Hyaluronan is a major component of the pericellular matrix surrounding tumor cells, including colon carcinomas. Elevated cyclooxygenase-2 levels have been implicated in several malignant properties of colon cancer. We now show for the first time a strong link between hyaluronan-CD44 interaction and cyclooxygenase-2 in colon cancer cells. First, we have shown that increased expression of hyaluronan synthase-2 induces malignant cell properties, including increased proliferation, anchorage-independent growth, and epithelial-mesenchymal transition in HIEC6 cells. Second, constitutive hyaluronan-CD44 interaction stimulates a signaling pathway involving ErbB2, phosphoinositide 3-kinase/AKT, β-catenin, and cyclooxygenase-2/prostaglandin E2 in HCA7 colon cancer cells. Third, the HA/CD44-activated ErbB2 → phosphoinositide 3-kinase/AKT → β-catenin pathway stimulates cell survival/cell proliferation through COX-2 induction in hyaluronan-overexpressing HIEC6 cells and in HCA7 cells. Fourth, perturbation of hyaluronan-CD44 interaction by hyaluronan oligomers or CD44-silencing RNA decreases cyclooxygenase-2 expression and enzyme activity, and inhibition of cyclooxygenase-2 decreases hyaluronan production suggesting the possibility of an amplifying positive feedback loop between hyaluronan and cyclooxygenase-2. We conclude that hyaluronan is an important endogenous regulator of colon cancer cell survival properties and that cyclooxygenase-2 is a major mediator of these hyaluronan-induced effects. Defining hyaluronan-dependent cyclooxygenase-2/prostaglandin E2-associated signaling pathways will provide a platform for developing novel therapeutic approaches for colon cancer.

Cyclooxygenase (COX),3 or prostaglandin G/H synthetase, which catalyzes the rate-limiting step in the synthesis of prostaglandins and other eicosanoids from arachidonic acid, is considered a promising target for cancer prevention and therapy. Of the two COX isoforms, COX-1 is constitutively present in tissues, whereas COX-2 is inducible. COX-1 is expressed in normal intestine and remains unchanged in intestinal tumors. In contrast, COX-2 is undetectable in normal intestine but is elevated in up to 85% of colorectal adenocarcinoma cells by a variety of pro-inflammatory stimuli and growth factors (1, 2). COX-2 overexpression can occur as a consequence of EGFR-induced phosphoinositide 3-kinase (PI3K) signaling in papillomas (2). In contrast, in cells transformed by mutant-activated Ras overexpression, increased EGFR signaling and transforming growth factor-β overproduction do not account for increased COX-2 expression (3). Recent evidence indicates that elevated expression of COX-2 is required for increased invasiveness, cellular adhesion, and inhibition of apoptosis in colon cancer. Increasing evidence suggests that COX-2 and COX-2-derived prostaglandin E2 (PGE2) are also involved in the control of angiogenesis (4).

Hyaluronan (HA), a multifunctional anionic polysaccharide, is composed of 2,000–25,000 disaccharide units of glucuronic acid and N-acetylgalactosamine (10^5 to 10^7 Da). HA has a structural role in many connective tissues and is associated with the pericellular matrix surrounding proliferating and motile cells in normal and pathological systems, where it has both structural and signaling functions (5–11). Moreover, a large body of literature indicates that high levels of HA exacerbate chronic inflammation in Crohn disease (12, 13), in bleomycin-induced lung inflammation (14), in diabetic neuropathy (15), in atherosclerosis (16), and in graft-versus-host disease (17). Recent evidence associates HA with malignant cell activities in vivo and in vitro (11, 18). HA levels are elevated and predictive of malignant

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1 The abbreviations used are: COX, cyclooxygenase; HA, hyaluronan; O-HA, HA oligomers; RTK, receptor tyrosine kinase; PGE2, prostaglandin E2; EMT, epithelial-mesenchymal transition; CA-, constitutively active; DN-, dominantly negative; siRNA, small interfering RNA; PI3K, phosphoinositide 3-kinase; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; EGFR, epidermal growth factor receptor; RTK, receptor tyrosine kinase; DMEM, Dulbecco’s modified Eagle’s medium; MMP, matrix metalloproteinase.

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progression in several human cancers (19, 20), although squamous adenocarcinomas have a low percentage of HA-positive cells (21). Also, despite the fact that high levels of stromal HA predict tumor progression (10), recent data suggest that stromal HA, but not tumor cell-produced HA, inhibits rather than promotes tumor progression (22). This is most likely because HA turnover may be important in tumor malignancy, *i.e.* high levels of HA coupled with degradation to smaller sized HA fragments may be required for induction of pro-malignant signaling (23–25). Manipulations of HA levels in cells and perturbation of endogenous HA-CD44 protein interactions in animal models have directly implicated HA in tumor progression (10). Rat colon adenocarcinomas have high levels of HA, and HA enhances colorectal tumor cell proliferation and motility in vitro and in vivo (26, 27). HA-CD44 interaction influences growth, adhesion, and invasion of colon carcinoma cells (26–28).

We and others have shown that interaction of HA with CD44 induces formation of complexes containing CD44 and ErbB2 or EGFR in a variety of tumor cells (28, 29). We have shown recently that inhibition of constitutive HA–tumor cell interactions in malignant colon and other carcinoma cells by HA oligomers (O-HA), soluble HA-binding proteins (soluble CD44), or CD44 siRNA suppresses constitutive ErbB2 or ligand-dependent activation of several receptor tyrosine kinases (RTKs), *e.g.* IGF1R, receptor-β, EGFR, and c-MET (30). On the other hand, we showed that these RTKs are activated in phenotypically normal intestinal epithelial HIEC6 cells by experimentally increasing HA production (29, 30). We also showed that O-HA not only compete for interaction of endogenous HA with CD44 but also affect HA production. We conclude that HA serves a general function in RTK activation in these and other cancer cells, thus leading to increased activity of cell survival and proliferation signaling pathways and increased oncogenic properties (29, 30).

