Prostaglandin E2 (PGE2), the most abundant COX-2–derived prostaglandin found in colorectal cancer, promotes tumor cell proliferation and survival via multiple signaling pathways. However, the role of PGE2 in tumor hypoxia is not well understood. Here, we show a synergistic effect of PGE2 and hypoxia on enhancing angioptiogen-like protein 4 (ANGPTL4) expression and that elevation of ANGPTL4 promotes colorectal cancer growth. PGE2 induces ANGPTL4 expression at both the mRNA and protein levels under hypoxic conditions. Moreover, hypoxia induces one of the PGE2 receptors, namely EP1. Activation of EP1 enhances ANGPTL4 expression, whereas blockade of EP1 by an antagonist inhibits PGE2 induction of ANGPTL4 under hypoxic conditions. Importantly, overexpression of ANGPTL4 promotes cell proliferation and tumor growth in vitro and in vivo. In addition, treatment with ANGPTL4 recombinant protein increases colorectal carcinoma cell proliferation through effects on STAT1 signaling. The MAP kinase and Src pathways mediate ANGPTL4-induced STAT1 expression and activation. These results are relevant to human disease because we found that the expression of ANGPTL4 and STAT1 are elevated in 50% of human colorectal cancers tested and there is a positive correlation between COX-2 and ANGPTL4 as well STAT1 expression in colorectal carcinomas. Collectively, these findings suggest that PGE2 plays an important role in promoting cancer cell proliferation via ANGPTL4 under hypoxic conditions.

Introduction

Recent evidence confirms a reduction in risk for colorectal and other cancers in individuals who regularly take aspirin or other nonsteroidal anti-inflammatory drugs. These drugs are potent inhibitors of COX-1 and COX-2. Elevation of COX-2 was found in approximately 50% of colorectal adenomas and 85% of adenocarcinomas (1, 2) and is associated with worse survival among patients with colorectal cancer (3). COX-2 converts free arachidonic acid into prostanoids, including prostaglandins (PG) and thromboxanes (TX). Prostaglandin E2 (PGE2) is the most abundant prostaglandin found in colorectal cancer, and is associated with worse survival among patients with colorectal cancer (3). COX-2 converts free arachidonic acid into prostanoids, including prostaglandins (PG) and thromboxanes (TX). Prostaglandin E2 (PGE2) is the most abundant prostaglandin found in colorectal cancer (4). An increasingly large body of evidence has shown that PGE2 mediates the tumor-promoting effects of COX-2 in colorectal cancer (5). PGE2 treatment dramatically increased both small- and large-intestinal adenoma burden in ApcMin/+ mice and significantly enhanced azoxymethane–induced colon tumor incidence and multiplicity (6, 7). PGE2 promotes tumor growth by stimulating EP receptor signaling with subsequent enhancement of cellular proliferation, promotion of angiogenesis, inhibition of apoptosis, and stimulation of invasion/motility (8).

Hypoxia is one of the most common and critical factors identified thus far in the regulation of cancer progression and metastasis (9, 10). Adaptation of tumor cells to hypoxia is achieved largely by undergoing genetic changes that enable them to survive and become more malignant or invasive (11). Hypoxia induces COX-2 expression and PGE2 levels via hypoxia-inducible factor (HIF)-1, a major transcription factor that regulates the hypoxic response, in colorectal carcinoma cells (12, 13). Increased levels of PGE2 during hypoxia, in turn, potentiates HIF-1 transcriptional activity and promotes colorectal carcinoma cell growth and survival (12), suggesting that cross-talk exists between PGE2 and hypoxia. However, the mechanism through which PGE2 promotes colorectal cancer progression in the hypoxic tumor microenvironment is unclear. We postulated that PGE2 might mediate a pivotal tumor cellular adaptive response to hypoxia with the implication for connecting tumor hypoxia to cancer progression.

Angiopoietin-like protein 4 (ANGPTL4) is a secretory protein that is cleaved into an N-terminal coiled-coil fragment (N-ANGPTL4) and a C-terminal fibrinogen-like domain (C-ANGPTL4) that modulates the disposition of circulating triglycerides (14). Although the role of ANGPTL4 in lipid metabolism has been well characterized, its role in cancer progression is poorly understood. Although recent studies suggest that ANGPTL4 is involved in cancer progression, the precise role of ANGPTL4 in angiogenesis and cancer progression is still debated. For example, ANGPTL4 has been reported...
to have proangiogenic effects and antiangiogenic effects in different models (15–17). Moreover, ANGPTL4 mediates TGF-β–induced lung metastasis of breast cancer (18) but inhibits metastasis of melanoma cells (19). These conflicting data support the need of additional research to address the role of ANGPTL4 in cancer progression.

