

## Experimental cancer therapy using restoration of NAD<sup>+</sup>-linked 15-hydroxyprostaglandin dehydrogenase expression

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### Abstract

Preclinical and clinical evidence shows that cyclooxygenase-2 (Cox-2)-mediated prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) overexpression plays an important role in tumor growth, metastasis, and immunosuppression. It has been shown that expression of NAD<sup>+</sup>-linked 15-hydroxyprostaglandin dehydrogenase (15-PGDH), a key enzyme responsible for PGE<sub>2</sub> inactivation, is suppressed in the majority of cancers, including breast and colon carcinoma. We have developed adenoviral vectors (Ad) encoding the 15-PGDH gene under control of the vascular endothelial growth factor receptor 1 (VEGFR1/flt-1; Adflt-PGDH) and the Cox-2 (Adcox-PGDH) promoters. The purpose of this study was to investigate cytotoxicity *in vitro* and therapeutic efficacy *in vivo* of 15-PGDH-mediated cancer therapy. The levels of PGE<sub>2</sub> and VEGF expression were correlated with PGE<sub>2</sub> receptor and Cox-2 and flt-1 expression in cancer cells. The *in vitro* study showed that Ad-mediated 15-PGDH expression significantly decreased proliferation and migration of cancer cells. Animal breast and colon tumor therapy studies showed that 15-PGDH gene therapy produced a significant delay in 2LMP and LS174T tumor growth. Combined therapy using 15-PGDH and anti-VEGF antibody (bevacizumab) significantly increased inhibition of growth of LS174T tumor xenografts in comparison with agents alone. These results suggest that 15-PGDH-mediated regulation of PGE<sub>2</sub> catabolism in the tumor microenvironment represents a novel approach for therapy

of human breast and colon cancer. [Mol Cancer Ther 2009;8(11):3130–9]

### Introduction

Preclinical and clinical evidence shows that prostaglandins (PG) play an important role in the growth of tumors. Prostaglandin endoperoxide synthase 2 (cyclooxygenase-2; Cox-2) is the rate-limiting enzyme involved in the oxidative transformation of arachidonic acid into PGH<sub>2</sub>, which represents the precursor of several bioactive molecules, including PGE<sub>2</sub>, PGD<sub>2</sub>, PGF<sub>2</sub>α, PGI<sub>2</sub> (prostacyclin), and TXA<sub>2</sub> (thromboxane A<sub>2</sub>; refs. 1, 2). Cox-2 is overexpressed in the majority of epithelial tumors, including breast and colon cancers. Aberrant upregulation of Cox-2 expression is correlated with prognostic markers that reflect a poor chance for survival, which includes tumor size, axillary node metastases, tumor grade, ductal histology, receptor negative disease, and overexpression of HER-2 (3, 4). Furthermore, elevated Cox-2 expression was significantly associated with reduced survival and recognized as an independent prognostic factor in colorectal cancer patients (5). Cox-2 is involved in activation of tumor-associated angiogenesis and in the inhibition of cancer cell apoptosis (6, 7). Moreover, elevated Cox-2 expression has recently been shown to correlate with distant breast cancer metastases (8). Inhibition of the Cox-2 pathway decreased colorectal metastasis growth in the liver (9–11). It was shown that activation of the Cox-2 signaling pathway leads to intestinal epithelial and colon tumor cell proliferation (12).

Growth, progression, invasion, and metastasis of cancers are accompanied by the formation of new blood vessels. Therefore, tumor-associated angiogenesis is an acquired capability of malignant tumors. High levels of PGE<sub>2</sub> expression in tumors are associated with increased angiogenesis (13). It has been shown that PGE<sub>2</sub> induces the expression of several proangiogenesis factors including vascular endothelial growth factor (VEGF; ref. 14) and basic fibroblast growth factor (bFGF; ref. 15) in blood vessel endothelial cells (16). VEGF and bFGF also induce Cox-2-mediated PGE<sub>2</sub> expression in endothelial cells, which raises the possibility of amplification of PGE<sub>2</sub> regulation of angiogenesis through a positive feedback loop (17). The addition of the anti-VEGF monoclonal antibody (mAb) bevacizumab (Avastin) to chemotherapy in the treatment of metastatic colorectal cancer has resulted in an increase of progression-free survival and overall survival (18).

NAD<sup>+</sup>-linked 15-hydroxyprostaglandin dehydrogenase (15-PGDH) catalyzes the oxidation of 15(S)-hydroxyl group of PGE<sub>2</sub> resulting in the formation of 15-keto metabolites with greatly reduced biological activities. Expression of 15-PGDH, a key enzyme responsible for PGE<sub>2</sub> inactivation, is suppressed in the majority of cancers, including breast and

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colorectal cancers (19–21). Thus, overexpression of Cox-2 and repression of 15-PGDH provide complementary pathways to increase levels of PGE<sub>2</sub> in the tumor microenvironment.

We have developed adenoviral vectors (Ad) encoding the human 15-PGDH gene under control of the VEGF receptor 1 (VEGFR-1/flt-1) and Cox-2 promoter element Adflt-PGDH and Adcox-PGDH, respectively. We investigated whether Ad-targeted 15-PGDH therapy produces cytotoxicity against cancer cells *in vitro* and in s.c. human breast and colon xenograft models. The results show that Ad-mediated overexpression of 15-PGDH significantly inhibited growth of the human breast and colon xenografts in athymic nude mice. Also, the antitumor effect 15-PGDH gene therapy was enhanced by coadministration of bevacizumab (Avastin).

## Materials and Methods

### Cells, Reagents, and Adenoviral Vectors

The 2LMP and LCC6 breast cancer cell lines (both were obtained from Dr. M. Lippman, Georgetown University, Washington, DC), MDA-MB-231 breast cancer cells, colon adenocarcinoma cells LS174T (American Type Culture Collection), and embryonic kidney cells HEK293 (Microbix Biosystems) were cultured in DMEM/F12 (Mediatech) containing 10% fetal bovine serum (Summit Biotechnology). Normal established breast epithelial cells MCF 10A (American Type Culture Collection) were cultured in mammary epithelial growth medium (Clontech) containing 100 ng/mL cholera toxin (Calbiochem). Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics and were grown in endothelial growth medium-2 growth media (Clonetics). Avastin anti-VEGF recombinant IgG1 mAb was obtained from Genentech.

Replication-deficient E1- and E3-deleted Ad vectors were constructed using standard molecular cloning methods (Supplementary materials).

