

Cytochrome P450 2J2 Promotes the Neoplastic Phenotype of Carcinoma Cells and Is Up-regulated in Human Tumors

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Abstract

Cytochrome P450 (CYP) arachidonic acid epoxygenase 2J2 converts arachidonic acid to four regioisomeric epoxyeicosatrienoic acids, which exert diverse biological activities in cardiovascular system and endothelial cells. However, it is unknown whether this enzyme highly expresses and plays any role in cancer. In this study, we found that very strong and selective CYP2J2 expression was detected in human carcinoma tissues in 101 of 130 patients (77%) as well as eight human carcinoma cell lines but undetectable in adjacent normal tissues and nontumoric human cell lines by Western, reverse transcription-PCR, and immunohistochemical staining. In addition, forced overexpression of CYP2J2, and CYP BM3F87V or addition of epoxyeicosatrienoic acids (EET) in cultured carcinoma cell lines *in vitro* markedly accelerated proliferation by analyses of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, cell accounts, and cell cycle analysis, and protected carcinoma cells from apoptosis induced by tumor necrosis factor α (TNF- α) in cultures. In contrast, antisense 2J2 transfection or addition of epoxygenase inhibitors 17-ODYA inhibited proliferation and accelerated cell apoptosis induced by TNF- α . Examination of signaling pathways on the effects of CYP2J2 and EETs revealed activation of mitogen-activated protein kinases and PI3 kinase-AKT systems and elevation of epithelial growth factor receptor phosphorylation level. These results strongly suggest that CYP epoxygenase 2J2 plays a previously unknown role in promotion of the neoplastic cellular phenotype and in the pathogenesis of a variety of human cancers. (Cancer Res 2005; 65(11): 4707-15)

Introduction

Release of arachidonic acid from the cell membrane by activated phospholipases renders it accessible for metabolism by several enzymes involved in the biosynthesis of eicosanoids (1, 2). These include prostaglandin endoperoxide H synthases (cyclooxygenases) that lead to generation of prostaglandins and thromboxane; lipoxygenases that lead to generation of leukotrienes, lipoxins, and hydroxyeicosatetraenoic acids (HETE); and a host of cytochromes P450 (P450) that produces HETEs and epoxyeicosatrienoic acids (EET). Changes in the expression level and distribution pattern of enzymes involved in eicosanoid biosynthesis

may have profound effects on tissue homeostasis and may contribute to the pathogenesis of numerous diseases. This may be especially relevant in carcinogenesis, where alterations of the expression and activities of cyclooxygenases and/or lipoxygenases have been implicated in a variety of human and rodent cancers (3–5). However, whereas considerable attention has been afforded to the roles of cyclooxygenase- and lipoxygenase-derived eicosanoids in carcinogenesis, relatively little data is available regarding the potential roles of P450-derived eicosanoids in this process.

Epoxygenation of arachidonic acid by multiple mammalian P450s results in the generation of four regioisomeric EETs (5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET), although differences in the catalytic efficiencies of individual P450 isoforms results in different EET profiles for each (6, 7). Cytochrome P450 2J2 (CYP2J2), a predominant human P450 arachidonic acid epoxygenase that generates all four EETs (8), has been detected at the mRNA and/or protein level in heart, lung, liver, kidney, ileum, jejunum, and colon (8). More recently, immunohistochemical analysis has confirmed CYP2J2 expression in these organs and also identified its presence in the stomach, pancreas, and pituitary gland (9). Importantly, P450-derived EETs have been implicated in a variety of physiologic processes that are relevant to cancer pathogenesis, including regulation of intracellular signaling pathways, gene expression, cellular proliferation, and inflammation, among others (10). However, the effects of EETs on these processes in relation to carcinogenesis have not been directly assessed.

The present study examined the potential involvement of CYP2J2 and its EET products in a host of processes important to cancer cell behavior and tumor pathogenesis. Using a variety of experimental approaches, we show that CYP2J2 and EETs are positive regulators of carcinoma cell proliferation and that they inhibit apoptosis. Furthermore, we show that CYP2J2 promotes tumor formation in an *in vivo* murine xenograft model and that its expression is elevated in established human carcinoma cell lines and in an assortment of human tumor samples. These observations suggest that CYP2J2 promotes the neoplastic phenotype of carcinoma cells and may represent a novel biomarker and potential target for therapy of human cancers.

Materials and Methods

Materials. Chemicals and reagents were obtained as follows: Trizol reagent and cell culture medium from Life Technologies (Eggenstein, Germany); reverse transcription-PCR (RT-PCR) kit from Takara Bio Co. Ltd. (Dalian, China); antibodies against epidermal growth factor receptor (EGFR), p-EGFR, phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), p-MAPK, Bcl-2, Bcl-xL, Bax, and nuclear factor κ B (NF- κ B) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); antibodies against AKT and p-AKT from Cell Signaling Technology (Beverly, MA);

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antibody against β -actin from Neomarkers (Fremont, CA); horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit immunoglobulin G and rabbit anti-mouse immunoglobulin G) from KPL (Gaithersburg, MD); enhanced chemiluminescence reagents from Pierce, Inc. (Rockford, IL); polyvinylidene difluoride (PVDF) membranes, prestained protein markers, and SDS-PAGE gels from Bio-Rad, Inc. (Hercules, CA); PD98059, apigenin, 17-ODYA, H7, and LY294002 from Cayman Chemical Co. (Ann Arbor, MI); 8,9-EET, 11,12-EET, and 14,15-EET from Sigma Chemical Co. (St. Louis, MO). Polyclonal antibodies against CYP2J2 were developed as described (11) and showed no cross-reactivity with other P450 isoforms. Antibody against CYP102 F87V was used as described (12). All other reagents were purchased from standard commercial suppliers.

Cell culture and cell viability and proliferation assays. Tca-8113, A549, HepG2, Ncl-H446, LS-174, SiHa, U251, ScaBER, HEK293, and HT1080 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM with 10% fetal bovine serum (FBS) at 37°C in a 95% air/5% CO₂ atmosphere at constant humidity. Cells were grown to 60% confluence, the medium was removed, and the cells were washed thrice with PBS and incubated with FCS-free DMEM at 37°C for 12 hours to allow for synchronization. Direct addition of 8,9-EET, 11,12-EET, or 14,15-EET (100 nmol/L) into the medium, or infection of cells with rAAV-CYP2J2, rAAV-CYP102 F87V, rAAV-antiCYP2J2, or rAAV-GFP (packed and purified as described previously, ref. 12; ~100 virions per cell) was done. For the construction of rAAV-antiCYP2J2, CYP2J2 cDNA was subcloned into rAAV plasmid pUF1 in antisense way and resultant plasmid was used for rAAV-antiCYP2J2 package as described above. Twelve hours after treatment with EETs or 1 week after infection, cells were harvested for cell number counts under a light microscope, for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cell viability to reflect cell proliferation as described previously (13, 14), and for determination of CYP102 F87V and CYP2J2 protein expression by Western blot analysis.