In this study, ANGPTL4 is identified as a novel focal point of the cross-talk between PGE2 and hypoxia signaling pathways in colorectal cancer cells. Our results show that PGE2 and hypoxia synergistically induce ANGPTL4 expression via EP1. Importantly, we show that the elevation of ANGPTL4 promotes tumor growth in vitro and in vivo. Moreover, we identified STAT1 as a new target of ANGPTL4 and found that inhibition of STAT1 attenuated ANGPTL4 induction of colorectal cancer cell proliferation. We further elucidated the ANGPTL4 signaling pathways in response to STAT1 expression and activation. The maximal induction of ANGPTL4 required both hypoxia and PGE2, indicating the complex nature of interactions within the tumor microenvironment that affect cancer signaling pathways.

Materials and Methods

Reagents

PGE2, 17-phenyl-trinor-PGE2, and SC-19220 were purchased from Cayman Chemical Company. U73122, BAPTA-AM, PD98059, SU6656, and diphenylene iodonium (DP1) were purchased from EMD Chemicals. ONO-AE3-208 was kindly provided by Ono Pharmaceutical Co. Full-length and C-terminal ANGPTL4 recombinant proteins were obtained from R&D Systems.

Cell culture and treatment

LS-174T, HT-29, and HCT-116 were purchased from the American Type Culture Collection. All cells were routinely maintained in McCoy’s 5A medium containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin in a 5% CO2 humidified incubator at 37°C. Cells were exposed to hypoxia by placing them in a mixed-gas incubator at 37°C. The cells were incubated with STAT1 antibody recognizing STAT1, Src, phospho-Src (Cell Signaling Technology), ANGPTL4 (R&D Systems), ERk, phospho-ERk (Santa Cruz Biotechnology), and Actin (Sigma-Aldrich). Secondary antibody conjugated to horseradish peroxidase (Vector Laboratories Inc.) was used at 1:2,000 to detect primary antibodies, and enzymatic signals were visualized by chemiluminescence.

Quantitative real-time PCR

Total RNA was isolated by using TRIzol (Invitrogen). cDNA was synthesized from 2 μg of total RNA with High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems) and mixed with TaqMan Gene Expression Assay Mix, sterile water, and TaqMan Fast Universal PCR Master Mix (Applied Biosystems). Quantitative real-time PCR (qRT-PCR) was carried out using a 7900 HT Fast (Applied Biosystems) system, and expression of target genes mRNA relative to 18s rRNA was calculated.

Measurement of ANGPTL4 secretion

Cells were exposed to PGE2 or hypoxic conditions, conditioned medium was collected, and then the amount of ANGPTL4 secreted into medium was measured using Human ANGPTL4 DuoSet ELISA kit (R&D Systems, Inc.).

DNA construction

ANGPTL4 cDNA (Origene) was amplified with pfuUltra Hotstart DNA polymerase (Stratagene). The PCR products were digested with XhoI and NotI and were cloned into pBMN-I-GFP (addgen).

Establishment of stable cell line

pBMN-I-GFP and pBMN-I-GFP-ANGPTL4 retroviral vectors were transfected into Phoenix cells in 60-mm dishes using Lipofectamine reagent (Invitrogen) according to the manufacturer’s protocol. Culture medium containing virus particles was collected 48 hours later and was added to LS-174T cells. Cells infected were sorted by green fluorescent protein (GFP) positivity to eliminate uninfected cells.

Western blotting

Whole cell lysates were prepared for Western blot analyses using lysis buffer containing 20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% TX-100, 1 mmol/L EDTA, pH 8.0, and 1 mmol/L phenylmethylsulfonylfluoride. Samples were denatured in a SDS sample buffer. Total proteins were separated by loading 20 μg of total cell lysate on a denaturing 10% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked with 5% nonfat dry milk in PBS containing 0.1% Triton X-100 and incubated with primary antibodies that recognize STAT1, Src, phospho-Src (Cell Signaling Technology), ANGPTL4 (R&D Systems), ERk, phospho-ERk (Santa Cruz Biotechnology), and Actin (Sigma-Aldrich). Secondary antibody conjugated to horseradish peroxidase (Vector Laboratories Inc.) was used at 1:2,000 to detect primary antibodies, and enzymatic signals were visualized by chemiluminescence.

