Potential Role of Microsomal Prostaglandin E Synthase-1 in Tumorigenesis*

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Microsomal prostaglandin E₂ synthase-1 (mPGES-1) is a stimulus-inducible enzyme that functions downstream of cyclooxygenase (COX)-2 in the PGE₂-biosynthetic pathway. Given the accumulating evidence that COX-2-derived PGE₂ participates in the development of various tumors, including colorectal cancer, we herein examined the potential involvement of mPGES-1 in tumorigenesis. Immunohistochemical analyses demonstrated the expression of both COX-2 and mPGES-1 in human colon cancer tissues. HCA-7, a human colorectal adenocarcinoma cell line that displays COX-2 and PGE₂-dependent proliferation, expressed both COX-2 and mPGES-1 constitutively. Treatment of HCA-7 cells with an mPGES-1 inhibitor or antisense oligonucleotide attenuated, whereas overexpression of mPGES-1 accelerated, PGE₂ production and cell proliferation. Moreover, cotransfection of COX-2 and mPGES-1 into HEK293 cells resulted in cellular transformation manifested by colony formation in soft agar culture and tumor formation when implanted subcutaneously into nude mice. cDNA array analyses revealed that this mPGES-1-directed cellular transformation was accompanied by changes in the expression of a variety of genes related to proliferator, morphology, adhesion, and the cell cycle. These results collectively suggest that aberrant expression of mPGES-1 in combination with COX-2 can contribute to tumorigenesis.

Clinical, genetic, and biochemical evidence has suggested that prostaglandin (PG) E₂ produced via the cyclooxygenase (COX)-2-dependent pathway plays a crucial role in the development of colorectal cancer and possibly other cancers (1). Non-steroidal anti-inflammatory drugs, which inhibit COX-2, reduce the incidence of colorectal cancer (2–4). The major prostanooid produced by several types of cancer is PGE₂, which is produced by three biosynthetic reactions involving phospholipase A₂ (PLA₂), COX, and terminal PGE₂ synthase (PGES). PGE₂ promotes survival and motility of colon cancer cells in vitro and promotes tumorigenesis and angiogenesis in vivo (5–7). High levels of constitutive expression of COX-2 have been found in various cancer cells and tissues (8, 9), and studies employing overexpression, antisense suppression, and specific inhibitors of COX-2 have demonstrated that COX-2 contributes to the progression of several types of cancer (10–12).

More direct evidence for the role of COX-2 and its product PGE₂ in colorectal tumorigenesis has been provided by gene targeting studies. Gene disruption of either COX-2 (13) or the PGE receptor EP2 (14) results in reduction of the number and size of intestinal polyps in Apc mutant mice, a model for human familial adenomatous polyposis. In another model, disruption of the genes for the PGE receptors EP1 (15) or EP4 (16) suppresses the development of colorectal cancer induced by carcinogen. Moreover, gene knockout of cytosolic PLA₂α (cPLA₂α), which supplies the substrate arachidonic acid to COX-2, also leads to reduced polyposis in Apc mutant mice (17, 18).

PGES catalyzes the conversion of PGH₂, which is produced from arachidonic acid by COX-1 or COX-2, to PGE₂. Recent advances in this field have led to identification of at least three PGES enzymes, including cytosolic PGES (cPGES) (19), microsomal PGES (mPGES) -1 (20–22), and mPGES-2 (23). Among them, microsomal PGES-1 (mPGES-1) has received much attention, as this enzyme is induced by proinflammatory stimuli, down-regulated by anti-inflammatory glucocorticoids, and functionally coupled with COX-2 in marked preference to COX-1 (20–22). In comparison, cPGES (the heat shock protein-associated protein p23) is constitutively and ubiquitously expressed and is selectively coupled with COX-1 (19). mPGES-2 does not show homology with mPGES-1 and has a unique N-terminal hydrophobic domain and a glutaredoxin-like domain (23), although its cellular function has not yet been addressed.

mPGES-1 is a member of the MAPEG (for membrane-associated proteins involved in eicosanoid and glutathione metabolism) superfamily, to which other proteins involved in arachidonic acid metabolism, such as 5-lipoxygenase-activating protein (FLAP) and leukotriene C₄ synthase, also belong (20–22). Induced expression of mPGES-1 has been postulated to be associated with various pathophysiological events in which COX-2-derived PGE₂ has been implicated, such as rheumatoid arthritis (24), febrile response (25), reproduction (26, 27), bone metabolism (21), and Alzheimer’s disease (28). A recent gene targeting study of mPGES-1 has shown that PGE₂ production by lipopolysaccharide-stimulated peritoneal macrophages depends almost entirely on this enzyme (29). Induced expression of mPGES-1 is regulated by the NF-IL-6 pathway (29) or the mitogen-activated protein kinase pathway (30), the latter of

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The abbreviations used are: PG, prostaglandin; COX, cyclooxygenase; PLA₂, phospholipase A₂; mPGES-1, microsomal prostaglandin E₂ synthase-1; MAPEG, membrane-associated proteins involved in eicosanoid and glutathione metabolism; TBS, Tris-buffered saline; IL, interleukin; HEK, human embryonic kidney.
which may switch on the inducible transcription factor Egr-1 that in turn binds to the proximal GC box in the mPGES-1 gene promoter, leading to mPGES-1 transcription (31).