To study the effect of inhibitors of P450 epoxygenase activity (17-ODYA, 50 μ mol/L), PI3K (LY294002, 10 μ mol/L), MAPK (apigenin, 25 μ mol/L), MAPK kinase (MEK, PD98059, 50 μ mol/L), and protein kinase C (PKC, H7, 10 μ mol/L), cells were allowed to attach overnight, and medium was changed to 1% fetal bovine serum the following day. Individual inhibitors were added directly to the cell medium and incubated for 2 hours. Individual EETs were added and cells assayed 12 hours later for viability and proliferation, as described above. Ethanol (0.25%) was used as a vehicle for all compounds.

Determination of 14,15-dihydroxyeicosatrienoic acid. For measurement of EETs and dihydroxyeicosatrienoic acid in the cultured cells, the cells infected with the different rAAVs for 7 days in 150-mm dishes were scraped in cold PBS (pH 7.2) with triphenylphosphine after washing with PBS and then homogenized and sonicated over ice. Eicosanoids were extracted from the cell homogenates thrice with ethyl acetate after acidification with acetic acid. After evaporation, the samples were dissolved in *N,N*-dimethylformamide (AMRESCO, Solon, OH) and concentrations of the stable EET metabolite 14,15-dihydroxyeicosatrienoic acid (DHET) was determined by an ELISA kit (Detroit R&D, Detroit, MI) according to the manufacturer's instructions.

Determination of apoptosis and cell cycle by flow cytometry. Cells infected with rAAV-GFP, rAAV-CYP2J2, rAAV-antiCYP2J2, or rAAV-CYP102 F87V were studied 1 week following infection, whereas cells treated with individual EETs (100 nmol/L) were studied 12 hours following their addition. In both cases, cells (6×10^5) were plated in 6-well plates in serum-containing medium and treated with tumor necrosis factor α (TNF- α , 50 ng/mL) for 12 hours to induce apoptosis. Cells were then harvested with trypsin/EDTA, resuspended in binding buffer [10 mmol/L HEPES/NaOH (pH 7.4), 140 mmol/L NaCl, and 2.5 mmol/L CaCl₂], and incubated with FITC-conjugated Annexin V and 1 μ g/mL propidium iodide (Annexin V-FITC kit; Bender MedSystems, San Bruno, CA), according to the manufacturer's protocol. Cells were then analyzed with a FACStar-Plus flow cytometer (Becton Dickinson, Franklin Lakes, NJ). To exclude necrotic cells, only cells that were Annexin V positive and propidium iodide negative were counted for early stages of apoptosis.

For analysis of cell cycle distribution, cells (1.5×10^6) were cultured as above for 12 hours, were fixed with 70% ethanol, and incubated with phosphate-citrate buffer (4 mmol/L citric acid and 192 mmol/L Na₂HPO₄) for 30 minutes at room temperature. After centrifugation, cell pellets were resuspended in PBS containing propidium iodide and RNase (10 μ g/mL each) and incubated for 20 minutes at room temperature. Quantification of sub-G₁ DNA content was determined using the CELLQuest program.

For determination of expression levels of apoptosis-related proteins, cells cultured and treated as above were harvested and lysed. Expression levels of NF- κ B, Bcl-2, Bcl-xL, and Bax proteins in cell lysates were quantified by Western blotting.

Murine xenograft model of tumor growth. All animal studies were approved by the Animal Research Committee of Tongji College and were done according to the guidelines of the NIH. Athymic BALB/C mice, 3 to 4 weeks of age, were housed on a 12-hour light/dark cycle and allowed *ad libitum* access to food and water. Mice were randomly divided into five groups before receiving cell transplants. Tca-8113 cells were infected with rAAV-GFP, rAAV-antiCYP2J2, rAAV-CYP2J2, or rAAV-CYP102 F87V and left for 4 days. Cells were washed twice with $1 \times$ PBS, trypsinized, and collected in MEM supplemented with FBS. Cell counts were determined using a hemocytometer and the cells were diluted to 1×10^{10} cells/L. Mice were injected s.c. with 500 μ L of one of the cell suspensions (5×10^6 cells per site). As a control, one group of mice was injected with uninfected Tca-8113 cells. Mice were monitored daily for 32 days, and tumors were measured once every 5 days (beginning 7 days after injection) with microcalipers. Tumor volumes were calculated as length \times width² \times 0.5236. At the end of the experiment (day 32), mice were sacrificed by sodium pentobarbital overdose (90 mg/kg i.p.) and the tumors dissected, weighed, and snap-frozen.

Tissue procurement and analysis of CYP2J2 expression. After obtaining informed consent, tissue samples from 130 patients with various tumors were collected during surgical procedures at Tongji Hospital. All human research protocols were approved by the Clinical Research Committees of Tongji Medical College and were carried out according to the guidelines of the NIH. Tissue samples included 35 esophageal carcinomas (31 esophageal squamous cell carcinomas and 4 esophageal adenocarcinomas), 71 lung carcinomas (37 squamous cell carcinomas, 26 adenocarcinomas, 8 small cell pulmonary carcinomas), 5 breast carcinomas, 5 stomach carcinomas, 10 liver carcinomas, and 4 colon adenocarcinomas. Eighty-seven patients were men and 43 were women, ages 54 ± 9 years (range, 32-75). Both tumor tissue and surrounding normal tissue were obtained from each patient. A piece from each tissue sample was immediately frozen in liquid nitrogen and stored at -80°C . Remaining tissue samples were fixed in 10% neutral buffered formalin, processed

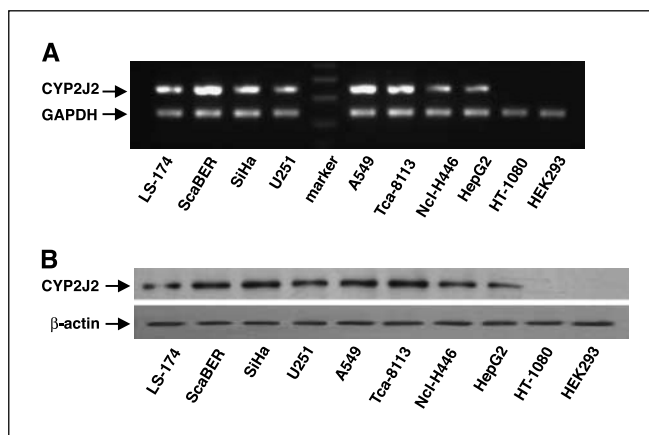


Figure 1. CYP2J2 expression is increased in human carcinoma (LS-174, ScaBER, SiHa, U251, A549, Tca-8113, Ncl-H446, and HepG2) versus noncarcinoma (HT-1080 and HEK293) cell lines. *A*, CYP2J2 mRNA levels detected by RT-PCR. *B*, CYP2J2 protein expression detected by Western blot.

