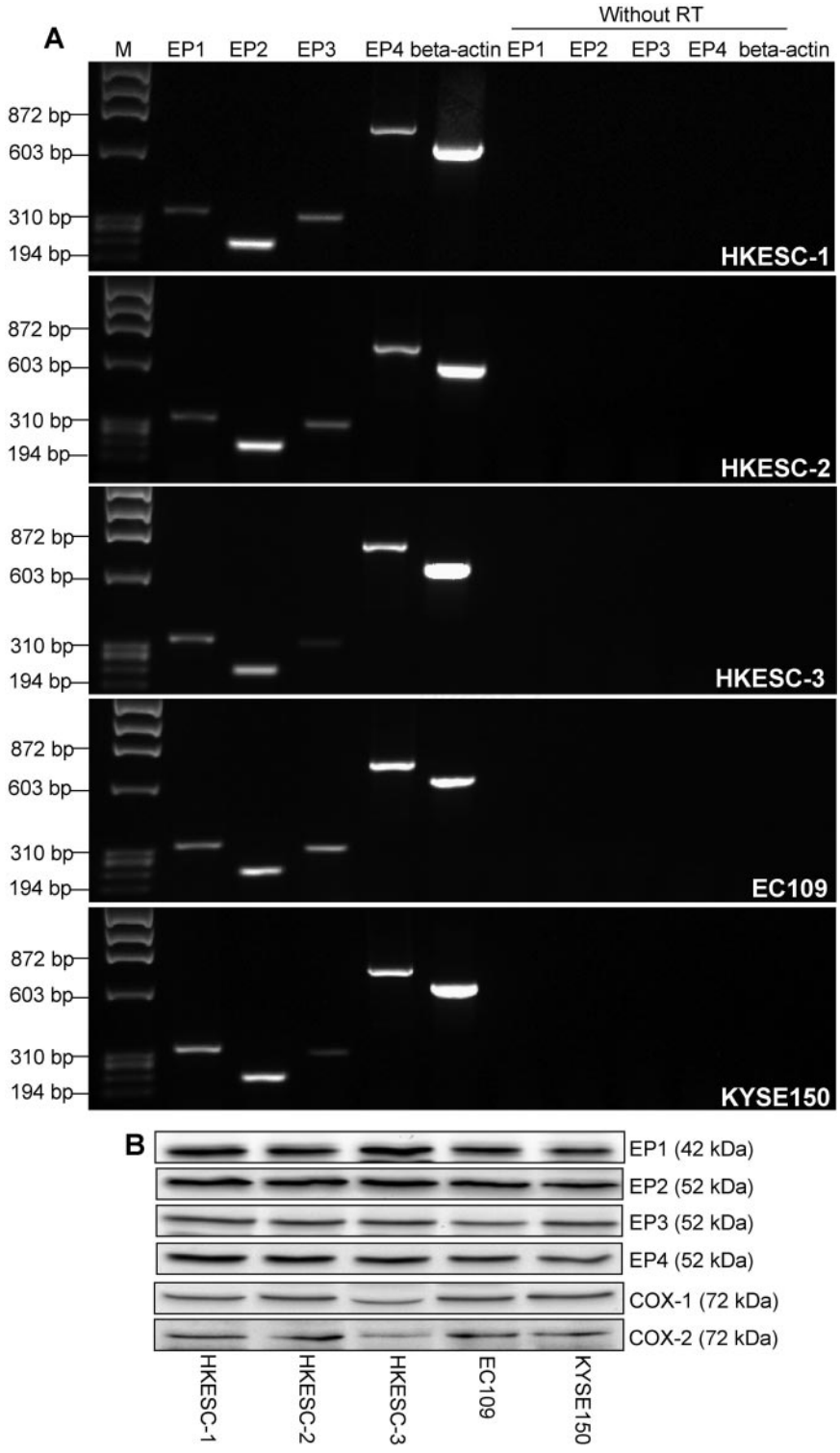