### RNA Preparation and Reverse Transcriptase-PCR

To determine VEGFR1/flt-1, EP receptor, Cox-2, and 15-PGDH mRNA expression, cells were lysed in TRIzol reagent (Invitrogen), and total RNA was extracted using RNeasy Mini Kit (Qiagen), following standard protocol, and quantified spectrophotometrically using a MBA 2000 spectrophotometer (Perkin Elmer). cDNA was synthesized using random hexamer primers and an Omniscript RT kit (Qiagen). The first-strand cDNA was used as the template for PCR. For amplification of cDNA encoding the target genes the following forward (f) and reverse (r) primers were used: flt-1 f: GAG GAT TCT GAC GGT TTC; flt-1 r: CCT GTC AGT ATG GCA TTG; 15-PGDH f: GTA AAG CTG CCC TGG ATG AG; 15-PDGH r: AAC AAA GCC TGG ACA AAT GG; EP1 f: TCT ACC TCC CTG CAG CGG CCA CT; EP1 r: GAA GTG GCT GAG GCC GCT GTG CCG GGA; EP2 f: ATG GGC AAT GCC TCC AAT GAC TCC CAG; EP2 r: CTC CAG GGA ACA ATT TCA AAA T; EP3 f: GAG CAC TGC AAG ACA CAC ACG GAG; EP3 r: GAT CTC CCA TGG TAT TAC TGA CAA; EP4 f: CCT CCT GAG AAA GAC AGT GCT; EP4 r: AAG ACA CTC TCT GAG TCC T; COX-2 f: TGA AAC CCA CTC

CAA ACA CAG; and COX-2 r: TCA TCA GGC ACA GGA GGA AG. The human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA was used as an internal standard for template loading of PCR by using primers: GAPDH f: TCC CAT CAC CAT CTT CCA; and GAPDH r: CAT CAC GCC ACA GTT TCC. PCR was done in the Eppendorf MasterCycler (Eppendorf). PCR product was analyzed by 1% agarose electrophoresis with 0.01 μg/mL ethidium bromide (Bio-Rad) staining.

### Measurement of PGE<sub>2</sub> Expression

Cells were infected with 50% tissue culture infectious dose (TCID<sub>50</sub>)/cell of Adflt-PGDH, Adcox-PGDH, or Adflt-Luc. Cell culture supernatants were collected at 48 h after treatment, centrifuged to remove floating cells, and stored at –20°C. The amount of PGE<sub>2</sub> released in the medium was measured with enzyme immunoassay kit (Cayman Chemical) following the manufacturer's instructions. The raw PGE<sub>2</sub> data were normalized according to the number of viable cells in each sample, determined by trypan blue staining. Cells were counted using a hemacytometer.

### Measurement of VEGF Expression

Cells were infected with 50 TCID<sub>50</sub>/cell of Adflt-PGDH, Adcox-PGDH, or Adflt-Luc. For generation of conditioned medium, cells were incubated for 48 h after treatment. Cell culture supernatants were collected and VEGF concentrations were assessed using a quantitative ELISA (R&D systems) following the manufacturer's instructions. The raw VEGF data were normalized according to the number of viable cells in each sample.

### Luciferase Assay

To determine VEGFR1/flt-1, Cox-2, and cytomegalovirus promoter activity, cells were infected with 5 TCID<sub>50</sub>/cell of Adflt-Luc, Adcox-Luc, or Adcmv-Luc, respectively. After 48 h the cells were treated with reporter lysis buffer (Promega), freeze-thawed once, and then these samples were mixed with luciferase assay reagent (Promega) and Luc activity was measured using Berthold Lumat LB9501. Luc activities were normalized for the number of viable cells in each sample and expressed as relative light units.

### Western Blot

To investigate the levels of recombinant protein expression, 2LMP cells were infected with 50 TCID<sub>50</sub>/cell of Adcox-PGDH, Adflt-PGDH, or Adflt-Luc. Cells and conditioned media were collected at 48 h after infection and subjected to Western blot analysis using anti-15-PGDH rabbit polyclonal antibody (Novus Biological). Cells were washed in TBS and homogenized in lysis buffer. Equal amounts of sample were loaded in each lane and separated on SDS-PAGE followed by transfer to a polyvinylidene difluoride membrane. The membrane was blocked with 5% nonfat milk in TBS. The membrane was incubated with specific and secondary antibodies, and then processed and treated with ECL plus Western Blotting Detection System (Amersham Biosciences).

### Clonogenic Survival Assay

At 3 h after infection with 50 TCID<sub>50</sub>/cell of Adflt-PGDH, Adcox-PGDH, or Adflt-Luc, cells were diluted to an appropriate cell density, placed into 6-well culture plates, and

cultured an additional 15 d. Cells were then fixed and stained with 2% (w/v) crystal violet in 70% ethanol. Colonies comprising  $\geq 50$  cells were counted. The plating efficiencies were calculated as the number of colonies divided by the number of test cells plated for each data point. Plating efficiencies were referenced back to the Adflt-Luc-treated control plating efficiency to determine the surviving fraction for Adflt-PGDH and Adcox-PGDH.

#### Cell Migration Assay

Migration assay was done using tissue culture plate inserts with 12.0  $\mu\text{m}$  tissue culture-treated Millcell polycarbonate membranes (Millipore). Cells were infected with 50 TCID<sub>50</sub>/cell of Adflt-PGDH, Adcox-PGDH, or Adflt-Luc. After overnight incubation the cells were harvested and added to the upper chambers at concentrations of  $5 \times 10^4$  cells/well. After incubation for 72 h nonmigrated cells on the upper side of the tissue culture plate insert were removed with a cotton swab, and the migrated cells attached to the lower side of the membrane were released using trypsin digest and counted using a hemacytometer.

#### S.c. Human Breast Tumor Xenograft Model

Female athymic nude mice were purchased from the Frederick Cancer Research Facility and housed under aseptic conditions in microisolator cages, and experiments were carried out according to the protocols approved by the Institutional Animal Care and Use Committee. For tumor cell inoculation the mice were anesthetized by xylazine/ketamine (90/10 mg/kg), and  $5 \times 10^6$  2LMP breast cancer cells were injected s.c. into the flanks of 8-week-old mice. When tumors reached 4 to 6 mm in diameter (day 0), groups of 8 to 9 mice were injected intratumorally with PBS, Adflt-Luc, Adflt-PGDH, or Adcox-PGDH at  $1 \times 10^8$  TCID<sub>50</sub>/tumor on days 0, 5, 10, and 15.

#### S.c. Human Colon Tumor Xenograft Model

To investigate the therapeutic efficacy of combination 15-PGDH-mediated therapy and anti-VEGF therapy,  $5 \times 10^6$  LS174T colon cancer cells were injected s.c. into the flanks of 8-week-old mice. When tumors reached 4 to 6 mm in diameter (day 0), groups of 8 to 9 mice were injected intratumorally with PBS, Adflt-Luc, or Adflt-PGDH at  $1 \times 10^8$  TCID<sub>50</sub>/tumor on days 0, 4, and 8. Avastin was administered i.p. at 5 mg/mouse on days 2 and 6. Tumor size was monitored twice a week using Vernier calipers. Tumor volumes ( $\text{mm}^3$ ) were calculated as  $\text{width}^2 \times \text{length} \times 0.5$ . Percent change from baseline at day 0 was computed by comparing the baseline value to the tumor size on each day of measurement. The mathematical model was fitted to tumor growth data to estimate tumor doubling time. The end point criterion for the experimental therapy study was a doubling in tumor volume from the initiation of treatment (day 0). Percent change from baseline at day 0 was computed by comparing the baseline value to the tumor volume on each day of measurement.

