

## ORIGINAL ARTICLE

# Cellular density-dependent down-regulation of EP4 prostanoid receptors via the up-regulation of hypoxia-inducible factor-1 $\alpha$ in HCA-7 human colon cancer cells

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## Keywords

Cell density, COX-2, EP4 prostanoid receptor, GPCRs, HCA-7 cells, HIF-1 $\alpha$ , human colon cancer, PGE<sub>2</sub>.

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## Abstract

Increases in prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and cyclooxygenase-2 (COX-2) levels are features of colon cancer. Among the different E-type prostanoid receptor subtypes, EP4 receptors are considered to play a crucial role in carcinogenesis by, for example, inducing COX-2 when stimulated with PGE<sub>2</sub>. However, EP4 receptor levels and PGE<sub>2</sub>-induced cellular responses are inconsistent among the cellular conditions. Therefore, the connections responsible for the expression of EP4 receptors were investigated in the present study by focusing on cell density-induced hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ). The expression of EP4 receptors was examined using immunoblot analysis, quantitative polymerase chain reaction, and reporter gene assays in HCA-7 human colon cancer cells with different cellular densities. The involvement of HIF-1 $\alpha$  and its signaling pathways were also examined by immunoblot analysis, reporter gene assays, and with siRNA. We here demonstrated that EP4 receptors as well as EP4 receptor-mediated COX-2 expression levels decreased with an increase in cellular density. In contrast, HIF-1 $\alpha$  levels increased in a cellular density-dependent manner. The knockdown of HIF-1 $\alpha$  by siRNA restored the expression of EP4 receptors and EP4 receptor-mediated COX-2 in cells at a high density. Thus, the cellular density-dependent increase observed in HIF-1 $\alpha$  expression levels reduced the expression of COX-2 by decreasing EP4 receptor levels. This novel regulation mechanism for the expression of EP4 receptors by HIF-1 $\alpha$  may provide an explanation for the inconsistent actions of PGE<sub>2</sub>. The expression levels of EP4 receptors may vary depending on cellular density, which may lead to the differential activation of their signaling pathways by PGE<sub>2</sub>. Thus, cellular density-dependent PGE<sub>2</sub>-mediated signaling may determine the fate/stage of cancer cells, i.e., the surrounding environments could define the fate/stage of malignancies associated with colon cancer.

## Abbreviations

ANOVA, analysis of variance; BSA, bovine serum albumin; CoCl<sub>2</sub>, cobalt chloride; COX, cyclooxygenase; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGR-1, early growth response factor-1; EP, E-type prostanoid receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HIF, hypoxia inducible factor; HRE, hypoxia response element; PCR, polymerase chain reaction; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

## Introduction

One major pathophysiological feature of colon cancer is increased levels of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and cyclooxygenase-2 (COX-2), an enzyme that produces prostanoids including PGE<sub>2</sub> (Eisinger *et al.* 2007). The actions of PGE<sub>2</sub> are known to be mediated by its cognate receptors, namely EP receptors (Woodward *et al.* 2011). G-protein-coupled EP receptors consist of four subtypes: EP1 to EP4 receptors (Woodward *et al.* 2011). Based on the findings of knockout-mice studies and research using pharmacological reagents, all EP receptors are generally considered to play a role in carcinogenesis (Woodward *et al.* 2011). However, of the four EP receptor subtypes, EP4 receptors and their signaling pathways are suggested to be critically involved in carcinogenesis because of their wide distribution in the body and various pathophysiological roles (Konya *et al.* 2013; Yokoyama *et al.* 2013). In contrast to EP2 receptors, conventional G<sub>zs</sub> protein-coupled EP4 receptors have been shown to activate a number of important signaling pathways including G<sub>zi</sub> protein-mediated signaling (Konya *et al.* 2013; Yokoyama *et al.* 2013). These signaling pathways include phosphatidylinositol 3-kinase-, extracellular signal-regulated kinases (ERKs)-,  $\beta$ -catenin-, and/or  $\beta$ -arrestine-mediated pathways, which may be key regulators in development of cancer (Fujino and Regan 2003; Konya *et al.* 2013; Yokoyama *et al.* 2013). EP4 receptor signaling has also been shown to increase the expression of PGE<sub>2</sub> synthase and cyclin D1 by inducing the expression of early growth response factor-1 (EGR-1) (Fujino *et al.* 2003; Fujino and Regan 2003) as well as COX-2 (Hull *et al.* 2004; Yoshida *et al.* 2013). Therefore, these signaling pathways may establish a positive feedback loop in which an increase in the synthesis of PGE<sub>2</sub> may further drive the activation of EP4 receptors, as reported previously (Fujino and Regan 2003; Regan 2003).

A previous study indicated that the expression of EP4 receptors may be up-regulated in colorectal cancer and adenoma (Hawcroft *et al.* 2007). However, relatively higher mRNA expression levels for EP4 receptors have also been detected in normal colon tissue than those for the other EP receptor subtypes (Gustafsson *et al.* 2007). The intense stimulation of EP4 receptors in normal colonic epithelial cells provoked a loss in barrier integrity, which may play a role in the early onset and/or progression of colitis following inflammation (Lejeune *et al.* 2010). Colitis, especially ulcerative colitis, is a considerable risk factor for colon cancer. A previous study also reported that the expression of COX-2 was detected from a very early stage in carcinoma growth (Fujita *et al.* 1998). Since EP4 receptors are the key receptor subtype for the induction of colitis and COX-2 (Hull *et al.* 2004;

Yoshida *et al.* 2013), EP4 receptors may play important roles in the early stages of development of colon cancer, in other words, in the pro-tumorigenic progression of intestinal epithelial cells, as has been suggested previously (Chell *et al.* 2006; Hawcroft *et al.* 2007).

Although increases in PGE<sub>2</sub> and COX-2 expression levels have been documented extensively in the development of colon cancer, contradictory findings have been reported in terms of the effects of PGE<sub>2</sub> in colon cancer cell lines. For example, the dose-dependent proliferative activity of PGE<sub>2</sub> was previously observed in HT-29 human colorectal cancer cells (Qiao *et al.* 1995), whereas another study showed that PGE<sub>2</sub> had no effect on the proliferation of HT-29 cells (Cassano *et al.* 2000). Moreover, a bell-shaped proliferation response was noted in SW1116 human colon cancer cells when PGE<sub>2</sub> concentrations were increased (Qiao *et al.* 1995). These differences in the effects of PGE<sub>2</sub> have been attributed to the cell-line-specific differential expression of EP receptor(s), use of different proliferation assays, and/or differential activation of EP receptors at different PGE<sub>2</sub> concentrations (Lesuffleur *et al.* 1990; Cassano *et al.* 2000; Hull *et al.* 2004). Meanwhile, the positive effects of PGE<sub>2</sub> on cell growth were observed in colorectal adenoma and carcinoma cell lines treated with lower concentrations of PGE<sub>2</sub> (Chell *et al.* 2006). In contrast, the growth of colorectal adenoma cell lines, but not carcinoma cell lines, was inhibited at higher doses of PGE<sub>2</sub> (Chell *et al.* 2006). These findings suggest that, at some stage of carcinogenesis, an increase in PGE<sub>2</sub> levels may become inhibitory to tumor cells, i.e., PGE<sub>2</sub> may be able to switch colon cancer cells from being tumor-promoting to growth-inhibitory, as described previously (Chell *et al.* 2006). However, the actual reason(s) and/or underlying mechanism(s) for these differences in cellular responses remain unknown.

Hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) is an oxygen-sensitive transcriptional factor that has significant roles in the cellular physiology and pathophysiology of diseases such as the development of cancer (Semenza 2003; Mabjeesh and Amir 2007; Rohwer *et al.* 2013). Although the up-regulated expression of HIF-1 $\alpha$  is known to be evoked by responses to a reduction in the availability of oxygen, the functional regulation of HIF-1 $\alpha$  has also been reported under normoxic conditions (Kuschel *et al.* 2012). Thus, hypoxia may represent one of the factors among several HIF-1 $\alpha$ -inducing stimuli that regulate the expression of HIF-1 $\alpha$  (Paltoglou and Roberts 2005). In fact, in spite of the absence of hypoxic areas, increases in the expression of the HIF-1 $\alpha$  protein have been reported in the most common cancer types (Kuschel *et al.* 2012). Furthermore, HIF-1 $\alpha$  was previously shown to be induced in response to an increase in cellular density (Paltoglou and Roberts 2005), and this induction was even evoked

when cells were grown under standard normoxic conditions (Wiesener et al. 1998).

In the present study, we demonstrated that the cellular density-dependent induction of HIF-1 $\alpha$  protein expression was accompanied by and responsible for the decrease observed in the expression of the EP4 receptors in HCA-7 human colon cancer cells. PGE<sub>2</sub>-induced COX-2 expression was previously shown to be regulated by the activation of EP4 receptors (Yoshida et al. 2013); therefore, an increase in the expression of HIF-1 $\alpha$  eventually reduced the expression of COX-2 by decreasing EP4 receptor levels. These results may provide one explanation for the discrepant actions of PGE<sub>2</sub>, such as its effects on proliferation, as described above, which may be due to the altered expression of EP4 receptors followed by alternations in the expression of COX-2 that may eventually change the levels of PGE<sub>2</sub> produced as well as its actions. Although cellular proliferation may not be solely regulated by the turnover of PGE<sub>2</sub>/COX-2, some of the aforementioned conversions in cellular responses were triggered and regulated by cellular-density-dependent alternations in the expression of HIF-1 $\alpha$ , which may cause cells to respond differently to PGE<sub>2</sub> depending on the surrounding environment.

## Materials and Methods

### Cell culture and imaging

The human colon cancer cell line HCA-7 was cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St Louis, MO) containing 10% fetal bovine serum (Thermo Scientific, Waltham, MA), 100 IU/mL penicillin (Meiji Seika, Tokyo, Japan), and 100  $\mu$ g/mL streptomycin (Meiji Seika). All materials were obtained from Wako Pure Chemical (Osaka, Japan) unless otherwise stated. Cell images were obtained using a Nikon Diaphot microscope (Nikon, Tokyo, Japan) with a Nikon D70 digital camera (Nikon) and processed using Nikon Capture 4 (Nikon).

### Western blotting

Cells were cultured at low ( $3 \times 10^4$  cells/each 6 well), middle ( $1 \times 10^5$  cells/each 6 well), and high ( $3 \times 10^5$  cells/each 6 well) densities in 6-well plates, and medium was replaced with fresh Opti-MEM prior to immunoblotting experiments (Invitrogen, Carlsbad, CA) containing 100 IU/mL penicillin and 100  $\mu$ g/mL streptomycin. Cells were then treated with 1  $\mu$ mol/L PGE<sub>2</sub> (Cayman, Ann Arbor, MI) or 50 ng/mL epidermal growth factor (EGF) (Higeta Shoyu, Tokyo, Japan) for 6 h to induce COX-2 and phosphorylated ERKs. In experiments using HIF-1 $\alpha$  siRNA, cells were transiently transfected using Lipofecta-

mine RNAi MAX Reagent (Invitrogen) and 15  $\mu$ mol/L HIF-1 $\alpha$  siRNA (sc-35561; Santa Cruz Biotechnology, Santa Cruz, CA) for 8 h, and medium was then replaced with fresh Opti-MEM containing 100 IU/mL penicillin and 100  $\mu$ g/mL streptomycin for 40 h. To induce COX-2, cells were treated with 1  $\mu$ mol/L PGE<sub>2</sub> (Cayman) for another 6 h. Cells were scraped into lysis buffer consisting of 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 5 mmol/L EDTA (pH 8.0), 1% Igepal CA-630 (MP Bio-medicals, Aurora, OH), 0.5% sodium deoxycholate, 10 mmol/L sodium fluoride, 10 mmol/L disodium pyrophosphate, 0.1% SDS, 0.1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 10  $\mu$ g/mL leupeptin (Sigma), and 10  $\mu$ g/mL aprotinin and transferred to microcentrifuge tubes. Samples were rotated for 30 min at 4°C and centrifuged at 16,000g for 15 min. Aliquots of samples containing 40–100  $\mu$ g of protein were electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes as described previously (Fujino et al. 2003). The membranes were incubated for 1 h at room temperature in 5% nonfat milk. Incubations were conducted for 16 h at 4°C in 5% bovine serum albumin (BSA) (Sigma) containing a 1:2000 dilution of an anti-COX-2-antibody (sc-1745; Santa Cruz Biotechnology); 1:1000 dilution of an anti- $\beta$ -tubulin-antibody (T4026; Sigma); 1:1000 dilution of an anti-phospho-ERK1/2-antibody (#9106; Cell Signaling Technology, Danvers, MA); a mixture of 1:500 dilution of an anti-ERK1-antibody and 1:20,000 dilution of an anti-ERK2-antibody (sc-93 and sc-154, Santa Cruz Biotechnology); 1:2000 dilution of an anti-human EP4 receptor-antibody (#101775; Cayman); an anti-EGF receptor-antibody (sc-003, Santa Cruz Biotechnology); or 1:1000 dilution of an anti-HIF-1 $\alpha$ -antibody (#3761; Cell Signaling Technology). After being incubated with primary antibodies, membranes were washed three times and incubated for 2 h at room temperature with a 1:4000 dilution of appropriate secondary antibodies conjugated with horseradish peroxidase under the same blocking conditions as those for the primary antibodies (Fujino et al. 2003). After washing three times, immunoreactivity was detected and visualized with chemiluminescence imaging system, LAS-1000 (Fuji Film, Tokyo, Japan). To ensure the equal loading of proteins, membranes were stripped and re-probed with the anti- $\beta$ -tubulin-antibody or anti-ERK1/2-antibodies under the conditions described above.