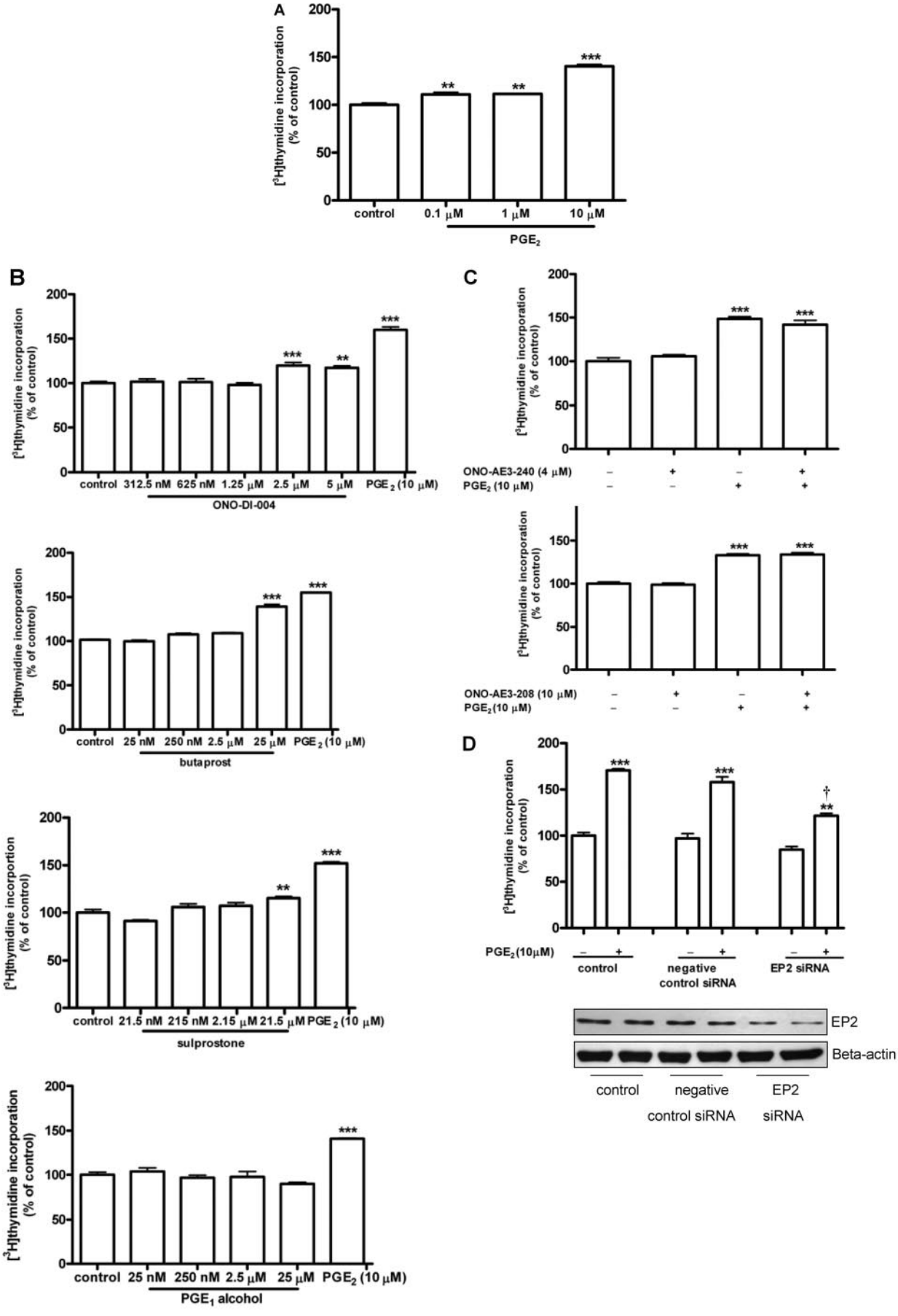


