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Prostaglandin E₂ Induces Human Enhancer of Filamentation 1 to Promote Proliferation of Colorectal Carcinoma Cells

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Abstract

Elevated expression of cyclooxygenase-2 (COX-2) and one of its downstream enzymatic products, prostaglandin E₂ (PGE₂) have been directly linked to colorectal carcinogenesis in a number of ways. Among which, PGE₂ promotes cell proliferation, cell cycle progression, and thus tumor growth. All of the mechanism(s) by which PGE₂ signaling regulates cell growth are not completely understood. Here, we demonstrate that PGE₂ treatment induces human enhancer of filamentation 1 (HEF1) expression and its link with cell cycle machinery in colorectal cancer cells. PGE₂ rapidly stimulated the expression of HEF1 mRNA and protein in colorectal cancer cells. Both PGE₂ treatment and HEF1 overexpression resulted in similar effects on cell proliferation, cell cycle progression, and tumor growth. Moreover, knockdown of HEF1 using shRNA suppressed PGE₂-driven cell proliferation and cell cycle progression. Cell cycle alterations involved HEF1 fragmentation as well as co-distribution of HEF1 and Aurora A along spindle asters during cell division. Furthermore, HEF1 co-immunoprecipitated with and activated Aurora A. Intriguingly, HEF1 expression was increased in 50% of human colorectal cancers compared with expression in paired normal tissue. These data suggest that PGE₂ induces HEF1 expression, which in turn promotes cell cycle progression through its interaction and activation of Aurora A. Clearly, HEF1 is a downstream mediator of PGE₂ action during colorectal carcinogenesis.

Keywords

PGE₂; HEF1/NEDD9/Cas-L; colorectal cancer; cell proliferation; cell cycle

Introduction

Prostaglandin E₂ (PGE₂) is the most abundant prostaglandin found in colorectal cancer tissue and is a downstream product of an 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 the downstream mediators of PGE₂ on promoting cell proliferation and tumor growth, which include PI3K/Akt pathway, Ras/MAPK pathway, β -catenin/TCF-4, and PPAR δ (3–6). Obviously, more work is needed to reveal the details by which PGE₂ signaling affects cell proliferation.

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. TGF- 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 demonstrated that HEF1 becomes fragmented and moves from the cytoplasm and focal adhesions during mitosis to centrosomes and mitotic spindles, where it interacts with and activates Aurora A to promote cell cycle progression (12–14).

Since 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 demonstrate 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 (Ann Arbor, MI). Cell proliferation reagent WST-1 was purchased from Roche Applied Science (Indianapolis, IN). Antibody to HEF1 (2B11) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). AuroraA and phospho-Aurora A antibodies were purchased from Cell Signaling Technology (Danvers, MA). Ki-67 antibody was purchased from DAKO (Carpinteria, CA). β -Actin antibody was obtained from Sigma-Aldrich (St. Louis, MO). 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 (Huntsville, AL). Lentivirus packaging vectors pMD2.G and psPAX2 (Addgene plasmids 12259 and 12260) were purchased from Addgene (Cambridge, MA). 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 (Manassas, VA). HCA-7 cells were a generous gift from Susan Kirkland. 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 media for 48h and treated with or without 1 μ M PGE₂ for 0, 2, 4, 8, and 24 h with three replicates per group. Total RNAs were extracted with Trizol (Invitrogen, Carlsbad, Ca) 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 two-fold change) as compared with control (0 h) were selected for further study.

Quantitative PCR

Quantitative PCR (Q-PCR) was performed as described previously (15). Primers used were: human *Hef1* forward 5'-GATGGGTGTCTCCAGCCTAA-3' and reverse 5'-

GGATCTGGTGGGAGTCTTCA-3', human *COX-2* forward 5'-CCCTTGGGTGTCAAAGGTAA-3' and reverse 5'-GCCCTCGTTATGATCTGTC-3', and human β -actin forward, 5'-AGAAAATCTGGCACCACACC-3' and reverse, 5'-AGAGGCGTACAGGGATAGCA-3'.

Northern blot analysis

Northern blot analysis was performed as previously described (15). Blot was hybridized in Hybrisol I (InterGen, Burlington, MA) with a ^{32}P -labeled human *Hef1* cDNA in coding region. The blot was exposed to film. The dot density on developed film was measured using NIH ImageJ software.

