HEF1 Is a Crucial Mediator of the Proliferative Effects of Prostaglandin E₂ on Colon Cancer Cells

Dianren Xia¹, Vijaykumar R. Holla¹, Dingzhi Wang¹, David G. Menter¹, and Raymond N. DuBois¹,²

Abstract

Prostaglandin E₂ (PGE₂), one of the downstream products of cyclooxygenase-2 enzymatic activity, promotes colorectal carcinogenesis in part by stimulating cell division. In this study, we define a critical mechanism in this process by showing that the prometastatic adapter protein human enhancer of filamentation 1 (HEF1; NEDD9) links PGE₂ to the cell cycle machinery in colorectal cancer cells. PGE₂ rapidly induced expression of HEF1 mRNA and protein in colorectal cancer cells. HEF1 overexpression elicited the same effects as PGE₂ treatment on cell proliferation, cell cycle progression, and tumor growth. Conversely, HEF1 knockdown suppressed PGE₂-driven cell proliferation and cell cycle progression. Cell cycle alterations involved HEF1 fragmentation as well as co-distribution of HEF1 and cell cycle kinase Aurora A along spindle asters during cell division. Moreover, Aurora A co-immunoprecipitated with HEF1 and was activated by HEF1. Consistent with a role for HEF1 in colorectal carcinogenesis, we found elevated expression of HEF1 in 50% of human colorectal cancers examined, relative to paired normal tissues. These findings establish that PGE₂ induces HEF1 expression, which in turn promotes cell cycle progression through its interaction with and activation of Aurora A. Further, they establish that HEF1 is a crucial downstream mediator of PGE₂ action during colorectal carcinogenesis. Cancer Res 70(2); 824–31. ©2010 AACR.

Introduction

Prostaglandin E₂ (PGE₂) is the most abundant prostaglandin found in colorectal tissue and is a downstream product of the inducible enzyme cyclooxygenase-2 (COX-2). A significant increase in COX-2 gene expression has been linked to colorectal carcinogenesis (1). Inhibition of COX-2 activity by nonsteroidal anti-inflammatory drugs, particularly selective COX-2 inhibitors such as celecoxib, can suppress colorectal adenoma recurrence (2). PGE₂ is the primary mediator of COX-2 in promoting cancer progression. Many studies have shown that PGE₂ promotes cell proliferation and tumor growth in colorectal cancer (3–6). Several pathways or molecules have been implicated as downstream mediators of PGE₂ in promoting cell proliferation and tumor growth, which include the phosphoinositide 3-kinase (PI3K)/Akt pathway, the Ras/mitogen-activated protein kinase (MAPK) pathway, β-catenin/T-cell factor 4, and peroxisome proliferator-activated receptor 6 (3–6). Obviously, more work is needed to reveal the details by which PGE₂ signaling affects cell proliferation.

Human enhancer of filamentation 1 (HEF1) is a scaffold protein that encodes multiple protein interaction domains. It has been implicated in numerous biological activities including mediating integrin-dependent signals at focal adhesions. HEF1 is preferentially expressed in epithelial cells and lymphocytes and undergoes substantial regulation during progression through the cell cycle (7). The regulation of HEF1 expression is not fully understood. Transforming growth factor-β and all-trans retinoic acid have been shown to upregulate HEF1 expression at the transcriptional level (8–10). Serum can also increase HEF1 expression (11). HEF1 localizes not only at focal adhesions but also at the centrosomes and mitotic spindles following stimulation by intrinsic and extrinsic cues (7). Studies have shown that HEF1 becomes fragmented and moves from the cytoplasm and focal adhesions to centrosomes and mitotic spindles during mitosis, where it interacts with and activates Aurora A to promote cell cycle progression (12–14).

Because both PGE₂ and HEF1 have been implicated in promoting cell proliferation or cell cycle progression in certain contexts, we hypothesized that PGE₂ induces HEF1 expression to promote cell cycle progression and growth of colorectal cancers. In the present study, we show that HEF1 functions as an important downstream target of PGE₂, which promotes the proliferation of colorectal cancer cells.