Cell viability assay

Ninety-six–well plates were seeded with 5,000 cells per well and cells were treated without or with ANGPTL4 in serum-free medium for 3 days. Cell viability was determined using Cell Proliferation Reagent WST-1 (Roche Applied Science).

Immunocytochemistry

Cells were treated with ANGPTL4, washed with PBS, and fixed with 4% paraformaldehyde for 30 minutes at room temperature, and subsequent blocking with PBS containing 1% bovine serum albumin and 0.1% TX-100 for 30 minutes at room temperature. The cells were incubated with STAT1 antibody (Cell Signaling Technology) for 2 hours at room temperature, followed by biotinylated secondary antibody (Vector Laboratories Inc.) for 1 hour, then Fluorescein streptavidin (Vector Laboratories Inc.) for 1 hour, then Fluorescein streptavidin (Vector Laboratories Inc.) for 30 minutes, and then 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) for 5 minutes to visualize nuclei. Cells were examined under a fluorescence microscope (Nikon ECLIPSE TE300) to determine localization of STAT1.

Immunohistochemistry

Paraffin-embedded specimens were treated with xylene and ethanol to remove the paraffin. The slides were immersed in Borg decloaker solution (Biocare Medical, Inc.) and boiled in a pressure cooker at 125°C for 5 minutes for antigen retrieval. Endogenous peroxidase activity was blocked by incubation in
3% H2O2 containing PBS solution for 10 minutes. The slides were blocked with 5% normal goat serum and incubated with anti-STAT1 (Cell Signaling), HIF-1α (BD Biosciences), and ANGPTL4 (Adipobioscience) at 4°C overnight. After washing with PBS, the slides were incubated with Goat anti-Rabbit HRP (Vector Laboratories). After washing, the slides were developed with 3,3-diaminobenzidine reagent (Vector Laboratories) followed by counterstaining with hematoxylin.

RNA interference
siGENOME SMARTpool siRNAs targeting STAT1 (M-003543-01-000) were purchased from Dharmacon, Inc. LS174T cells were transfected with 20 nmol/L of STAT1 siRNA or nontargeting siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s specifications. The efficacy of knockdown was confirmed by Western blot analysis.

Xenograft study
All mice were housed and treated in accordance with protocols approved by the Institutional Animal Care and Use Committee at The University of Texas MD Anderson Cancer Center. LS-174T cells (5 × 10^6) selected for the stable expression of ANGPTL4 (ANGPTL4/LS-174T) or control GFP (GFP/LS-174T) were injected s.c. into the flanks of nude mice. The tumor size was measured starting from 13 to 28 days after injection. After the mice were euthanized using CO2 asphyxiation, necropsies were done to remove tumors and measurements were taken of tumor weight and size.

Gene expression data of colon cancer patients
Human colorectal carcinoma specimens were obtained from the Tissue Procurement and Banking Facility (TPBF) at The University of Texas MD Anderson Cancer Center. Equal amounts of mRNA were analyzed by qRT-PCR for COX-2, ANGPTL4, and STAT1. Microarray data from Moffit Cancer Center (Moffit cohort, GSE17536, N = 177) were downloaded from GEO (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE17536). Kaplan–Meier plots and log-rank test were used to estimate patient prognosis.

Statistical analysis
Each experiment was done at least 3 times, and data are presented as the mean ± SE. Statistical significance was determined using Student t test, 1-factor ANOVA, or 2-factor ANOVA, where applicable. In Figs. 1–4, * indicates P < 0.05; ** indicates P < 0.01; and *** indicates P < 0.001.