Recent studies have demonstrated that exogenous addition of HA induces COX-2 through interaction with CD44 in endothelial cells (31) and that COX-2 overexpression is a proximal mediator of CD44-dependent invasion in human non-small cell lung cancer and human renal cell carcinoma cells (32, 33). Most studies of the role of HA in the malignant properties of various cancer cells have addressed the effect of exogenously added HA rather than the function of endogenous tumor cell-produced HA (34, 35). However, exogenously added HA does not necessarily mimic the effects of endogenously produced HA rather than the function of endogenous tumor cell-produced HA (34, 35). However, exogenously added HA does not necessarily mimic the effects of endogenously produced HA rather than the function of endogenous tumor cell-produced HA (34, 35). However, exogenously added HA does not necessarily mimic the effects of endogenously produced HA rather than the function of endogenous tumor cell-produced HA (34, 35). However, exogenously added HA does not necessarily mimic the effects of endogenously produced HA rather than the function of endogenous tumor cell-produced HA (34, 35). However, exogenously added HA does not necessarily mimic the effects of endogenously produced HA rather than the function of endogenous tumor cell-produced HA (34, 35). However, exogenously added HA does not necessarily mimic the effects of endogenously produced HA rather than the function of endogenous tumor cell-produced HA (34, 35). However, exogenously added HA does not necessarily mimic the effects of endogenously produced HA rather than the function of endogenous tumor cell-produced HA (34, 35). However, exogenously added HA does not necessarily mimic the effects of endogenously produced HA rather than the function of endogenous tumor cell-produced HA (34, 35).

**EXPERIMENTAL PROCEDURES**

**Materials**—HA oligomers (O-HA) were prepared as described previously (18). HA polymer (200 kDa) was from ICN Biomedicals. Antibodies against human CD44 (HCAm), vimentin, cytokeratin, β-catenin, glyceraldehyde-3-phosphate dehydrogenase, and goat polyclonal antibody against COX-2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against ErbB2 and p-ErbB2 were from Upstate Biotechnology; rabbit polyclonal antibodies against AKT and p-AKT were from Cell Signaling Technology, Inc. (Danvers, MA); rabbit polyclonal antibody to β-galactosidase (ab616) was from Abcam Inc. (Cambridge, MA); antibody against β-actin, β-tubulin, COX-2 inhibitor NS398, and 2-(p-iiodophenyl)-3-(p-nitrophenoxy)-5-phenyltetrazolium salt, and chondroitinase ABC were from Sigma. The secondary antibodies used were horseradish peroxidase-conjugated with anti-rabbit IgG, anti-goat IgG, and anti-mouse IgG from Amersham Biosciences, and horseradish peroxidase-anti-biotin antibody was purchased from Zymed Laboratories Inc. Bovine HA-binding protein, chitin oligosaccharide mixture was from North Star Bioproducts (Cape Cod, MA). Human recombinant epidermal growth factor was purchased from R & D Systems. Enhanced chemiluminescence reagents (Western Lightning Chemiluminescence Reagent Plus) were from PerkinElmer Life Sciences. AG825 (an ErbB2 inhibitor) was from EMD Biosciences (La Jolla, CA). DMEM (high glucose), RPMI 1640 medium, Ham’s F-12, penicillin/streptomycin, glutamine, and Versene were from Invitrogen. Fetal bovine serum was purchased from Atlanta Biologicals. Phosphate-buffered saline (PBS) was from Cambrex. Plasmids for dominant-negative (DN) AKT, pUSEamp-myr-p110 PI3K (constitutively active (CA) PI3K), and pUSEamp plasmid were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Dominant-negative p110 PI3K was a gift from Dr. L. Cantley (Brigham and Women’s Hospital, Boston) and subcloned into pUSEamp. pSV2-ErbB2 (V659E) CA-ErbB2 was a gift from Dr. J. Schlegel, Freiburg, Germany. p-IRE2-antisense (As)-COX-2 plasmid was a gift from Dr. Michael C. Archer, Toronto, Ontario, Canada. pRES2 was from Clontech. The mouse HAS2 cDNA construct pCI-neo-HAS2 was obtained from Dr. A. Spicer (University of California, Davis). pCI-neo and pSV-β-galactosidase plasmids were from Promega. A DN mutant of AKT (T308A, S473A)-adenovirus (DNAKT-Ad) (42) and β-galactosidase-adenovirus (β-gal-Ad) were gifts from Dr. K. Walsh (Boston University). Unless specified, all other reagents were the highest grade from Sigma.

**Cell Culture**—HCA7 (colony 29) human colon carcinoma cells (European Collection of Cell Cultures, UK) were maintained in DMEM, 10% fetal bovine serum, 2 mM glutamine, and 11 mg/ml pyruvate. The HIEC-6 human intestinal cell line (I.-F. Beaulieu, University of Sherbrook, Quebec, Canada) was maintained in DMEM (high glucose), 4% fetal bovine serum, 20 mM HEPES buffer (pH 7.4), 50 units/ml penicillin, 50 μg/ml streptomycin, 10 μg/ml insulin, and 5 ng/ml human recombinant...
epidermal growth factor (43), and used between the 15th and 17th passage in this study. Cell lines were grown at 37 °C in 5% CO₂.

**Cell Proliferation Assay**—Cell proliferation was determined by using cell titer 96R Aqueous One Solution cell proliferation assay kit (Promega) according to the manufacturer’s instructions. Cells (5,000/well) were plated in a 96-well tissue culture plate, grown for 72 h, and assayed for cell proliferation by adding 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)2-(4-sulfophenyl)-2H-tetrazolium, inner salt, a tetrazolium compound. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)2-(4-sulfophenyl)-2H-tetrazolium, inner salt (was bioreduced by the cells into a formazan product that is soluble in the medium, and the absorbance at 490 nm in 96-wells was measured by an ELISA plate reader.

**Anchorage-independent Growth Assay**—This assay was performed in 6-well plates following the procedure as described (44). Briefly, the wells were coated with 0.6% agarose in PBS. The single-cell suspension was prepared in PBS and suspended in RPMI containing 5% fetal bovine serum. A 3 ml cell suspension containing 12,000 cells was mixed with 300 μl of 3% agarose, and 1 ml was layered onto 0.6% solidified agarose and allowed to solidify. Culture medium (3 ml) was added on the top layer of each well and changed every 72 h until colonies were visible (within 7–10 days) under an inverted microscope. The colonies were stained with 2-(p-iodophenyl)-3-(p-nitropheryl)-5-phenyltetrazolium salt at 1.0 mg/ml (45). The cell clusters appeared as deep brick-red against a colorless background and were counted under the microscope and photographed in a digital camera.

**RNA Silencing and HAS2 Adenovirus, β-Galactosidase Adenovirus, and DN-AKT Adenovirus Infection**—Human CD44 siRNA (46) and β-catenin siRNA (47) were prepared by Dharmaco. The siRNA transfection was carried out at 100 pmol using Oligofectamine (Invitrogen) according to the manufacturer’s instructions. Cells were transfected with the siRNA in 6-well plates with cells at 70–90% confluence. The cells were then incubated at 37 °C in 5% CO₂ for 24 h, replated in 150-mm dishes, and allowed to grow for 48 h in complete medium. The recombinant HAS2 adenovirus (HAS2-Ad) was produced, and β-galactosidase-adenovirus (β-gal-Ad), DN-AKT was amplified at Tufts University and used as described previously (29, 46). To validate transfection efficiency of various cDNAs used in this study, we co-transfected HIEC6 cells, HIEC6-HAS2-Ad cells, and HCA7 cells with pSV-β-galactosidase plasmid.