#### Immunohistochemical Analysis of Tumors for CD31 Expression

The formalin-fixed and paraffin-embedded 5- $\mu\text{m}$ -thick sections of tumor samples were incubated overnight with rat anti-mouse CD31/PECAM1 polyclonal antibody (BD Biosciences Pharmingen). The sections were then incubated

with donkey anti-rat antibody secondary antibody (Jackson ImmunoResearch Laboratories). Antigen-antibody complexes were visualized by incubation with 3,3-diaminobenzidine substrate and counterstained with diluted Harris hematoxylin. Tumor vessels containing CD31-positive (brown) cells were quantified by microscopy (original magnification,  $\times 200$ ) of at least 5 random fields/tumor, and were calculated as relative microvessel density.

#### Statistical Methods

All error terms are expressed as the SD of the mean. Significance levels for comparison of differences between groups in the *in vitro* experiments were analyzed by Student's *t* test. The differences were considered significant when  $P < 0.05$ . All reported *P* values are two-sided. In the animal model tumor therapy studies, the treatment groups were compared with respect to tumor size and percent of original tumor size over time. To test for significant differences in tumor size between treatment groups, ANOVA test was conducted. When the ANOVA indicated that a significant difference existed ( $P < 0.05$ ), multiple comparison procedures were used to determine where the differences lay.

## Results

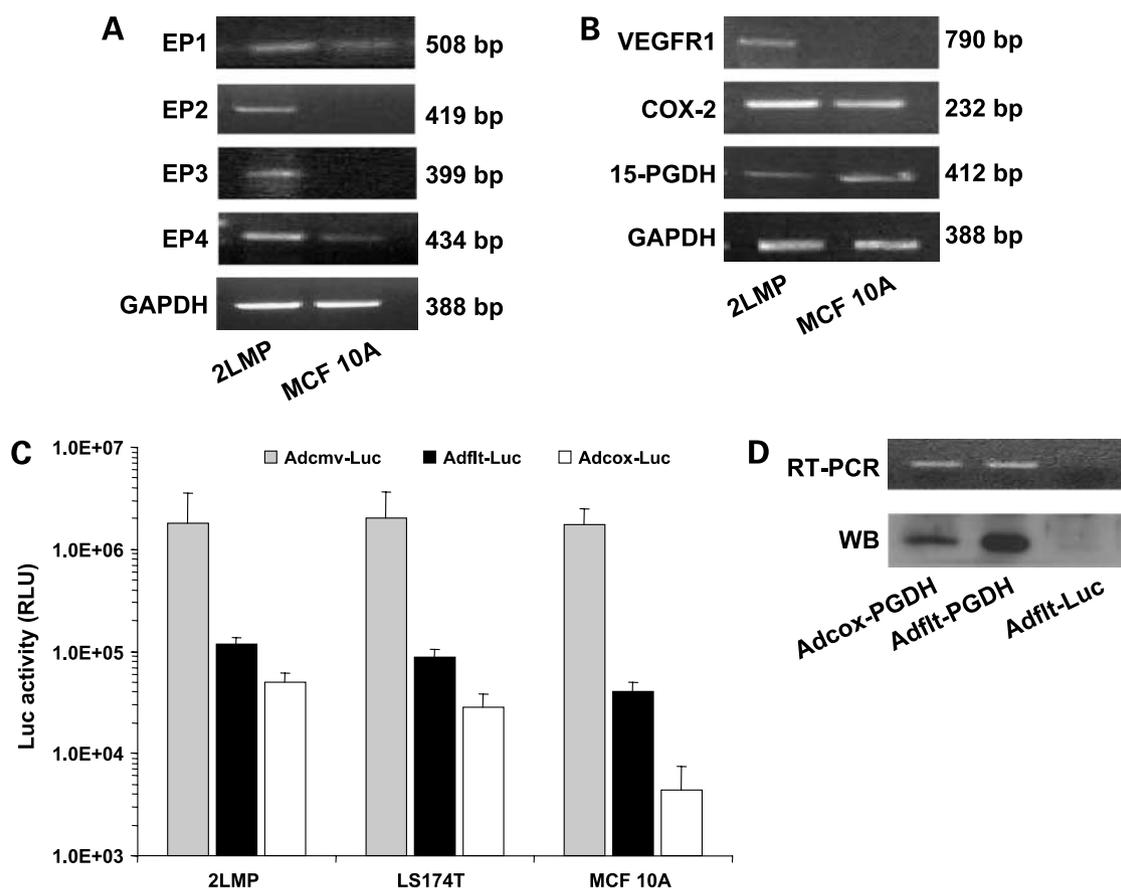
### PGE<sub>2</sub> and VEGF Secretion Are Associated with Expression of Prostaglandin Receptors, Cox-2, and VEGFR1 in Cancer Cells

Recent studies have shown a strong interaction between the Cox-2 and the VEGF signaling pathways. In this study, 2LMP breast cancer cells and MCF 10A normal mammary gland epithelial cells were tested for PGE<sub>2</sub> EP1-4 receptor, Cox-2, and VEGFR1 mRNA expression, as well as VEGF and PGE<sub>2</sub> protein secretion. Expression of PGE<sub>2</sub> receptors EP1-4 in 2LMP and MCF 10A cells was investigated by reverse transcriptase-PCR (RT-PCR). As shown in Fig. 1A, 2LMP cancer cells expressed all four types of PGE<sub>2</sub> receptors, whereas MCF 10A cells showed undetectable levels of EP2 and EP3 as well as lower levels of EP1 and EP4 receptor mRNA expression compared with 2LMP cells.

Importantly, MCF 10A cells showed undetectable levels of VEGFR1 expression, and showed lower-level Cox-2 mRNA expression in comparison with 2LMP breast cancer cells as measured by RT-PCR (Fig. 1B). However, RT-PCR analysis revealed a higher level of 15-PGDH expression in MCF 10A cells in comparison with 2LMP cells (Fig. 1B).

Also, quantitative analysis of EP receptors, and Cox-2 and VEGFR1/flt-1 mRNA expression in an expanded panel of breast cancer cells was done using real-time RT-PCR. The results of real time RT-PCR analysis, summarized in Supplementary Table S1 (supplementary materials), are confirmed higher levels of EP receptor, Cox-2, and VEGFR1 mRNA expression in all tested cancer cells in comparison with MCF 10A cells. In contrast, the levels of 15-PGDH expression were significantly higher in normal breast cells in comparison with cancer cells (Supplementary Table S1).