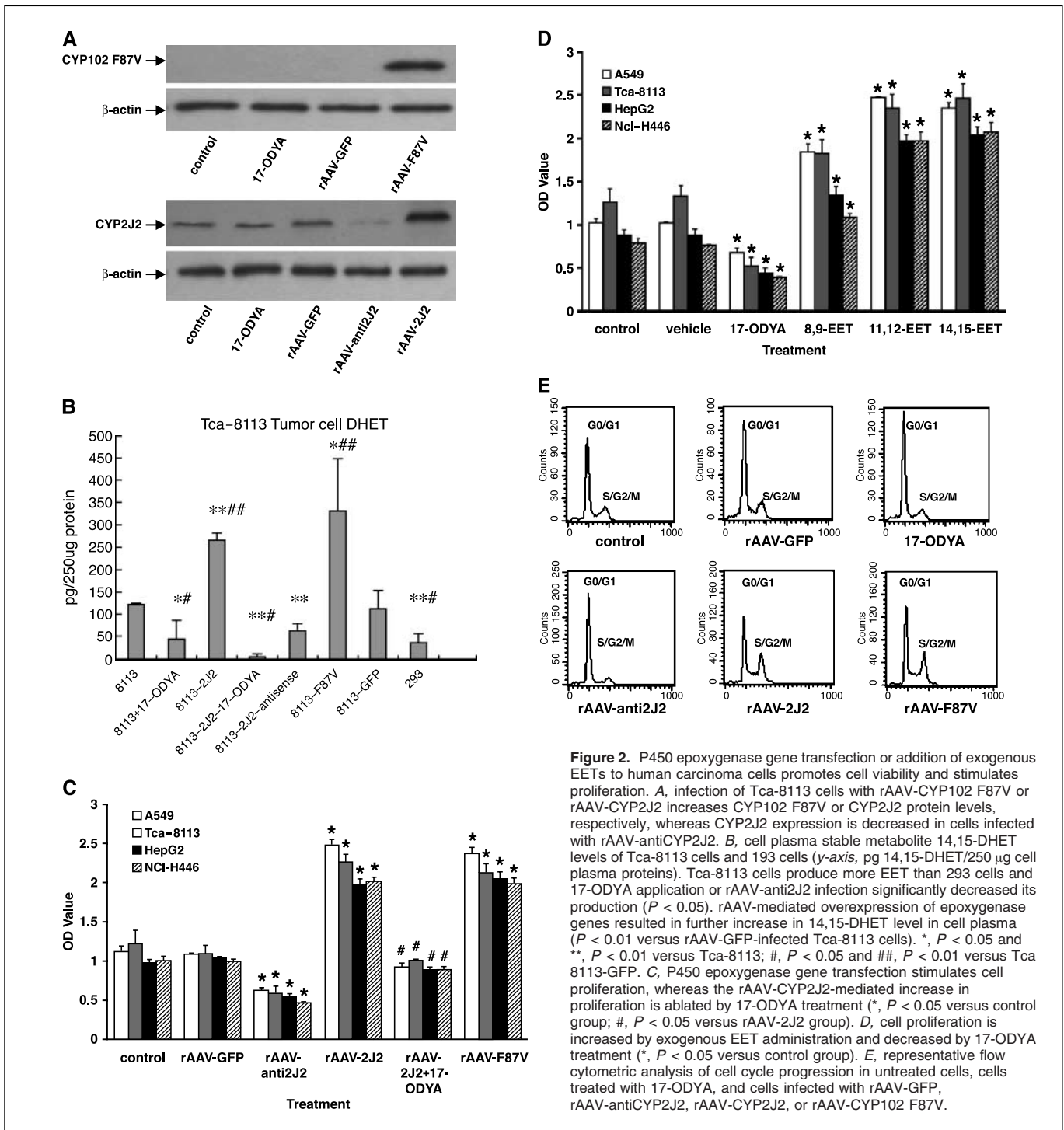


Figure 2. P450 epoxygenase gene transfection or addition of exogenous EETs to human carcinoma cells promotes cell viability and stimulates proliferation. **A**, infection of Tca-8113 cells with rAAV-CYP102 F87V or rAAV-CYP2J2 increases CYP102 F87V or CYP2J2 protein levels, respectively, whereas CYP2J2 expression is decreased in cells infected with rAAV-antiCYP2J2. **B**, cell plasma stable metabolite 14,15-DHET levels of Tca-8113 cells and 193 cells (*y*-axis, pg 14,15-DHET/250 μg cell plasma proteins). Tca-8113 cells produce more EET than 293 cells and 17-ODYA application or rAAV-anti2J2 infection significantly decreased its production ($P < 0.05$). rAAV-mediated overexpression of epoxygenase genes resulted in further increase in 14,15-DHET level in cell plasma ($P < 0.01$ versus rAAV-GFP-infected Tca-8113 cells). *, $P < 0.05$ and **, $P < 0.01$ versus Tca-8113; #, $P < 0.05$ and ##, $P < 0.01$ versus Tca 8113-GFP. **C**, P450 epoxygenase gene transfection stimulates cell proliferation, whereas the rAAV-CYP2J2-mediated increase in proliferation is ablated by 17-ODYA treatment (*, $P < 0.05$ versus control group; #, $P < 0.05$ versus rAAV-2J2 group). **D**, cell proliferation is increased by exogenous EET administration and decreased by 17-ODYA treatment (*, $P < 0.05$ versus control group). **E**, representative flow cytometric analysis of cell cycle progression in untreated cells, cells treated with 17-ODYA, and cells infected with rAAV-GFP, rAAV-antiCYP2J2, rAAV-CYP2J2, or rAAV-CYP102 F87V.

through graded ethanol solutions, embedded in paraffin, and immunostained for CYP2J2. Paired nontumor and tumor tissues were further examined by RT-PCR and Western blot analysis for CYP2J2.

Analysis of CYP2J2 expression by reverse transcription-PCR and Western blot. Total RNA and protein were isolated from frozen tissue samples or from cell cultures with Trizol reagent. Semiquantitative analysis of the expression of CYP2J2 mRNA was done using a multiplex RT-PCR technique. Expression of mRNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. One microgram of total RNA was reverse-transcribed using the Takara Bio

RT-PCR kit according to the manufacturer's protocol. PCR was done in a 25-μL reaction mixture containing 5 μL of cDNA template, 1× PCR buffer, 1.5 mmol/L MgCl₂, 0.8 mmol/L deoxynucleotide triphosphates, 1 unit of Taq DNA polymerase, and 100 nmol/L of each primer for CYP2J2 (sense primer, 5'-TATCATGCTCGGGCGATGG-3'; antisense primer, 5'-AAGCG-TTCTCCGAAGGTG-3') or for GAPDH (sense primer, 5'-CCTTGCCTC-CAGACAATGC-3'; antisense primer, 5'-CCACGACATACCTCAGCAC-3'). Expected amplicon sizes were 597 bp for CYP2J2 and 340 bp for GAPDH. The conditions for PCR were one cycle of denaturation at 94°C for 5 minutes followed by 35 cycles of 94°C for 45 seconds, 56°C for 45 seconds,

and 72°C for 1 minute, with a final extension at 72°C for 7 minutes. PCR products were resolved in 1.5% agarose gels stained with ethidium bromide. The relative intensity of CYP2J2 compared with GAPDH was calculated for each sample by densitometry.

Protein concentrations of tissue and cell extracts were determined by the Bradford method (Bio-Rad). Twenty micrograms of protein extracted from each sample were subjected to electrophoresis in 8% polyacrylamide slab gels. After transfer to PVDF membrane and blocking with 5% nonfat milk powder, blots were probed with the CYP2J2 antibody (1:750), followed by incubation with HRP-conjugated secondary antibody (rabbit anti-goat, 1:800). CYP2J2 proteins were visualized by enhanced chemiluminescence and quantified by densitometry.