Results
PGE2 enhances ANGPTL4 expression in colorectal carcinoma cells under hypoxic conditions
To help mimic the conditions present in the tumor microenvironment, we examined the role of PGE2 under normoxic and hypoxic conditions. We conducted transcriptome analysis with RNA isolated from LS-174T cells exposed to PGE2 and/or hypoxia. LS-174T cells normally have low COX-2 levels and do not produce PGE2 under normoxic and hypoxic conditions (data not shown). Analysis of our microarray data revealed that ANGPTL4 was one of the PGE2 downstream targets in cells exposed to hypoxia. Multiple lines of evidence have shown that hypoxia induces ANGPTL4 expression in several cell lines including monocytes and adipocytes (20–22). As expected, hypoxia increased ANGPTL4 expression at both mRNA and protein levels in LS-174T cells (Fig. 1A and B). Strikingly, PGE2 treatment dramatically enhanced ANGPTL4 expression under hypoxic conditions at both mRNA and protein levels in a dose-dependent manner in LS-174T cells, whereas PGE2 had no effect on ANGPTL4 expression under normoxic conditions (Fig. 1A and B). The synergistic effect of PGE2 and hypoxia on ANGPTL4 protein expression was also observed in HCT-116 and HT-29 colorectal carcinoma cells (Fig. 1C). These results show that PGE2 and hypoxia synergistically induce ANGPTL4 expression in colorectal cancer cells.

PGE2 mediates PGE2-stimulated ANGPTL4 expression during hypoxia
To explore why PGE2 induces ANGPTL4 expression only under hypoxic conditions but not under normoxic conditions, we first examined the effects of hypoxia on PGE2 receptor levels. There are 4 known PGE2 receptors (EP1, EP2, EP3, and EP4) that are typical G protein-coupled receptors. Because recent reports showed that hypoxia induced PGE2 expression in osteoblastic cells (23), we examined the effect of hypoxia on EP1 expression in colorectal cancer cells. Indeed, hypoxia specifically increased only EP1 expression and not other PGE2 receptors in LS-174T cells (Fig. 2A). Because LS-174T cells express high levels of EP4, we examined which receptor mediates the effects of PGE2 on upregulation of ANGPTL4 expression. Treatment of LS-174T cells with an EP1 agonist, SC19220, blocked PGE2-induced ANGPTL4 induction under hypoxic conditions, whereas an EP4 agonist, ONO-AE3-208, had no effect on ANGPTL4 expression (Fig 2B). Consistent with these results, a selective EP1 agonist (17-PT PGE2) has the same effects as PGE2 on ANGPTL4 expression (Fig. 2C). Because EP1 is coupled to the Gq/PLC/Ca2+ pathway (24), we tested whether inhibition of this pathway attenuates PGE2 induction of ANGPTL4. Treatment of LS-174T cells with a PLC inhibitor (U73122) and a Ca2+ chelating agent (BAPTA) partially suppressed PGE2-induced ANGPTL4 expression under hypoxic conditions, respectively (Fig. 2D). These results reveal that EP1 is induced by hypoxia and mediates PGE2 induction of ANGPTL4.

ANGPTL4 promotes tumor growth in vitro and in vivo
To determine the biologic role of ANGPTL4 in regulating cancer cell proliferation, LS-174T cells were treated with full-length recombinant ANGPTL4 (F-ANGPTL4) and C-ANGPTL4, respectively. Our results showed that C-ANGPTL4 more dramatically promoted LS-174T cell proliferation than F-ANGPTL4 (Fig. 3A, top). In addition, C-ANGPTL4 exhibited similar effects on regulating HCT-116 and HT-29 cell proliferation (Fig. 3A, bottom). In contrast, both F-ANGPTL4 and C-ANGPTL4 had no effects on regulating LS-174T cell apoptosis (data not shown). To confirm the proliferative effect of ANGPTL4, we established LS-174T cells that overexpressed ANGPTL4 (ANGPTL4/LS-174T) and control cells (GFP/LS-174T). C-ANGPTL4 (37 kDa) was easily detected in the
conditioned medium of ANGPTL4/LS-174T cells (Fig. 3B, top). Because ANGPTL4 is proteolytically cleaved upon secretion from cells, F-ANGPTL4 was strongly detected in the cell lysate but not the conditioned medium (data not shown). Consistent with above results, overexpression of ANGPTL4 enhanced cell proliferation (Fig. 3B, bottom). These results are consistent with our observation that PGE2 increases cell proliferation under hypoxic conditions (Fig. 3C).

To validate our in vitro results in vivo, we injected ANGPTL4/LS-174T or GFP/LS-174T cells into the flanks of nude mice. The tumor size was measured starting from 13 to 28 days after injection. Tumor weight and C-ANGPTL4 levels were also measured after mice were euthanized. As expected, C-ANGPTL4 levels were higher in the tumor tissue of mice injected with cells expressing ANGPTL4 than in the controls (Fig. 3D, left). Moreover, cells expressing ANGPTL4 formed larger tumors than the control cells (Fig. 3D, middle and right), suggesting that elevation of ANGPTL4 promotes colorectal cancer growth. Taken together, these results suggest that ANGPTL4 accelerates colorectal cancer growth by enhancing cell proliferation.