**Preparation of Cell Lysates**—Cells were washed twice in cold PBS after treatment, harvested with Versene, and then washed twice in cold PBS. The cell pellet was lysed in buffer containing 1% Nonidet P-40, 0.5 mM EDTA, 5 mM sodium orthovanadate, 10% (v/v) glycerol, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, and 50 mM HEPES (pH 7.5), and then stored as described previously (30). Protein concentration in the cell lysate was determined by Folin’s phenol reagent (48).

**Treatment with ErbB2 Inhibitor AG825 and COX-2 Inhibitor NS398**—For specific inhibition of ErbB2, cells were preincubated in a serum-free complete medium for 24 h and then in 10 mM NaN₃ and 10 mM deoxyglucose in glucose-free RPMI medium for 30 min to slow metabolism, after which they were treated with 0.0–2.0 μM AG825 (49) for 2 h. For selective inhibition of COX-2, cells were preincubated in a serum-free complete medium for 24 h and then treated with NS398 (5 μM) (50). After treatment the cells were washed in PBS, lysed, and then immunoblotted.

**SDS-PAGE**—Western blotting of cell lysates was carried out as described earlier (30). Intensity of the bands was quantified by densitometry using ImageJ (NIH) program.

**HA Assay by ELISA**—HA released in serum-free culture media collected after 24 h of incubation was assayed using an ELISA-like assay (51). All incubations were performed at room temperature. Briefly, 4-fold 1:2 dilutions of medium were mixed with an equal volume of biotinylated HA-binding protein (1 μg/ml stock) in a total volume of 100 μl and incubated for 1 h. Control (buffer only) and various concentrations of standard HA were incubated similarly. Each dilution was added in triplicate. The mixture (100 μl) was added to HA-coated wells in a Maxisorp plate (Nunc) and incubated 1 h. After washing, horseradish peroxidase-anti-biotin antibody at 1:5000 dilution (100 μl) was added. After 1 h, the plate was washed, and 100 μl of substrate solution (containing 7 mg of o-phenylenediamine and 5 μl of 30% hydrogen peroxide in 12 ml of citrate (0.05 M) phosphate (0.1 M) buffer (pH 5.0)) was added and incubated with shaking in the dark for 5 min. The reaction was stopped by adding 50 μl of sulfuric acid (2 M). The plate was read at 490 nm in an ELISA plate reader. The concentration of HA in the medium was calculated from a standard curve.

**Caspase-3 Assay**—We used a caspase-3 assay kit from Biovision Research Products (Mountain View, CA). The assay was performed according to the manufacturer’s instructions (18). Cell lysates were prepared in the supplied lysis buffer, and extracts containing 200 μg of protein were incubated with DEVD-p-nitroanilide as substrate. The enzyme activity was determined by measuring the absorbance of the colored product, p-nitroanilide, at 405 nm spectrophotometrically.

**Preparation of Stable Transfectants**—All transfections were carried out using an AMAXA Nucleofector II following instructions from the manufacturer. The HAS2 stable transfectant of HIEC6 (HIEC6-HAS2) and vector transfectant (HIEC6-v) clones were prepared using linearized pcDNA-Has2 construct and pCI-neo plasmid and selected in the presence of geneticin at 500 μg/ml (Invitrogen).

**Immunohistochemistry**—HIEC6-v and HIEC6-HAS2 cells were plated on polylysine-coated coverslips and incubated at 37 °C. The next day, cells were fixed, permeabilized, and allowed to react with antibodies (1:1000 dilution) against vimentin, cytokerin, and β-catenin and then counterstained with secondary antibodies conjugated with Texas Red (1:2000 dilution). After washing, the coverslips were attached to glass slides using a drop of antifade solution. The outer rim of the coverslips were closed with clear fingernail polish and stored at 4 °C until analysis.

**PGE₂ Assay**—PGE₂ released in cultured media of treated and untreated HIEC6-v clones, HIEC6-HAS2 clones, and HCA7 cells was measured by competitive enzyme immunoassay (EIA kit, Cayman Chemical) following the manufacturer’s instructions. Briefly, dilutions of culture media were incubated with a
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fixed amount of PG-acetylcholinesterase conjugate (tracer) in wells coated with PG antiserum. Because of the competition between tracer and PGE$_2$ in the media, the amount of tracer bound to PG-antiserum is inversely proportional to the PGE$_2$ concentration of the mixture in the well. The bound esterase activity was determined by adding Ellman’s reagent containing the substrate and measuring the absorbance at 412 nm. A standard curve was prepared by using different amounts of PGE$_2$ in medium without serum. The amount of PGE$_2$ was calculated by comparing the absorbance value with the standard.

RESULTS AND DISCUSSION

Increased HA Production Induces Activation of Cell Survival Properties and EMT in Human Intestinal Epithelial HIEC6 Cells—Previously, we showed that increased HA production stimulates cell survival activities leading to increased drug resistance and anchorage-independent growth in breast carcinoma cells (46, 52, 53). Thus we evaluated the effects of increased HA production on cell proliferation, anchorage-independent growth, and activation of cell survival-related proteins in HIEC6 cells. Because cytoplasmic stabilization of β-catenin and other events associated with EMT contribute to colon cancer progression (54, 55), we also examined the effects of increased HA production on these parameters.

Overexpression of HAS2 with a recombinant HAS2 adenovirus caused an ~2–3-fold increase in HA production in these cells under the conditions used (30). This increase in HA caused ~2-fold increase in cell proliferation (Fig. 1A).

Because β-catenin signaling correlates with increased invasiveness and proliferation (56, 57), and since β-catenin and COX-2 are important for colon cancer progression (58–60), we next examined the effect of increased HA production on the distribution of β-catenin, a common marker of EMT (61, 62). In addition, the control HIEC6-β-gal-Ad cells formed epithelial islands (Fig. 1B, left panel) and stained strongly for cytokeratin but not for vimentin (data not shown), whereas the HAS2-overexpressing HIEC6-HAS2-Ad cells became scattered (Fig. 1B, right panel) and stained strongly for vimentin and not for cytokeratin (data not shown). These changes in cellular organization and cytoskeletal characteristics because of overproduction of HA are also characteristic of EMT (61, 62) suggesting that increased HA is sufficient to induce this potentially oncogenic phenotype in normal HIEC6 cells.