The levels of PGE<sub>2</sub> were significantly higher in conditioned medium of 2LMP breast cancer cells in comparison with MCF-10A cells ( $30.4 \pm 5.1$  pg/mL and  $11.3 \pm 3.5$  pg/mL,



**Figure 1.** Expression of PGE<sub>2</sub> receptors, Cox-2, and VEGFR1 *in vitro*. 2LMP breast cancer cells and MCF 10A mammary gland epithelial cells were collected, and total RNA was extracted. The levels of (A) PGE<sub>2</sub> receptor (EP1-4) and (B) Cox-2, VEGFR1/flt-1, and GAPDH (loading control) mRNA expression were determined using RT-PCR. C, Luc expression in cancer cells. Cells were infected with Adcmv-Luc, Adflt-Luc, and Adcox-Luc at 5 TCID<sub>50</sub>/cell. Luc expression was analyzed at 48 h after infection. D, Ad-mediated expression of 15-PGDH. The expression of 15-PGDH in 2LMP cells at 48 h after infection with 50 TCID<sub>50</sub>/cell of Adflt-PGDH, Adcox-PGDH, or Adflt-Luc was examined using RT-PCR and Western blot (WB) analysis. One representative of three different experiments is shown.

respectively) measured by ELISA. Similarly, the levels of VEGF evaluated using ELISA were significantly higher in conditioned medium of 2LMP cells ( $19.7 \pm 4.2$  ng/mL) in comparison with MCF 10A cells ( $7.1 \pm 2.3$  ng/mL).

#### 15-PGDH Overexpression Inhibits Proliferation and Migration of Cancer Cells and HUVECs

Initially, cells were infected with Adcmv-Luc, Adflt-Luc, or Adcox-Luc, and the cytomegalovirus, VEGFR1, and Cox-2 promoter activity was evaluated using luciferase (Luc) assay. The levels of VEGFR1- and Cox-2-driven Ad-mediated Luc expression were higher in 2LMP breast cancer cells as well as LS174T colon cancer cells compared with MCF 10A normal mammary gland epithelial cells (Fig. 1C).

To identify Ad-mediated 15-PGDH expression, 2LMP breast cancer cells were infected with Adflt-PGDH, Adcox-PGDH, or Adflt-Luc. The preliminary study showed that infection of 2LMP and LS174T cancer cells with 50 TCID<sub>50</sub>/cell of Adflt-Luc has a negligible effect on levels of 15-PGDH and Cox-2 expression as well as on the levels of PGE<sub>2</sub> and VEGF secretion in comparison with uninfected control. Thus, for *in vitro* study Adflt-Luc was used as con-

trol. The infected cells were collected at 48 hours after infection and then analyzed by RT-PCR as well as protein expression by SDS-PAGE followed by immunoblotting analysis. The results (Fig. 1D) show higher levels of 15-PGDH mRNA and protein expression in Adflt-PGDH- and Adcox-PGDH-infected cells as compared with Adflt-Luc-infected control cells. Similar results were obtained with LS174T cells (data not shown).

Next, we investigated whether Ad-mediated 15-PGDH overexpression affects PGE<sub>2</sub> and VEGF expression in cancer cells. 2LMP and LS174T cells were infected with Adcox-PGDH, Adflt-PGDH, or Adflt-Luc, and then the levels of PGE<sub>2</sub> and VEGF were measured by ELISA. As shown in Fig. 2A, infection of 2LMP cells with Adcox-PGDH and Adflt-PGDH resulted in decreased PGE<sub>2</sub> concentration by 2.0-fold and 3.2-fold ( $P < 0.05$ ), respectively, compared with conditioned media from Adflt-Luc-infected control cells. PGE<sub>2</sub> secretion in LS174T cells following Adcox-PGDH and Adflt-PGDH infection was reduced by 1.5-fold and 1.6-fold ( $P > 0.05$ ), respectively, in comparison with Adflt-Luc-infected cells.

In comparison with Adflt-Luc-infected cells, the levels of VEGF were significantly reduced in conditioned media from Adcox-PGDH- and Adflt-PGDH-infected 2LMP cells, by 2.9-fold and 1.9-fold, respectively ( $P < 0.05$ ), and by 4.4-fold and 5.7-fold for Adcox-PGDH- and Adflt-PGDH-infected LS174T cells, respectively ( $P < 0.01$ ; Fig. 2B).

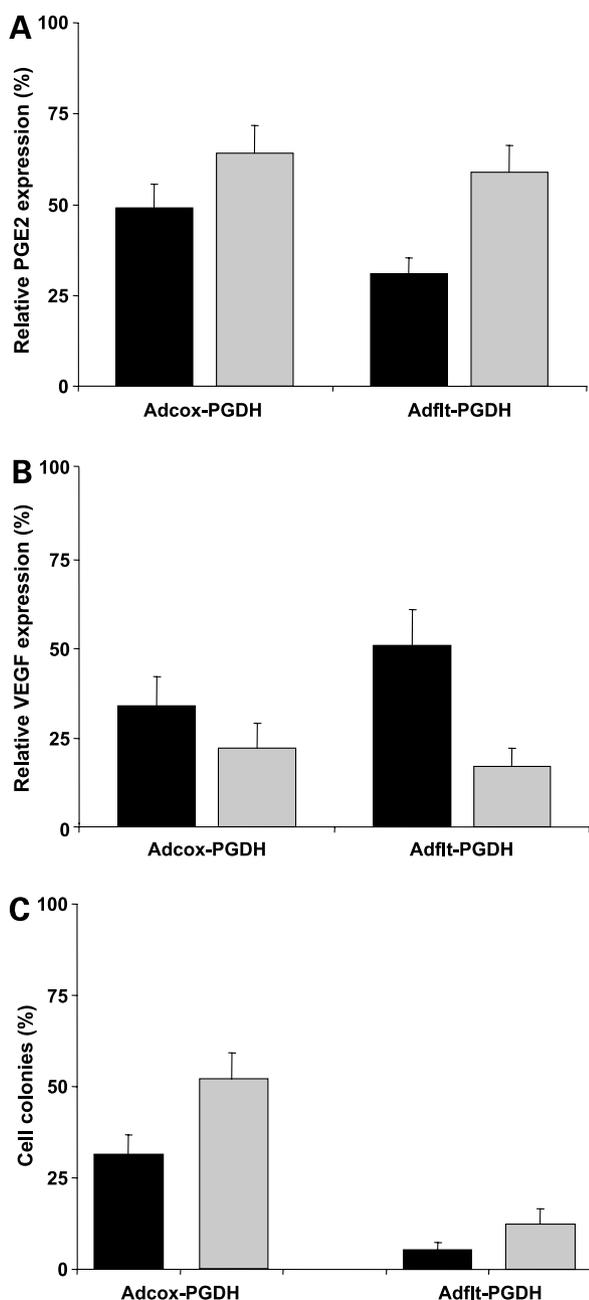
To determine 15-PGDH-mediated cytotoxicity, 2LMP and LS174T cancer cells were infected with Adcox-PGDH, Adflt-PGDH, or Adflt-Luc, and subjected to a clonogenic survival assay. As shown in Fig. 2C, there was 3.0-fold and 1.9-fold ( $P < 0.01$ ) reduction in the number of colonies for Adcox-PGDH-infected 2LMP and LS174T cells, respectively, as

compared with Adflt-Luc-infected control cells. Adflt-PGDH infection of 2LMP and LS174T cells significantly reduced the number of colonies 18.2-fold and 8.1-fold ( $P < 0.001$ ), respectively, in comparison with Adflt-Luc-infected cells.