CYP2J2 immunohistochemistry. Four-micrometer-thick tissue sections were cut, deparaffinized in xylene, and rehydrated. Following heat-mediated antigen retrieval, slides were incubated with the CYP2J2 antibody (final concentration, 5 µg/mL) for 1 hour at room temperature. Slides were washed thrice (15 minutes each) with PBS at room temperature followed by incubation with secondary antibody (rabbit anti-goat, 1:400 dilution) for 1 hour at room temperature. Following three more washes with PBS (15 minutes each) at room temperature, immunostaining was visualized with diaminobenzidine (Sigma) for 3 to 5 minutes. For positive controls, sections of colon cancer expressing the CYP2J2 protein were included in each staining procedure. For negative controls, nonimmunized rabbit immunoglobulin G or TBS was substituted for the primary antibody to distinguish false-positive responses from nonspecific binding to immunoglobulin G or the secondary antibody. Additionally, preabsorbed antibody with an excess amount of recombinant CYP2J2 protein abolished the staining. For each sample, the staining procedure was repeated a second time to confirm results.

Statistical analysis. Data are presented as group means ± SE. *In vitro* experiments were done a total of four to six times, and each experiment was done with triplicate replications of each treatment group. Statistical comparisons among groups were done by the Wilcoxon test, the Student's *t* test, or ANOVA as appropriate. In all cases, statistical significance was defined as $P < 0.05$.

Results

P450 epoxygenases and epoxyicosatrienoic acids stimulate proliferation. We examined a variety of established human carcinoma cell lines (LS-174, ScaBER, SiHa, U251, A549, Tca-8113, Ncl-H446, and HepG2) and two human noncarcinoma cell lines (HT-1080 and HEK293) for expression of CYP2J2 mRNA and protein. Expression of both mRNA and protein were observed in all eight tumor cell lines, albeit to varying degrees of intensity, whereas no expression was detectable in the nontumor cell lines (Fig. 1A and B). Based on these observations, we surmised that CYP2J2 may contribute to the neoplastic phenotype of carcinoma cells. Thus, we next determined the effects of CYP2J2 overexpression and exogenous EET administration in a subset of these cell lines.

Four of the carcinoma cell lines (Tca-8113, A549, Ncl-H446, and HepG2), which differed in expression levels of CYP2J2, were selected for further study. Cells were infected with a recombinant adeno-associated virus vector containing the CYP2J2 cDNA in a sense direction (rAAV-CYP2J2), the CYP2J2 cDNA in an antisense direction (rAAV-antiCYP2J2), the CYP102 F87V cDNA

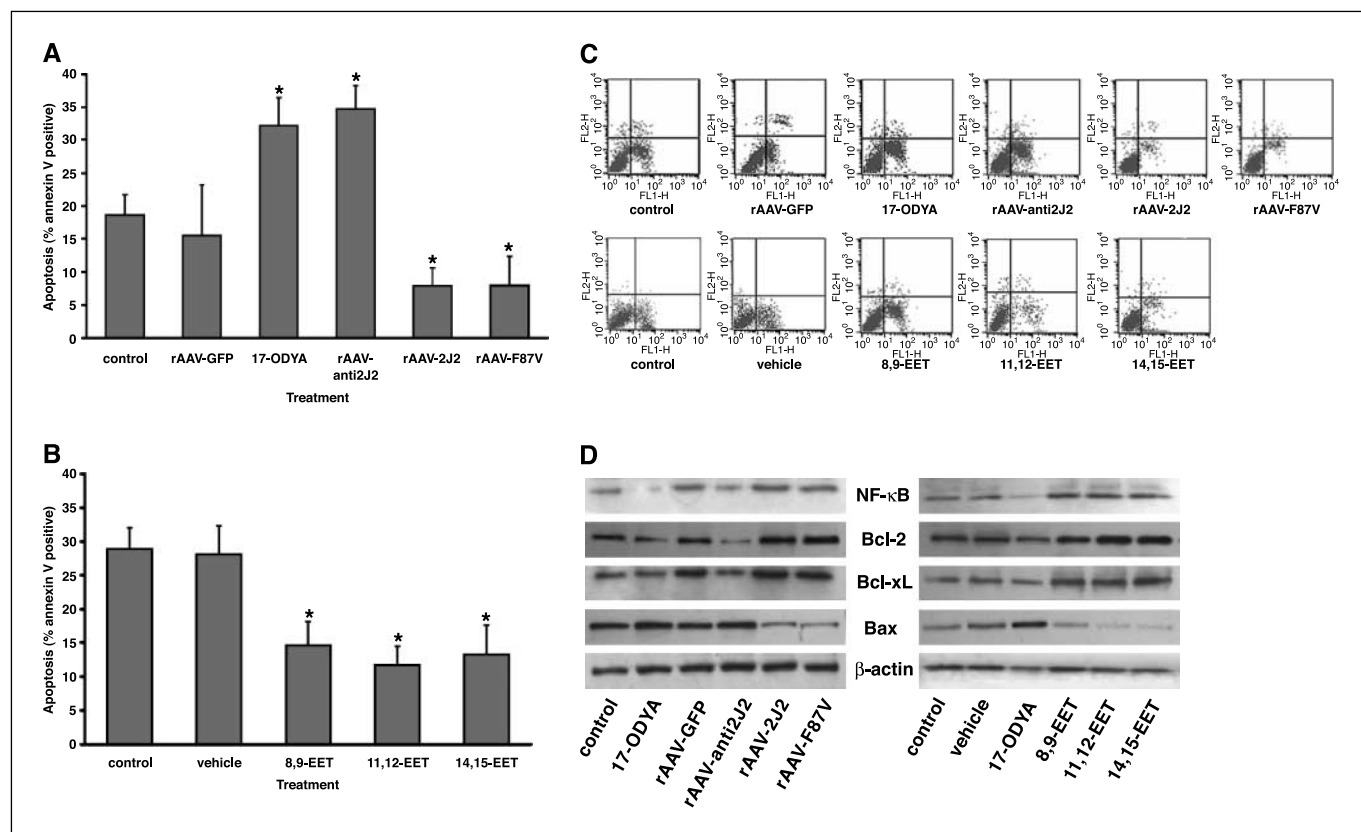


Figure 3. P450 epoxygenase gene transfection or addition of exogenous EETs to human carcinoma cells decreases apoptosis induced by TNF- α . *A*, apoptosis of Tca-8113 cells is decreased by P450 epoxygenase gene transfection and increased by 17-ODYA treatment or rAAV-antiCYP2J2 infection (*, $P < 0.05$ versus control group). *B*, exogenous administration of EETs decreases apoptosis (*, $P < 0.05$ versus control group). *C*, representative scatterplots wherein the lower right quadrant of each scatterplot are Annexin V-positive (apoptotic) cells. *D*, representative Western blots showing altered levels of NF- κ B, Bcl-2, Bcl-xL, and Bax following P450 epoxygenase gene transfection or exogenous administration of EETs.