Materials and Methods

Materials and reagents. PGE₂ was obtained from Cayman Chemical. Cell proliferation reagent WST-1 was purchased from Roche Applied Science. Antibody to HEF1 (2B11) was purchased from Santa Cruz Biotechnology. Aurora A and
phospho-Aurora A antibodies were purchased from Cell Signaling Technology. Ki-67 antibody was purchased from DAKO. β-Actin antibody was obtained from Sigma-Aldrich.

Retroviral HEF1 expression vector (LZRS-Ires-HEF1) and control vector (LZRS-Ires-GFP) were kind gifts from Dr. Lynda Chin (Dana-Farber Cancer Institute, Boston, MA). shRNA vector targeting HEF1 (pGIPZ-shHEF1) and control nonsilencing vector (pGIPZ-shCon) were purchased from Open Biosystems. Lentivirus packaging vectors pMD2.G and psPAX2 (Addgene plasmids 12259 and 12260) were purchased from Addgene. Human colorectal tumor specimens were obtained as described previously (15).

**Cell culture.** LS-174T, LoVo, Caco-2, and DLD-1 cells were purchased from the American Type Culture Collection. HCA-7 cells were a generous gift from Susan Kirkland (Department of Histopathology, Imperial College London, London, United Kingdom). These cells were maintained in McCoy's 5A medium containing 10% fetal bovine serum in a 5% CO2 atmosphere.

**Microarray analysis.** LS-174T cells were cultured in serum-free medium for 48 h and treated without or with 1 μmol/L PGE2 for 0, 2, 4, 8, and 24 h with three replicates per group. Total RNAs were extracted with Trizol (Invitrogen) and subjected to microarray analysis using Affymetrix Human Genome U133 Plus 2.0 Array. Resulting data were analyzed by GeneSpring GX and GeneTraffic softwares. Those genes with significant change in expression (at least 2-fold change) as compared with control (0 h) were selected for further study.

**Quantitative PCR.** Quantitative PCR was done as described previously (15). Primers were human Hef1 forward, 5′-GATGGGTGTCTCAGCCTAA-3′, and reverse, 5′-GGATCTCGTGAGTTCTTA-3′; human COX-2 forward, 5′-CCCTTGGGTTGTCAGTGTA-3′, and reverse, 5′-GCCCTCGTATGATGCT-3′; and human β-actin forward, 5′-AGAAAAATCTGGCACCA-3′, and reverse, 5′-AGAGGCTACAGGGATA-3′.

**Northern blot analysis.** Northern blot analysis was done as described previously (15). Blot was hybridized in Hybrisol I (InterGen) with a 32P-labeled human Hef1 CDNA in coding region. The blot was exposed to film. The dot density on developed film was measured using NIH Image software.

**Western blot analysis.** Western blot analysis was done as described previously (16). HEF1 can be cleaved into smaller fragments (1–405 amino acids, 44 kDa; the full length of HEF1 is 835 amino acids, 105 kDa) under certain conditions (12). The HEF1 antibody [2B11 (Santa Cruz Biotechnology), which is raised against amino acids 82–325 of human HEF1] can recognize both the full length and the small fragment of HEF1.

**Proliferation assay.** Ninety-six-well plates were seeded with 3,000 cells per well in 0.1 mL of growth medium. After cells were allowed to attach overnight at 37°C, they were washed twice with PBS and then incubated in serum-free medium for 2 d. In dose-response studies, cells were treated with different concentrations of PGE2 in serum-free medium for 3 d. Time-point experiments involved a single 1 μmol/L concentration of PGE2. Cell growth was determined by adding 10 μL of WST-1 proliferation reagent per well in the last 4 h or 20 μL of bromodeoxyuridine (BrdUrd) label (Calbiochem) per well in the last 24 h, following the assay protocol. Absorbance was measured at 450 nm using a SpectraMax M5 microplate reader ( Molecular Devices).