A possible linkage of mPGES-1 with tumorigenesis has been provided by a recent observation that mPGES-1 is constitutively expressed in several cancers, most of which also express COX-2 constitutively (32–34). In this study, we have used colon cancer cell lines and mPGES-1-transfected cells to examine the expression of mPGES-1 in colorectal cancer tissues and cells and evaluate its potential role in tumorigenesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human embryonic kidney (HEK) 293 cells (Human Science Research Resources Bank) and human colon adenocarcinoma HCA-7 cells (a generous gift from Dr. M. Tsujii (Osaka University) and Dr. R. DuBois (Vanderbilt University Medical Center and VA Medical Center) were cultured in RPMI 1640 medium (Nissui Pharmaceutical Co.) containing 10% (v/v) fetal calf serum (Bioserum). The cDNAs for human mPGES-1 and its mutant R1105S (21), human COX-1 and COX-2 (35), and human cPGES (19) were described previously, cDNA probes for human rhøA and human c-myc were donated by Dr. M. Shibanuma (Showa University). HEK293 cells stably expressing COX-2 and/or mPGES-1 were described previously (21). The enzyme immunosassay kit for PGE2 and PGF2α, the COX-2 antibody, and the COX-2 inhibitor NS-398 were purchased from Calbiochem. The MAPEG inhibitor MK-886 was from Toronto Research Chemicals. The rabbit anti-human α-FLAG, goat anti-human COX-1 and goat anti-human COX-2, goat anti-human rhøA and goat anti-human eznin antibodies were purchased from Santa Cruz. Mouse monoclonal antibody against human ErbB3 and mouse monoclonal anti-FLAG antibodies were from Sigma. Rabbit antibody against human Egr-1 was provided by Dr. H. Naraba (National Cardiovascular Center Research Institute, Japan). Fluorescein isothiocyanate-conjugated anti-FLAG IgG, Cy3-conjugated anti-rabbit IgG, and horseradish peroxidase-conjugated anti-goat-, mouse-, and -rabbit IgGs were purchased from Zymed Laboratories Inc.. Rabbit antisera for human mPGES-1 was prepared as described previously (19). Human terluekin (IL)-1β was purchased from Genzyme. LipofectAMINE 2000, oligofectamine, Opti-MEM medium, TIRzol reagent, genetin, hygroycin, zeocin, and the pcDNA3.1 series of mammalian expression vectors were obtained from Invitrogen.

**Preparation of Antibody against Human mPGES-1**—Human mPGES-1 cDNA was subcloned into the bacterial expression vector pET21c (Novagen) and transformed into the competent cell BL21-D3 (Strategen). After culture with 0.5 mM isoprropyl-β-D-thiogalactosidase (IPTG) in a 2-liter Erlenmeyer flask at 25 °C, the bacterial cells were collected by centrifugation and disrupted by sonication with a Branson sonifier (10 s, 3 times, 50% duty) in 150 mM Tris-HCl (pH 8.0) containing 150 mM NaCl. After centrifugation of the sonicates at 100,000 g at 4 °C, the membrane fractions were used as an enzyme source. An aliquot (10 μg of protein equivalents) was incubated with 0.5 μg of PGE2, 30 μg at 24 °C in 0.1 ml of 0.1 mM Tris-HCl (pH 8.0) containing 1 mM glutathione and 5 μg of indomethacin. After stopping the reaction by the addition of 100 mM FeCl3, PGE2 contents in the reactions were quantified by use of the enzyme immunoassay kit. Protein concentrations were determined by the bichinonic acid protein assay kit (Pierce) with bovine serum albumin as a standard.