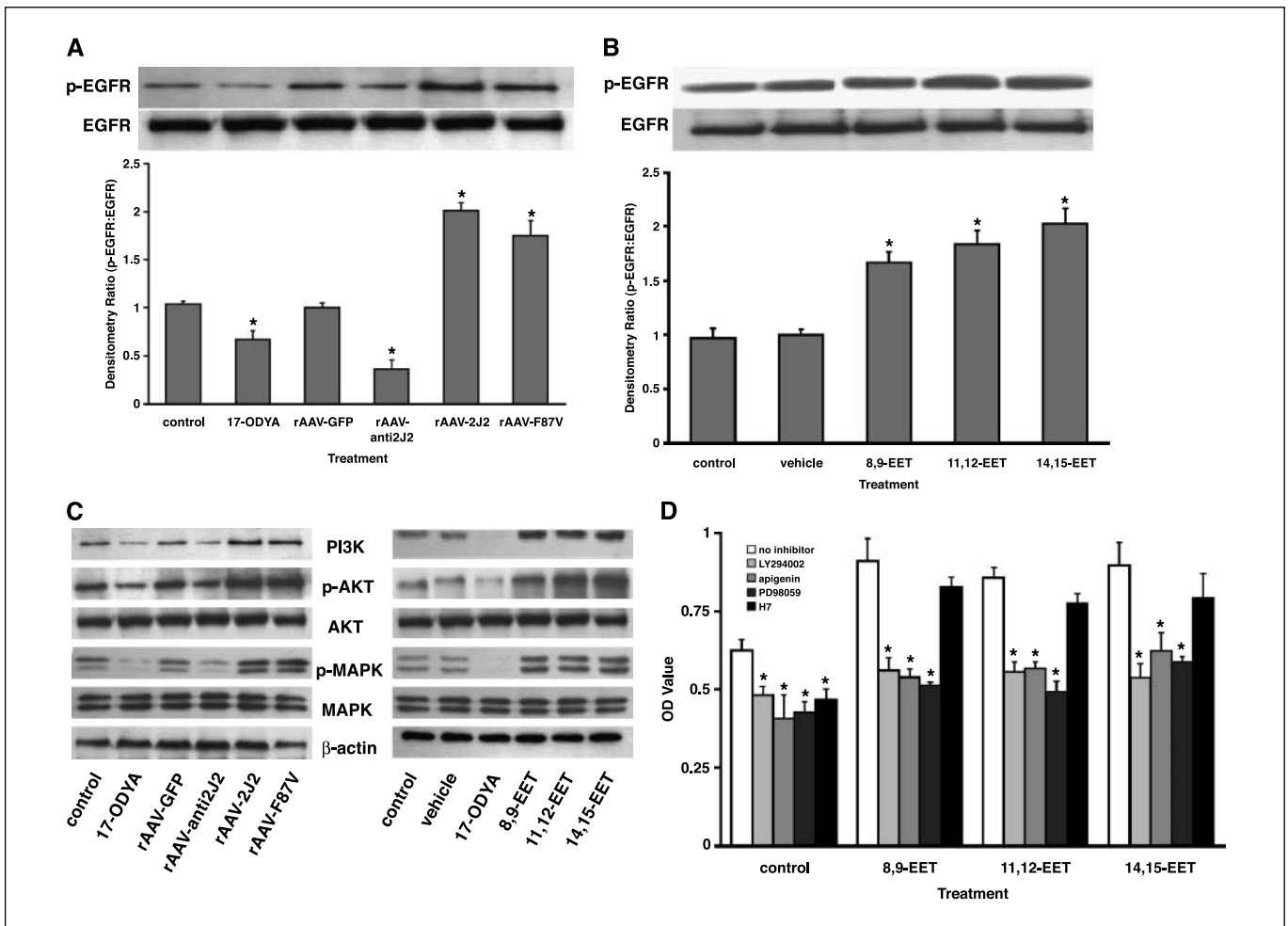


Figure 4. Effects of P450 epoxygenase gene transfection or exogenous EET administration on the EGF signaling pathway in human carcinoma cells. *A*, increased phosphorylation of the EGFR in Tca-8113 carcinoma cells following P450 epoxygenase gene transfection (*, $P < 0.05$ versus control group). *B*, increased phosphorylation of the EGFR in Tca-8113 carcinoma cells following exogenous administration of EETs (*, $P < 0.05$ versus control group). *C*, representative Western blots showing alteration of intracellular PI3K, AKT, and MAPK signaling pathways following P450 epoxygenase gene transfection or exogenous administration of EETs. *D*, cellular proliferation is stimulated by EETs and is decreased by inhibitors of PI3K (LY294002), MAPK (apigenin), and MEK (PD98059) but not of PKC (H7) (*, $P < 0.05$ versus no inhibitor treatment).

(rAAV-CYP102 F87V), or the GFP cDNA (rAAV-GFP) at ~100 virus particles per cell. The cells were examined for gene expression 1 week later. In all four cell lines, expression of CYP102 F87V (a mutated P450 that generates 14*S*,15*R*-EET almost exclusively (15) and CYP2J2 were increased by rAAV-CYP102 F87V or rAAV-CYP2J2 infection, respectively, whereas CYP2J2 expression was decreased by infection with rAAV-antiCYP2J2 (representative results for Tca-8113 cells are shown in Fig. 2A). To further investigate the functional role of the CYP2J2 and CYPF87V in the cancer cells, we measured the concentrations of the major CYP2J2- and F87V-dependent epoxidation product from arachidonic acid. Given the instability of the EETs, we determined concentration of the stable metabolite 14,15-DHET after extraction from the cell homogenates. The results showed that DHET concentrations were significantly higher in cancer cell lines than 293 cells (122.33 ± 3.51 versus 33.33 pg/250 g protein), epoxygenase inhibitor application of 17-ODYA and rAAV-anti-2J2 infection dramatically decreases production of the metabolite, but infection and overexpression of rAAV-2J2 and rAAV-F87V further increased

the DHET levels (130.33 ± 48.40 versus 266.66 ± 16.50 in rAAV-2J2 infected cells, 332.33 ± 117.01 in rAAV-F87V infected cells, and 256.33 ± 26.26 pg/250 g protein, $P < 0.01$; Fig. 2B).

Cell proliferation, determined by the MTT assay, was decreased by rAAV-antiCYP2J2 infection (Fig. 2C). Conversely, infection with rAAV-CYP2J2 or rAAV-CYP102 F87V increased proliferation, whereas administration of the epoxygenase inhibitor 17-ODYA reduced the rAAV-CYP2J2-mediated increase of proliferation to control levels (Fig. 2C). Proliferation was also increased by exogenous administration of EETs for 12 hours (Fig. 2D), whereas administration of 17-ODYA alone decreased proliferation (Fig. 2D). The effects of epoxygenases on cell cycle progression in Tca-8113 cells were assessed using flow cytometry. One week after infection with rAAV-CYP2J2, rAAV-antiCYP2J2, rAAV-CYP102 F87V, or rAAV-GFP, the cells were stained with propidium iodide for analysis. Infection with rAAV-CYP2J2 or rAAV-CYP102 F87V increased the percentage of cells in S-G₂-M phase (63.77% and 62.38%, respectively) compared with uninfected, rAAV-GFP-infected, or rAAV-antiCYP2J2-infected cells (30.37%, 29.9%, and 13.07%, respectively; Fig. 2E). Correspondingly, rAAV-CYP2J2 or

rAAV-CYP102 F87V reduced the percentage of cells in G₀-G₁ phase (36.26% and 37.62%, respectively) compared with uninfected, rAAV-GFP-infected, or rAAV-antiCYP2J2-infected cells (69.63%, 70.1%, and 86.93%, respectively; Fig. 2E). Collectively, these results suggest that P450 epoxygenases and their EET products promote viability and stimulate proliferation of carcinoma cells.