We and others have shown that interaction of endogenous HA with CD44 has a profound influence on cell survival properties (29, 30). We have also shown that endogenous HA-CD44 interaction regulates the stability of signaling complexes containing CD44 and RTKs in a variety of tumor cells, and this complex is essential for maintaining major cell survival pathways like PI3K and MAPK (29, 30, 52). Based on these studies, we next examined the effects of elevated HA on ErbB2, Erk1/2, and AKT activation in HIEC6-HAS2-Ad cells. The degree of stimulation was found to be dependent on incubation time with HAS2 adenovirus and on cell numbers. In the experiment shown in Fig. 1C, 0.5 × 10$^5$ HIEC6 cells were infected separately with β-gal-Ad (control) and HAS2-Ad and grown for 48 h; the apparent stimulation of p-ErbB2, pErk1/2, and p-AKT by increased HA was ~2–4-fold (Fig. 1C). These results further suggest that increased HA induces activation of cell survival proteins in HIEC6-HAS2-Ad cells compared with HIEC6-β-gal-Ad cells.

A hallmark characteristic of transformed cells is their ability to form colonies in soft agar. However, HIEC6-HAS2-Ad cells did not form colonies in soft agar, possibly because they only produce elevated levels of HA temporarily. Therefore, we stably transfected HIEC6 cells with a PCI-neo-HAS2 plasmid to create HIEC6-HAS2 clones that produced high levels of HA permanently. These clones produced ~14-fold more HA than the vector-transfected cells (Fig. 2A). HIEC6-HAS2 clones produce ~50-fold (~250 colonies/well) more colonies in soft agar than the vector transfected cells (HIEC6-v) (~5 colonies/well) (Fig. 2B). In addition, HIEC6-HAS2 clones show other characteristics associated with EMT, i.e. increased vimentin, MMP2, and MMP9 expressions compared with HIEC6-v clones (Fig. 2C). The results in Figs. 1 and 2 indicate that HAS2-overexpressing HIEC6-Ad cells and HIEC6-HAS2 clones acquired many properties of mesenchymal cells as well as an invasive phenotype by overexpressing invasion-associated genes such as $\alpha V$, MMP2, and MMP9 (63).

Increased HA Production Stimulates COX-2 Expression and Activity in HIEC6 Cells—Previously addressed in colon cancer cells. Thus we examined the effect of increased HA on COX-2 expression and enzyme activity, as measured by PGE$_2$ production, in HIEC6 cells. We found that increased HA production in HIEC6-HAS2-Ad cells up-
regulates COX-2 expression as measured by Western blotting (Fig. 3A, lane 3 versus lane 1). However, unlike results obtained with endothelial cells (31), exogenously added hyaluronan polymer in HIEC6 cells did not increase COX-2 expression (Fig. 3A, lane 2 versus lane 1) indicating the fact that mere interaction of unoccupied hyaluronan receptors on the epithelial cell surfaces does not transmit necessary signaling information into cells. We also examined the effect of two antagonists of HA: CD44 interactions, namely O-HA (100 μg/ml) and CD44 siRNA (100 pmol) to determine whether the effects of increased HA in HIEC6-HAS2-Ad cells could be reversed. Both CD44siRNA and O-HA, as well as the COX-2 inhibitor NS398 (5 μM), were found to reverse the increase in HA-induced COX-2 expression to levels near to or below that expressed by controls (Fig. 3A). The densitometric analysis of these results is shown in Fig. 3B as fold change in COX-2 protein expression normalized with respect to the loading control β-galactosidase. The increase in COX-2 expression was reversed >100% by CD44 siRNA (Fig. 3B, bar 6 versus bar 4), >100% by O-HA (Fig. 3B, bar 5 versus bar 3), and >100% by NS398 (Fig. 3B, bar 7 versus bar 3). In parallel with the effects on COX-2 expression, HAS2 transfection caused an increase in PGE2 production indicating the presence of enzymatically active COX-2; this increase was observed both in HIEC6-HAS2-Ad cells (~2-fold compared with HIEC6-β-gal cells; Fig. 3C) and in the stable HIEC6-HAS2 clones (~15–16-fold compared with HIEC6-v clones; Fig. 3E). This increase in PGE2 production was reversed by O-HA, CD44 siRNA, and COX-2 inhibitor NS398 (Fig. 3C). The difference between HIEC6-HAS2-Ad and stable HIEC6-HAS2 clones is that the latter has more sustained production of HA (Fig. 3E) thus promoting the changes in phenotype as seen in Fig. 2.

To rule out effects of contaminants on COX-2 expression in the O-HA preparation, we performed the following control experiments in three experiments.

FIGURE 2. Stable transfection of HIEC6 cells with HAS2 induces transformed properties. HIEC6 cells were stably transfected with linearized pCI-neo empty vector or pCI-neo-HAS2 (73). A, amounts of HA produced by pCI-neo-vector stable transfectant (HIEC6-v; control) and by pCI-neo-HAS2 stable transfectant clones (HIEC6-HAS2 clones) were measured in culture media after 48 h of incubation. Results are given for three experiments as means ± S.E. The HIEC6-HAS2 clones show a marked increase in HA production (~14-fold) compared with HIEC6-v clones. B, above cell clones were grown for 7–10 days in soft agar, and colony formation was detected by adding a tetrazolium salt (45). Colonies with diameters >0.2 mm were counted (18, 52). HIEC6-HAS2 clones show a marked increase (~50-fold) in number of colonies (~250 colonies/well) compared with HIEC6-v clones (~5 colonies/well). C, cell lysates were prepared from these clones and processed for Western blot analysis for vimentin, MMP2, MMP9, and β-actin (loading control). Overexpression of HA in HIEC6-HAS2 clones induces elevated expression of these proteins, which are markers of classical EMT. Results in A–C suggest that HIEC6-HAS2 clones have hallmark properties for mesenchymal and invasive phenotypes. Results are expressed as mean ± S.D. for three experiments. B and C are representative of three experiments.