STAT1 is important for ANGPTL4-mediated cell proliferation

Although several recent reports have shown the role of ANGPTL4 in cancer progression (15, 25, 26), little is known about how ANGPTL4 regulates cell proliferation and what downstream targets of ANGPTL4 are involved in regulating cell proliferation. The microarray analysis revealed that C-ANGPTL4 treatment increased IFN-related gene expression, such as STAT1, IFI35, IFI9, and IRF1. These genes were validated by qRT-PCR (data not shown). We further showed that C-ANGPTL4 treatment induced STAT1 expression at both the mRNA and protein levels as well as its nuclear localization (Fig. 4A, top and middle). Immunohistochemical staining further revealed that STAT1 expression was elevated in tumor tissue derived from mice implanted with ANGPTL4/LS-174 cells as compared with the GFP control (Fig. 4A, bottom). Because STAT1 is overexpressed in head and neck and lung cancer and has been reported to have a proliferative effect (27, 28), we examined the contribution of STAT1 to C-ANGPTL4-induced cell proliferation. Knockdown of STAT1 significantly suppressed C-ANGPTL4-induced cell proliferation (Fig. 4B). Because extracellular signal–regulated kinase (ERK) and Src are known to be the critical signaling molecules for cell proliferation or survival (29), we determined whether these pathways are involved in C-ANGPTL4 induction of STAT1 expression. As shown in Fig. 4C, PGE2 was able to enhance the phosphorylation of Src and ERK as well as STAT1 expression under hypoxic conditions. Moreover, C-ANGPTL4 treatment induced phosphorylation of Src and ERK in time-dependent manners (Fig. 4D, top), whereas pharmacologic inhibitors of Src (SU6656) and ERK (PD98059) blocked STAT1 expression stimulated by C-ANGPTL4, respectively (Fig. 4D, middle). Recently, ANGPTL4 has been reported to stimulate NADPH-oxidase–dependent production of \( \text{O}_2^- \), which in turn triggers the Src and ERK pathway (30). Therefore, we examined whether ANGPTL4 regulated Src and ERK activation and STAT1 expression via upregulating \( \text{O}_2^- \) production. Indeed, inhibition of \( \text{O}_2^- \) production by an NADPH oxidase inhibitor
inhibitor (DP1) suppressed ANGPTL4 stimulated the phosphorylation of Src and ERK and the expression of STAT1 (Fig. 4D, bottom). Collectively, these results indicate that STAT1 is a downstream target of C-ANGPTL4 for regulating cancer cell proliferation and that STAT1 induction is dependent on NADPH-oxidase-mediated production of $O_2^-$, Src, and MAP kinase pathways.

**ANGPTL4 and STAT1 are elevated in human colorectal cancer specimens**

Because increased levels of COX-2 and a downstream product, PGE$_2$, have been reported in most human colorectal cancers (1) and hypoxia is one of the key elements in the tumor microenvironment that promotes cancer development and progression (9, 10), we examined ANGPTL4 levels in human colorectal cancer and the correlation of ANGPTL4 with COX-2 and STAT1 in human colorectal cancer. ANGPTL4 mRNA levels were elevated in 16 of 30 cancer specimens as compared with those in adjacent normal mucosa (Fig. 5A). Moreover, ANGPTL4 expression in human colorectal cancer specimens correlates with COX-2 expression ($r = 0.2433, P = 0.0056$). In addition, high STAT1 expression was found in 17 of 30 tumor tissues and is also correlated with ANGPTL4 expression ($r = 0.6861, P < 0.0001$). To support these findings, we used a public microarray database to retrieve gene expression data of colorectal cancer patients. Patients in the Moffitt cohort ($N = 177$) were dichotomized according to expression level of ANGPTL4. The overall survival rate of patients with higher expression of ANGPTL4 was significantly worse than that of those with lower expression of ANGPTL4 ($r = 0.434, P < 0.0001$) and STAT1 ($r = 0.232, P = 0.001$) in the Moffitt cohort (Fig. 5C). Therefore, these results suggest that elevation of ANGPTL4 could be relevant to human colorectal cancer progression.