**Retrovirus and lentivirus production and stable transfection.** LZRS-Ires-HEF1 and control LZRS-Ires-GFP retroviral vectors were transfected into Phoenix cells, or pGIPZ-shHEF1
and pGIPZ-shCon along with package vectors psPAX2 and pMD2.G were transfected into 293T cells in 60-mm dishes using Lipofectamine reagent (Invitrogen) according to the manufacturer’s protocol. After overnight incubation, the transfection medium was removed and replaced by 3 mL of growth medium. Culture medium containing virus particles was collected 24 h later and passed through a 0.45-μm filter to remove cell debris. Cells were plated in a 60-mm dish 24 h before they were infected. Medium containing virus was added to the cells and repeated one more time with freshly collected virus particles 24 h later. During the viral infection process, the final concentration of polybrene (Sigma) in these experiments was adjusted to 4 μg/mL for Phoenix cells and 8 μg/mL for 293T cells. Puromycin (2 μg/mL) was added for 5 d, or after 3 d of infection, cells were sorted by green fluorescent protein (GFP) positivity to eliminate uninfected cells.

**Cell cycle analysis.** Cells were cultured in serum-free medium for 2 d. Fresh serum-free medium containing 1 μmol/L PGE2 was added for 24 h. Cells were collected by trypsinization and fixed with 100% ethanol on ice for 20 min. After centrifugation (500 × g for 3 min), cells were stained with propidium iodide (Invitrogen) in 1 mL of staining solution containing 50 μg/mL propidium iodide, 100 μg/mL RNase A (DNase-free), and 70% ethanol prepared in PBS. After a 30-min incubation, the stained cells were subjected to fluorescence-activated cell sorting (FACS) for cell cycle analysis.

**Immunofluorescence.** Immunofluorescence studies involved plating cells on coverslips in 10% serum medium. After overnight growth, cells were washed twice with PBS, fed with serum-free medium, and incubated at 37°C for 2 more days. After treatment with 1 μmol/L PGE2 in fresh serum-free medium for 24 h, cells were fixed with 4% paraformaldehyde.

**Figure 2.** Alteration of HEF1 expression affected PGE2-induced proliferation in LS-174 T cells. A, PGE2 promoted cell proliferation. LS-174T and HCA-7 cells were treated with PGE2, and cell proliferation was monitored using WST-1 proliferation reagent [LS-174T cells (left) and HCA-7 cells (right)] or a BrdUrd proliferation kit (LS-174T cells; middle) over a 3-d period. *, P < 0.05, compared with nontreatment control. B, knocking down of HEF1 decreased the proliferation of LS-174T cells. Cells were stably transfected with vectors containing nonsilencing control shRNA (shCon) or HEF1 shRNA (shHEF1). HEF1 expression in the presence of PGE2 was examined by Western blotting (left). LS-174T/shCon and LS-174T/shHEF1 cells were treated with PGE2 for 3 d. Cell proliferation was measured using WST-1 proliferation reagent (P = 0.04; * indicates P < 0.05). C, overexpression of HEF1 increased the proliferation of LS-174T cells. Cells were stably transfected with vectors containing GFP control (LS-174T/GFP) or HEF1 (LS-174T/HEF1). HEF1 expression was examined by Western blotting (left). LS-174T/GFP and LS-174T/HEF1 cells were treated using increasing concentrations of PGE2 for 3 d (middle; P = 0.005; * indicates P < 0.05) or using a single concentration (1 μmol/L PGE2) over a series of time points (right; P = 0.039; * indicates P < 0.05). Cell proliferation was determined using WST-1 proliferation reagent.
for 15 min at room temperature, permeabilized with methanol at −20°C for 10 min, blocked with 3% bovine serum albumin, and incubated with antibodies using standard protocols. Primary antibodies included mouse anti-HEF1 monoclonal antibody (2B11, Santa Cruz Biotechnology; 1:100) and rabbit anti–Aurora A polyclonal antibody (Cell Signaling Technology; 1:100). Secondary antibodies antimouse Alexa-488 and antirabbit Alexa-594 (Invitrogen; 1:100,000) were applied along with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen; 1:100,000) for 1 h at room temperature.