**SDS-PAGE and Immunoblotting**—Cell lysates (2 × 105 cell equivalents) were subjected to SDS-PAGE using 7.5–12.5% gels under reducing conditions. The separated proteins were electroblotted onto nitrocellulose membranes (Schleicher and Schuell) with a semi-dry blottter (MilBlot-SEE system; Millipore). After blocking with 5% (w/v) skim milk in TBS-Tween (1:5,000 dilution for mPGES-1 and T/B, 1:20,000 dilution for cPGES, cPLA2, and COX-2), and 1:5,000 dilution for FLAG, appropriate antibodies were added, incubated for 1 h, and then washed with TBS-Tween (3 × 5 min each, and incubated for 2 h with the first antibodies in TBS (1:50 dilution for COX-1 and FLAG epitope in TBS-Tween) for 2 h, followed by incubation with horseradish peroxidase-conjugated anti-rabbit (for mPGES-1 and cPGES), anti-goat (for COXs), or anti-mouse (for FLAG) IgG (1:5,000 dilution) to detect the respective antibodies. The membranes were washed with TBS-Tween (3 × 5 min each), and then exposed to chemiluminescent- or colorimetric-reactive substrate (ECL reagent; Amersham Biosciences) or the CSA system staining kit (for mPGES-1; DAKO). The resulting blots were then probed with the respective cDNA probes that had been labeled with [32P]dCTP (Amersham Biosciences) by random priming (Takara Biomedical). All hybridizations were carried out as described previously (35).

**RNA Blotting**—Approximately equal amounts (~5 μg) of total RNA obtained from the cells were applied to separate lanes of 1.2% (w/v) formaldehyde-agarose gels, electrophoresed, and transferred to Immobilon-N membranes (Millipore). The resulting blots were then probed with the respective cDNA probes that had been labeled with [32P]dCTP (Amersham Biosciences) by random priming (Takara Biomedical). All hybridizations were carried out as described previously (35).

**Immunohistochemistry**—The tissue sections were incubated with Taget Retrieval Solution (DAKO) as required, incubated for 10 min with 3% (v/v) H2O2, washed 3 times with TBS for 5 min each, incubated with 5% (v/v) skim milk for 30 min, washed 3 times with TBS-Tween for 5 min each, and incubated with the first antibody for 1 h. After washing with TBS-1 (500 ml) and 1,200 dilutions for anti-mPGES-1 and anti-COX-2 antibodies, respectively. Then the sections were treated with the LSAB2 staining kit (for COX-2; DAKO) or the CSA system staining kit (for mPGES-1; DAKO).
Fig. 1. Immunohistochemical detection of mPGES-1 and COX-2 in human colorectal tumor tissues. Paraffin-embedded sections of human colorectal adenocarcinoma (A and B) and adenoma (C and D) tissues as well as normal tissues (E and F) were subjected to immunostaining with anti-mPGES-1 (A, C, and E) and anti-COX-2 (B, D, and F) antibodies. After immunostaining, the sections were counterstained with hematoxylin and eosin. A, B and E, ×400; C, D, and F, ×200.

Results

mPGES-1 Is Expressed in Human Colorectal Cancer Tissues—Surgically resected human colorectal adenocarcinoma (Fig. 1, A and B) and adenoma (Fig. 1, C and D) tissues were fixed with formaldehyde, embedded in paraffin, and serial sections were immunostained with antibodies against mPGES-1 (Fig. 1, A, C, and E) and COX-2 (Fig. 1, B, D, and F). In the sections shown in Fig. 1, virtually all adenocarcinoma cells (panels A and B) and adenoma cells (panels C and D) were positively stained with anti-mPGES-1 and anti-COX-2 antibodies. Signal for mPGES-1 was distributed throughout the cytoplasm with a punctate pattern, whereas that of COX-2 was enriched in the luminal side of the nuclei. Considering that mPGES-1 is an integral membrane protein, it is likely that the observed mPGES-1 immunostaining reflects its distribution in the endoplasmic reticulum membrane. Immunoreactive signals for mPGES-1 and COX-2 were barely seen in normal colon sections (Fig. 1, E and F). Of several colon cancer tissue sections examined, 9 of 9 adenocarcinoma tissues and 5 of 7 adenoma tissues were positive for mPGES-1 immunoreactivity. Furthermore, 5 of 9 mPGES-1-positive adenocarcinoma tissues and 4 of 5 mPGES-1-positive adenoma tissues were also COX-2-positive.

mPGES-1 Is Involved in the Growth of Human Colon Adenocarcinoma Cell Line HCA-7—Fig. 2A depicts the expression of the PGE2-biosynthetic enzymes cPLA2α, COX-1, and -2, cPGES and mPGES-1 in 3 human colon cancer cell lines (HCA-7, WiDr, and HCT116) as well as in HEK293 cells. Of these cell lines, only HCA-7, a colon adenocarcinoma cell line that has been reported to exhibit COX-2- and PGE2-dependent growth (4, 36, 37), expressed COX-2 and mPGES-1 constitutively. mPGES-1 expression was also detected in WiDr and HCT116 cells, although rather more weakly than in HCA-7 cells. Indirect immunofluorescent cytostaining analysis by confocal microscopy revealed the colocalization of COX-2 and mPGES-1 in the perinuclear area (Fig. 2B). All these cell lines expressed cPLA2α and cPGES constitutively, whereas COX-1 expression was restricted to HCA-7 and WiDr cells (Fig. 2A). Stimulation of HCA-7 cells with IL-1β, a proinflammatory cytokine that increases the expression of COX-2 and mPGES-1 in various cell types (20–22, 24, 25), did not alter the expression of COX-2 (not shown) and mPGES-1 (Fig. 2C). We therefore chose HCA-7 cells as a model for further study.