Western blot analysis

Western blot analysis was performed as described previously (16). HEF1 can be cleaved into smaller fragments (1–405 amino acids, 44kD; The full length of HEF1 is 835 amino acids, 105 kD) under certain conditions (12). The HEF1 antibody used (Santa Cruz Biotechnology, 2B11, which is raised against amino acids 82–398 of human HEF1) can recognize both the full length and the small fragment of HEF1.

Proliferation assay

Ninety-six well plates were seeded with 3000 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 (SFM) for 2 days. In dose-response studies, cells were treated with different concentrations of PGE₂ in SFM for 3 days. Time-point experiments involved a single 1- μM concentration of PGE₂. Cell growth was determined by adding 10 μL of WST-1 proliferation reagent in the last 4 h or 20 μL of Brdu Label (Calbiochem, San Diego, Ca) in the last 24 h per well following the assay's protocol. Absorbance was measured at 450 nm using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA).

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, Carlsbad CA) according to the manufacturer's protocol. After an overnight incubation, the transfection medium was removed and replaced by 3 mL of growth medium. Culture medium containing virus particles were 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 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, St. Louis, MO) in these experiments was adjusted to 4 $\mu\text{g/mL}$ for Phoenix cells and 8 $\mu\text{g/mL}$ for 293T cells. Puromycin (2 $\mu\text{g/mL}$) was added for 5 days, or after 3 days of infection, cells were sorted by green fluorescent protein (GFP) positivity to eliminate uninfected cells.

Cell cycle analysis

Cells were cultured in SFM for 2 days. Fresh SFM containing 1 μM PGE₂ was added for 24 h. Cells were collected by trypsinization and fixed with 100% ethanol on ice for 20 min. After centrifugation (500 $\times$ g for 3 min), cells were stained with propidium iodide (PI; Invitrogen) in 1 mL of staining solution containing 50 $\mu\text{g/mL}$ PI, 100 $\mu\text{g/mL}$ 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 SFM, and incubated at 37°C for 2 more days. After treatment with 1 μ M PGE₂ in fresh SFM for 24 h, cells were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with methanol at -20°C for 10 min, blocked with 3% BSA, and incubated with antibodies using standard protocols. Primary antibodies included mouse monoclonal antibody anti-HEF1 (2B11) (Santa Cruz Biotechnology, 1:100) and rabbit polyclonal antibody anti-Aurora A (Cell Signaling; 1:100). Secondary antibodies anti-mouse Alexa-488 and anti-rabbit Alexa-594 (Invitrogen, 1:100,000) were applied along with 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 performed 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, 1:200, Santa Cruz Biotechnology) and anti-Ki-67 (1:50, DAKO, Carpinteria, CA). Secondary antibody was incubated at room temperature for 1 h followed by diaminobenzidine chromogen (Vector Laboratories) and haematoxylin counter staining.

Co-immunoprecipitation

Co-immunoprecipitation analysis was performed as described previously (16) using anti-HEF1 (2B11; Santa Cruz Biotechnology) or anti-Aurora A antibodies (Danvers, MA).

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 (5×10^5) selected for the stable expression of HEF1 (LS-174T/HEF1) or control GFP (LS-174T/GFP) were injected subcutaneously into the flanks of nude mice. Three weeks after injection, mice were euthanized using CO₂ asphyxiation, necropsies were performed to remove tumors, and measurements were taken of tumor weight and size.

Statistical analysis

Each experiment was performed at least 3 times, and data are presented as the mean $\pm$ S.E. Statistical significance was determined using a Student's *t* test, one-factor ANOVA, or two-factor ANOVA wherever applied. *P* values < 0.05 were 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 demonstrated a 4-fold increase in *HEF1* mRNA expression. To confirm the microarray results, Q-PCR and Western blot analysis revealed that PGE₂ rapidly induced *HEF1* expression at both RNA (Fig. 1A) and protein (Fig. 1B, upper panels and supplemental Fig. 1) levels. The induction of *HEF1* mRNA expression was significant and persisted for at least 24 h. At the protein level, HEF1 expression peaked between 6 and 12 h. HEF1 expression was also increased in HCA-7 cells (Fig. 1B, lower panel). Other colorectal cancer cell lines that express low basal levels of HEF1 also responded to PGE₂ treatment by up-regulating HEF1 protein expression, but not to the same degree as 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.

