

Cyclooxygenase-2 Transcription Is Regulated by Human Papillomavirus 16 E6 and E7 Oncoproteins: Evidence of a Corepressor/Coactivator Exchange

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Abstract

Cyclooxygenase (COX-2) is overexpressed in human papillomavirus (HPV)-induced diseases, including cervical cancer. Although HPV E6 and E7 oncoproteins have been causally linked to cervical carcinogenesis, their effects on COX-2 gene expression are unknown. Increased levels of COX-2 mRNA, protein, and prostaglandin E₂ synthesis were detected in HPV16 E6- and E7-expressing cervical cancer cells (CaSki and SiHa) compared with an uninfected cervical cancer cell line (C33A). HPV16 E6 and E7 oncoproteins induced COX-2 transcription by activating the epidermal growth factor receptor (EGFR)→Ras→mitogen-activated protein kinase pathway. Interestingly, HPV16 oncoproteins stimulated EGFR signaling, in part, by inducing the release of amphiregulin, an EGFR ligand. The inductive effects of HPV16 E6 and E7 were mediated by enhanced binding of activator protein-1 to the cyclic AMP (cAMP)-responsive element (−59/−53) of the COX-2 promoter. The potential contribution of coactivators and corepressors to HPV16 E6- and E7-mediated induction of COX-2 was also investigated. Chromatin immunoprecipitation assays indicated that E6 and E7 oncoproteins induced the recruitment of phosphorylated c-Jun, c-Fos, UbcH5, and cAMP-responsive element binding protein-binding protein/p300 to the COX-2 promoter. In contrast, E6 and E7 inhibited the binding of the histone deacetylase 3-nuclear receptor corepressor (NCoR) complex to the COX-2 promoter. Moreover, overexpression of NCoR blocked E6- and E7-mediated stimulation of the COX-2 promoter. Taken together, these results indicate that HPV16 E6 and E7 oncoproteins stimulated COX-2 transcription by inducing a corepressor/coactivator exchange. To our knowledge, this study also provides the first evidence that NCoR can function as a repressor of COX-2 gene expression. [Cancer Res 2007;67(8):3976–85]

Introduction

Cervical cancer is the second most common cause of death from cancer among women worldwide and remains a major cause of mortality among women of reproductive age in developing countries (1). In the United States, ~10,000 women are diagnosed with cervical cancer each year (2). During the past 20 years, strong epidemiologic and experimental evidence has linked infection with certain high-risk types of human papillomavirus (HPV) to the

development of cervical cancer (3). HPV16 accounts for ~50% of all cervical malignancies (4). The high-risk HPV types encode two oncoproteins, E6 and E7, both of which are involved in cellular transformation (5). E6 and E7 stimulate cell proliferation by interfering with the function of regulatory proteins in cells, including the tumor suppressors p53 and Rb (6, 7). In addition to modulating tumor suppressors, HPV E6 and E7 oncoproteins have other effects that contribute to carcinogenesis (8, 9). Recently, prophylactic vaccines have been developed to prevent cervical cancer caused by HPV types 16 and 18 (10). Unfortunately, these vaccines are unlikely to be of significant benefit to the large number of individuals with established lesions who are already infected with oncogenic HPV. A detailed understanding of the mechanisms that are modulated by HPV E6 and E7 oncoproteins could strengthen the rationale for the use of targeted therapies to prevent or treat cervical cancer in infected individuals.

Cyclooxygenases (COX) catalyze the first step in the synthesis of prostaglandins (PG) from arachidonic acid. There are two isoforms of COX. COX-1 is constitutively expressed in most tissues and seems to mediate various physiologic functions (11). By contrast, COX-2 is undetectable in most normal tissues but is rapidly induced by oncogenes, growth factors, cytokines, and tumor promoters (12–15). Multiple lines of evidence suggest that COX-2 has a significant role in carcinogenesis. COX-2 is overexpressed in transformed cells and in various malignancies, including cervical cancer (14, 16–19). In transgenic mice, overexpression of COX-2 led to neoplastic changes in the breast, skin, and pancreas (20–22). Tumor formation and growth were reduced in animals that were engineered to be COX-2 deficient (23–25) or treated with a selective COX-2 inhibitor (16, 26–29). Treatment with celecoxib, a selective COX-2 inhibitor, has proven efficacy in the treatment of colorectal polyps in humans (30, 31). Recently, celecoxib was also found to be active in the treatment of women with high-grade cervical dysplasia (32). Several different mechanisms can potentially explain the link between COX-2 and malignancy. COX-2-derived PGs can stimulate cell proliferation, promote angiogenesis, increase invasiveness, and inhibit immune surveillance and apoptosis (16, 33–37).