Fig. 1. Expression of EP receptors in human esophageal squamous cell carcinoma cells. A, results from RT-PCR revealed that the transcripts of all four EP receptor subtypes, EP1 to EP4 receptors, were present in a panel of esophageal squamous cell carcinoma cell lines (HKESC-1, HKESC-2, HKESC-3, EC109, and KYSE150). Direct PCR amplifications of mRNA without prior reverse transcription were used as a 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.

eration (Fig. 2B). These data indicated that the EP2 receptor, and to a lesser extent EP1 receptor, were involved in mediating the stimulatory effect 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), whereas the 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. Because the EP2 receptor agonist butaprost strongly increased HKESC-1 cell proliferation compared with other agonists, the role of the EP2 receptor in PGE_2-induced cell proliferation was further investigated by RNA interference experiments. Using specific siRNA, down-regulation of the EP2 receptor significantly attenuated PGE_2-induced proliferation in HKESC-1 cells (Fig. 2D, top). The efficacy of EP2 receptor depletion was further verified by Western blot analy-



sis, 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. Because phosphorylation of Akt and Erk1/2 has been suggested to mediate the growth-promoting effect of PGE_2 in other cancer cell types (Leng et al., 2003; Han and Wu, 2005; Krysan et al., 2005; Cherukuri et al., 2007), 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 c-Jun amino-terminal kinase (JNK), 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. 3, B and C). Because the EP2 receptor seemed 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 μM also markedly increased Erk1/2 phosphorylation after 10 min of 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). Therefore, we measured the mRNA expression levels of these AP-1 components in HKESC-1 cells treated with or without PGE_2. As shown in Fig. 4, A and B, 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, the 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. 3, C and D). Similar to PGE_2 treatment, the change in c-Fos expression was the most prominent among the up-regulated genes, up to a 10-fold increase compared with control. We also observed that the time-course changes in FosB, Fra-1, and c-Jun 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, whereas PGE_2 can activate all four EP receptor subtypes that EP1, EP3, and EP4 receptors may also involve in regulating the expression of these genes.

PGE_2 Induced c-Fos Protein Expression That was Abolished by MAPK/Erk Kinase 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 conditions was almost undetectable, whereas 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 μM completely abolished PGE_2-induced c-Fos expression (Fig. 5B). Likewise, the 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. Because 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, 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).

Discussion

Overexpression of COX-2 and the subsequent elevation of PGE_2 levels have been implicated in the pathogenesis of human esophageal squamous cell carcinoma (Morgan, 1997; Zimmermann et al., 1999; Zhi et al., 2006). In this study, 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 four other 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. (1999) also demonstrates

Fig. 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 PGE_2 (A), EP1 receptor agonist ONO-DI-004 (B), 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 pretreated for 1 h with specific EP3 receptor antagonist ONO-AE3-240 (C) or EP4 receptor antagonist ONO-AE3-208 before treatment with 10 μM 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 the EP2 receptor siRNA, cells were treated with 10 μM 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. β-Actin was used to evaluate protein loading. Data are presented as mean ± S.E.M. ($n = 3$) of a representative experiment performed in triplicate. **, $p < 0.01$ and ***, $p < 0.001$ versus the respective control groups; †, $p < 0.001$versus the PGE_2-treated group.