FIGURE 3. HA regulates COX-2 expression and PGE2 production in HIEC6 intestinal epithelial cells. A, HIEC6 cells were infected overnight with a recombinant HAS2 adenovirus (HIEC6-HAS2-Ad cells) or a control β-galactosidase adenovirus (HIEC6-β-gal-Ad cells) and co-transfected with pSV-β-galactosidase plasmid to normalize for transfection efficiency. HIEC6-pSV-β-gal-transfected cells were treated with high molecular weight hyaluronan (100 μg/ml, 16 h); 200 kDa from ICN Biomedicals). HIEC6-HAS2-Ad cells were treated with control siRNA (sc siRNA, 100 pmol), CD44 siRNA (100 pmol), O-HA (100 μg/ml, 16 h), or COX-2 inhibitor NS398 (5 μM, 16 h). After treatment, cells were lysed and processed for Western blot analysis for COX-2 and β-galactosidase. HIEC6-HAS2-Ad cells produced increased COX-2 levels compared with controls, but treatment with exogenous HA polymer had little or no effect. CD44 siRNA, O-HA, and NS398 completely reversed this increase; CD44 siRNA and NS398 reduced COX-2 to levels below basal expression by the control cells. B, densitometric quantitation of the results of three experiments similar to that in A. The densitometric value for each COX-2 band (mean ± S.D.) was normalized with respect to the transfection control β-galactosidase. Western blot results in A are representative of these three independent experiments. The variability in expression of COX-2 under each of the conditions in these experiments is 3–10%. The differences between various conditions is statistically significant in each case ( p < 0.001). C, PGE2 secretion into the culture media of the treated and untreated HIEC6 cells from various treatments was measured using an EIA kit (Cayman Chemical). CD44 siRNA inhibits PGE2 production by 75%, whereas O-HA inhibits by 40–50% in these experiments. D, specificity of the effect of O-HA on COX-2 expression. The HIEC6-HAS2-Ad cells (lane b) (control); HIEC6-β-gal-Ad cells, lane a were grown for 24 h with 100 μg/ml of O-HA (lane c), with O-HA after treatment at 100 °C for 10 min (lane d), with O-HA after treatment with heat-inactivated (100 °C for 10 min) chondroitinase ABC (Sigma, 2 unit/ml) for 1 h at 37 °C (lane e), or with O-HA after treatment with active chondroitinase ABC (Sigma, 2 unit/ml) for 1 h at 37 °C, and then heat-inactivated (100 °C for 10 min) (lane f). Cell lysates from these cells were immunoblotted for COX-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (loading control) antibodies. E, HIEC6-v and HIEC6-HAS2 stably transfected clones were grown for 48 h, and PGE2 secretion into the culture media were measured as in C. The results show that HIEC6-HAS2 clones, which possess highly elevated levels of HA (Fig. 2A), produce increased levels of PGE2, that are comparable with HCA7 colon cells (Fig. 4C). Results are presented as means ± S.D. of three independent experiments.
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**FIGURE 4.** Endogenous HA and CD44 regulate COX2 expression in HCA7 colon cancer cells. A, cell lysates were prepared from HCA7 cells that were untreated or treated with control siRNA (scr siRNA, 100 pmol), CD44 siRNA (100 pmol), O-HA (100 μg/ml for 16 h), or NS398 (5 μM for 16 h), and then processed for Western blotting for COX-2 and β-galactosidase. CD44 siRNA and NS398 inhibited COX-2 expression by 60–80%. O-HA showed lesser inhibition (40–50%) than CD44 siRNA. B, densitometric quantitation of the results of the COX-2 protein expression in three independent experiments performed with HCA7 cells. The densitometric value for each COX-2 band (mean ± S.D.) was normalized with respect to the β-galactosidase loading control. The variability in expression of COX-2 under each of the conditions in these experiments is 4–10%. The differences between various conditions is statistically significant in each case (p < 0.001). C, HA secreted into the culture media of HCA7 cells treated with control siRNA, chitin oligosaccharide (100 μg/ml; control for O-HA treatment), chondroitin sulfate (100 μg/ml; control for O-HA treatment), O-HA, CD44siRNA, or NS398, or without treatment, were measured using an ELISA-like method (51). O-HA inhibits HA production by 40%, whereas CD44siRNA has no effect on HA production. NS398 inhibits HA production by 60%. D, PGE2 secretion into the culture media of untreated HCA7 cells or HCA7 cells treated with A was measured using an EIA kit (Cayman Chemical). CD44 siRNA (100 pmol) inhibits PGE2 production by 80–90%, whereas O-HA at 100 μg/ml has ~25% inhibitory effect on PGE2 production in these experiments. Results are presented as mean ± S.D. of three independent experiments.

We showed that heating the O-HA to 100 °C for 10 min had no effect on their ability to inhibit COX-2 induction (Fig. 3D, lane d versus lane e). We treated the O-HA with chondroitinase ABC, which would degrade the O-HA to unsaturated disaccharides that are not recognized by CD44 (64, 65). HA-oligomers treated with heat-inactivated chondroitinase ABC was used as a control (Fig. 3D, lane e). As expected, the chondroitinase ABC-digested O-HA followed by heat inactivation (100 °C for 10 min) did not inhibit COX-2 activation (Fig. 3D, lane f versus lane e). These results show that increased HA interaction with CD44 induces phenotypic changes in COX-2 expression and enzyme activity that may favor tumor cell survival processes.

**Elevated Endogenous HA Constitutively Regulates COX-2 Expression and Activity in HCA7 Colon Carcinoma Cells**—COX-2 is significantly elevated in colon carcinoma and plays a role in colon carcinoma pathogenesis; however, little is known about proximal mechanisms that operate to induce COX-2. Given the above observations on the effects of experimentally increased HA production on COX-2 in HIEC6 cells, we tested whether the constitutively elevated HA (Fig. 4C, bar 1) and high levels of CD44 (30) in HCA7 malignant colon carcinoma cells play an important role in the aberrantly elevated expression and activity of COX-2 in these cells (66). Indeed, the augmented expression of COX-2 was substantially down-regulated, i.e. by 40–80%, by CD44 siRNA and O-HA (Fig. 4B, bars 3 and 4 versus bars 1 and 2). The densitometric analysis of these results is shown in Fig. 4B as fold change in COX-2 protein normalized with respect to the loading control β-galactosidase expression.

The effect of CD44siRNA was comparable with NS398 (Fig. 4, A, B, and D) as was also shown in the case of HIEC6 cells (Fig. 3, A–C).