### Quantitative polymerase chain reaction

Cells were cultured at low, middle, and high densities in 6-well plates, and medium was replaced with fresh Opti-MEM containing 100 IU/mL penicillin and 100  $\mu$ g/mL streptomycin prior to immunoblotting experiments. Total

RNAs were isolated using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. After quantities were determined, cDNAs were prepared from 5  $\mu$ g of total RNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA), and the expression of mRNA was determined using TaqMan Gene Expression Assay primers for the human EP4 receptor (Context Sequence, TCCATCCCCTCGTGGTGGCAGTAT; Target Exon, 2; Chromosome, 5; NCBI gene reference, NM\_000958.2) (Applied Biosystems, Foster City, CA). Polymerase chain reaction (PCR) reactions consisted of 40 cycles of 95°C for 15 sec and 60°C for 45 sec in an ABI 7500 thermal cycler (Applied Biosystems). Threshold values (Ct) were determined automatically by the system software and relative mRNA expression levels were analyzed using the comparative  $\Delta\Delta$ Ct method, as described previously (Ji et al. 2010). Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

### Luciferase assay

The human EP4 promoter luciferase reporter plasmid was constructed using the same methods as described previously (Kambe et al. 2008). Briefly, -1236 to -42 base pairs of the human EP4 promoter region were amplified by PCR from human genomic DNA (Promega, Madison, WI) using the primers 5'-GCAGATGGGAAGAGGTTTTT-3' (sense) and 5'-TTCTCCTCCTCCAAGTTTCC-3' (antisense), as reported previously (Kambe et al. 2008). Using the product of the first PCR (1197 bp) as a template, a second round of PCR was then performed to introduce restriction enzyme sites; Nhe I (upstream) and Hind III (downstream), using the following; 5'-GGGCTAGCCTG CAGATGGGAAGAGGTTTTTCCAGGA ATTTAAA-3' (sense) and 5'-GGAAGCTTTGGAGCTCGC GTGCTGGGCCTTTCCACCCTCTGTACAACTTTTCT CCTCCT-3' (antisense) (Kambe et al. 2008). To construct the human EP4 promoter luciferase plasmid, pGL3/EP4R-Promoter-Luc, the product of the second round of PCR (1248 bp), and the pGL3-basic luciferase vector (Promega) were digested with Nhe I and Hind III restriction enzymes (TOYOBO, Osaka, Japan) and then purified and ligated, followed by sequencing and verification. Cells were cultured at low, middle, and high densities in 6-well plates, and medium was replaced with fresh Opti-MEM containing 100 IU/mL penicillin and 100  $\mu$ g/mL streptomycin prior to the experiments. Cells were transiently transfected with firefly pGL3/EP4R-Promoter-Luc, the hypoxia response element (HRE) luciferase reporter plasmid (pGL3/HRE-Luc27) (Ji et al. 2010), or Renilla human EP2 or EP3 receptor promoter luciferase reporter plasmids (S713225, S722267; SwitchGear Genomics, Menlo Park, CA), and either with human HIF-1 $\alpha$  siRNA,

renilla luciferase control plasmids, pRL-CMV (Promega), or the firefly luciferase control plasmid; pGL3-CMV (CMV promoter of pRL-CMV was replaced with the pGL3 vector) using GeneJuice (Novagen/Merck, Darmstadt, Germany) reagent. After approximately 8 h, the transfection reagents were removed by a medium change and cells were incubated for a further 8 h. Cells were then lysed and assayed using the Dual luciferase reporter assay system (Promega) according to the manufacturer's instructions with the GL-200 luminometer (Microtech Nichion, Chiba, Japan). Data were normalized by calculating the ratios of the firefly luciferase scores to the corresponding renilla luciferase values or vice versa.

### cAMP assay

Cells were cultured at low, middle, and high densities in 6-well plates and were then switched to Opti-MEM containing 100 IU/mL penicillin and 100  $\mu$ g/mL streptomycin 16 h prior to the experiments. Cells were treated with 0.1 mg/mL isobutylmethylxanthine (Sigma) for 20 min followed by either vehicle (Me<sub>2</sub>SO) or 1 nmol/L to 10  $\mu$ mol/L PGE<sub>2</sub> for 60 min at 37°C. Experiments were terminated by removing medium and placing the cells on ice. The formation of cAMP was measured as described previously (Fujino et al. 2002). The amount of cAMP present was calculated from a standard curve prepared using unlabeled cAMP. The amounts of cAMP produced by HCA-7 cells in Opti-MEM were approximately 0.5 to 1 pmol/3  $\times$  10<sup>4</sup> cells within 24 h of changing medium (vehicle-treated control of low density cells).

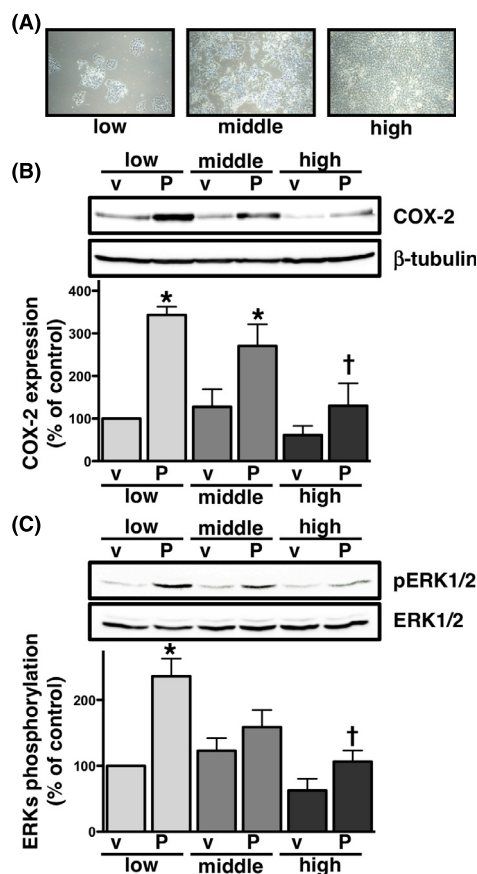
### Statistical analysis

Data are expressed as the mean  $\pm$  SD or mean  $\pm$  SEM. Statistical analyses were performed using Prism 5 for Mac OS X software (GraphPad Software, La Jolla, CA). The multiple comparison tests in the analysis of variance (ANOVA) were the Newman-Keuls multiple comparisons test. An ANOVA and *t*-test were used to evaluate three or more independent experiments. Significance was assumed at *P* < 0.05.

## Results

### PGE<sub>2</sub>-stimulated COX-2 expression and phosphorylation of ERKs was decreased by an increase in cellular density

HCA-7 human colon cancer cells were cultured at low, middle, and high densities, as described in the Materials and Methods (Fig. 1A). To examine the effects of cellular density on PGE<sub>2</sub>-induced COX-2 expression, HCA-7 cells



**Figure 1.** Effects of cellular density on PGE<sub>2</sub>-induced COX-2 expression (B) or phosphorylation of ERKs (C) in HCA-7 cells. Cells were cultured at low ( $3 \times 10^4$  cells/each 6 well), middle ( $1 \times 10^5$  cells/each 6 well), and high ( $3 \times 10^5$  cells/each 6 well) densities (A). Each cellular density was treated with either vehicle (v) or 1  $\mu$ mol/L PGE<sub>2</sub> (P) for 6 h (B) or 15 min (C) at 37°C and then subjected to immunoblot analysis with an antibody against COX-2 (B) or phospho-ERKs 1 and 2 (pERK1/2) (C), as described in the Materials and Methods. The blots were stripped and re-probed with an antibody against  $\beta$ -tubulin (B) or ERKs 1 and 2 (ERK1/2) (C). The bar graphs represent the ratio of COX-2 to  $\beta$ -tubulin (B) or pERK1/2 to total ERK1/2 (C) as assessed with pooled densitometric data (mean  $\pm$  SD) from more than three independent experiments. Data are normalized to the ratio of COX-2 to  $\beta$ -tubulin (B) or pERK1/2 to total ERK1/2 (C) of vehicle-treated controls at the low cellular density as 100%. \* $P < 0.05$ , ANOVA, significantly different from the vehicle treatment. † $P < 0.05$ , ANOVA, significantly different from PGE<sub>2</sub>-stimulated HCA-7 cells at the low cellular density.

cultured of different densities were treated with 1  $\mu$ mol/L PGE<sub>2</sub> for 6 h. As shown in Figure 1B, stimulating cells with PGE<sub>2</sub> significantly induced the expression of COX-2 at the low cellular density. COX-2 was also significantly induced by the treatment with PGE<sub>2</sub> at the middle cellular density, whereas the production of COX-2 was significantly lower at the high cellular density than at the low