**Immunohistochemistry.** General procedures for immunohistochemical staining were followed. Briefly, after dewaxing and rehydrating, slides were boiled in citrate buffer using an EZRetriever microwave (Biogenex; 98°C for 5 min). Blocking was done with 5% normal horse serum and 1% normal goat serum in PBS. Primary antibodies were incubated at 4°C overnight. Antibodies used were anti-HEF1 (2B11, Santa Cruz Biotechnology; 1:200) and anti–Ki-67 (DAKO; 1:50). Secondary antibody was incubated at room temperature for 2 h followed by diaminobenzidine chromogen (Vector Laboratories) and hematoxylin counterstaining.

**Co-immunoprecipitation.** Co-immunoprecipitation analysis was done as described previously (16) using anti-HEF1 (2B11; Santa Cruz Biotechnology) or anti–Aurora A antibodies (Cell Signaling Technology).

**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 M.D. Anderson Cancer Center. LS-174T cells (5 × 10⁵) selected for the stable expression of HEF1 (LS-174T/HEF1) or control GFP (LS-174T/GFP) were injected s.c. into the flanks of nude mice. Three weeks after injection, the mice were euthanized using CO₂ asphyxiation; necropsies were done to remove tumors; and measurements were taken of tumor weight and size.

**Statistical analysis.** Each experiment was done at least three times, and data are presented as the mean ± SE. Statistical significance was determined using Student’s t test, one-factor ANOVA, or two-factor ANOVA, where applicable. P < 0.05 was considered statistically significant.

**Results**

**PGE₂ induces HEF1 expression in colorectal cancer cells.** To identify potential target genes regulated by PGE₂ in colorectal cancer, LS-174T cells were treated with or without PGE₂ and subjected to microarray analysis, which showed a 4-fold increase in HEF1 mRNA expression (17, 18). To confirm the microarray results, quantitative PCR and Western blot analysis revealed that PGE₂ rapidly induced HEF1 expression at both the RNA (Fig. 1A) and protein levels (Fig. 1B, top; Supplementary Fig. S1). The induction of HEF1 mRNA expression was significant and persisted for at least 24 hours. At the protein level, HEF1 expression peaked between 6 and 12 hours. HEF1 expression was also increased in HCA-7 cells (Fig. 1B, bottom). Other colorectal cancer cell lines that express low basal levels of HEF1 also responded to PGE₂ treatment by upregulating HEF1 protein expression, but not to the same degree as that observed in LS-174T and HCA-7 cells (Fig. 1C). Because HEF1 induction was most dramatic in LS-174T cells, subsequent experiments were carried out using this cell line.

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protein expression decreased by about 50% with HEF1 shRNA compared with control shRNA (Fig. 2B, left). Although the knockdown did not completely eliminate the expression of HEF1 protein, cell proliferation was significantly reduced (Fig. 2B, right). These data suggest that suppression of HEF1 expression limits the ability of parental LS-174T cells to respond to PGE2-induced cell proliferation. To further show that HEF1 mediates the effect of PGE2 on cell proliferation, HEF1 protein was overexpressed in LS-174T/HEF1 cells compared with LS-174T/GFP cells (Fig. 2C, left). In the absence of PGE2, stable HEF1 overexpression in LS-174T/HEF1 cells led to increased cell proliferation compared with GFP alone in control cells (Fig. 2C, middle; 0 μmol/L). PGE2 treatment increased the proliferation of both LS-174T/GFP and LS-174T/HEF1 cells in a dose- and time-dependent manner (Fig. 2C, middle and right). Overexpression of HEF1 in another colorectal cancer cell line, LoVo, also resulted to increased cell proliferation as in LS-174T cells (Supplementary Fig. S2). These data suggest that increased HEF1 expression increases cell proliferation similar to that observed with PGE2 treatment (Fig. 2A).