P450 epoxygenases and epoxyeicosatrienoic acids protect human carcinoma cells from apoptosis induced by tumor necrosis factor- α . Given the observed effects on carcinoma cell proliferation, we next determined whether P450 epoxygenases and EETs altered apoptosis, an event known to be dysregulated in neoplastic cells. As a representative of the carcinoma cell lines, Tca-8113 cells that were infected with rAAV-CYP2J2, rAAV-antiCYP2J2, rAAV-CYP102 F87V, or rAAV-GFP 1 week prior, or that were incubated with EETs for 12 hours, were stimulated with TNF- α and analyzed by flow cytometry. Apoptosis, as assessed by Annexin V staining, was reduced in cells infected with rAAV-CYP2J2 or rAAV-CYP102 F87V and increased in rAAV-antiCYP2J2-infected or 17-ODYA-treated cells (Fig. 3A). All three EETs also resulted in decreased apoptosis following TNF- stimulation (Fig. 3B). Representative flow cytometry scatterplots from all treatment groups are shown in Fig. 3C. Western blot analyses revealed that infection of cells with rAAV-CYP2J2 or rAAV-CYP102 F87V, or addition of EETs to the cell media, increased levels of NF- κ B and of the antiapoptotic proteins Bcl-2 and Bcl-xL, but decreased levels of the proapoptotic protein Bax (Fig. 3D). Together, these observations indicate that P450 epoxygenases and EETs protect carcinoma cells from apoptosis induced by TNF- α through regulatory effects on proapoptotic and antiapoptotic protein expression.

P450 epoxygenases and epoxyeicosatrienoic acids lead to phosphorylation of epidermal growth factor receptor and activate downstream PI3 κ and mitogen-activated protein kinase signaling pathways in human carcinoma cells. The aberrant regulation of various growth factors and their signaling pathways, and the resultant effects on cell survival and proliferation, is recognized as playing an important role in the progression of many cancers. Therefore, using Tca-8113 cells as a representative carcinoma cell line, we investigated the influence of P450 epoxygenases and EETs on the expression of a variety of growth factors including EGF, vascular endothelial growth factor, insulin-like growth factor, and transforming growth factor- α . Protein expression levels of all of the growth factors examined were not altered by P450 epoxygenase gene transfection or by EET exposure (data not shown), nor were receptor levels for these growth factors changed by any of the experimental protocols. However, whereas EGFR levels were not altered, phosphorylated EGFR was increased by infection with rAAV-CYP2J2 or rAAV-CYP102 F87V and by exposure to EETs (Fig. 4A and B). Conversely, rAAV-antiCYP2J2 infection or treatment with 17-ODYA decreased EGFR phosphorylation. These data are consistent with the concept that EGFR activation plays a critical role in maintaining the neoplastic phenotype of cancer cells (16). We then examined the status of the downstream PI3K/AKT and MAPK signaling pathways, as activation of these has also been suggested to promote maintenance of neoplastic cell stability (17, 18). We observed a similar profile as above, in that P450 epoxygenase gene transfection or EET exposure increased levels of PI3K and of phosphorylated AKT and MAPK, whereas rAAV-antiCYP2J2 and 17-ODYA decreased these levels (Fig. 4C). Thus, consistent with the effects on cell proliferation and apoptosis

documented earlier, P450 epoxygenases and EETs seem to exert positive regulatory effects on intracellular signaling pathways important to these processes in carcinoma cells.

To examine the importance of activation of these signaling pathways in EET-induced enhancement of cell survival and proliferation, the MTT proliferation assay was done with cells incubated with individual EETs in the presence or absence of inhibitors of PI3K, MAPK, MEK, and PKC. With the exception of the PKC inhibitor, all inhibitors tested decreased the proliferative effect of EETs on Tca-8113 carcinoma cells (Fig. 4D), indicative of an important role for these intracellular signaling pathways in the positive regulation of cell proliferation by EETs.

P450 epoxygenases enhance murine xenograft tumor formation and growth. Having established that P450 epoxygenases and EETs exert important regulatory effects on carcinoma cells *in vitro*, the potential influence of CYP2J2 on tumor formation was assessed in an *in vivo* model. Tca-8113 cells were infected with rAAV-CYP2J2, rAAV-antiCYP2J2, rAAV-CYP102 F87V, or rAAV-GFP. Four days later, athymic BALB/C mice were injected s.c. with cells from one of the transfection protocols (5×10^6 cells per mouse) or with uninfected Tca-8113 cells as a control. A significant enhancement of tumor growth rate and size was observed in mice injected with either rAAV-CYP2J2- or rAAV-CYP102 F87V-infected cells, with tumors appearing earlier (day 6 versus day 8.5 in control and rAAV-GFP groups) and having greater volume than those in the other groups (Fig. 5A and B). In contrast, tumors in mice injected with rAAV-antiCYP2J2-infected cells appeared later

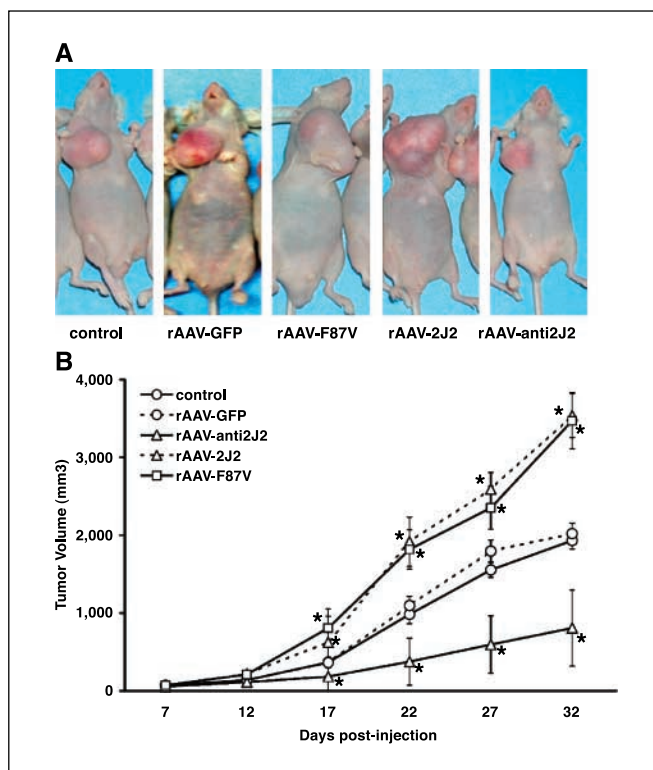


Figure 5. Enhancement of *in vivo* tumor formation by P450 epoxygenase gene transfection. **A**, representative examples of tumors 32 days following s.c. injection of control or transfected carcinoma cells shows increased tumor size in P450 epoxygenase-transfected groups and decreased tumor size in the rAAV-antiCYP2J2 group. **B**, tumor volumes in mice injected with control or transfected carcinoma cells (*, $P < 0.05$ versus control and rAAV-GFP groups).