To examine the role of PGE2 produced by the COX-2/mPGES-1 pathway in the growth of HCA-7 cells, we first examined the effects of NS-398, a well known COX-2 inhibitor (38), and MK-886, an mPGES-1 inhibitor that also inhibits other MAPEG proteins such as FLAP and LTC4 synthase (22), on cell growth and PGE2 production. Treatment of HCA-7 cells with NS-398 almost completely abolished PGE2 production, accompanied by ~40% reduction of cell growth (Fig. 2D). When HCA-7 cells were treated with MK-886 at a concentration completely inhibiting mPGES-1 enzymatic activity in vitro (data not shown), there was a ~40% reduction in cell growth (as in the case of NS-398 treatment), even though the inhibition of PGE2 production was only partial (~60%) (Fig. 2E).

To assess the contribution of mPGES-1 to cell growth and PGE2 production in a more comprehensive way, we used an antisense oligonucleotide for mPGES-1 to reduce its expression. Treating the cells with an mPGES-1-specific antisense oligonucleotide resulted in marked reduction of mPGES-1 protein expression with no appreciable change in COX-2 expression (Fig. 3A). In this setting, both cell growth (Fig. 3B) and PGE2 production (Fig. 3C) were reduced partially, as observed in the experiments with MK-886 (Fig. 2E). The production of PGE2 was unaltered by antisense treatment (Fig. 3D), verifying that the antisense acted specifically on mPGES-1 but not on upstream enzymes or other terminal enzymes. Control oligonucleotide did not affect mPGES-1 expression, PGE2 production, or cell growth (not shown). These results suggest that PGE2 produced via the COX-2/mPGES-1 pathway is partially involved in the proliferation of HCA-7 cells.

We next examined if, conversely, overexpression of mPGES-1 would facilitate the growth of HCA-7 cells. To this end, C-terminal FLAG-tagged mPGES-1 cDNA was transfected into HCA-7 cells by lentivirus-mediated gene transfer. As shown in Fig. 4A, the virus-infected cells expressed FLAG-tagged mPGES-1 protein just above the position of endogenous mPGES-1, whereas the constitutive expression of the upstream enzymes cPLA2α and COX-2 was unaltered. PGE2 activity in vitro in the membrane fraction of cell lysates (Fig. 4B) and PGE2 release into medium during culture (Fig. 4C) were markedly increased in mPGES-1-transfected cells relative to control cells. Furthermore, the cell growth rate of mPGES-1-transfected cells was significantly faster than that of control cells (Fig. 4D).
Transformation of HEK293 Cells by Overexpression of mPGES-1—As shown in Fig. 5A, HEK293 cells cotransfected with COX-2 and mPGES-1 grew more rapidly than parental cells over the entire culture periods. COX-2/mPGES-1 cotransfection into HEK293 cells led to cell aggregation, rounding and piling up, and both enzymes were colocalized in the perinuclear region in the aggregated cells (Fig. 5B). These morphological changes were less pronounced in replicate COX-2/mPGES-1–cotransfected cells cultured in the continued presence of NS-398, added immediately after transfection, or in cells cotransfected with COX-2 and mPGES-1-R110S, which has very weak enzyme activity (21) (Fig. 5B). These results suggest that the catalytic functions of COX-2 and mPGES-1 are both required for triggering cellular transformation. However, addition of NS-398 to already transformed COX-2/mPGES-1-expressing clones failed to reverse their growth and aggregated morphology (not shown), indicating that transformation of HEK293 cells by COX-2/mPGES-1 cotransfection is an irreversible event.

As anchorage-independent growth is considered to be an in vitro test for tumorigenesis, we examined the growth of COX-2/mPGES-1-cotransfected HEK293 cells in a semisoft agar medium. As demonstrated in Fig. 6A, the COX-2/mPGES-1-coexpressing cells exhibited marked anchorage-independent growth, as manifested by the appearance of a number of large colonies. On the other hand, cells expressing COX-2 alone or cells coexpressing COX-1 and mPGES-1 formed fewer and smaller colonies, and parental cells did not grow appreciably in soft agar (Fig. 6A). Quantification of the numbers and sizes of colonies formed in this colony assay is summarized in Fig. 6B.