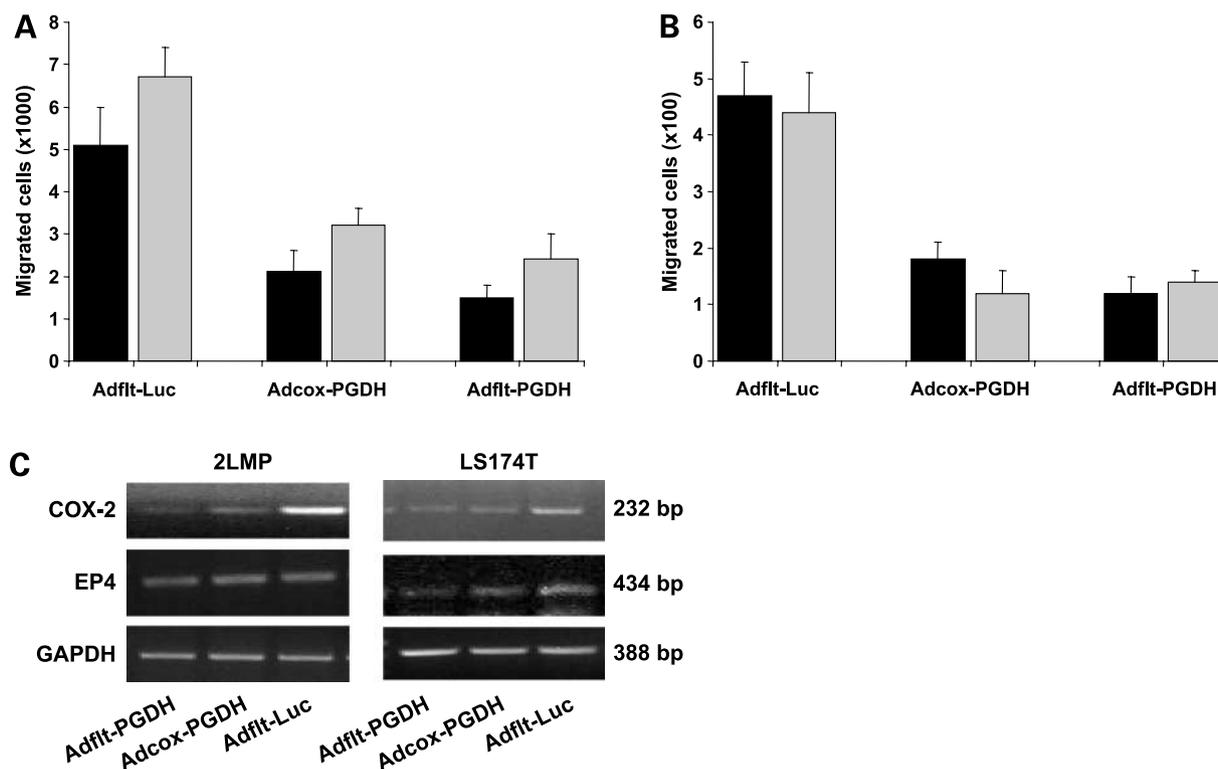
To further evaluate whether Ad-mediated 15-PGDH expression inhibits cancer cell migration, the conditioned media obtained from 2LMP and LS174T cancer cells infected with Adflt-Luc, Adcox-PGDH, or Adflt-PGDH were applied to the lower chambers of culture plate transwell inserts, and uninfected cells were seeded to the upper chambers of transwell inserts. Conditioned media from Adcox-PGDH- and Adflt-PGDH-infected cancer cells inhibited 2LMP cell migration by 2.4-fold and 3.4-fold ( $P < 0.05$ ), respectively, and migration of LS174T cells by 2.1-fold and 2.6-fold ( $P < 0.05$ ), respectively, compared with conditioned media from Adflt-Luc infected cells (Fig. 3A).

Also, we determined human blood vessel endothelial cell migration using conditioned media obtained from cancer cells infected with Adflt-Luc, Adcox-PGDH, or Adflt-PGDH. The conditioned media were applied to the lower chambers of culture plate transwell inserts, and HUVECs were seeded to the upper chambers of transwell inserts. As shown in Fig. 3B, conditioned media from Adcox-PGDH- and Adflt-PGDH-infected 2LMP cancer cells inhibited HUVEC migration by 2.6-fold and 3.9-fold ( $P < 0.05$ ), respectively, whereas conditioned media from Adcox-PGDH- and Adflt-PGDH-infected LS174T cells decreased migration of endothelial cells by 3.7-fold and 3.1-fold ( $P < 0.05$ ), respectively, compared with conditioned media from Adflt-Luc-infected cancer cells.

Next, we investigated the PGE<sub>2</sub> receptor expression in cancer cells following Ad-mediated 15-PGDH overexpression. 2LMP and LS174T cells were infected with Adflt-PGDH, Adcox-PGDH, or Adflt-Luc, and expression of PGE<sub>2</sub> receptor and Cox-2 mRNA was investigated by RT-PCR. The results presented in Fig. 3C show that Adflt-PGDH and Adcox-PGDH decreased levels of Cox-2 mRNA in both cancer cell lines and EP4 mRNA expression in LS174T cells in comparison with Adflt-Luc-infected cells.



**Figure 2.** Ad-mediated 15-PGDH expression inhibits proliferation and migration of cancer cells. **A**, 15-PGDH decreased PGE<sub>2</sub> expression in cancer cells. 2LMP (black) and LS174T (gray) cells were infected with 50 TCID<sub>50</sub>/cell of Adcox-PGDH, Adflt-PGDH, or Adflt-Luc. Cell culture supernatants were collected at 48 h after infection, and PGE<sub>2</sub> protein released in the medium was measured with ELISA. Data are presented as percentage of PGE<sub>2</sub> concentration in comparison with conditioned medium from Adflt-Luc-infected cells. **B**, 15-PGDH overexpression decreased VEGF secretion in cell culture medium of cancer cells. 2LMP (black) and LS174T (gray) cancer cells were infected with 50 TCID<sub>50</sub>/cell of Adcox-PGDH, Adflt-PGDH, or Adflt-Luc. Cell culture supernatants were collected at 48 h after infection, and VEGF concentration in the medium was measured with ELISA. Data are presented as percentage of VEGF concentration in comparison with conditioned medium from Adflt-Luc-infected cells. **C**, clonogenic survival assay of human cancer cells. 2LMP (black) and LS174T (gray) cells were infected with 50 TCID<sub>50</sub>/cell of Adcox-PGDH, Adflt-PGDH, or Adflt-Luc. Cells were diluted to an appropriate cell density and placed into 6-well culture plates, and colonies were counted at 15 d after treatment. Data are presented in comparison with Adflt-Luc-infected cells. Presented are mean values  $\pm$  SD of three independent experiments, each done in six replicates.