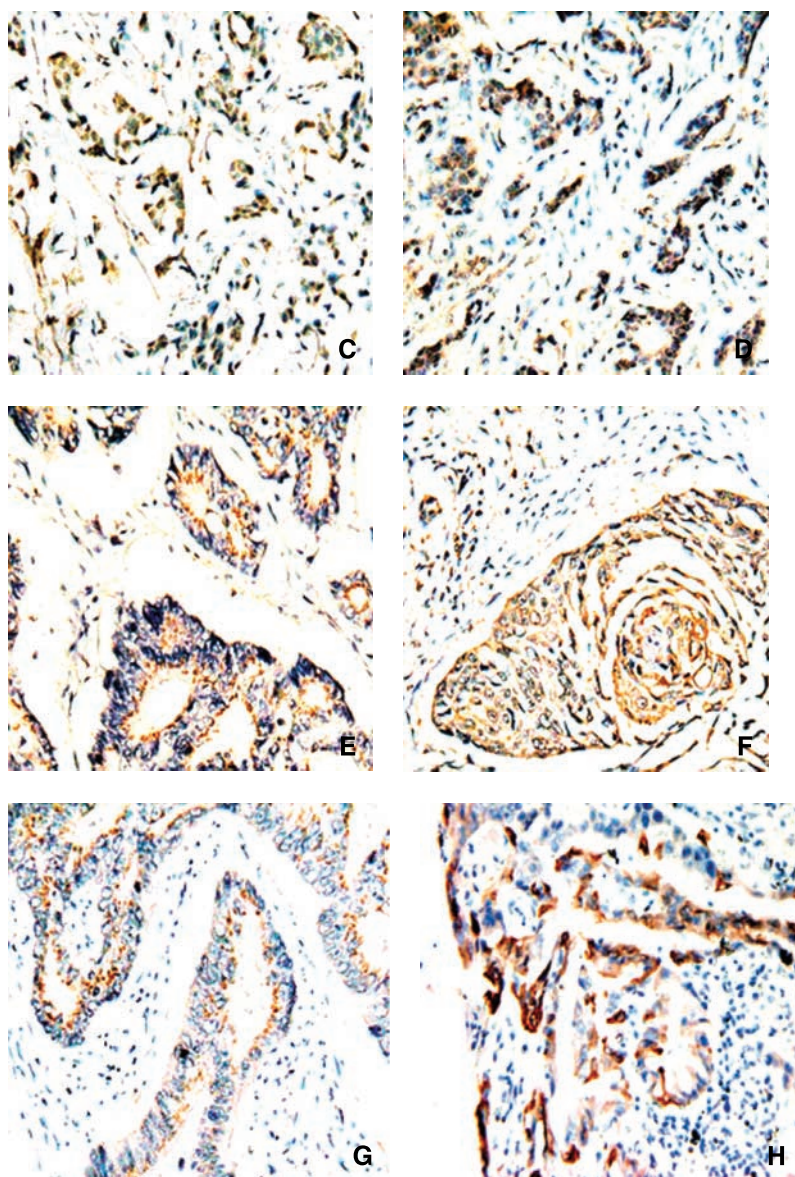
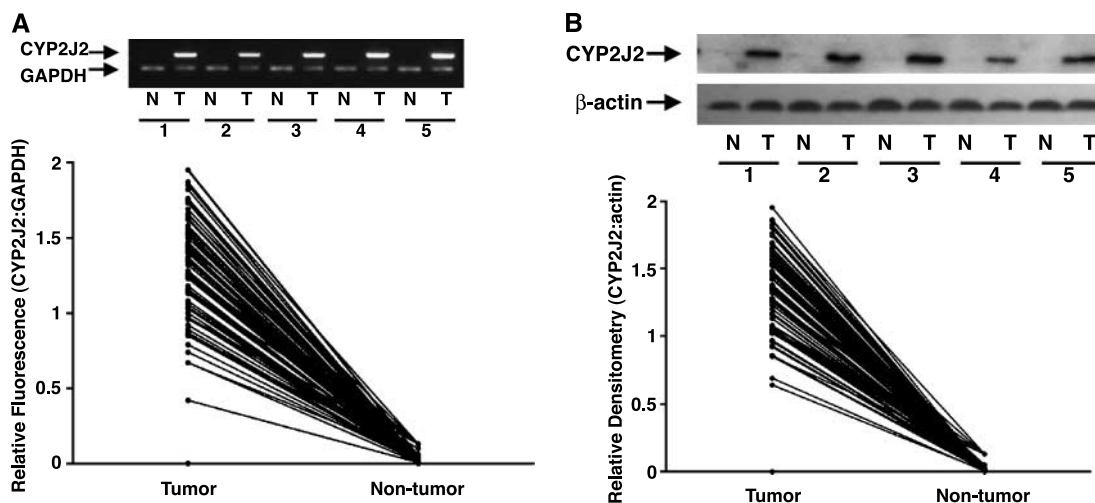


Figure 6. CYP2J2 expression is increased in human tumor versus nontumor tissue. *A*, expression levels of CYP2J2 mRNA in esophageal squamous cell carcinoma (*T*) and adjacent nontumor (*N*) tissues. *B*, expression levels of CYP2J2 protein in esophageal squamous cell carcinoma (*T*) and adjacent nontumor (*N*) tissues. Associated graphs in (*A*) and (*B*) show CYP2J2 mRNA and protein levels in all tumor and normal tissues examined. *C-H*, immunostaining for CYP2J2 in human tumor samples from breast (*C*), stomach (*D*), esophagus (*E*), liver (*F*), colon (*G*), and lung (*H*).

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(day 10) and were significantly smaller than those in all other groups over the course of the study. Furthermore, two of the mice in this group did not develop any detectable tumors. Thus, P450 epoxygenases in general, and CYP2J2 in particular, seem to enhance the development of tumorigenesis in an *in vivo* xenograft tumor model.