Alteration of HEF1 expression affects PGE₂-induced proliferation in LS-174T cells

PGE₂ treatment led to increased cell proliferation in LS-174T and HCA-7 cells in a dose-dependent manner (Fig. 2A). To examine whether HEF1 plays a role in PGE₂-induced cell proliferation, HEF1 expression was knocked down using shRNA in LS-174T cells. HEF1 expression decreased about 50% with *HEF1* shRNA compared with control shRNA (Fig. 2B, left panel). Although the knockdown did not completely eliminate the expression of HEF1 protein, cell proliferation was significantly reduced (Fig. 2B, right panel). These data suggest that the suppression of HEF1 expression limits the ability of parental LS-174T cells to respond to PGE₂-induced cell proliferation.

To further demonstrate that HEF1 mediates the effect of PGE₂ on cell proliferation, HEF1 protein was overexpressed in LS-174T/HEF1 cells and compared to LS-174T/GFP cells (Fig. 2C, left panel). 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 panel, 0 μ M). PGE₂ treatment increased the proliferation of both LS-174T/GFP and LS-174T/HEF1 cells in a dose- and time-dependent manner (Fig. 2C, right two panels). Overexpression of HEF1 in another colorectal cancer cell line LoVo also resulted to increased cell proliferation as in LS-174T cells (Supplemental Fig. 2). These data suggest that increased HEF1 expression increases cell proliferation similar to 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 demonstrate the role of HEF1 in PGE₂ induced cell cycle progression, we first examined whether HEF1 also promotes cell cycle progression by using the HEF1 overexpressed LS-174T/HEF1 cells. As expected, overexpression of HEF1 in LS-174T cells shifted the number of cell 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 SFM but blocked PGE₂-induced cell cycle progression (Fig. 3C). These results suggest that HEF1 accelerates and 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- μ M 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, upper panel). 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, lower panel). Co-immunoprecipitation analysis results indicated a direct interaction between HEF1 and Aurora A and that PGE₂ treatment increased Aurora A phosphorylation in LS-174T cells (Fig. 4D,

left panel). These data suggested that PGE₂ stimulated 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). IHC 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%–80% of colorectal cancers (20), we hypothesized that HEF1 expression is up-regulated in colorectal cancer as well. Q-PCR analysis revealed that *HEF1* expression increased in at least 7 of 15 tumor specimens compared with adjacent normal mucosa (Fig. 6A and supplemental Fig. 3A). 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 (Supplemental Fig. 2B, $r = 0.6235$, $P = 0.013$).

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 result in fewer 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 on these biological functions.

Reports from our laboratory, as well as others, show that PGE₂ affects cell proliferation via activating MAPK or PI3K/Akt pathways (3,6,21,22). How these pathways link to cell cycle and 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 (Fig. 1B and 2A). Increased HEF1 expression had a similar effect on the proliferation of LS-174T cells not treated with PGE₂ (Fig. 2C, middle panel, 0 μ M). 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 helps mediate the biological effects of PGE₂ during cell proliferation and cell cycle progression. Furthermore, mechanistically HEF1 fragmented, relocated, and co-distributed with Aurora A along spindle asters during cell division (Fig. 4A and 4C). HEF1 co-immunoprecipitated with and activated Aurora A (Fig. 4D), which is required for the G₂-to-M transition during cell division. Thus, HEF1 bridges the effect of PGE₂ on cell proliferation and tumor growth with cell division.

HEF1 protein relocated 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 demonstrated 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 stage of the cell cycle. This could be one of the reasons why only a small group of cells responded in cell cycle progression to PGE₂ treatment or HEF1 alteration (Fig. 3). Here we demonstrated that a single bioactive lipid PGE₂ has the same effect as serum on HEF1 induction, fragmentation, and relocalization and Aurora A activation.

In conclusion, we provide evidence that HEF1 is induced by PGE₂ and mediates its effects on promoting cell proliferation by enhancing cell cycle progression. Given that HEF1 can redistribute between the focal adhesions of migrating cells or 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

PGE₂-induced HEF1 expression in colorectal cancer cells. *A*, *HEF1* was induced by PGE₂ in LS-174T cells. LS-174T cells were treated with different doses of PGE₂ for 24 h or examined using a single 1-μM dose at different time points. After total RNA was collected, *HEF1* expression was examined using Q-PCR. *B*, LS-174T and HCA-7 cells were treated with PGE₂ as in *A*. HEF1 expression was examined using Western blot analysis. *C*, Other colorectal cancer cell lines also up-regulated HEF1 in response to PGE₂. A panel of colorectal cancer cell lines was treated with 1 μM PGE₂ for 24 h, and protein lysates were analyzed for HEF1 expression (upper panel). LoVo cells were treated as described in panel *B*, and Western blotting was performed for HEF1 (lower panel).