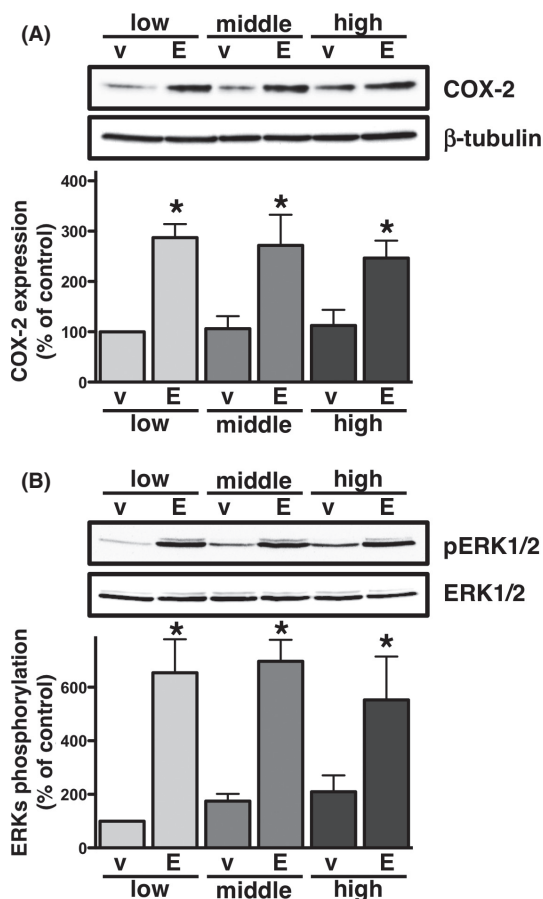
cellular density. The induction of COX-2 was previously reported via the activation of ERKs in HCA-7 cells (Yoshida et al. 2013); therefore, the PGE<sub>2</sub>-stimulated phosphorylation of ERKs was examined at different cellular densities. As with the induction of COX-2, the treatment with PGE<sub>2</sub> significantly induced the phosphorylation of ERKs at the low cellular density (Fig. 1C). However, PGE<sub>2</sub> only slightly induced the phosphorylation of ERKs at the middle cellular density. In contrast, the phosphorylation of ERKs was significantly reduced by the PGE<sub>2</sub> treatment at the high cellular density. To ensure the equal loading of proteins, blots were stripped and re-probed with antibodies to  $\beta$ -tubulin or ERKs in Figure 1B or C, respectively. As shown in the lower panels of Figure 1B and C, nearly identical amounts of proteins were present under both experimental conditions.

### COX-2 expression and phosphorylation of ERKs were not decreased by an increase in cellular density when cells were stimulated with EGF

Previous studies demonstrated that stimulating cells with EGF induced COX-2 expression as well as the phosphorylation of ERKs (Yoshida et al. 2013; Tomas et al. 2014). As shown in Figure 2A, stimulating cells with EGF significantly induced the production of COX-2 at equivalent levels to the PGE<sub>2</sub> stimulation at the low cellular density. However, in contrast to the PGE<sub>2</sub> stimulation, the expression of EGF-stimulated COX-2 was also significantly induced at the middle and high densities to the same extent as that observed at the low cellular density. Similar results were found for the phosphorylation of ERKs when cells were treated with EGF, as shown in Figure 2B. Although basal levels increased slightly in a cellular density-dependent manner, cellular density may not be involved in the induction of COX-2 expression or phosphorylation of ERKs in HCA-7 cells with the EGF treatment, unlike the PGE<sub>2</sub> treatment.

### The expression and function of EP4 receptors were cellular density-dependently down-regulated

The induction of COX-2 expression by PGE<sub>2</sub> was previously shown to involve the activation of EP4 receptors, which was followed by the transactivation of EGF receptors (Yoshida et al. 2013). Since EGF receptor-mediated COX-2 expression was not affected by cellular density, the most plausible target that was functionally changed by cellular density was an upstream component from the EGF receptor, that is, the receptor for PGE<sub>2</sub> in HCA-7 cells. Therefore, the expression of EP4 receptors was



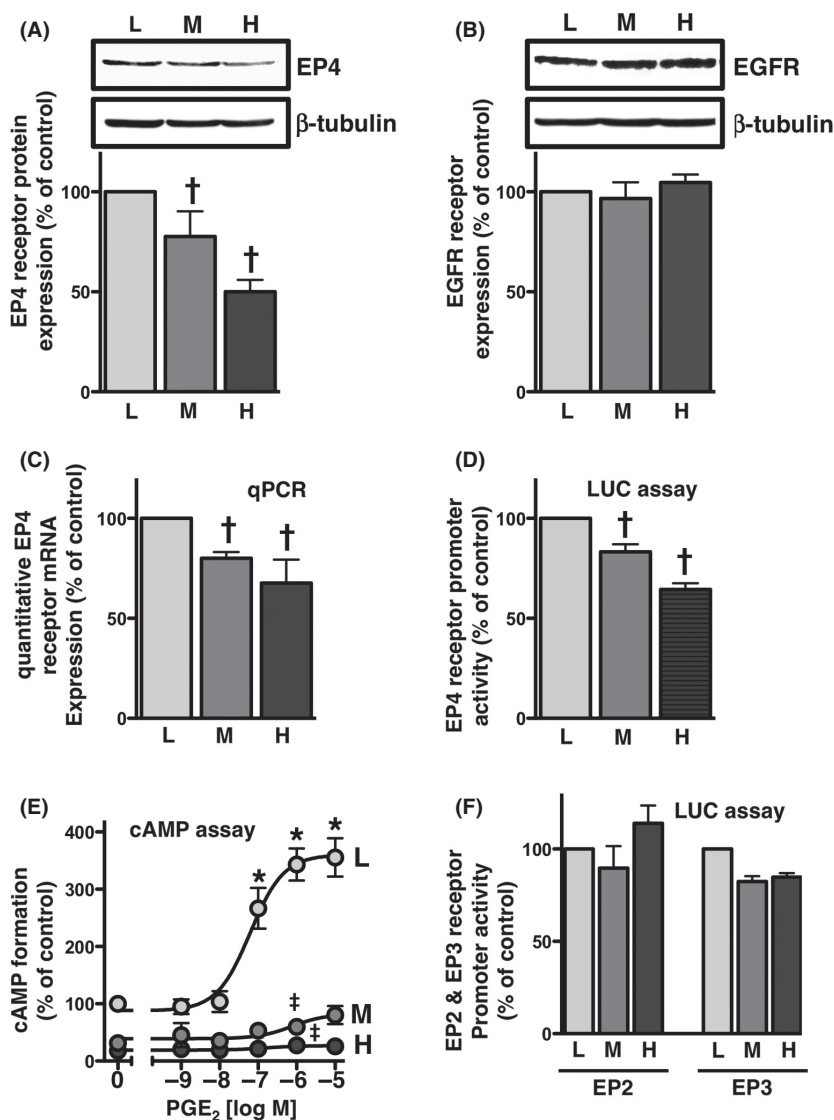
**Figure 2.** Effects of cellular density on EGF-induced COX-2 expression (A) or phosphorylation of ERKs (B) in HCA-7 cells. Cells were cultured at low ( $3 \times 10^4$  cells/each 6 well), middle ( $1 \times 10^5$  cells/each 6 well), and high ( $3 \times 10^5$  cells/each 6 well) densities. Each cellular density was treated with either vehicle (v) or 50 ng/mL of EGF (E) for 6 h (A) or 15 min (B) at 37°C and then subjected to immunoblot analysis with an antibody against COX-2 (A) or phospho-ERKs 1 and 2 (pERK1/2) (B) as described in the Materials and Methods. The blots were stripped and re-probed with an antibody against  $\beta$ -tubulin (A) or ERKs 1 and 2 (ERK1/2) (B). The bar graphs represent the ratio of COX-2 to  $\beta$ -tubulin (A) or pERK1/2 to total ERK1/2 (B) as assessed with pooled densitometric data (mean  $\pm$  SD) from more than three independent experiments. Data are normalized to the ratio of COX-2 to  $\beta$ -tubulin (B) or pERK1/2 to total ERK1/2 (C) of vehicle-treated controls at the low cellular density as 100%. \* $P < 0.05$ , ANOVA, significantly different from the vehicle treatment.