In addition to COX-2, the constitutive level of PGE2 in HCA7 cells was also high (Fig. 4D, bar 1) and was reduced by ~80–90% using CD44 siRNA or NS398 (Fig. 4D, bars 6 and 7 versus bars 1 and 2). The O-HA inhibited COX-2 expression by 40–50% (Fig. 4, A, lane 3 versus lane 1, and B, bar 3 versus bar 1), a lesser extent than the CD44 siRNA (Fig. 4A, lane 4 versus lane 2, and B, bar 4 versus bar 2). The usual dose of O-HA (100 μg/ml) caused an ~25% reduction (Fig. 4D, bar 5 versus bars 1–3) in PGE2 production. We also used a higher dose of O-HA (200 μg/ml) and found that this dose inhibits PGE2 production by 50–60% (data not shown). We also tested the effect of various reagents related to HA oligomers. We used chitin oligosaccharides as a negative control because they are similar to the HA oligomers in size and in chemical composition. They are a polymer of N-acetylgalcosamine and are sufficiently closely related to HA that HA synthase can produce chitin oligosaccharide (67). We found that they do not have a significant effect on PGE2 production (Fig. 4D). Hyaluronan polymer (data not shown) and chondroitin sulfate also do not have significant effect on HA secretion, and COX-2 enzyme activity, i.e. PGE2 production (Fig. 4, C and D). These results (Fig. 4, C and D, bars 3 and 4 versus bars 1 and 2; and data not shown) extend our previous findings that chitin oligomers, chondroitin sulfate, and polymeric HA do not mimic the effects of O-HA on various signaling parameters (18). To rule out the effects of contaminants in the O-HA preparation, we showed that chondroitinase ABC treatment ablates the effects of O-HA, whereas heat-inactivated O-HA and heat-inactivated chondroitinase ABC-treated O-HA have similar inhibitory effects on COX-2 protein expression and activities (data not shown) as shown previously for other signaling activities in HCA7 cells (30). Therefore, we conclude that perturbation of endogenous HA–CD44 interaction by HA–CD44 antagonists (CD44 siRNA, and O-HA) suppresses COX-2 expression and PGE2 production in HCA7 cells.

We also examined the effects of the reagents used above on production of HA itself. As expected from previous results with HIEC6 cells (30), CD44siRNA had no effect on HA secretion (Fig. 4C, bar 6 versus bar 2), whereas O-HA inhibited HA production by ~40% (Fig. 4C, bar 5 versus bars 1–3). Surprisingly, NS398 also inhibited HA production significantly, i.e. by ~60% (Fig. 4C, bar 7 versus bar 1). The latter result suggests that COX-2 may regulate HA production as well as the reverse, thus suggesting the possibility of a positive feedback loop. To further
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We investigated whether COX-2 is a downstream target of a HA-CD44 interaction requires participation of ErbB2, AKT, and β-catenin.

We tested whether HA regulates COX-2 via its effects on an ErbB2 → PI3K/AKT → β-catenin axis. We found that increased HA stimulates activation of AKT and ErbB2 and expression of β-catenin in parallel with its effect on COX-2 expression in HIE6C cells (Fig. 5A, lane 2 versus lane 1). Each of these increases was reversed by O-HA and CD44 siRNA (Fig. 5A, lanes 4 and 5 versus lanes 2 and 3). The densitometric analysis of the results (Fig. 5C) indicates that both CD44siRNA and O-HA reversed the effects of up-regulation of HA synthesis on COX-2, p-AKT, p-ErbB2, and β-catenin to levels approximately equal to or lower than controls, although CD44siRNA was usually more potent than the O-HA. In HCA7 cells, these activities are constitutively elevated and are inhibited to similar extents by DN-AKT, an ErbB2 inhibitor AG825, and CD44 siRNA (Fig. 5B, lanes 2–4 versus lane 1). Indeed CD44siRNA is even more efficient than the AG825 in reducing COX-2 and p-ErbB2, whereas DN-AKT, AG825, and CD44siRNA have about the same effects on p-AKT and β-catenin expression (Fig. 5D). These results indicate that the induction of COX-2 expression by endogenous HA-CD44 interaction requires participation of ErbB2, AKT, and β-catenin.

We then investigated whether COX-2 is a downstream target of a HA-CD44 → ErbB2-PI3K/AKT → β-catenin signaling pathway, using the HCA7 cells. We inhibited β-catenin synthesis by transient transfection of these cells with β-catenin siRNA, which decreased COX-2 by 60–80% (Fig. 6A, lane 3 versus lane 1). The inhibition of COX-2 and β-catenin was not restored by co-transfection with CA-PI3K (Fig. 6A, lane 4 versus 3). However, CA-PI3K did cause an increase in COX-2 and β-catenin expression when used alone (Fig. 6, A, lane 5 versus lane 2, and C). These results strongly suggest that β-catenin and COX-2 are placed downstream of PI3K, and that both PI3K and β-catenin regulate COX-2 expression in HCA7 cells. Similarly, data in Fig. 6B shows that DN-PI3K transfection suppressed COX-2, p-AKT, and β-catenin expression by >70% (Fig. 6B, lane 3 versus lane 1). However, this suppression was not reversed by co-transfection with CA-ErbB2 (Fig. 6B, lane 4 versus lane 3). CA-ErbB2 transfection alone increases p-ErbB2 and p-AKT expression over endogenous levels of these proteins but has less effect on endogenous β-catenin and COX-2 expression in HCA7 cells (Fig. 6, B, lane 5 versus lane 2, and D). The variable degree of stimulation of p-ErbB2, p-AKT, COX-2, and

investigate this possibility, we examined the effects of antisense COX-2 on HA production in HIE6C-HAS2 clones, as well as the effect of transfection with sense COX-2 on HA production in HIE6C cells. We found that increased COX-2 stimulated HA production and antisense COX-2 decreased HA production (data not shown), supporting this postulate. Further work on this positive feedback loop is ongoing in our laboratory.

Role of ErbB2, AKT, and β-Catenin in Mediating Effects of HA on COX-2 Expression—Our studies on colon, prostate, and breast carcinoma cells demonstrate that interaction of endogenous HA with CD44 induces phosphorylation of multiple RTKs (30) and formation of a multimeric signaling complex present constitutively in lipid rafts (29). These interactions lead to stimulation of the PI3K/AKT cell survival pathway (29, 46). In colorectal cancer, both COX-2 and the WNT signaling cascade are active. The WNT signaling cascade stabilizes β-catenin by inactivating GSK3-β (68). Furthermore, the PI3K/AKT pathway can lead to inactivation of GSK3-β (69). Overexpression of CD44 is an early event in the colorectal adenoma-carcinoma sequence (70–72). Because elevated HA in HIE6C-HAS2 clones regulates several properties required for the transformed phenotype (Fig. 2, A–C), and because increased HA in HIE6C-HAS2-Ad-infected cells regulates activation of ErbB2 and AKT (Fig. 1C), and influences the distribution of β-catenin (Fig. 1B), we tested whether HA regulates COX-2 via its effects on an ErbB2 → PI3K/AKT → β-catenin axis. We found that increased HA stimulates activation of AKT and ErbB2 and expression of β-catenin in parallel with its effect on COX-2 expression in HIE6C cells (Fig. 5A, lane 2 versus lane 1). Each of these increases was reversed by O-HA and CD44 siRNA (Fig. 5A, lanes 4 and 5 versus lanes 2 and 3). The densitometric analysis of the results (Fig. 5C) indicates that both CD44siRNA and O-HA reversed the effects of up-regulation of HA synthesis on COX-2, p-AKT, p-ErbB2, and β-catenin to levels approximately equal to or lower than controls, although CD44siRNA was usually more potent than the O-HA. In HCA7 cells, these activities are constitutively elevated and are inhibited to similar extents by DN-AKT, an ErbB2 inhibitor AG825, and CD44 siRNA (Fig. 5B, lanes 2–4 versus lane 1). Indeed CD44siRNA is even more efficient than the AG825 in reducing COX-2 and p-ErbB2, whereas DN-AKT, AG825, and CD44siRNA have about the same effects on p-AKT and β-catenin expression (Fig. 5D). These results indicate that the induction of COX-2 expression by endogenous HA-CD44 interaction requires participation of ErbB2, AKT, and β-catenin.