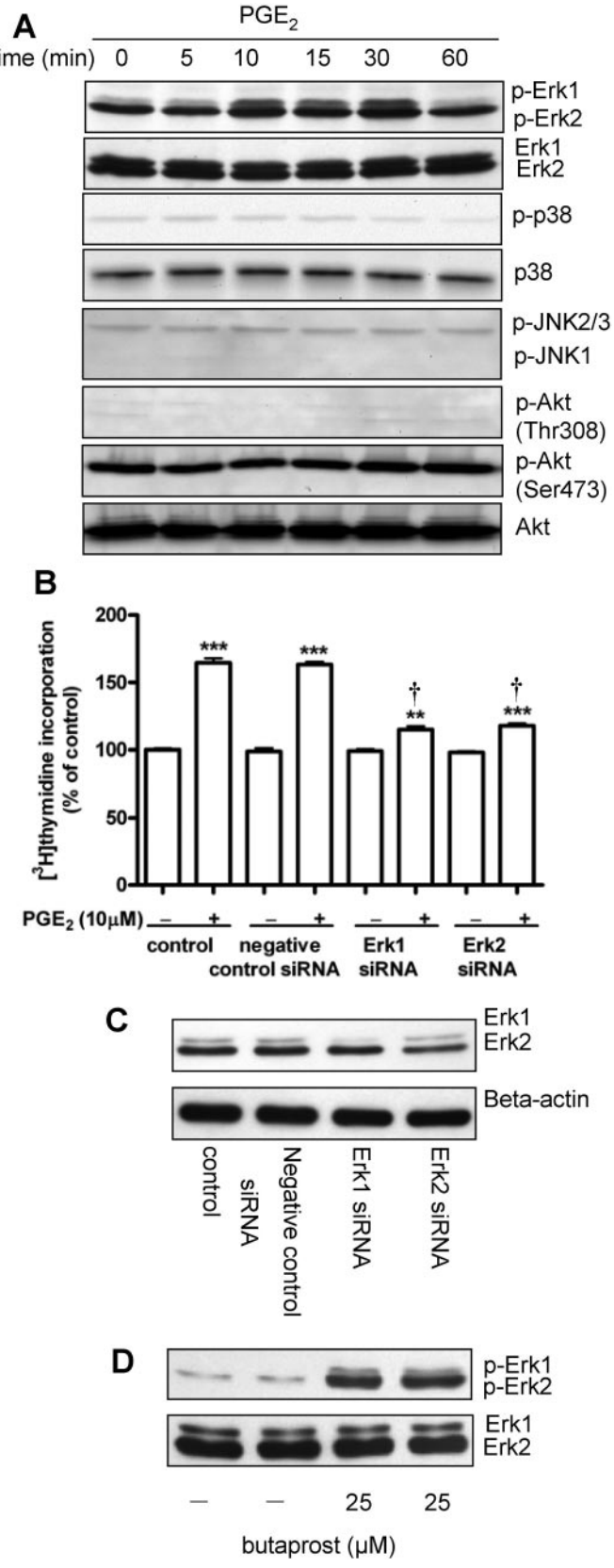


Fig. 3. Stimulatory effect of PGE_2 and the EP2 receptor agonist butaprost on phosphorylation of Erk1/2 in HKESC-1 cells. A, serum-deprived cells were lysed after 10 μM PGE_2 stimulation, and lysates were probed with phospho-Erk1/2, Erk1/2, phospho-p38, *p*-38, phospho-JNK, phospho-Akt (Thr308), phospho-Akt (Ser473), and 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 μM 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 ± S.E.M. ($n = 3$) of a representative experiment performed in triplicate. **, $p < 0.01$ and ***, $p < 0.001$ versus the respective control groups; †, $p < 0.001$ 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. β-Actin was used to evaluate protein loading. Data shown are representative of three independent experiments. D, serum-deprived cells were lysed after 25 μM 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.

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. These observations indicate that PGE_2 exerts its procarcinogenic 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 the 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, whereas knockdown of the EP2 receptor attenuates the PGE_2-induced proliferative response (Fig. 2, B and D). Up-regulation of EP receptors has been reported in rat Barrett's metaplasia, a premalignant lesion of esophageal adenocarcinoma, induced by duodenal contents reflux (Jang et al., 2004). In this study, we provide direct evidence that the 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 the 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 (Han and Roman, 2004; Castellone et al., 2005; Donnini et al., 2007).