When parental and COX-2/mPGES-1-expressing HEK293 cells, as well as HCA-7 cells used as a positive control, were injected subcutaneously into athymic nude mice, COX-2/mPGES-1-expressing HEK293 cells and HCA-7 cells, but not parental HEK293 cells, formed large solid tumors after 3 months (Fig. 7A). Histopathologic examination of a fraction of whole tumor tissues from COX-2/mPGES-1-expressing HEK293 cell xenografts is shown in Fig. 7B. At the site of implantation, a whitish nodular tumor was formed in the subcutaneous tissue and exhibited a well circumscribed mass (Fig. 7B, panel a). The tumor cells had swollen nuclei of round or polygonal shape with spindled mitosis, and their cytoplasm was scarce and chromophobic (Fig. 7B, panel b). These characteristic features of the tumor suggest its malignancy. The tumor cells

Fig. 2. Expression of mPGES-1 in HCA-7 cells. A, the expression of PGE_2-biosynthetic enzymes in HEK293, HCA-7, WiDr, and HCT116 cells, as assessed by immunoblotting. Equal protein (20 μg protein equivalent) from the cell lysates were separated by SDS-PAGE and Western blotted at the same time with the corresponding antibody to allow for a direct comparison. B, subcellular locations of COX-2 (green) and mPGES-1 (red) as assessed by confocal microscopy. Yellow color (merge) indicates the co-localization of both enzymes. Fluorescent signals were overlaid on phase-contrast microscopy. C, expression of mPGES-1 in HCA-7 cells after treatment with IL-1β for the indicated periods. D and E, effects of 10 ng/ml NS-398, a COX-2 inhibitor (D), and 20 μM MK-886, a MAPEG inhibitor (E), on growth of and PGE_2 production by HCA-7 cells after 4 days of culture (n = 3, *, p < 0.05).

Fig. 3. Effects of mPGES-1 antisense oligonucleotide on HCA-7 cells. A, expression of mPGES-1 and COX-2 proteins in HCA-7 cells with (+) or without (−) treatment with an mPGES-1-specific antisense oligonucleotide, as assessed by immunoblotting. B–D, effects of the antisense treatment on cell growth (B), PGE_2 production (C), and PGF_2α production (D) after culture for 48 h (n = 3, *, p < 0.05).

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were proliferating with scanty interstitium that was mainly composed of capillaries (Fig. 7B, panel c, arrow). These capillaries consisted of swollen endothelial cells, and were likely to be newly formed vessels. Immunohistochemistry for mPGES-1 (Fig. 7B, panel d) and COX-2 (Fig. 7B, panel e) revealed the location of both enzymes around the nuclei in tumor cells. The HCA-7 xenograft tumor appeared as a nodular mass and was well demarcated (Fig. 7C, panel a). Tumor with a thin fibrous capsule was visible in the subcutaneous region (Fig. 7C, panel b, arrow). The tumor cells had scarce cytoplasm and hyperchromatic nuclei of various sizes with sporadic mitosis, and were partially proliferating with tubular formation. These features indicated adenocarcinoma differentiation (Fig. 7C, panel c). Immunoreactivities for mPGES-1 (Fig. 7C, panel d) and COX-2...
Identification of a Panel of Genes Altered by Overexpression of mPGES-1—To further elucidate the mechanisms of cellular transformation by co-overexpression of COX-2/mPGES-1, we surveyed a panel of genes, the expression of which was significantly altered in COX-2/mPGES-1-cotransfected HEK293 cells relative to cells expressing COX-2 alone, by cDNA array analysis. As summarized in Table I, increased genes included those required for cytoskeletal regulation (the small G protein RhoA), cell growth (the receptor tyrosine kinases ErbB3 and Flt1, the cyclin-dependent kinase CDK5, and the tumor necrosis factor signaling molecule TRAF-1), gene transcription (c-Myc, GATA4, and YL-1), and so on. Conversely, decreased genes included the H9251 subunit of protein phosphatase 1, cytoskeleton regulators (tubulin H9252 and ezrin), cell adhesion regulators (several integrins and H92511-catenin), the transcription factor Egr-1, and thyromosins (Table I).

We then performed Northern and Western blot analyses to verify that the expression of these genes identified by cDNA array analysis indeed differ between COX-2- and COX-2/mPGES-1-transfected HEK293 cells and between parental and mPGES-1-transfected HCA-7 cells. A representative result of the blotting analyses is shown in Fig. 8. In line with the cDNA array analysis, the expression levels of rhoA, c-myc, and ErbB3 were markedly increased, whereas those of ezrin and Egr-1 were decreased, in COX-2/mPGES-1-cotransfected cells relative to COX-2-transfected cells (Fig. 8A). Moreover, increased expression of rhoA, c-myc, and ErbB3 was also observed in mPGES-1-transfected HCA-7 cells as compared with parental cells (Fig. 8B).