Figure 2. EP1 mediates PGE$_2$-induced ANGPTL4 expression during hypoxia. A, LS-174T cells were exposed to hypoxia for 12 hours and qRT-PCR was done to determine the expression of EP1, EP2, EP3, and EP4 receptors (left). LS-174T cells were exposed to hypoxia, and EP1 protein levels were analyzed by Western blotting over times as indicated (right). Actin was used as a protein loading control. B, LS-174T cells were pretreated with EP1 (10 $\mu$mol/L SC-19220) or EP4 (10 $\mu$mol/L ONO-AE3-208) antagonists for 1 hour and then were treated with PGE$_2$ under normoxic or hypoxic conditions, following qRT-PCR and ELISA to detect ANGPTL4 mRNA and protein. C, ANGPTL4 levels were determined in the growth medium with a commercial ELISA after treatment with the indicated concentration of EP1 agonist (17-PT-PGE$_2$) under normoxic or hypoxic conditions. D, cells were pretreated with PLC inhibitor (10 $\mu$mol/L U73122) or Ca$^{2+}$-chelator (1 $\mu$mol/L BAPTA-AM) for 1 hour and then treated with PGE$_2$ under normoxia/hypoxia for 48 hours. ANGPTL4 levels were analyzed by ELISA. *P < 0.05; ** P < 0.01; ***P < 0.001.
Discussion

Hypoxia in the tumor microenvironment contributes to cancer progression and metastasis by activating adaptive programs that promote cancer cell proliferation, survival, and motility as well as tumor-associated angiogenesis. It has been reported that there is a cross-talk between the COX-2 pathway and signaling pathways affected by hypoxia. For example, hypoxia induces COX-2 expression in colorectal cancer cells and vascular endothelial cells (12, 31). Reciprocally, COX-2–derived PGE2 regulates HIF-1 expression and transcriptional activity in those cells (12, 32). Although a connection between COX-2 and hypoxia has been established, the precise mechanisms by which PGE2 and hypoxia synergistically promote cancer progression are largely unclear. In this study, we identify ANGPTL4 as a novel PGE2 downstream target under hypoxic conditions in colorectal cancer cells. We also found that hypoxia specifically enhanced EP1 expression in colorectal carcinoma cells without significant effects on EP2, EP3, or EP4 (Fig. 2A). EP1 has been reported to promote cell survival and proliferation in different types of cancer cells (34, 35). A role for EP1 in colorectal tumorigenesis has been further confirmed in animal models. For example, inhibition of EP1 signaling by genetic deletion or treatment of an EP1-selective antagonist reduced intestinal tumor burden in ApcMin/+ mice (36), showing that EP1 contributes to cancer development. Our findings suggest that EP1 contributes to cancer development. Our results suggest that the COX-2-PGE2 pathway plays an important role in adapting cancer cells to the tumor microenvironment such as hypoxia that promotes cancer progression.

PGE2 exerts its biologic effects in an autocrine or paracrine manner by binding to its cognate cell surface receptors, EP1, EP2, EP3, and EP4. All 4 EP receptors have been implicated in regulating cancer progression in certain contexts. These receptors are categorized into 3 groups. EP1 couples to Gq to increase intracellular calcium, EP2 and EP4 couple to Ga to induce intracellular cyclic AMP, and EP3 couples to Gz to decrease cyclic AMP. EP receptors have distinct functions because each receptor has a specific tissue distribution and induces a specific signaling pathways (some redundancy may exist; ref. 33). Here, we found that hypoxia specifically enhanced EP1 expression in colorectal carcinoma cells without significant effects on EP2, EP3, or EP4 (Fig. 2A). EP1 has been reported to promote cell survival and proliferation in different types of cancer cells (34, 35). A role for EP1 in colorectal tumorigenesis has been further confirmed in animal models. For example, inhibition of EP1 signaling by genetic deletion or treatment of an EP1-selective antagonist reduced intestinal tumor burden in ApcMin/+ mice (36), showing that EP1 contributes to cancer development. Our findings suggest that the COX-2-PGE2 pathway plays an important role in adapting cancer cells to the tumor microenvironment such as hypoxia that promotes cancer progression.
results are supported by the observation that hypoxia specifically increases EP1 expression in bone cells. Hypoxia-induced EP1 may play a role in bone remodeling and repair in regions of compromised or damaged bone, where O\textsubscript{2} tension is low (23). Our data showed that only hypoxia-induced EP1 mediated PGE\textsubscript{2} induction of ANGPTL4, although EP4 expression is high in both normoxic and hypoxic conditions (Fig. 2). This result explains why PGE\textsubscript{2} only induced ANGPTL4 under hypoxic conditions but not under normoxic conditions. Moreover, our results from immunochemistry studies showed that ANGPTL4