MAPK cascades (Erk1/2, p38, and JNK) and the phosphatidylinositol 3-kinase/Akt pathway are key signaling molecules involved in the regulation of cell proliferation, survival, and differentiation. Therefore, it 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. 3, B and C), suggesting that phosphorylation of Erk1/2 but not the other two members of MAPK cascades is required for the mitogenic effect of PGE_2. It is interesting to note that 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 (Zimmermann et al., 1999; Zhi et al., 2006). Therefore, it is possible that COX-2-derived PGE_2 may enhance a positive feedback loop to stimulate cell prolifera-

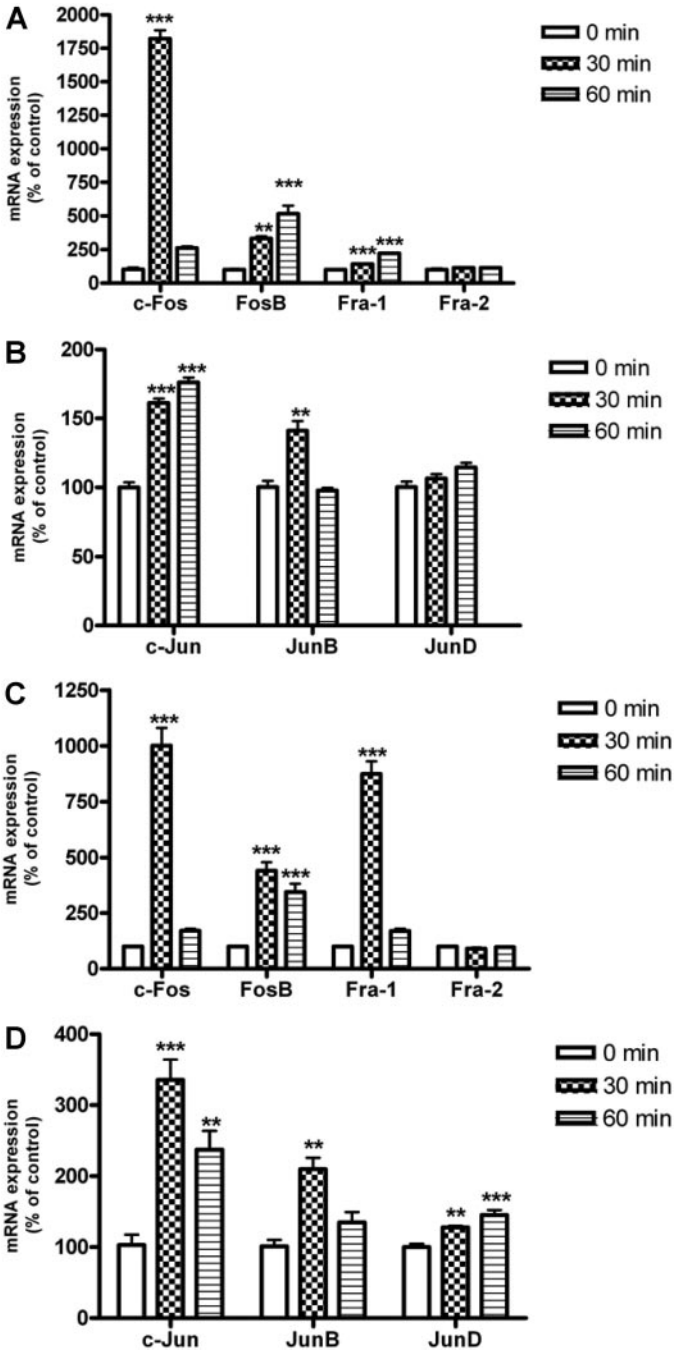


Fig. 4. Effects of PGE_2 and the EP2 receptor agonist butaprost on the mRNA expression of members of Fos and Jun families in HKESC-1 cells as determined by quantitative real-time PCR. The mRNA expression of members of Fos (A) and Jun families (B) (expressed as percentage of control) was up-regulated in response to treatment with 10 μM PGE_2 for 30 and 60 min. The mRNA expression of members of the Fos (C) and Jun (D) families (expressed as percentage of control) showed similar increases in response to treatment with the EP2 receptor agonist butaprost (25 μM) for 30 and 60 min. β-Actin was used as an internal control for normalization. Data are presented as mean ± S.E.M. ($n = 3$) of a representative experiment performed in triplicate. **, $p < 0.01$ and ***, $p < 0.001$ versus the respective control groups.