DISCUSSION

Involvement of COX-2 and its main product, PGE2, in cancer development has been well documented by several studies. There is a reduction in the relative risk of colorectal cancer in individuals taking non-steroidal anti-inflammatory drugs (2, 3). COX-2 levels are increased in 90% of human colorectal cancers and 50% of premalignant adenomas (1). COX-2 expression in colorectal carcinoma cells provides a growth and survival advantage and increases tumor invasiveness, and PGE2 increases growth and motility of colorectal carcinoma cells through the EP4 receptor signaling pathway (8–12). Genetic disruption of cPLA2 (17, 18), COX-2 (13), or the EP2 receptor (14) results in decreased number and/or neoplastic growth of colorectal polyps in APC mutant mice. In addition to colorectal cancer, transgenic overexpression of COX-2 in mouse mammary gland results in spontaneous development of mammary tumor (10). Similarly, transgenic mice with keratin 5 promoter-driven COX-2 overexpression in basal epidermal cells exhibit a preneoplastic skin phenotype (39). PGE2 regulates COX-2-dependent, CD44- and matrix metalloproteinase-2-mediated invasion in non-small cell lung cancer in an autocrine/paracrine manner via EP4 receptor signaling (40). Given these observations, the aim of this study was to evaluate the potential contribution of mPGES-1, which lies downstream of the COX-2-dependent PGE2-biosynthetic pathway, to tumorigenesis. Elevated expression of mPGES-1 in human cancers has recently been demonstrated in non-small cell lung cancer (32) and endometrial adenocarcinoma (33). In this study, we performed immunohistochemistry of human colon cancer and adenoma tissues with anti-COX-2 and anti-mPGES-1 antibodies and found that both enzymes are coex-
pressed in many, even if not all, of malignant and benign colorectal tumor cells (Fig. 1). While this study was underway, Yoshimatsu et al. (34) reported that mPGES-1 is overexpressed in 80% of human colorectal tumors and adenomas, in line with our present observation.

To assess the role of mPGES-1 in growth of colon cancer cells in cell culture, we took advantage of HCA-7 cells, a human colon cancer cell line that has often been used to investigate COX-2-dependent tumorigenesis (4, 36, 37). Proliferation of HCA-7 cells is reduced by COX-2 inhibitors, and this is reversed by exogenous PGE2 (4, 36). PGE2 also prevents COX-2 inhibitor-induced apoptosis by inducing expression of Bcl-2, and increases clonogenicity in HCA-7 cells (37). In this study, we found that both mPGES-1 and COX-2 are constitutively, but not inducibly, expressed in HCA-7 cells (Fig. 2A). Although the transcriptional regulations of COX-2 and mPGES-1 are not entirely identical (24, 31), recent observations that the expression of COX-2 in HCA-7 cells is transcriptionally regulated by the NF-IL-6-regulatory element (41) and that mPGES-1 induction in lipopolysaccharide-stimulated mouse macrophages is ablated in NF-IL-6-deficient mice (29) suggest a role for this transcription factor in the expression of both COX-2 and mPGES-1 in HCA-7 cells.

Importantly, studies using MK-886, an inhibitor for MAPEG proteins including mPGES-1 (Fig. 2, C and D), an mPGES-1-specific antisense oligonucleotide (Fig. 3) and overexpression of mPGES-1 (Fig. 4) together suggest that mPGES-1 is involved in PGE2 production and proliferation of HCA-7 cells, even if partially. Somewhat paradoxically, under conditions where the COX-2 inhibitor NS-398 almost completely abolished PGE2 production, reduction of cell growth by NS-398 was still only partial, whereas reduction of PGE2 production and cell growth by MK-886 or the mPGES-1 antisense was partial and parallel (Figs. 2 and 3). A likely explanation for these observations is that the PGE2 produced via the COX-2/mPGES-1 pathway contributes to cell growth, whereas there is an additional COX-
2-dependent but mPGES-1-independent PGE₂-biosynthetic route that is unrelated to the mitogenic response. Polarized, vectorial production of PGE₂ toward basolateral and apical directions in HCA-7 cells (36) may support the existence of distinct PGE₂ pools. Considering that the growth-stimulating effect of PGE₂ on HCA-7 cells depends, at least in part, on EP4 (16), the PGE₂ produced by COX-2/mPGES-1 in the perinuclear region may be preferentially presented to this G-protein-coupled PGE₂ receptor through an unknown mechanism. Alternatively, the perinuclear PGE₂ (or possibly other unknown metabolites) produced by COX-2/mPGES-1 may act on certain nuclear receptors that in turn promote cell growth. Indeed, several eicosanoids have been shown to stimulate the peroxisome proliferator-activated receptor family of nuclear receptors (43, 44). Nonetheless, different sensitivity of PGE₂ production by HCA-7 cells to COX-2 and mPGES-1 inhibitors and mPGES-1 antisense oligonucleotide strongly argues that this cell line may contain an alternative COX-2-dependent PGE₂-biosynthetic route that involves other PGE₂ enzyme(s). Although cPGES is abundantly expressed in HCA-7 cells (Fig. 2A), this enzyme has been reported to be coupled rather specifically with COX-1, not COX-2 (19). mPGES-2, a recently identified enzyme whose transcript is detected in human colon (22), may represent a second PGE₂ that can be coupled with COX-2, a possibility that is now under investigation.