**Figure 3.** 15-PGDH inhibited migration of cancer cells and HUVECs. **A**, 2LMP (black) and LS174T (gray) cells were infected with 50 TCID<sub>50</sub>/cell of Adflt-Luc, Adcox-PGDH, or Adflt-PGDH. Cell-conditioned medium was collected at 48 h after infection. Cells were seeded in the upper chamber, conditioned medium was added in the lower compartment, and cells were allowed to migrate for 96 h. The migrated cells attached to the lower chamber were counted using a hemacytometer. Presented are mean values  $\pm$  SD of the number of cells in three independent experiments, each done in three replicates. **B**, 15-PGDH inhibited migration of endothelial cells. Cancer cells were infected with 50 TCID<sub>50</sub>/cell of Adflt-Luc, Adcox-PGDH, or Adflt-PGDH. Cell-conditioned medium was collected at 48 h after infection. HUVECs were seeded in the upper chamber, conditioned medium from 2LMP (black) and LS174T (gray) cells was added in the lower compartment, and cells were allowed to migrate for 120 h. The migrated endothelial cells attached to the lower chamber were counted using a hemacytometer. Presented are mean values  $\pm$  SD of the number of cells in three independent experiments, each done in three replicates. **C**, cancer cells were infected with 50 TCID<sub>50</sub>/cell of Adflt-Luc, Adcox-PGDH, or Adflt-PGDH. The cells were collected at 48 h after infection, and total RNA was extracted. The levels of Cox-2, EP4, and GAPDH (loading control) mRNA expression were determined using RT-PCR.

At the same time 15-PGDH overexpression produced no alteration in levels of expression of VEGFR1 and EP1-3 receptor mRNA in 2LMP and LS174T cancer cells (data not shown).

#### Ad-mediated 15-PGDH Therapy of Breast Tumor Xenografts

To investigate the antitumor effect of the 15-PGDH-mediated therapy of breast tumor xenografts, 2LMP cells were s.c. injected into the flank of athymic nude mice. Before treatment, the mean tumor sizes in groups at baseline were not significantly different between treatment groups ( $P > 0.05$ ) and the within-treatment variances ( $P > 0.05$ ). The preliminary dose escalation study showed that multiple intratumoral injections of 2LMP and LS174T tumor xenografts with  $1 \times 10^8$  TCID<sub>50</sub> Adflt-Luc had a negligible effect on tumor growth. Consistency of delivery of Adflt-Luc vector into s.c. xenografts was confirmed using luciferase assay (3 tumors/group) at 3 days after the last injection (data not shown). *In vivo* tumor therapy with  $1 \times 10^8$  TCID<sub>50</sub> of each Ad occurred on days 0, 5, 10, and 15, beginning 5 days after tumor cell injection. The baseline mean and SD for tumor volumes on day 0 was  $86.0 \pm 9.1$  mm<sup>3</sup>. The mean time to

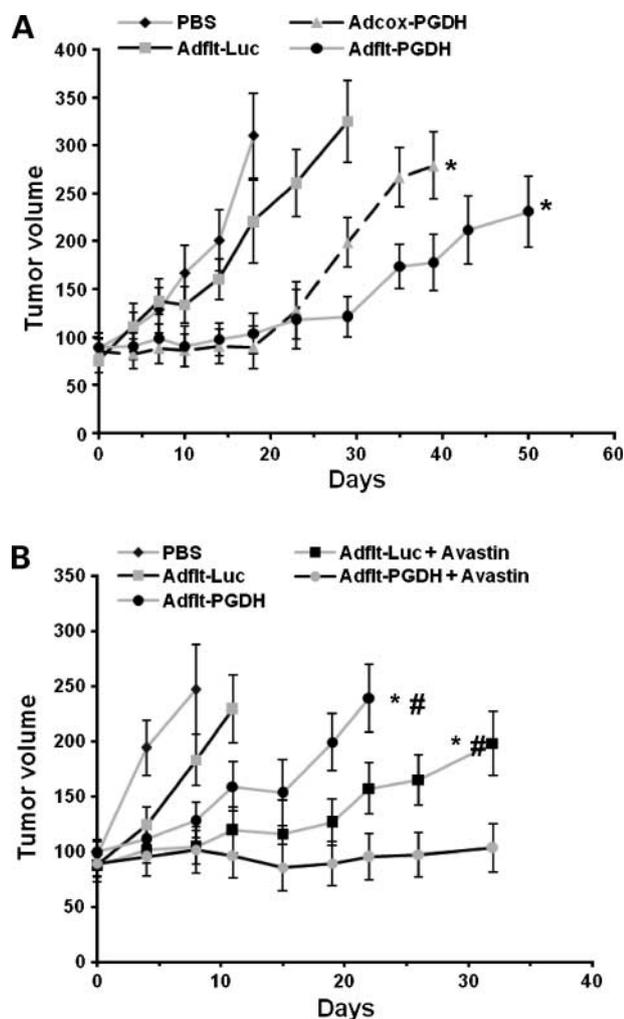
tumor doubling for the PBS-, Adflt-Luc-, Adcox-PGDH-, and Adflt-PGDH-treated groups were 11, 12, 28, and 39 days, respectively (Fig. 4A). Comparisons of mean time to tumor doubling of the group treated with Adcox-PGDH versus Adflt-Luc and Adflt-PGDH versus Adflt-Luc showed significant differences between the groups ( $P < 0.05$ ).

To determine whether the inhibitory effect of Ad-mediated 15-PGDH therapy on tumor growth was linked to the suppression of tumor angiogenesis, we examined the distribution of the endothelial cell-specific antigen CD31/PECAM1. 2LMP s.c. xenograft-bearing mice received treatment with PBS, Adflt-Luc, Adcox-PGDH, and Adflt-PGDH, as described above, and 3 days after the last injection, the tumors with surrounded tissue (3 tumors/group) were excised, fixed in 10% neutral buffered formalin, and stained with anti-CD31 antibody. Microscopic examination of CD31-stained tumor sections showed that compared with PBS-injected control mice, Adcox-PGDH and Adflt-PGDH injection decreased the number of positive cells (Supplementary Fig. S1; supplementary materials). The quantitative evaluation of tumor-associated vascularity showed that the number of CD31-positive cells in 2LMP tumor xenografts treated

with Adcox-PGDH and Adflt-PGDH decreased 1.9 ( $12.1 \pm 3.7$ ) and 2.7-fold ( $8.4 \pm 3.1$ ;  $P < 0.05$ ), respectively, over that of Adflt-Luc-treated control tumors ( $22.5 \pm 4.2$ ), with PBS-treated xenografts at  $21.8 \pm 5.0$ .