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 panel and HCA-7 cells, right panel) or a Brdu proliferation kit (LS-174T cells, middle panel) over a 3-day period. * indicates $P < 0.05$ compared with nontreatment control. **B**, Knocking down of HEF1 decreased the cell 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 panel). LS-174T/shCon and LS-174T/shHEF1 cells were treated with PGE₂ for 3 days. 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 panel). LS-174T/GFP and LS-174T/HEF1 cells were treated using increasing concentrations of PGE₂ for 3 days (middle panel, $P = 0.0051$, * indicates $P < 0.05$) or using a single concentration (1 μ M PGE₂) over a series of time points (right panel, $P = 0.039$, * indicates $P < 0.05$). Cell proliferation was determined using WST-1 proliferation reagent.

Figure 3.

HEF1 promoted cell cycle progression in LS-174T cells. **A**, PGE₂ increased cell cycle progression in LS-174T cells. Cells were serum starved and treated with or without 1 μ M PGE₂ for 24 h followed by cell cycle analysis using FACS ($P < 0.01$, * indicates $P < 0.05$). **B**, Overexpression of HEF1 increased cell cycle progression in LS-174T cells. Serum-starved LS-174T/GFP and LS-174T/HEF1 cells were analyzed for cell cycle changes by FACS ($P = 0.046$, * indicates $P < 0.05$). **C**, Knocking down of HEF1 decreased cell cycle progression in LS-174T cells. LS-174T/shCon and LS-174T/shHEF1 cells were treated with 1 μ M PGE₂ for 24 h, and the cell cycle was analyzed by FACS ($P = 0.042$, * indicates $P < 0.05$).

Figure 4.

HEF1 fragmentation and relocalization for Aurora A activation. *A*, PGE₂ increased HEF1 fragmentation in LS-174T cells. LS-174T cells were treated with PGE₂, and Western blotting was performed as described in Fig. 1*B*. Fragmented HEF1 has a molecular size of about 44 kD. *B*, PGE₂ increased the number of cells in metaphase. LS-174T cells were treated with or without 1 μM PGE₂ for 24 h and incubated with Aurora A antibody for immunofluorescent staining as described in Materials and Methods. Cells in metaphase were counted under a microscope. Five fields were counted and averaged ($P = 0.014$, * indicates $P < 0.05$). *C*, HEF1 relocated to the spindle aster area in LS-174T cells. LS-174 T cells growing on coverslips were treated with or without 1 μM PGE₂ for 24 h and examined for the immunofluorescent

localization of HEF1 (green), Aurora A (red), or the intercalation of DAPI into DNA (blue). Insets provide magnified images of spindle asters and condensed chromosomes. *D*, HEF1 co-immunoprecipitated with Aurora A and increased Aurora A phosphorylation. LS-174T cells were treated with or without 1 μ M PGE₂ for 24 h. HEF1 was pulled down with anti-HEF1 antibody from whole-cell lysates. Aurora A or p-Aurora A were detected by Western blotting (left panel). Right panel, Western blotting of whole-cell lysates.

Figure 5.

Increases in HEF1 expression promoted tumor growth in nude mice. LS-174T/GFP and LS-174T/HEF1 cells (5×10^5) were subcutaneously injected into the flanks of nude mice ($n = 5$). Three weeks later, mice were euthanized and necropsied, and measurements were taken for tumor weight (A, $P = 0.0188$, * indicates $P < 0.05$) and size (B, $P = 0.0458$, * indicates $P < 0.05$). B, Distribution of HEF1 and Ki-67 in tumors formed from LS-174T/HEF1 cell. Immunohistochemical staining for HEF1 and Ki-67 was performed with sectioned tissues from the tumors described in A. C, HEF1 co-immunoprecipitated with Aurora A in tumors grown in vivo. Cell lysates from the tumors described in A were used for precipitation with anti-Aurora A and blotted with anti-HEF1 after separating with SDS-PAGE.

Figure 6.

HEF1 expression in colorectal cancer. *A*, *HEF1* mRNA expression in human tumor and matched normal tissues using 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.