In another model using HEK293 cells, cotransfection of COX-2 and mPGES-1 led to cellular transformation, as demonstrated by rapid proliferation, morphological change, piling up, and aggregation in normal culture, large colony formation in soft agar culture, and formation of solid tumors in nude mice (Figs. 5–7). Immunochemical examination of the tumor revealed tissue invasion by COX-2/mPGES-1-derived tumor cells as well as angiogenesis (Fig. 7), consistent with the angiogenic effect of COX-2 and PGE₂ (11, 45, 46). Growth promotion and morphological change of COX-2/mPGES-1-transfected HEK293 cells in culture were less pronounced if NS-398 was added immediately after COX-2/mPGES-1 transfection, or if a catalytically inactive mPGES-1 mutant was transfected in place of native enzyme (Fig. 5C). Once transformed, however, neither NS-398 nor MK-886 reversed the growth and aberrant morphology of COX-2/mPGES-1-cotransfected HEK293 cells. These observations indicate that, even though the COX-2/mPGES-1 catalytic product triggers cellular transformation, it may not be required for subsequent maintenance of a transformed phenotype in this setting. A similar event has been observed in ECV endothelial cells transfected with COX-1 (not COX-2, which induced apoptosis in these cells), where aggressive growth of COX-1-transfected ECV cells was no longer inhibited by indomethacin (47). Unlike HCA-7 cells (4, 36), simple addition of exogenous PGE₂ to HEK293 cells did not induce cellular transformation, as mentioned previously (21). The following possibilities could be considered: (i) continuous production of high levels of PGE₂ around the perinuclear area by COX-2/mPGES-1 is critical for inducing transformation; (ii) some additional components, which act cooperatively with PGE₂ to induce cellular transformation, are induced by COX-2/mPGES-1 overexpression; and (iii) some unknown substances produced by COX-2/mPGES-1 may be involved in transformation.

To gain insights into mPGES-1-promoted cellular transformation, we sought to identify mPGES-1-regulated genes by cDNA array technology (Table I and Fig. 8). The genes identified so far can be categorized into several groups: (i) genes for signaling molecules related to cell proliferation and differentiation; (ii) genes for transcription factors; (iii) genes related to cytoskeletal regulation; (iv) cell adhesion molecules; and (v) genes with unknown functions. Some of the induced genes are proto-oncogenes that have the capacity to promote cellular transformation when transfected alone into cells.

Increased genes related to cell growth and differentiation include those encoding the receptor tyrosine kinases ErbB3 and Flt1, the cyclin-dependent protein kinase CDK5, the ribosomal proteins S3A and S19, the proliferation-associated nucleolar protein NOL1, and TRAF1. ErbB3, a ligand for heregulin, is a member of the epidermal growth factor receptor family and its overexpression has been frequently found in human tumors (48). Induced expression of ErbB3 is in line with a recent report that PGE₂ transactivates epidermal growth factor receptor, thereby switching on the mitogenic signaling pathway in gastric epithelial and colon cancer cell lines (49). Moreover, COX-2 is overexpressed in heregulin 2-positive breast cancer (50). Induction of Flt1, a receptor for vascular endothelial cell growth factor (51, 52), may be linked to angiogenesis, which has been associated with COX-2- and PGE₂-dependent tumor development (11, 45, 46).

Cyclin-dependent protein kinases generally play crucial roles in cell cycle progression (53). Apart from cell cycle control, CDK5 phosphorylates a diverse list of substrates and regulates a range of cellular processes, including cell adhesion and motility (54, 55). Ribosomal proteins are integral components of the basal cellular machinery involved in protein synthesis and have been found to play roles in regulating cell growth and

FIG. 8. Altered expression of several genes in COX-2/mPGES-1-cotransfected HEK293 cells and mPGES-1-transfected HCA-7 cells. A, comparison between COX-2-expressing and COX-2/mPGES-1-expressing HEK293 cells. ErbB3, Egr-1, and ezrin were detected by Western blotting and c-myc and rhoA by Northern blotting. The Northern membrane was reprobed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe to confirm equal loading of samples into each lane. B, comparison between parental and mPGES-1-transfected HCA-7 cells. The results of ErbB3 (Western blotting), c-myc and rhoA (Northern blotting) are shown. For Northern blot analysis, ribosomal RNA (rRNA), which was visualized with ethidium bromide, is also shown.
transformation (56, 57). NOL1 expression is associated with cell proliferation during G1-S phases and represents a biological marker indicative of tumor aggressiveness, particularly the late events of colorectal tumor progression (58, 59). TRAF1 is a signal transducer of the tumor necrosis factor receptor family, which has been implicated in cell differentiation (60). Conversely, decreased expression of protein phosphatase 1, a tumor suppressor of which mutations have been found in lymphoma and hepatoma (61, 62), may also account for aggressive cell growth of COX-2/mPGES-1 cotransfectants.