examined using HCA-7 cells cultured at different cellular densities. As shown in Figure 3A, the expression of EP4 receptors in HCA-7 cells was significantly decreased in a cellular density-dependent manner. On the other hand, no significant differences were observed in the expression levels of EGF receptors at the different cellular densities (Fig. 3B). To confirm that the reduction in the protein expression of the EP4 receptors was due to the down-

regulation of EP4 receptors at the transcriptional level, we examined the expression levels of EP4 receptor mRNAs by quantitative PCR and the promoter activity of EP4 receptor genes by a luciferase assay using a reporter plasmid containing the EP4 receptor promoter region. As shown in Figure 3C and D, mRNA expression levels as well as promoter activities of the EP4 receptors were significantly decreased in a cellular density-dependent manner. Since EP4 receptors conventionally couple to the  $G_{zs}$  protein, the effects of cellular density on the receptor function of EP4 was then examined using the cAMP assay. HCA-7 cells cultured at different cellular densities were treated with 1 nmol/L to 10  $\mu$ mol/L of PGE<sub>2</sub> for 1 h. As shown in Figure 3E, the formation of cAMP was dose-dependently increased at the low cellular density; however, the effects of PGE<sub>2</sub> on cAMP formation at both the middle and high cellular densities were negligible. Therefore, the PGE<sub>2</sub>-induced formation of cAMP was significantly reduced in a cellular density-dependent manner. Of the four EP receptor subtypes, EP4 receptors are reported to be expressed predominantly in HCA-7 cells (Yokoyama et al. 2013). However, HCA-7 cells have also been shown to express the mRNAs of EP2, EP3, and EP4 receptors (Fujino et al. 2011). Therefore, we attempted to confirm whether the reduction observed in the formation of cAMP at the middle and high cellular densities was due to the decreased expression of  $G_{zs}$ -coupled EP2 receptors and/or induction of  $G_{zi}$ -coupled EP3 receptors. EP2 and EP3 receptor promoter activities were examined at different cellular densities, using the luciferase reporter gene assay. As shown in Figure 3F, neither a significant reduction in EP2 promoter activity nor significant induction of EP3 promoter activity were observed at the different cellular densities. These results suggested that the decrease observed in the formation of cAMP at the middle and high cellular densities may have been due to the reduced expression of EP4 receptors, as shown in Figure 3A, C, and D.

### The expression of HIF-1 $\alpha$ was cellular density-dependently up-regulated

HIF-1 $\alpha$ , a DNA binding complex generally shown to be regulated by oxygen, is also reported to be induced by a high cellular density (Wiesener et al. 1998; Paltoglou and Roberts 2005). As shown in Figure 4A, the increase observed in the expression of the HIF-1 $\alpha$  protein was confirmed to be dependent on cellular density. To determine if the increase in HIF-1 $\alpha$  protein levels was involved in activating the target gene as a transcriptional factor, HIF-1 $\alpha$ -mediated transcriptional activity was examined using the HRE-contained luciferase reporter plasmid (Ji et al. 2010). Figure 4B shows that the luciferase



**Figure 3.** EP4 receptors were down-regulated in a cellular density-dependent manner in HCA-7 cells. Cells were cultured at low (L;  $3 \times 10^4$  cells/each 6 well), middle (M;  $1 \times 10^5$  cells/each 6 well), and high (H;  $3 \times 10^5$  cells/each 6 well) densities. Each cellular density was then subjected to immunoblot analysis with an antibody against EP4 (A) or EGFR (B), quantitative PCR (C), the EP4 promoter-luciferase assay (D), PGE<sub>2</sub>-stimulated cAMP assay (E), or EP2 or EP3 promoter-luciferase assay (F), as described in the Materials and Methods. (A, B) The blots were stripped and re-probed with an antibody against  $\beta$ -tubulin. The bar graphs represent the ratio of COX-2 to  $\beta$ -tubulin as assessed with pooled densitometric data (mean  $\pm$  SD) from more than three independent experiments. Data are normalized to the ratio of COX-2 to  $\beta$ -tubulin at the low cellular density as 100%. (C) The RNA of HCA-7 cells at each cellular density was isolated and used for quantitative real-time PCR with primers specific for either human EP4 or GAPDH, as described in the Materials and Methods. Data were analyzed by comparative methods relative to the expression of GAPDH and were normalized to the results obtained at the low cellular density as 100%. Data are the mean  $\pm$  SD from three independent experiments. (D, F) Each cellular density was transiently transfected with human EP4 receptor promoter- (D), human EP2 promoter-, or EP3 promoter- (F) responsive luciferase reporter genes and control pRL-CMV or pGL3-CMV plasmids, and luciferase activity was determined as described in the Materials and Methods. Data were analyzed by comparative methods relative to the results of control luciferase activity levels, and were normalized to the results obtained at the low cellular density as 100%. Data are the mean  $\pm$  SD from three independent experiments. (E) Each cellular density was treated with the indicated concentrations of PGE<sub>2</sub> for 60 min. cAMP formation was determined as described in the Materials and Methods. Data are the means  $\pm$  SEM of three independent experiments, with each being performed in duplicate. Data were normalized to the vehicle-treated control at the low cellular density as 100%. \**P*, †*P* < 0.05, ANOVA, significantly different from the vehicle-stimulated HCA-7 cells at the low cellular density. ‡*P* < 0.05, ANOVA, significantly different from PGE<sub>2</sub>-stimulated HCA-7 cells at the low cellular density (the analyzed results were shown for 1  $\mu$ mol/L PGE<sub>2</sub> only).

transcriptional activity of HRE was dependent on increases in cellular density; therefore, the increase observed in HIF-1 $\alpha$  levels was associated with an enhancement in the activity of its transcriptional potential in HCA-7 cells cultured at a high cellular density.