**PGE2 treatment and HEF1 expression influence cell proliferation by modulating cell cycle progression.** To study the mechanism by which PGE2 increases cell proliferation, we performed FACS analysis with PGE2-treated LS-174T cells. Treatment with PGE2 shifted the number of parental LS-174T cells from the G1 phase into the S-G2 phase of the cell cycle (Fig. 3A). To show the role of HEF1 in PGE2-induced cell cycle progression, we first examined whether HEF1 also promotes cell cycle progression by using the HEF1-overexpressing LS-174T/HEF1 cells. As expected, overexpression of HEF1 in LS-174T cells shifted the number of parental LS-174T cells from the G1 phase into the S-G2 phase of the cell cycle (Fig. 3A).
of cells from the G1 phase into the S-G2 phase (Fig. 3B). Next, we examined whether decrease of HEF1 attenuates the effect of PGE2 on cell cycle progression. Indeed, knockdown of HEF1 minimally effected the cell cycle progression of parental LS-174T cells in serum-free medium but blocked PGE2-induced cell cycle progression (Fig. 3C). These results suggest that HEF1 mediates the effect of PGE2 on the progression of cells from the G1 to the S-G2 phase of the cell cycle.

**PGE2 treatment and HEF1 expression influence cell cycle progression by interacting with Aurora A.** HEF1 has been shown to regulate cell cycle progression by fragmenting and relocalizing to spindle asters and activating Aurora A (11, 12, 19). PGE2 treatment led to increased levels of fragmented HEF1 found in LS-174T cells (Fig. 4A). Further examination of these cells by immunofluorescence showed that a single 1 μmol/L dose of PGE2 increased the number of cells observed in metaphase (Fig. 4B). Immunofluorescence analyses also revealed that HEF1 localized in the cytoplasm of quiescent LS-174T cells (Fig. 4C, top). In contrast, close examination of mitotic cells indicated increased instances of metaphase or anaphase along with elevated levels of HEF1 (green) that had redistributed to the area surrounding spindle asters (Fig. 4C). Aurora A (red) staining showed a pattern similar to that of HEF1. When these red and green staining patterns were combined into merged images, the presence of yellow areas indicated that these two proteins were colocalized in LS-174T cells (Fig. 4C, bottom). Co-immunoprecipitation analysis indicated a direct interaction between HEF1 and Aurora A and PGE2 induction of Aurora A phosphorylation in LS-174T cells (Fig. 4D, left). These data suggest that PGE2 stimulates HEF1 fragmentation and relocalization to spindle asters and the activation of Aurora A to enhance cell cycle progression.

**HEF1 increases tumor growth in vivo.** LS-174T/HEF1 or LS-174T/GFP cells were injected into the flanks of nude mice to validate whether our in vitro observations would translate into similar effects in vivo. The mice were euthanized 3 weeks after injection, and tumor weight and size were measured. Cells expressing HEF1 resulted in a significant increase in both tumor weight and size compared with those expressing the GFP control (Fig. 5A). Immunohistochemical staining revealed that HEF1 and Ki-67 stained the same population of cells in those tumors (Fig. 5B). Furthermore, HEF1 interacted with Aurora A in the tumors formed from LS-174T/HEF1 cells (Fig. 5C). These results indicate that HEF1 also interacts with Aurora A to promote cell proliferation and tumor growth in vivo.

**HEF1 expression is increased in colorectal cancers.** Given that COX-2 overexpression is observed in approximately 70% to 80% of colorectal cancers (20), we hypothesized that HEF1 expression is upregulated in colorectal cancer as well. Quantitative PCR analysis revealed that HEF1 expression increased in at least 7 of 15 tumor specimens compared with adjacent normal mucosa (Fig. 6A; Supplementary Fig. S3A). Meanwhile, higher HEF1 expression (tumor/normal ratio >1) was found in 15 of 30 tumor tissues using Northern blot analysis (Fig. 6B). Overall, HEF1 expression increased in about 50% of colorectal cancers compared with normal tissues and correlated with COX-2 expression (Supplementary Fig. S3B; r = 0.6235, P = 0.013).

**Discussion**

Elevated expression of COX-2 and concomitant overproduction of PGE2 have been directly linked to colorectal carcinogenesis. COX-2 inhibitors can suppress colorectal carcinogenesis, but long-term use can cause adverse effects in a subset of patients (1). Our research has focused on identifying and characterizing the effector molecules downstream of PGE2-driven signaling involved in colorectal carcinogenesis, with the ultimate goal of developing approaches that have the same benefit but with fewer resulting side effects. Here we report that PGE2 rapidly stimulated the expression...
of HEF1 in colorectal cancer cells. Both PGE2 treatment and HEF1 expression elicited similar effects on cell proliferation and tumor growth. The alteration of HEF1 levels by PGE2 influenced cell proliferation and cell cycle progression, suggesting that HEF1 can act as a downstream effector of PGE2 in these biological functions.