Transcription factors whose expressions are altered by COX-2/mPGES-1 include c-Myc, GATA4, and YL-1 (increased) as well as Egf-1 (decreased). Induced expression of c-Myc, a helix-loop-helix transcription factor, is of great importance in controlling cell growth and vitality (63), and is commonly amplified in many human tumors (64). Involvement of c-Myc in colon cancer development downstream of the Wnt/APC/β-catenin signaling pathway has been documented (65, 66). Moreover, the activation of the Wnt/APC/β-catenin signaling pathway results in transcriptional up-regulation of COX-2 in cancer cells (67). GATA-4, a member of the GATA transcription factor family, plays a role in the regulation of cell migration and its overexpression has been found in several types of cancer (68). YL-1, also reported as transcription factor-like 1, has been implicated in anchorage-independent cell growth (69). Egf-1, a COX-2/mPGES-1-decreased gene, is an inducible transcription factor that binds to GC-rich elements and plays a crucial role in transcriptional activation of the mPGES-1 gene (31). Hence, the reduction of Egf-1 expression in COX-2/mPGES-1 cotransfectants may be a reflection of negative feedback regulation of mPGES-1 expression.

Altered genes encoding proteins for cytoskeletal regulation include RhoA (increased), ezrin, tubulin, and annexins (decreased), which may be responsible for the marked morphological change in COX-2/mPGES-1 cotransfectants. The small G protein RhoA regulates various aspects of actin filament rearrangement and has a key role in growth of tumors (70, 71). Ezrin plays structural and regulatory roles in the assembly and stabilization of specialized plasma membrane domains, particularly in surface projections such as microvilli and membrane ruffles where it links the microfilaments to the plasma membrane (72). Annexins interact with cytoskeletal proteins and have been implicated in diverse cellular responses including differentiation and membrane fusion (73). Because annexins are capable of inhibiting cPLA₂α (74), decreased expression of annexins may lead to increased activation of cPLA₂α, thereby amplifying the COX-2/mPGES-1-dependent PG₂ biosynthesis. Indeed, cPLA₂α has often been associated with cellular transformation (75) and cPLA₂α knockout decreases the incidence of polyposis in Apc mutant mice (17, 18). There were decreases in the expression of several cell adhesion molecules, such as integrins and α₂-catenin, which are critical components for focal adhesion (76, 77). Thus, decreased expression of these focal adhesion proteins is consistent with the reduced anchorage dependence of COX-2/mPGES-1 cotransfectants.

The most remarkably decreased genes in COX-2/mPGES-1 cotransfectants are those for thymosins. Prothymosin α is a small highly acidic protein found in the nuclei of virtually all mammalian tissues, and its high conservation in mammals and wide tissue distribution suggest an essential biological role (42). Although the exact mechanism of action of thymosins remains elusive, the one constant has been their relationship with the proliferating state of the cell and its requirement for cellular growth and survival. Overall, although the mechanisms by which overexpression of COX-2/mPGES-1 alters the expression of these genes, and whether these genes, alone or in combination, induce transformation of HEK293 cells, are currently unclear, the present findings shed light on unexplored aspects of the combined action of COX-2 and mPGES-1 in tumorigenesis. Indeed, increases in some of the genes identified in COX-2/mPGES-1-transfected HEK293 cells were also observed in mPGES-1-transfected HCA-7 cells (Fig. 8B), suggesting that our findings could be applicable to at least some types of colorectal cancer. Evaluation of the effect of COX-2/mPGES-1 in non-transformed intestinal cell lines will further insight into this crucial issue.

In summary, this study demonstrates that mPGES-1, in concert with COX-2, can be associated with cellular transformation and cancer development. Future studies using mPGES-1 knockout mice or mPGES-1-specific inhibitors would open further insights into the role of this critical PG₂ biosynthetic terminal enzyme in tumorigenesis. Importantly, our observation that mPGES-1 is overexpressed in colorectal tumors provides the basis for future studies that will evaluate whether mPGES-1 is a bona fide therapeutic target.

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Potential Role of Microsomal Prostaglandin E Synthase-1 in Tumorigenesis
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