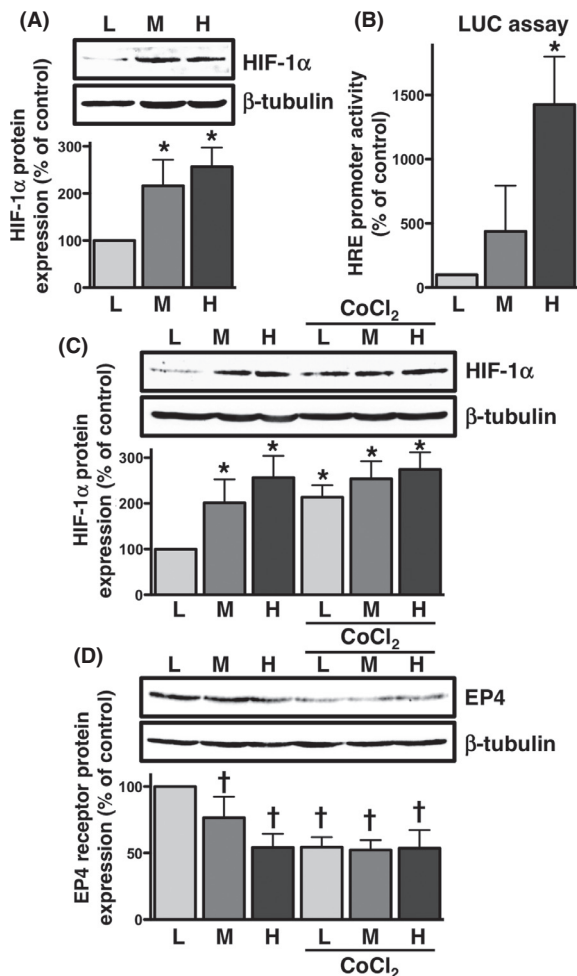
### Enhanced expression of HIF-1 $\alpha$ by CoCl<sub>2</sub> was accompanied by a decrease in EP4 receptor expression

Cobalt chloride (CoCl<sub>2</sub>) is known to be a chemical inducer of HIF-1 (Piret *et al.* 2002); therefore, the effects of CoCl<sub>2</sub> on HIF-1 $\alpha$  expression in HCA-7 cells cultured at different cellular densities were examined. As shown in Figure 4C, the expression of HIF-1 $\alpha$  was significantly increased by the treatment with CoCl<sub>2</sub>, especially at the low cellular density. Since certain levels of the HIF-1 $\alpha$  protein were intrinsically expressed at the middle and high cellular densities, no further increment was observed in its expression by the treatment with

CoCl<sub>2</sub>. The relationship between the increase in HIF-1 $\alpha$  expression and decrease in EP4 receptor expression was then examined by western blot analysis using different densities of cells treated with CoCl<sub>2</sub>. Figure 4D showed that the treatment with CoCl<sub>2</sub> eliminated the cellular density-dependent decrease in EP4 receptor expression. Thus, as inversely correlated with the increment in HIF-1 $\alpha$  as shown in Figure 4C, the expression of EP4 receptors was significantly decreased by the treatment with CoCl<sub>2</sub>. Therefore, a negative correlation may exist between the expression levels of HIF-1 $\alpha$  and EP4 receptors.

### Knockdown of HIF-1 $\alpha$ by siRNA reduced HRE luciferase activity and increased EP4 receptor expression and promoter activity

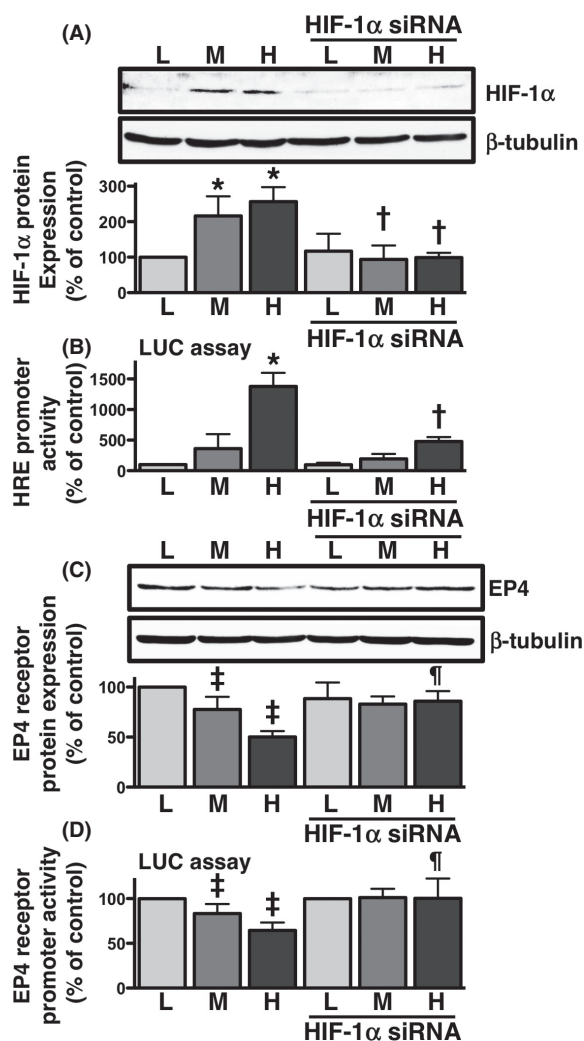
To confirm if the expression of HIF-1 $\alpha$  was involved in the expression of EP4 receptors, we used siRNA for HIF-1 $\alpha$  to examine the protein expression levels of EP4 receptors and their promoter transcriptional activities. The potency of siRNA for HIF-1 $\alpha$  was initially evaluated using western blot analysis and HIF-1 $\alpha$ -mediated



**Figure 4.** HIF-1 $\alpha$  was cellular density- and CoCl<sub>2</sub>-dependently up-regulated, and EP4 receptors were down-regulated by CoCl<sub>2</sub> in HCA-7 cells. Cells were cultured at low (L;  $3 \times 10^4$  cells/each 6 well), middle (M;  $1 \times 10^5$  cells/each 6 well), and high (H;  $3 \times 10^5$  cells/each 6 well) densities. Each cellular density was then subjected to immunoblot analysis with antibody against HIF-1 $\alpha$  (A, C) or the EP4 receptor (D), or the HIF-responsive HRE luciferase assay (B), as described in the Materials and Methods. A, The blots were stripped and re-probed with an antibody against  $\beta$ -tubulin. The bar graphs represent the ratio of HIF-1 $\alpha$  to  $\beta$ -tubulin as assessed with pooled densitometric data (mean  $\pm$  SD) from more than three independent experiments. Data were normalized to the ratio of HIF-1 $\alpha$  to  $\beta$ -tubulin at the low cellular density as 100%. (B) Each cellular density was transiently transfected with the HRE luciferase reporter genes and control pRL-CMV plasmid, and luciferase activity was determined as described in the Materials and Methods. Data were analyzed by comparative methods relative to the results of control renilla luciferase activity levels, and were normalized to the results obtained at the low cellular density as 100%. Data are the mean  $\pm$  SD from three independent experiments. (C, D) Each cellular density was treated with 100  $\mu$ mol/L CoCl<sub>2</sub> for 16 h, then subjected to immunoblot analysis with an antibody against HIF-1 $\alpha$  (C) or the EP4 receptor (D), as described in the Materials and Methods. The blots were stripped and re-probed with an antibody against  $\beta$ -tubulin. The bar graphs represent the ratio of HIF-1 $\alpha$  (C) or EP4 receptors (D) to  $\beta$ -tubulin as assessed with pooled densitometric data (mean  $\pm$  SD) from more than three independent experiments. Data are normalized to the ratio of HIF-1 $\alpha$  (C) or EP4 receptors (D) to  $\beta$ -tubulin at the low cellular density as 100%. \* $P < 0.05$ , ANOVA, significantly different from the vehicle treatment. † $P < 0.05$ , ANOVA, significantly different from the low cellular density control.