### Combination of Anti-VEGF mAb and 15-PGDH Therapy of Colon Tumor Xenografts

Recently the anti-VEGF mAb bevacizumab (Avastin) in combination with chemotherapy was approved for first- and second-line treatments of metastatic colorectal cancer



**Figure 4.** The experimental animal therapy study. **A**, antitumor effect of Ad-mediated 15-PGDH gene therapy in breast cancer xenografts. Treatment was started at the time of established tumor growth (day 0 equal to 5 d after tumor cell injection). Mice were injected intratumorally with PBS or with  $1 \times 10^8$  TCID<sub>50</sub> Adcox-PGDH, Adflt-PGDH, or Adflt-Luc on days 0, 5, 10, and 15. Data points represent the mean tumor volume for each group of animals. \*,  $P < 0.05$  in comparison with Adflt-Luc. **B**, efficacy of combination Ad-mediated 15-PGDH gene therapy with Avastin using human colon cancer xenograft model. Therapy was initiated 7 d after tumor cell injection (day 0). The mice were injected intratumorally with PBS, Adflt-Luc, or Adflt-PGDH at  $1 \times 10^8$  TCID<sub>50</sub>/tumor on days 0, 4, and 8. Avastin was administered i.p. at 5 mg/mouse on days 2 and 6. Data points represent the mean tumor volume for each group of animals. \*,  $P < 0.05$ , Adflt-PGDH alone and Adflt-Luc plus Avastin in comparison with Adflt-Luc alone; #,  $P < 0.05$ , Adflt-PGDH plus Avastin versus Adflt-PGDH alone or Adflt-Luc plus Avastin.

(18). We tested whether flt-1-driven overexpression of 15-PGDH alone and in combination with Avastin could delay growth of LS174T colon tumor xenografts in athymic nude mice. Tumor therapy was initiated on day 0, which corresponded to 7 days after tumor cell injection. The baseline mean and SD for tumor volumes on day 0 was  $96.7 \pm 12.0$  mm<sup>3</sup>. The mean times to tumor doubling for the PBS, Adflt-Luc, Adflt-PGDH, and Adflt-Luc in combination with Avastin-treated groups were 5, 8, 21, and 25 days, respectively, in comparison with Adflt-PGDH plus Avastin, which produced prolonged delay of LS174T tumor growth (approximately 130% of original tumor size at day 32, the last day of the study; Fig. 4B). Moreover, in the group treated with Adflt-PGDH in combination with Avastin, two tumors underwent complete regression, in contrast to no regressions with PBS-, Adflt-Luc-, Adflt-PGDH-, or Adflt-Luc plus Avastin-treated groups. Comparisons of mean time to tumor doubling of the groups treated with Adflt-PGDH versus Adflt-Luc, Adflt-Luc in combination with Avastin versus Adflt-Luc, and Adflt-PGDH in combination with Avastin versus Adflt-PGDH or Adflt-Luc plus Avastin showed significant differences between the groups ( $P < 0.05$ ).

Immunohistochemical analysis of LS174T tumors stained with anti-CD31 antibody showed fewer brown-stained CD31-positive endothelial cells (Supplementary Fig. S2; supplementary materials) and reduced microvessel density 1.7-, 3.5-, and 6.7-fold ( $P < 0.05$ ) in a cross-section of tumor from mice treated with Adflt-PGDH ( $19.7 \pm 5.3$ ), Adflt-Luc plus Avastin ( $9.2 \pm 2.7$ ), and Adflt-PGDH plus Avastin ( $4.9 \pm 2.1$ ), respectively, compared with Adflt-Luc-treated tumors ( $32.8 \pm 5.9$ ), with PBS-treated tumors at  $36.1 \pm 6.7$ . These results show the antiangiogenesis efficacy of 15-PGDH therapy against tumor-associated angiogenic vasculature *in vivo*.

## Discussion

The inducible Cox-2 is strongly implicated in both breast and colon neoplasia. Clinical trials are being conducted to study the use of Cox-2 inhibitors alone and in combination with other agents in the neoadjuvant, adjuvant, and metastatic treatment settings (22, 23). Despite tremendous promise of anti-Cox-2 therapy using selective Cox-2 inhibitors, which were developed to provide an alternative with reduced gastrointestinal risk to the traditional nonsteroidal anti-inflammatory drugs, these have been associated with an increased incidence of major cardiovascular toxicity due to inhibition of Cox-2-mediated PGI<sub>2</sub> expression (24). Thus, their utilization for cancer prevention and therapy is currently questionable, and suggest that further development of novel selective agents with lower toxicity is needed. It is reasonable that inhibition of only PGE<sub>2</sub>, rather than global PG biosynthesis, would avoid some of the potential gastrointestinal and cardiovascular side effects.

Thus, clearly other novel therapy strategies are desperately needed to achieve adequate cancer therapy when the ineffectiveness of the existing treatment regimens is well

recognized. The application of gene therapy to neoplastic diseases offers a promising approach to achieve targeted antitumor intervention. Further to this end, gene therapies for cancer may be combined with standard modalities to accomplish synergistic anticancer effects. The Adcox-PGDH and Adflt-PGDH vectors have been developed to function as therapeutic gene delivery vehicles by incorporating an expression cassette containing the human *15-PGDH* transgene. The employment of tumor-specific promoters for *15-PGDH* gene expression would increase therapeutic efficacy and avoid adverse side effects of anti-cancer treatment.

In a comparative study, MCF 10A, normal established breast epithelial cells, and 2LMP breast cancer cells, derived by passage of MDA-MB-231 tumor cells in ascites of nude mice (reflecting the metastatic state; refs. 25–27), were tested for PGE<sub>2</sub> and VEGF protein expression, as well as EP1-4, Cox-2, and VEGFR1 mRNA expression. The results of *in vitro* studies show that the levels of elevated PGE<sub>2</sub> and VEGF secretion in 2LMP breast cancer cell culture media were associated with high levels of expression of PGE<sub>2</sub> receptors, Cox-2, and VEGFR1 in comparison with MCF 10A normal cells. The high levels of EP1-4, Cox-2, and VEGFR1 mRNA were detected using quantitative RT-PCR in 2LMP, MDA-MB-231, and LCC6 breast cancer cells in contrast to relatively lower *15-PGDH* mRNA expression.