We then investigated whether COX-2 is a downstream target of a HA-CD44 → ErbB2-PI3K/AKT → β-catenin signaling pathway, using the HCA7 cells. We inhibited β-catenin synthesis by transient transfection of these cells with β-catenin siRNA, which decreased COX-2 by 60–80% (Fig. 6A, lane 3 versus lane 1). The inhibition of COX-2 and β-catenin was not restored by co-transfection with CA-PI3K (Fig. 6A, lane 4 versus 3). However, CA-PI3K did cause an increase in COX-2 and β-catenin expression when used alone (Fig. 6, A, lane 5 versus lane 2, and C). These results strongly suggest that β-catenin and COX-2 are placed downstream of PI3K, and that both PI3K and β-catenin regulate COX-2 expression in HCA7 cells. Similarly, data in Fig. 6B shows that DN-PI3K transfection suppressed COX-2, p-AKT, and β-catenin expression by >70% (Fig. 6B, lane 3 versus lane 1). However, this suppression was not reversed by co-transfection with CA-ErbB2 (Fig. 6B, lane 4 versus lane 3). CA-ErbB2 transfection alone increases p-ErbB2 and p-AKT expression over endogenous levels of these proteins but has less effect on endogenous β-catenin and COX-2 expression in HCA7 cells (Fig. 6, B, lane 5 versus lane 2, and D). The variable degree of stimulation of p-ErbB2, p-AKT, COX-2, and
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FIGURE 6. HA, ErbB2, PI3K/AKT, and β-catenin regulate COX-2 expression. A, effects of β-catenin siRNA transfection on COX-2 expression in HCA7 cells. Lanes 1 and 2, Western blots of lysates of HCA7 cells transiently transfected with control siRNA or pUSEamp empty vector control, respectively; lanes 3 and 4, transiently transfected with β-catenin siRNA without or with co-transfection with CA-PI3K respectively; lane 5, transiently transfected with CA-PI3K. β-Actin was a loading control. B, Catenin siRNA down-regulated COX-2 and, as expected, β-catenin expression. These inhibitions were not restored by co-transfection with CA-PI3K. B, effects of DN-PI3K transfection on COX-2 expression in HCA7 cells. Lanes 1 and 2, respectively; Western blots of empty vector pUSEamp or pSV2-transfected HCA7 cell lysates (controls); lanes 3 and 4, HCA7 cells transiently transfected with pUSEamp-DN-PI3K without or with co-transfection with CA-ErbB2, respectively; lane 5, HCA7 cells transiently transfected with CA-ErbB2. β-Tubulin was a loading control. DN-PI3K inhibited COX-2, β-catenin, p-AKT, p-ErbB2 expression, and these inhibitions were not restored by co-transfection with CA-ErbB2. However, CA-ErbB2 transfection alone increased p-ErbB2 and p-AKT expression over the endogenous levels of these proteins but has little effect on endogenous β-catenin and COX-2 expression in HCA7 cells. Results presented are representative of three experiments for A and B, C and D, the densitometric values for Western blots of COX-2, p-AKT, p-ErbB2, and β-catenin were normalized with respect to the β-actin (C) and β-tubulin (D) as loading controls. Results are presented as mean ± S.D. of three independent experiments. Variability in expression of p-ErbB2, p-AKT, COX-2, and β-catenin under each of the various conditions in these experiments is 4–10%. The differences in expression of these proteins with respect to the specific control are statistically significant (p < 0.001).

β-catenin by CA-PI3K or CA-ErbB2 (Fig. 6, A–D) may be due to several factors. For example, stimulation of these proteins by overexpression of PI3K or ErbB2 may have varied to some degree because of variation in efficiency of transient transfection; however, our analyses of this parameter revealed very small differences. A more likely explanation is that the efficacy of stimulation of expression of these proteins by transient transfection with CA-PI3K or CA-ErbB2 depends on their endogenous levels and the turnover rates of these proteins. Alternatively, some subpopulation of these proteins may be regulated by HA-CD44 → ErbB2 → PI3K/AKT pathway and others may not. Nevertheless, these results strongly suggest that PI3K is downstream of ErbB2 and regulates the COX-2 pathway. The reason for partial inhibition of ErbB2 by DN-PI3K (Fig. 6B) and DN-AKT (Fig. 5B) is presently not clear but may be due to decreased HA synthesis (46). Clearly, further work is needed to confirm whether PI3K/AKT regulates ErbB2 via HA synthesis, but our unpublished results support the possibility that COX-2 may mediate regulation of HA production via PI3K/AKT. The results of Fig. 5, A–D, and Fig. 6, A–D, strongly support a pathway in which HA-CD44 interaction, ErbB2, PI3K/AKT, β-catenin, and COX-2 are co-regulated, and place COX-2 and β-catenin downstream of HA-CD44, ErbB2, and PI3K/AKT.

COX-2 Mediates HA-induced Effects in HAS2-overexpressing HIEC6 Cells—In the sections above we have demonstrated that endogenous HA-CD44 interaction regulates COX-2 expression and enzyme activity via an ErbB2 → PI3K/AKT → β-catenin axis and induces a transformed phenotype in phenotypically normal HIEC6 intestinal cells. The change in phenotype includes increased proliferation and cell survival properties, acquisition of mesenchymal characteristics, anchorage-independent growth, and MMP production (Figs. 1 and 2). Because COX-2 is downstream of CD44, we next addressed the question whether COX-2 mediates some of these effects of increased HA production, specifically proliferation and survival, using antisense (AS)-COX-2 transfection in HIEC6-HAS2-Ad cells. The results show that antisense COX-2 reverses the increase in cell proliferation caused by increased HA production (Fig. 7A, 4th bar versus 3rd and 2nd bars). In parallel, AS-COX-2 caused a marked increase in caspase-3 activity in the HIEC6-HAS2-Ad cells (Fig. 7B, 4th bar versus 1st to 3rd bars). These results indicate that endogenous HA regulates cell survival/proliferation through COX-2 induction.