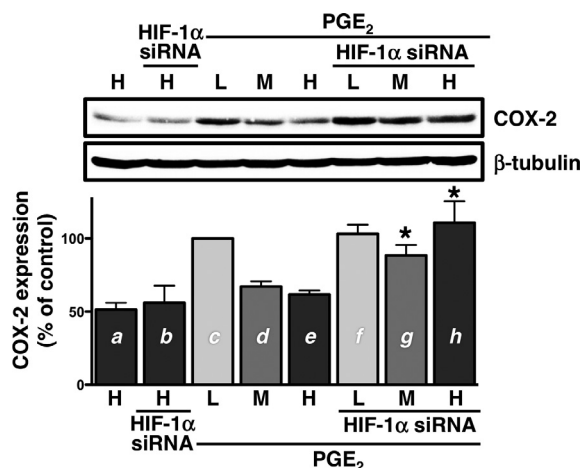
transcriptional activity with the HRE luciferase reporter plasmid. As shown in Figure 5A and B, the transfection of siRNA to HCA-7 cells significantly reduced HIF-1 $\alpha$  protein expression at the middle and high cellular densities and HRE promoter activity at the high cellular density. These results confirmed the potency of siRNA; therefore, its effects on the expression and promoter transcriptional activity of EP4 receptors were subsequently examined. The results of western blotting showed that blocking the expression and activity of HIF-1 $\alpha$  with siRNA inhibited the reduction in EP4 receptor protein expression and EP4 receptor promoter-mediated luciferase activity, and this increase was significant at the high cellular density, as shown in Figure 5C and D, respectively. Thus, these results suggested that HIF-1 $\alpha$  may inversely regulate the expression of EP4 receptors.



### Knockdown of HIF-1 $\alpha$ by siRNA restored PGE<sub>2</sub>-induced reductions in COX-2 expression in high cellular density-cultured cells

The effects of blocking HIF-1 $\alpha$  with siRNA on PGE<sub>2</sub>-induced COX-2 expression was confirmed, particularly in HCA-7 cells cultured at a high cellular density. As shown in Figure 6, the stimulation with PGE<sub>2</sub> did not induce the expression of COX-2 at a high density of HCA-7 cells (lane *e* vs. lane *a*), similar to that shown in Figure 1B. However, when cells were transfected with HIF-1 $\alpha$  siRNA, the expression level of COX-2 was increased (lane *h* vs. lane *e*) to a similar level to the PGE<sub>2</sub>-induced expression of COX-2 at the low cellular density (lane *h* vs. lane *c*). This effect of siRNA was also observed in cells cultured at the middle cellular density (lane *g* vs. lane *d*). In addition, the expression of COX-2 was unaffected in cells cultured at the high cellular density transfected with HIF-1 $\alpha$  siRNA alone without the PGE<sub>2</sub> stimulation (lane *b* vs. lane *a*). Thus, restoring the expression of COX-2 may reflect the restoration of EP4 receptor expression by offsetting the increment in cellular density-dependent HIF-1 $\alpha$  expression by siRNA, especially in HCA-7 cells cultured at the high cellular density.

**Figure 5.** Effects of HIF-1 $\alpha$  siRNA on HIF-1 $\alpha$  protein expression, HIF-responsive luciferase activity, EP4 receptor expression, and EP4 receptor promoter-responsive luciferase activity in HCA-7 cells cultured at different cellular densities. Cells were cultured at low (L;  $3 \times 10^4$  cells/each 6 well), middle (M;  $1 \times 10^5$  cells/each 6 well), and high (H;  $3 \times 10^5$  cells/each 6 well) densities. Each cellular density was transfected with HIF-1 $\alpha$  siRNA for 48 h, and cells were then subjected to immunoblot analysis with antibody against HIF-1 $\alpha$  (A) or EP4 receptors (C), or the luciferase assay for HIF-responsive HRE (B) and EP4 receptor promoter-responsive (D) activities, as described in the Materials and Methods. (A, C) The blots were stripped and re-probed with an antibody against  $\beta$ -tubulin. The bar graphs represent the ratio of HIF-1 $\alpha$  (A) or EP4 receptors (C) to  $\beta$ -tubulin as assessed with pooled densitometric data (mean  $\pm$  SD) from more than three independent experiments. Data were normalized to the ratio of HIF-1 $\alpha$  (A) or EP4 receptors (C) to  $\beta$ -tubulin at the low cellular density as 100%. (B, D) Each cellular density was transiently transfected with HIF-responsive HRE luciferase reporter genes (B) or EP4 receptor promoter luciferase reporter genes (D) and the control pRL-CMV plasmid 20 h after siRNA transfection, and luciferase activity was determined, as described in the Materials and Methods. Data were analyzed by comparative methods relative to the results of control renilla luciferase activity levels, and were normalized to the result obtained at the low cellular density as 100%. Data are the mean  $\pm$  SD from three independent experiments. \**P*, †*P* < 0.05, ANOVA, significantly different from control transfection at the low cellular density. ‡*P*, ¶*P* < 0.05, ANOVA, significantly different from each density control that was not transfected with siRNA.

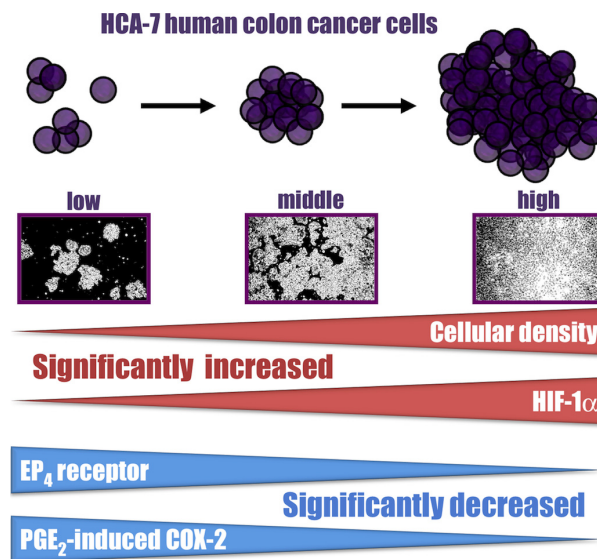


**Figure 6.** Effects of HIF-1 $\alpha$  siRNA on PGE<sub>2</sub>-induced COX-2 expression in high density cultured HCA-7 cells. Cells were cultured at low (L;  $3 \times 10^4$  cells/each 6 well), middle (M;  $1 \times 10^5$  cells/each 6 well), and high (H;  $3 \times 10^5$  cells/each 6 well) densities. Cells were transfected with HIF-1 $\alpha$  siRNA for 48 h, treated with either vehicle or  $1 \mu\text{mol/L}$  PGE<sub>2</sub> for 6 h at 37°C, and then subjected to immunoblot analysis with an antibody against COX-2, as described in the Materials and Methods. The blots were stripped and re-probed with an antibody against  $\beta$ -tubulin as assessed with pooled densitometric data (mean  $\pm$  SD) from more than three independent experiments. Data were normalized to the ratio of COX-2 to  $\beta$ -tubulin of PGE<sub>2</sub>-treated controls at the low cellular density (c) as 100%. \* $P < 0.05$ ,  $t$ -test, significantly different from d (without HIF-1 $\alpha$  siRNA at the middle cellular density) to g (with siRNA at the middle cellular density) and/or e (without HIF-1 $\alpha$  siRNA at the high cellular density) to h (with siRNA at the high cellular density), respectively.