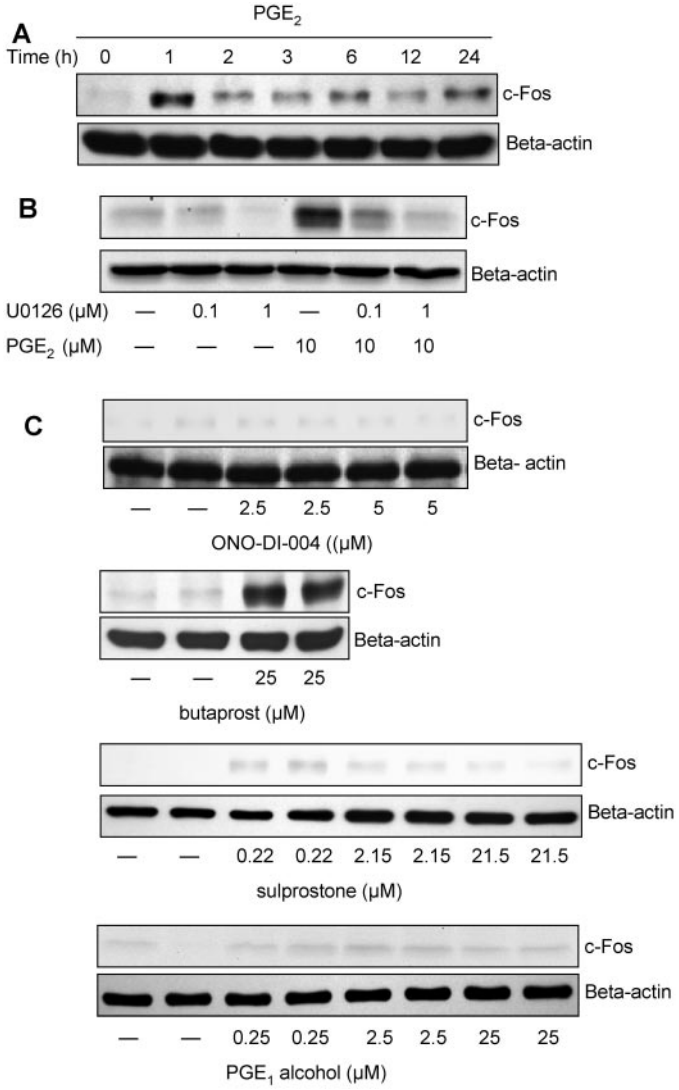


Fig. 5. Involvement of the EP2 receptor and Erk1/2 phosphorylation in PGE_2 induced c-Fos protein expression. A, serum-deprived HKESC-1 cells were exposed to 10 μM PGE_2 and collected at 0, 1, 2, 3, 6, 12, and 24 h for the determination of c-Fos protein expression by Western blot analysis. B, cells were pretreated with the MEK inhibitor U0126 for 1 h before treatment with 10 μM PGE_2 for an additional hour. Thereafter, cells were collected for determination of c-Fos protein level by Western blot analysis. Protein expression of c-Fos in response to EP1 receptor agonist ONO-DI-004 (C), 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. β-Actin was used to evaluate protein loading. Data shown are representative of three independent experiments.

tion in esophageal squamous cell carcinoma cells. Regulation of Erk1/2 activity by cAMP has been observed in some cell lines (Gerits et al., 2008). Because the EP2 receptor is a Gs protein-coupled receptor, it may regulate Erk1/2 activity via the cAMP pathway. In this respect, we investigated the effects of cAMP on activation of Erk1/2 in HKESC-1 cells. Although forskolin, an adenylate cyclase activator, increased the intracellular cAMP level more potently than the EP2 agonist butaprost and PGE_2 (Supplementary Fig. 1), it does not influence Erk1/2 phosphorylation and cell proliferation (Supplementary Fig. 2, A and B). Thus, a mechanism other than the cAMP pathway may be involved in EP2 receptor-mediated HKESC-1 cell proliferation. This theory needs further exploration. Apart from the MAPK cascade, Akt has been implicated in PGE_2-induced cholangiocarcinoma and hepatocellular carcinoma cell proliferation (Leng et al., 2003; Han and Wu, 2005). Akt becomes activated as a result of