High expression of CYP2J2 in human tumors but not in adjacent normal tissues. Finally, to assess the potential involvement of CYP2J2 in human carcinogenesis, the expression of CYP2J2 in human tumors and adjacent normal tissues was examined by RT-PCR, Western blot analysis, and immunohistochemistry. Tissue samples were procured from 130 patients with various types of carcinomas. Relative to adjacent normal tissue, increased tumor expression levels of CYP2J2 mRNA and protein were observed in 101 (77%) of the patients. In the other 29 patients, CYP2J2 mRNA and protein were not detectable in tumor or adjacent normal tissue. Increased CYP2J2 expression relative to adjacent normal tissue was observed in the majority of samples from all tumor types, including esophageal squamous cell carcinoma (20 of 31), esophageal adenocarcinoma (4 of 4), pulmonary squamous cell carcinoma (28 of 37), pulmonary adenocarcinoma (18 of 26), small cell pulmonary carcinoma (7 of 8), breast carcinoma (5 of 5), stomach carcinoma (5 of 5), liver carcinoma (10 of 10), and colon adenocarcinoma (4 of 4). Expression levels of mRNA and protein from tumor and adjacent nontumor tissue samples from individual patients are shown in Fig. 6A and B. Immunostaining of various tumor tissues revealed CYP2J2 expression to be predominantly localized in carcinoma cells, with expression in endothelial cells also detectable (Fig. 6C-H). To determine if hyperproliferation would alter CYP2J2 expression, we analyzed two inflammatory pseudotumor tissue samples and two chronic esophagitis samples by immunohistochemistry and found, with the exception of moderate endothelial staining, no detectable CYP2J2 expression in these proliferative, nontumor tissues (data not shown). Collectively, these results suggest that neoplastic cells selectively overexpress CYP2J2 relative to surrounding normal cells and that, rather than being tissue type- or tumor stage-specific, this overexpression is a general phenomenon in human carcinomas.

Discussion

In comparison with most normal human tissues, expression of CYP2J2 is relatively high in the heart and in endothelial and smooth muscle cells of the vasculature. CYP2J2-derived EETs are intimately involved in the regulation of vascular homeostasis (19) and are potential candidates for endothelium-derived hyperpolarizing factor. Additionally, EETs have been shown to exert antiapoptotic effects (20) and to stimulate cell proliferation (21, 22) in pig kidney epithelial and human umbilical vein endothelial cells. Whereas these characteristics are generally recognized as important in the pathogenesis of cancer, remarkably little is known regarding the involvement and contributions of P450-derived EETs in carcinogenesis. Results of the present study indicate that CYP2J2 and its EET products promote the neoplastic phenotype of carcinoma cells and that CYP2J2 expression is up-regulated in human carcinoma cell lines and in a variety of human tumor tissues. Together, these data suggest an important role for this enzyme and its epoxygenase products in tumor pathogenesis.

A variety of established human carcinoma cell lines were shown to express abundant CYP2J2 mRNA and protein under basal

conditions, whereas undetectable expression was found in non-carcinoma cell lines. Given the varied background of the carcinoma cell lines examined, up-regulation of CYP2J2 in all of them is suggestive of a common regulatory event that may contribute to the neoplastic phenotype of tumor cells of diverse origin. Several characteristics of carcinoma cells render them dangerous to the host, including their abilities to avert programmed cell death (apoptosis) and malignantly proliferate. Forced overexpression of CYP2J2 and CYP 102 F87V rendered carcinoma cells even more resistant to apoptosis and enhanced their migratory and proliferative properties, whereas enhancement of apoptosis and inhibition of proliferation were achieved with rAAV-antiCYP2J2 transfection or with administration of the epoxygenase inhibitor 17-ODYA. These observations highlight the importance of functional P450 epoxygenase activity on the observed phenotypic changes induced by CYP2J2. Moreover, exogenous administration of EETs mimicked the effects of CYP2J2 overexpression on carcinoma cells, further indicating that production of EETs by CYP2J2 was likely responsible for the observed outcomes.

It is interesting to note that transfection of CYP2J2 or CYP102 F87V elicited similar effects in all of the phenotypic and signaling pathway outcomes studied herein. Given that CYP102 F87V is a mutant P450 epoxygenase that generates 14,15-EET almost exclusively (15), whereas CYP2J2 generates all four EETs (6), the observed effects of CYP2J2 could conceivably be due solely to generation of 14,15-EET. Indeed, others have shown that 14,15-EET activates the EGFR and stimulates cellular proliferation (23), providing support for this possibility. However, exogenous administration of 8,9-EET or 11,12-EET (5,6-EET cannot be reliably tested *in vitro* due to its chemical instability) rendered carcinoma cells more resistant to apoptosis to a similar extent as did exogenous 14,15-EET administration. Furthermore, all three EETs elicited effects comparable to those of CYP2J2 or CYP102 F87V transfection. These observations suggest that whereas 14,15-EET alone can clearly elicit phenotypic alterations in carcinoma cells, 8,9-EET and 11,12-EET are able to exert similar effects. Therefore, the CYP2J2-induced phenotypic changes in carcinoma cells likely result from the combined generation of all three EETs that were examined and not solely from production of 14,15-EET.

Having shown a positive influence of CYP2J2-derived EETs on the phenotype of carcinoma cells *in vitro*, the effects of CYP2J2 on tumor pathogenesis were assessed in an *in vivo* murine xenograft model of tumor formation. Such models are commonly employed to assess potential anticancer drug candidates and other strategies aimed at combating tumor growth (24). Carcinoma cells overexpressing CYP2J2 generated tumors at a faster rate and that were of increased size relative to those generated from control carcinoma cells. As was the case for the *in vitro* effects of CYP2J2, similar outcomes were observed for *in vivo* tumor formation initiated by carcinoma cells infected with CYP102 F87V. Importantly, tumor formation and size were blunted by antiCYP2J2 transfection, thereby highlighting the significance of functional CYP2J2 in the resultant *in vivo* tumor formation and identifying a potential antitumor therapeutic target deserving of further attention.

In an assortment of human tumor samples from different organs, CYP2J2 expression was markedly elevated relative to surrounding nontumor tissue, where expression was low or undetectable. Notably, CYP2J2 expression was pronounced in nonvascular cells of tumors, indicating a shift from the normal

vascular expression pattern of the enzyme. Increased expression in cancerous versus surrounding normal tissue is not unique to CYP2J2, as a variety of P450s exhibit this discordant expression pattern (25). Indeed, recognition of increased expression of various P450s in the tumor microenvironment has led a number of investigators to explore the use of P450 tumor expression patterns for targeted therapy (26). Depending on the suspected or known role of a particular P450 in tumor pathogenesis, strategies may include exploitation of the catalytic activity of the enzyme to activate antitumor prodrugs, antisense therapy to nullify enzyme expression and activity, or other suitable techniques. To date, the metabolism of exogenous compounds by CYP2J2 has not been characterized. Thus, recognizing the positive regulatory effects of CYP2J2 and its EET products on carcinoma cells and tumor pathogenesis as documented in the present study, potential antitumor therapies based on the expression pattern of CYP2J2 would likely involve strategies aimed at reducing its activity in tumors whereas simultaneously minimizing alteration of its important contribution to vascular homeostasis in normal tissues.

Collectively, the results of the present investigation suggest important and previously unrecognized roles for CYP2J2 and its EET products in carcinogenesis. Our data (i) reveal a complex set of effects elicited by CYP2J2-derived EETs on carcinoma cells that enhances their neoplastic phenotype; (ii) implicate CYP2J2 epoxygenase activity in the pathogenesis of tumor formation *in vivo*; and (iii) identify CYP2J2 as a potential biomarker and target for therapy of human cancers of various origins. Further study of the roles of CYP2J2 in carcinogenesis may identify novel and improved approaches for the identification and treatment of cancers.

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