Although the underlying molecular mechanisms of regulation of *15-PGDH* expression remain to be characterized in more detail, it was shown that the loss of *15-PGDH* expression in colon cancer is most likely due to the upregulation and binding of the transcriptional repressor Snail to E-boxes located in the *15-PGDH* promoter (28). Also, ZEB1 and Slug are downstream targets of the epidermal growth factor receptor signaling pathway through activated ERK, and these transcriptional repressors are important for the regulation of *15-PGDH* expression in a subset of non-small cell lung cancer cells (29). It was shown that upregulation of *15-PGDH* induced by nonsteroidal anti-inflammatory drugs has the potential to inhibit growth of human glioma, in part, by upregulation of p21 possibly independent from Cox-2 enzymatic function (30). Recent studies identified a tumor suppressor activity of *15-PGDH* in colon and bladder cancers, and suggested epigenetic silencing of the enzyme by DNA methylation and histone modification (19, 31). Low *15-PGDH* levels were shown in the poorly differentiated, Cox-2-expressing MDA-MB-231 breast cancer cells isolated from pleural effusions as well as in primary breast cancers. Decreased *15-PGDH* levels were correlated with unfavorable prognostic factors (32). Restoration of *15-PGDH* expression produced a delay in breast tumor growth (33).

The results of *in vitro* studies showed that Ad-mediated *15-PGDH* overexpression produced increased cytotoxicity and inhibited cancer cell migration in comparison with Adflt-Luc. It is becoming more obvious from preclinical and clinical studies that overexpression of Cox-2 and repression of *15-PGDH* provide complementary pathways to increase PGE<sub>2</sub> levels in tumor. Ad-mediated restoration of *15-PGDH* expression was associated with reduction of

PGE<sub>2</sub> and VEGF concentration in cultured media and decreased levels of Cox-2 and EP4 mRNA expression in colon cancer cells. Although the role of PGE<sub>2</sub> receptors in cancer biology remains complex, it was shown that expression of EP4 plays an important role in colon and breast cancer development (34–38). However, additional studies are required to investigate molecular mechanisms of the therapeutic effect of *15-PGDH* overexpression in cancer cells.

Future studies also need to establish precise mechanisms of regulation of tumor angiogenesis via Ad-mediated *15-PGDH* overexpression. The *in vitro* study showed that cell conditioned medium from Adcox-PGDH- or Adflt-PGDH-infected cancer cells inhibited migration of HUVEC and decreased proliferation of endothelial cells (data not shown) in comparison with Adflt-Luc. Importantly, it was shown that PGE<sub>2</sub> induces the expression of VEGF (14) and bFGF (15) in blood vessel endothelial cells (16). On the other hand, both VEGF and bFGF can induce PGE<sub>2</sub> expression in endothelial cells, which raises the possibility of amplification of PGE<sub>2</sub> regulation of angiogenesis through a positive feedback loop (17). Thus, decreased levels of PGE<sub>2</sub> and VEGF in cultured media from *15-PGDH*-overexpressed cancer cells can, in part, produce inhibition of tumor-associated microvasculature.

The animal therapy studies showed that Ad-mediated *15-PGDH* therapy of breast and colon tumor xenografts produced growth inhibition. Importantly, a significant delay in tumor growth was associated with a decreased number of CD31-positive cells in Adcox-PGDH- and Adflt-PGDH-injected tumor xenografts in comparison with Adflt-Luc. Data from experimental and clinical studies indicate that growth of most cancers is strongly angiogenesis-dependent. High levels of PGE<sub>2</sub> expression in breast and colon tumors are associated with increased angiogenesis. Overexpression of PGE<sub>2</sub> induces VEGF production in tumor cells, which is associated with robust expression of its cognate VEGFR1/flt-1 receptor and results in activation of tumor-associated angiogenesis and in development of self-sufficiency in growth signaling in highly aggressive metastatic breast tumors (39). The antiangiogenic drug Avastin is widely being used for treatment of patients with colon cancer. The antitumor effect of Adflt-PGDH-mediated therapy of colon tumor xenografts was significantly improved by coadministration of Avastin.

Notably, the most effective growth inhibition of both breast and colon tumor xenografts occurred when expression of the *15-PGDH* gene was driven by the *flt-1* promoter. VEGF and its cognate receptor VEGFR1/*flt-1* are frequently overexpressed in human malignancies, including breast and colon carcinomas. Recent studies in breast cancer show that high levels of VEGF production in tumor cells is often associated with robust expression of its cognate VEGFR1/*flt-1* receptor in tumor-associated blood microvessels and tumor cells (40). The precise molecular mechanisms of regulation of carcinogenesis by VEGFR1 remains uncertain; it is obvious, however, that *flt-1* overexpression plays a dual role through activation of tumor-associated angiogenesis and in development of self-sufficiency in growth signaling,

which is involved in development of highly aggressive breast cancer phenotypes (41).

It was shown that high levels of VEGF/VEGFR1 were associated with reduced survival in breast cancer patients (42). Expression of flt-1 in colon cancer cells has been recently associated with induction of cell motility and invasion (43). Recently, we have shown that flt-1-driven expression of the tumor necrosis factor-related apoptosis-inducing ligand suppressed *in vivo* growth of human prostate tumors (44). Importantly, expression of flt-1 is inducible and significantly enhanced under hypoxic conditions or under exposure to oxidative stress (45). Both hypoxia and oxidative stress are hallmarks of the tumor microenvironment, and this may explain tumor specificity of the flt-1 promoter. Expression of flt-1 has been observed in tumor cells, endothelial cells, and in tumor-infiltrated CD11b myeloid cells. Inflammatory tumor-infiltrated CD11b myeloid cells are involved in both regulation of antitumor immune response and tumor-associated neovasculation. These cells have been shown to support angiogenesis *via* secretion of MMP-9 and VEGF (46). Interestingly, flt-1-driven expression of the 15-PGDH gene in the tumor microenvironment promotes significant reduction of VEGF secretion by tumor-infiltrated CD11b myeloid cells and also stimulates the antitumor immune response in immunocompetent mice bearing established syngeneic murine colon tumors (47).

Collectively, our data show that Ad-driven expression of 15-PGDH promotes inhibition of breast and colon cancer cell growth. The 15-PGDH-mediated anticancer effect is associated with reduction of PGE<sub>2</sub> and VEGF levels and decreased Cox-2 and PGE<sub>2</sub> receptor EP4 mRNA expression. The results of experimental therapy studies show that administration of Adcox-PGDH and Adflt-PGDH into mice with established tumors resulted in delay of tumor growth. The results of *in vivo* studies reveal that the inhibitory effect of 15-PGDH therapy on tumor growth was associated with the suppression of tumor-associated angiogenesis. Our studies show that the antitumor effect can be further enhanced when 15-PGDH therapy was combined with anti-VEGF mAb. These results suggest that 15-PGDH-mediated regulation of PGE<sub>2</sub> catabolism in the tumor microenvironment represents a novel approach for therapy of breast and colon cancer.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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