## Discussion and Conclusions

The present study revealed a clear inverse correlation between cellular density/HIF-1 $\alpha$  expression and EP4 receptor/PGE<sub>2</sub>-induced COX-2 expression, as depicted in Figure 7. As shown in Figure 1 and previously reported (Yoshida et al. 2013), PGE<sub>2</sub> has the ability to strongly induce expression of COX-2 via EP4 receptors at a low cellular density. However, the effect of PGE<sub>2</sub> on COX-2 induction was significantly reduced under the high cellular density condition. In addition, the results obtained at the middle cellular density were inconclusive, such that the outcomes were vague and unclear; they were similar to those at the high cellular density and other times to the low cellular density. Similar patterns have been also reported previously (Paltoglou and Roberts 2005).

Interestingly, even at higher, and possibly close to the maximum expression levels of HIF-1 $\alpha$  at the high cellular density, quantitative PCR, luciferase assay, and western blotting revealed that the expression levels of EP4 receptors only decreased by 50% (Fig. 3A, C, and D). HIF-1 $\alpha$



**Figure 7.** Schema for the cellular density-dependent regulation of HIF-1 $\alpha$ , EP4 receptor, and PGE<sub>2</sub>-induced COX-2 expression in HCA-7 cells. The expression of HIF-1 $\alpha$  increased in a cellular density-dependent manner, whereas that of the EP4 receptors and PGE<sub>2</sub>-induced COX-2 decreased.

has been implicated in the induction of SP-1 and EGR-1 transcriptional factors (Wong et al. 2010), which are known to be critical factors in regulation of the EP4 receptor promoters (Kambe et al. 2008). Thus, one possible explanation is that either the direct or indirect regulation of reductions in the expression of EP4 receptors by HIF-1 $\alpha$  may be accompanied by the induction of SP-1 and EGR-1 expression, which counteractively induces receptor expression. Although the expression of EP4 receptors was not completely reduced at the high cellular density, their functions were eliminated because the reduction in COX-2 expression (Fig. 1B), and also that in cAMP formation were almost completely ameliorated (Fig. 3E) by the PGE<sub>2</sub> stimulation. Therefore, although EP4 receptors may not be expressed on membranes as functional receptors at the high cellular density, they could be diffusely expressed in the cytoplasm, as reported previously in inflammatory cells (Lejeune et al. 2010). We do not yet have strong evidence for the localization of EP4 receptors in HCA-7 cells cultured with different cellular densities; therefore, the mechanism responsible for how the cellular density-dependent increases in HIF-1 $\alpha$  regulated EP4 receptor expression currently remains unknown. However, as shown in Figure 5, since the knockdown of HIF-1 $\alpha$  mRNA by siRNA recovered the expression of EP4 receptors, even at the high cellular density, the involvement of HIF-1 $\alpha$  in regulating the expression of EP4 receptors is clear.

An increase in the expression of COX-2 is generally considered to be a hallmark for the development of colon cancer; therefore, an increase in the expression of HIF-1 $\alpha$  may induce the expression of COX-2 aside from the reduction in EP4 receptor expression. Indeed, HIF-1 $\alpha$  was previously shown to up-regulate COX-2 expression by direct transcriptional regulation in HT-29 and HCT116 colon cancer cell lines under hypoxic conditions (Kaidi et al. 2006). In contrast, since COX-2 expression is down-regulated by transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), an increase in HIF-1 $\alpha$  could reduce COX-2 expression by acting on the HRE of the TGF- $\beta$ 1 promoter region, followed by the production/secretion of that cytokine, as reported previously (Takai et al. 2013; Hung et al. 2013). However, in our experimental conditions, as shown in Figure 6 lane b, the knockdown of HIF-1 $\alpha$  by siRNA did not alter the expression of COX-2. Moreover, the EGF-induced expression of COX-2 was not altered by an increase in the expression of HIF-1 $\alpha$ , as shown in Figure 2A, the EP4 receptor-independent, but HIF-1 $\alpha$ -mediated direct regulation of COX-2 expression may be not much involved in. Therefore, the PGE<sub>2</sub>-induced expression of COX-2 in HCA-7 cells may simply be regulated by EP4 receptor levels and their signaling pathways.

As described above, although the up-regulated expression of COX-2 is associated with the development of colon cancer, the underlying mechanism/meaning involving the cellular density-dependent loss of PGE<sub>2</sub>-induced COX-2 expression in HCA-7 cells remains unclear. COX-2 expression was previously detected from a very early stage in carcinoma growth (Fujita et al. 1998). Therefore, COX-2 expression may be induced in the early stages of colon cancer development, by PGE<sub>2</sub>-stimulated EP4 receptors, which may further induce COX-2 expression and cellular growth through a positive feedback loop. These results may be corroborated by previous findings in which an increment in HIF-1 $\alpha$  occurred at the early stages of carcinogenesis and strongly correlated with malignant progression such as angiogenesis and metastasis (Mabjeesh and Amir 2007). Thus, an increase in HIF-1 $\alpha$  may induce the expression of vascular endothelial growth factor (VEGF), thereby increasing neovascularization for cancer growth, as reported previously (Semenza 2003; Mabjeesh and Amir 2007; Rohwer et al. 2013). We previously demonstrated that VEGF was constitutively secreted in HCA-7 cells, a condition corresponding to the middle to high cellular densities (Fujino et al. 2011). Meanwhile, the induction of HIF-1 $\alpha$  by the increase in cellular growth should reduce the expression of EP4 receptors, as demonstrated in the present study. As we previously reported and stated earlier, HCA-7 cells also endogenously express EP3 prostanoid receptors (Fujino et al. 2011), and the

expression level of EP3 receptors is likely remain unchanged by cellular density, as shown in Figure 3F. Therefore, under these cellular conditions, exogenously applied PGE<sub>2</sub> stimulated EP3 receptors, as shown previously, but not EP4 receptors (Fujino et al. 2011), and this may have been because EP4 receptors had already lost their functions. Moreover, the stimulation of EP3 receptors evoked cellular migration by enhancing the expression of VEGF receptor-1, which is known to play an important role in establishing cellular migration/metastasis, as we previously reported (Fujino et al. 2011). Thus, at the high cellular density, HCA-7 cells enhanced the expression levels of VEGF by increasing HIF-1 $\alpha$  as well as VEGFR-1 expression, not by fewer EP4 receptors, but by the activation of stationary EP3 receptors that stimulate cellular migration such as invasion/metastasis. The exact meanings/reasons for a reduction in the expression of EP4 receptors by HIF-1 $\alpha$  at the high cellular density currently remain unclear. However, the decrease in COX-2 levels may promote colon cancer cells from the cancer growth stage at the low cellular density to the metastatic stage at the high cellular density by switching the responsive primary EP receptor subtypes from EP4 receptors to EP3 receptors. EP3 receptors are known to have one of the highest affinities for PGE<sub>2</sub> (Narumiya et al. 1999; Breyer et al. 2001); therefore, these receptors could be activated at even the low levels of PGE<sub>2</sub> produced by the reduced expression of COX-2.

Although the increase observed in the expression of EP4 receptors during carcinogenesis has been widely accepted (Chell et al. 2006; Hawcroft et al. 2007), other studies demonstrated that EP4 receptor levels were significantly higher in normal colon tissue than in tumor tissue (Gustafsson et al. 2007). One possible explanation for these differences is that the expression of EP4 receptors could be changed under certain conditions such as cell density. The results of this study were exclusively obtained from HCA-7 cells; therefore, additional experiments need to be conducted using other colon cancer cell lines and/or other cancer cells. However, the results of the present study suggest that this novel regulation mechanism of EP4 receptor expression by HIF-1 $\alpha$  may play important roles in determining the fate/stage of colon cancer via its surrounding environment.

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## Disclosures

None declared.

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