Reports from our laboratory, as well as those of others, show that PGE2 affects cell proliferation by activating the MAPK or PI3K/Akt pathways (3, 6, 21, 22). How these pathways link to cell division to regulate cell proliferation must be further defined. Here, we present the first data indicating that HEF1 can also mediate the effects of PGE2. PGE2 treatment induced HEF1 expression and increased the proliferation of serum-starved LS-174T and HCA-7 cells (Figs. 1B and 2A). Increased HEF1 expression had a similar effect on the proliferation of LS-174T cells not treated with PGE2 (Fig. 2C, middle, 0 μmol/L). Moreover, knockdown of HEF1 attenuated the effect of PGE2-enhanced cell proliferation (Fig. 2B). This was even more evident after HEF1 knockdown, which blocked the ability of PGE2 to promote cell cycle progression (Fig. 3C). Together, these results indicate that HEF1 mediates the biological effects of PGE2 during cell proliferation and cell cycle progression. Furthermore, HEF1 mechanistically fragmented, relocated, and co-distributed with Aurora A along spindle asters during cell division (Fig. 4A and C). HEF1 interacted with Aurora A and activated it (Fig. 4D), which is required for the G2-to-M transition during cell division. Thus, HEF1 mediates the effect of PGE2 on tumor growth through enhancing the cell cycle progression.

HEF1 protein relocalized from focal adhesions to the mitotic spindle asters in a cell cycle-regulated manner in MCF-7 cells (14). Increased HEF1 in G2-M phases was accompanied with increased level of fragmentation, and the resulting fragments moved from the cytoplasm or focal adhesions to the centrosomes, where these fragments interacted with and activated molecules required for mitotic progression, such as Aurora A (12, 13). These studies have clearly shown the involvement of HEF1 in the regulation of cell cycle progression by using chemically synchronized MCF-7 cells and serum stimulation. In the present study, we tried to synchronize LS-174T cells with serum starvation. However, LS-174T cells were able to grow and divide in the absence of serum (data not shown). Therefore, the cell population we used was a mixed group of cells in different stages of the cell cycle. This could be one of the reasons why only a small group of cells in cell cycle progression responded to PGE2 treatment or HEF1 alteration (Fig. 3). Here we showed that a single bioactive lipid, PGE2,

![Figure 6](https://cancerres.aacrjournals.org／) HEF1 expression in colorectal cancers. A, HEF1 mRNA expression in human tumor and matched normal tissues using quantitative PCR (Q-PCR) analysis. B, HEF1 mRNA expression in human tumor and matched normal tissues using Northern blot analysis. The dot density was measured using NIH ImageJ software and the density ratio of tumor/normal HEF1 was plotted on a bar graph. The cross line indicates a ratio equal to 1.
has the same effect as serum on HEF1 induction, fragmentation, and relocation and Aurora A activation.

In conclusion, we provide evidence that HEF1 is induced by PGE₂ and mediates its effects in promoting cell proliferation by enhancing cell cycle progression. Given that HEF1 can redistribute between the focal adhesions of migrating cells and the mitotic machinery as cells divide, it may act as a "molecular switch" that causes cells to either migrate or enter cell division. We are currently studying the possible involvement of HEF1 in PGE₂-stimulated migration, invasion, and metastasis of colorectal cancer cells. Together, these studies will add to our understanding of how PGE₂ regulates cell proliferation, cell cycle progression, cell migration, invasion, and metastasis. The eventual goal is to help identify the possible targets downstream of COX-2 for the prevention and treatment of colorectal cancer.

Disclosure of Potential Conflicts of Interest

R.N. DuBois: consultant/advisory board, Tragarra. The other authors disclosed no potential conflicts of interest.

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