Conclusions—The major findings of these experiments and our recent studies on several types of carcinoma cells are as follows. (a) HA interaction with CD44 constitutively promotes activation of several RTKs, specifically ErbB2, EGFR, platelet-derived growth factor receptor-β, IGF1R-β, and c-MET, as well as formation of large RTK-containing signaling complexes in malignant carcinoma cells (29, 30, 46), and these interactions lead to a number of downstream events necessary for malignant cell characteristics (11). (b) HA-overexpressing intestinal epi-

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to further examine the mechanism of HA regulation of COX-2 in the induction of cell proliferation and other malignant properties. These results highlight the potential importance of HA-induced COX-2 signaling as a therapeutic target in colon carcinoma.

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REFERENCES

1. Sheng, H., Shao, J., Dixon, D. A., Williams, C. S., Prescott, S. M., DuBois, R. N., and Beauchamp, R. D. (2000) J. Biol. Chem. 275, 6628–6635

2. Wu, R., Abramson, A. L., Shikowitz, M. J., Dannenberg, A. J., and Steinberg, B. M. (2005) Clin. Cancer Res. 11, 6155–6161

3. Du, J., Jiang, B., and Barnard, J. (2005) Cell 93, 705–716

4. Markwald, R. R., Fitzharris, T. P., Bank, H., and Bernanke, D. H. (1978) Dev. Biol. 62, 292–316

5. Toole, B. P. (2001) Semin. Cell Biol. 12, 79–87

6. Toole, B. P. (2002) Glycobiology 12, R37–R42

7. Lee, J. Y., and Spicer, A. P. (2000) Curr. Opin. Cell Biol. 12, 581–586

8. Turley, E. A., Noble, P. W., and Bourguignon, L. Y. (2002) J. Biol. Chem. 277, 4589–4592

9. Toole, B. P., Wight, T. N., and Tammi, M. (2002) J. Biol. Chem. 277, 4593–4596

10. Toole, B. P. (2004) Nat. Rev. Cancer 4, 528–539

11. de la Motte, C. A., Hascall, V. C., Calabro, A., Yen-Lieberman, B., and Strong, S. A. (1999) J. Biol. Chem. 274, 30747–30755

12. Majors, A. K., Austin, R. C., de la Motte, C. A., Pyeritz, R. E., Hascall, V. C., Kessler, S. P., Sen, G., and Strong, S. A. (2003) J. Biol. Chem. 278,
HA Regulates Activation of COX-2-mediated Cell Survival

47223–47231
14. Teder, P., Vandivier, R. W., Jiang, D., Liang, J., Cohn, L., Pure, E., Henson, P. M., and Noble, P. W. (2002) Science 296, 155–158

15. Wang, A., and Hascall, V. C. (2004) J. Biol. Chem. 279, 10279–10285

16. Chai, S., Chai, Q., Danielsen, C. C., Bjorth, P., Nyengaard, J. R., Ledet, T., Yamaguchi, Y., Rasmussen, L. M., and Wogensen, L. (2005) Circ. Res. 96, 583–591

17. Milinkovic, M., Antin, J. H., Hergrueter, C. A., Underhill, C. B., and Schröder, J. A. (2005) Cancer Res. 65, 1215–1223

18. Misra, S., Ghatak, S., Tammi, M., and Hyman, R. (2000) J. Biol. Chem. 275, 26967–26975

19. Anttila, M. A., Tammi, R. H., Tammi, M. I., Syrjanen, K. J., Saarikoski, S. V., Voutilainen, J., and Kosma, V. M. (2004) Cancer Res. 64, 15821–15828

20. Lipponen, P., Aaltomaa, S., Tammi, R., Tammi, M., Agren, U., and Kosma, V. (2001) Eur. J. Cancer 37, 849–856

21. Kosunen, A., Ropponen, K., Kellokoski, J., Pukkila, M., Virtanen, J., Ahonen, E., Johansson, R., Tammi, R., Tammi, M., Neudauer, C. L., McCarthy, J. B., and Bullard, K. M. (2004) Cancer Res. 64, 4569–4576

22. Laurich, C., Wheeler, M. A., Wilson, C. M., Iida, I., Eng, D., Simpson, M. A., McCarthy, J. B., and Bullard, K. M. (2004) J. Surg. Res. 122, 70–74

23. Bourguignon, L. Y., Gunja-Smith, Z., Iida, N., Zhu, H. B., Young, L. J., Muller, W. J., and Cardiff, R. D. (1998) J. Cell. Physiol. 176, 206–215

24. Bourguignon, L. Y., Gunja-Smith, Z., Iida, N., Zhu, H. B., Young, L. J., Muller, W. J., and Cardiff, R. D. (1998) J. Cell. Physiol. 176, 206–215

25. Misra, S., Ghatak, S., Toole, B. P., and Hyman, R. (2000) J. Biol. Chem. 275, 26967–26975

26. Fraser, J. R., Laurent, T. C., and Laurent, U. B. (1995) Anal. Biochem. 238, 83–90

27. Schaeffer, W. I., and Friend, K. (1976) Cancer Lett. 1, 259–262

28. Misra, S., Ghatak, S., and Toole, B. P. (2005) J. Biol. Chem. 280, 20310–20315

29. Hwang, J. T., Ha, J., and Park, O. J. (2005) Biochem. Biophys. Res. Commun. 332, 433–440

30. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275

31. Osherov, N., Gazit, A., Gilion, C., and Levitzki, A. (1993) J. Biol. Chem. 268, 11134–11142

32. Marieb, E. A., Zoltan-Jones, A., Li, R., Misra, S., Ghatak, S., Cao, J., Zucker, S., and Toole, B. P. (2004) Cancer Res. 64, 1229–1232

33. Park, T. E., Elder, D. J., and Paraskeva, C. (2000) Cancer Res. 60, 504–507

34. Haskel, V. C., and Toole, B. P. (2005) J. Biol. Chem. 280, 46879–46892

35. Brown, W. T., Hascall, V., and Toole, B. P. (2003) J. Biol. Chem. 278, 259–266

36. Teder, P., Vandivier, R. W., Jiang, D., Liang, J., Cohn, L., Pure, E., Henson, P. M., and Noble, P. W. (1998) Biochem. J. 332, 301–309

37. Spencer, A. P., Augustine, M. L., and McDonald, J. A. (1996) J. Biol. Chem. 271, 23400–23406