Figure 4. ANGPTL4 enhances cancer cell growth through STAT1. A, LS-174T cells were exposed to C-ANGPTL4 for 12 hours (mRNA) and 24 hours (protein) and qRT-PCR and Western blot were done for STAT1 mRNA and protein levels, respectively (top). Cells treated with vehicle (CTL) or C-ANGPTL4 were stained with antibody against STAT1 (green) and counterstained with DAPI (blue). Immunofluorescence was detected by a fluorescence microscope (Nikon ECLIPSE TE300; ×400; middle). Immunohistochemistry was done to detect STAT1 expression in tumor tissue generated by injection of ANGPTL4/LS-174 cells into the flanks of nude mice compared with the GFP control (bottom). B, cells were transfected with nontargeting and STAT1 siRNA, respectively. After 24 hours, transfecting cells were treated with C-ANGPTL4 for 3 days and subjected to Western blotting for STAT1 (top) and cell proliferation analysis (bottom). C, Western blot analysis was done in LS-174T cells exposed to normoxia/hypoxia or DMSO/1 \textmu mol/L PGE\textsubscript{2} for 24 hours to determine the expression of STAT1 and the phosphorylation of ERK and Src. D, Western blot analysis was carried out to examine the phosphorylation of ERK and Src in LS-174T cells in the presence or absence of C-ANGPTL4 (top). The cells were pretreated with 1 \textmu mol/L SU6656 (SU) or 10 \textmu mol/L PD98059 (PD) for 1 hour and then were treated with C-ANGPTL4. STAT1 levels were determined (middle). Cells were treated with 2 \textmu mol/L diphenylene iodonium (DP1) and C-ANGPTL4 and then the phosphorylation of ERK and Src (0.5 h) as well as the expression of STAT1 (24 h) were determined (bottom). **, P < 0.01; *, P < 0.05.
is expressed in hypoxic regions of the tumors (Supplementary Fig. S1) although HIF-1α, an indicator of hypoxia, and ANGPTL4 did not exactly colocalize in same cells but overlapped in the same area because HIF-1α is a transcription factor and ANGPTL4 is a ligand to be secreted and diffused in the tumors. Collectively, these findings imply that tumor hypoxia enhances PGE2 signaling through upregulating EP1 receptor expression, which may allow for adaptation of cancer cells to the tumor microenvironment.