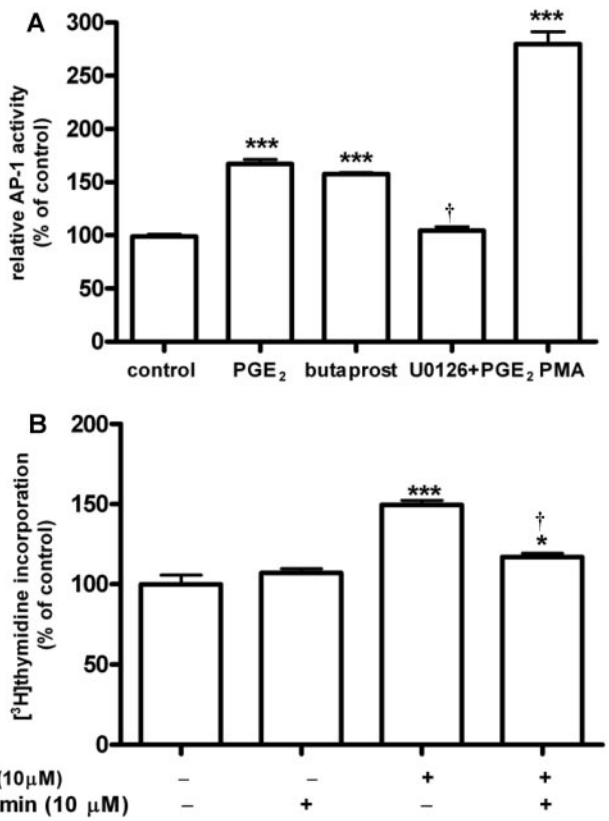


Fig. 6. Involvement of AP-1 activation in PGE_2-induced cell proliferation in HKESC-1 cells. A, cells were transfected with a ratio 10:1 of the pAP-1 (PMA)-luc plasmid and pRL-TK plasmid. After exposure to 10 μM PGE_2 or 25 μM butaprost for 6 h, cells were collected for determination of AP-1 activity. For investigating the role of Erk1/2 phosphorylation in PGE_2-induced AP-1 activation, cells were pretreated with 1 μM MEK inhibitor U0126 for 1 h before treatment with 10 μM PGE_2 for an additional 6 h. pAP-1 (PMA)-luc luciferase activities were normalized by pRL-TK luciferase activities for transfection efficiency. PMA at 100 nM was used as a positive control. Data are presented as mean ± S.E.M. ($n = 3$) of a representative experiment performed in triplicate. ***, $p < 0.001$ versus the respective control groups; †, $p < 0.001$ versus the PGE_2-treated group. B, serum-deprived cells were pretreated with 10 μM curcumin for 1 h before treatment with 10 μM PGE_2 for another 24 h. Cell proliferation was then examined by [^{3}H]thymidine incorporation assay. Data are presented as mean ± S.E.M. ($n = 3$) of a representative experiment performed in triplicate. *, $p < 0.05$ and ***, $p < 0.001$ versus the respective control groups; †, $p < 0.001$ versus the PGE_2-treated group.

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 multistage development of tumors (Liu et al., 2002; Young et al., 2003). 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 after 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, proinflammatory 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). In agreement, our study reveals that PGE_2 dramatically increased c-Fos expression and AP-1 transcriptional activity (Figs. 5A and 6A), both of which can be abolished by the MEK inhibitor U0126 (Figs. 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 (Figs. 4C and 5C), and AP-1 activity to a similar magnitude as PGE_2 exposure (Fig. 6A), indicating that the EP2 receptor mediates the effects of PGE_2 on these parameters. This conclusion is substantiated by the fact that the 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 the Erk/AP-1 pathway in PGE_2-induced cell proliferation through the 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 the 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 the EP2 receptor may represent a promising therapeutic strategy for the treatment of esophageal squamous cell carcinoma and deserves further clinical investigation.

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