The synergistic effects of hypoxia and other factors such as insulin, platelet-derived growth factor, and TNF-α targeting genes has been reported in multiple types of cells, including cancer cells (37, 38). In contrast to PGE2, these factors also induce the expression of target genes under normoxic conditions. Our results show that PGE2 induced ANGPTL4 expression only under hypoxic conditions. Several studies have revealed that ANGPTL4 is transcriptionally regulated in response to intracellular or extracellular signals (18, 39). Because HIF mediates hypoxia upregulation of ANGPTL4 in different types of cells and PGE2 enhances HIF-1 transcriptional activity (12, 13, 20–22), it is possible that HIF-1 mediates the effect of PGE2 on induction of ANGPTL4. However, our results show that inhibition of HIF-1 did not affect PGE2 induction of ANGPTL4 (data not shown). On the basis of the observations that ANGPTL4 is one of the peroxisome proliferator-activated receptor (PPAR) target genes (40) with several putative peroxisome proliferator response consensus sequences on its promoter (41) and PGE2 transactivates PPARδ activity (7), we postulate that PPARδ is a potential transcription factor that could regulate PGE2-mediated ANGPTL4 expression under hypoxic conditions. Further investigation is needed to test this hypothesis.
The functions of ANGPTL4 have been well established in regulating lipid metabolism through the inhibition of lipoprotein lipase (LPL). N-ANGPTL4, which assembles into multimeric structures, is essential to inhibit the activity of LPL (39). However, C-ANGPTL4 exists as a monomer and its functional role in monomeric form is still unclear. In addition to the role of ANGPTL4 in lipid metabolism, several recent studies have linked F-ANGPTL4 to angiogenesis and cancer development (15, 25, 26). However, few studies have delineated the biologic function of C- or N-ANGPTL4 in cancer progression. Here, we show that C-ANGPTL4 induces colorectal cancer cell proliferation more effectively than F-ANGPTL4 (Fig. 3A). In addition, our results show for the first time that C-ANGPTL4 induced colorectal cancer proliferation through upregulation of STAT1. STAT1 has been reported to stimulate cell growth and is overexpressed in head and neck and lung cancer (27, 28). In addition, activation of STAT1 promotes cell survival in Wilms tumor (27). Moreover, radiation induces STAT1 expression through activation of epidermal growth factor receptor in breast cancer cells (42). STAT1 has been reported to regulate the oncogenic genes Fos and Egr1 in the absence of STAT3 (43). However, little information regarding the mechanism by which STAT1 regulates cell proliferation and oncogenesis is available. Because our microarray data showed that C-ANGPTL4 increased several oncogenic genes, including Fos and Egr1 (data not shown), the effect of C-ANGPTL4 on these oncogenic genes via STAT1 may contribute to cancer cell proliferation. Moreover, we identified novel C-ANGPTL4 downstream targets, Src and ERK, which are critical for C-ANGPTL4 induction of STAT1 expression (Fig. 4). It has been well established previously that the Src and ERK are responsible for cell proliferation. Therefore, the role of ANGPTL4 in cancer development is likely conferred by C-ANGPTL4 through activating the signaling pathways which regulate cell proliferation. Although C- and F-ANGPTL4 have a fibrinogen-like domain of angiopoietin that binds to angiopoietin receptor Tie1 and Tie2, C- and F-ANGPTL4 do not have any significant affinity for those receptors (44). A recent report shows that C-ANGPTL4 interacts with integrin β1 and β5 and produces O₂⁻ to regulate anoikis resistance of cancer cells (30). Therefore, confirmation of the C-ANGPTL4 receptor such as integrins will add to our understanding of how C-ANGPTL4 regulates cell proliferation as well as angiogenesis and metastasis.

It is generally accepted that hypoxia in the tumor microenvironment promotes chemoresistance (45). It has been reported that COX-2 overexpressing cancer cells are more resistant to doxorubicin and gefitinib (46, 47). Moreover, STAT1 has been shown to play a role in chemoresistance and radioresistance. A recent study has shown that the IFN-related gene signature including STAT1 and its target genes is associated with resistance to DNA damage across multiple cancer cell lines and can affect resistance to chemotherapy (48). In addition, radioresistant tumor cells expressed higher STAT1 levels than control cells (28). In accordance with these published results, we found for the first time that STAT1 was upregulated by ANGPTL4, and our preliminary data showed that ANGPTL4 treatment suppressed oxaliplatin-induced cell death in LS-147T cells (data not shown). Furthermore, the expression of COX-2, ANGPTL4, and STAT1 in human colorectal cancer specimens seems to be correlated (Fig. 5). On the basis of these observations, it is conceivable that ANGPTL4 may contribute to the hypoxia or COX-2–induced drug resistance and may offer a therapeutic option for colorectal cancer patients.

In conclusion, our studies reveal the molecular mechanism by which PGE₂ signaling and hypoxia coordinately promote tumor growth. We found that PGE₂ enhanced ANGPTL4 expression via EP1 receptor signaling that was enhanced by hypoxia. Moreover, we provide in vitro and in vivo evidence showing that elevation of ANGPTL4 promotes tumor growth. These results indicate that PGE₂ plays an important role in promoting a cellular adaptive response to hypoxia for their growth via ANGPTL4. Knowledge about the role of PGE₂ in tumor hypoxia may enhance our understanding of how cancer cells adapt to hypoxia for their growth. It has not escaped our attention that other genes are also more dramatically induced under hypoxic conditions following PGE₂ treatment. This group of genes may reveal other important connections between inflammatory mediators and cancer progression in the tumor microenvironment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Marivonne Rodriguez, Sung-Nam Cho, and Hong Wu for technical assistance.

Grant Support

This work was supported by NIH Grants RO1DK 62112 (R.N. Dubois), P01-CA-77839 (R.N. Dubois), and R37-DK-47297 (R.N. Dubois).

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Received April 14, 2011; revised August 9, 2011; accepted August 26, 2011; published OnlineFirst September 21, 2011.

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Sun-Hee Kim, Yun-Yong Park, Sang-Wook Kim, et al.

Cancer Res  Published OnlineFirst September 21, 2011.

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