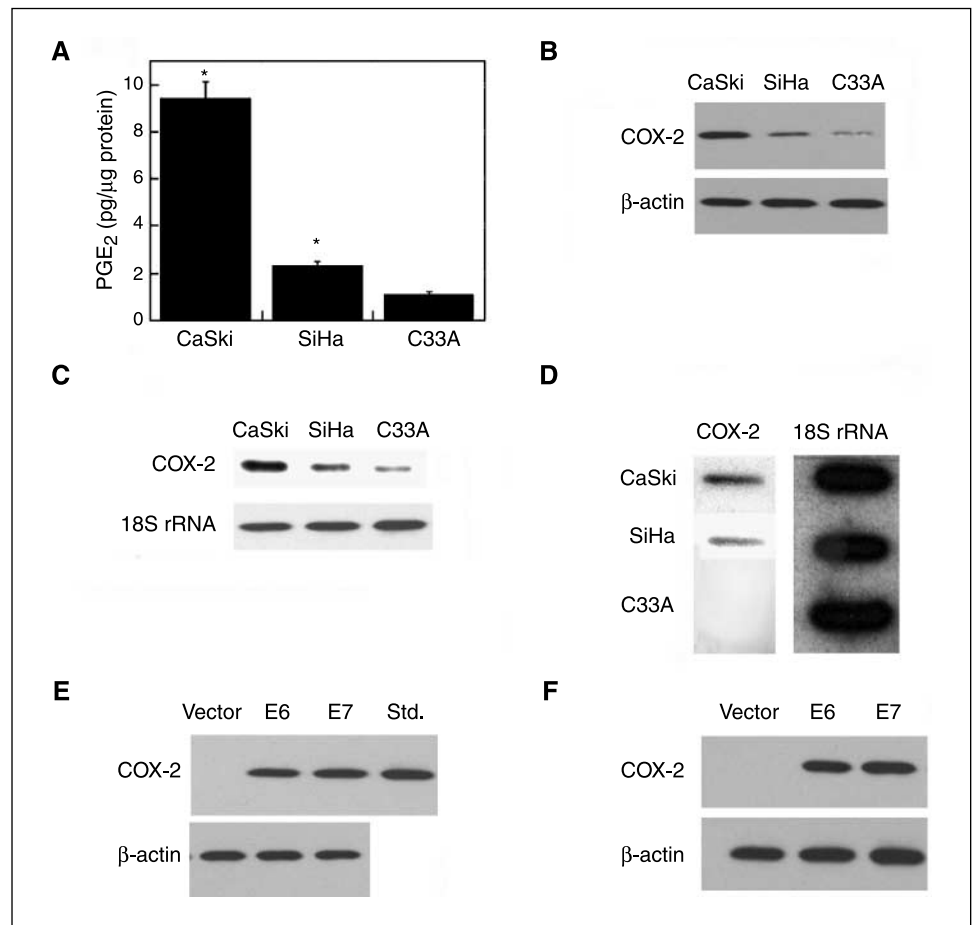
Although COX-2 is overexpressed in HPV-induced diseases, including cervical and penile cancers (18, 19, 38, 39), it is uncertain if this is a consequence of cell transformation or a direct effect of HPV E6 and E7 oncoproteins on COX-2 transcription. Hence, the main purpose of the current study was to determine whether HPV16 E6 and E7 oncoproteins induce COX-2 gene expression. We show that HPV16 E6 and E7 oncoproteins stimulated COX-2 transcription by activating the epidermal growth factor receptor (EGFR)→Ras→mitogen-activated protein kinase (MAPK)→activator protein-1 (AP-1) pathway. Importantly, overexpression of either HPV16 E6 or E7 also caused dissociation of nuclear receptor corepressor (NCoR) from the COX-2 promoter in association with recruitment of phosphorylated c-Jun/c-Fos and

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Figure 1. HPV16 E6 and E7 oncoproteins induce COX-2 transcription. **A**, CaSki, SiHa, and C33A cells were grown in culture medium for 6 h. This medium was then replaced with fresh medium containing 10 μ mol/L sodium arachidonate. Thirty minutes later, the medium was collected to determine amounts of PGE₂. Production of PGE₂ was determined by enzyme immunoassay. Columns, mean ($n = 6$); bars, SD. *, $P < 0.001$, compared with C33A cells. **B**, cellular lysate protein (500 μ g) was used to immunoprecipitate COX-2 or β -actin from the cells as described in Materials and Methods. Immunoprecipitates were then loaded on a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. The immunoblot was probed with antibodies specific for COX-2 or β -actin. **C**, total cellular RNA was isolated from CaSki, SiHa, and C33A cells. RNA (10 μ g) was added to each lane. The blot was hybridized with probes that recognized COX-2 mRNA and 18S rRNA. **D**, nuclei were isolated from CaSki, SiHa, and C33A cells. The COX-2 and 18S rRNA cDNAs were immobilized onto nitrocellulose membranes and hybridized with labeled nascent RNA transcripts. **E** and **F**, COX-2 expression was analyzed in C33A (**E**) or HEK293 (**F**) cells stably expressing vector alone or HPV E6 or E7. Cell lysate protein (100 μ g) was subjected to 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. The immunoblot was probed with antibodies specific for COX-2 or β -actin.



the coactivator cyclic AMP (cAMP)-responsive element binding protein-binding protein (CBP)/p300. To our knowledge, this study provides the first evidence that NCoR can repress *COX-2* gene expression, a finding that may help to explain why COX-2 is ordinarily not expressed in normal epithelium.

Materials and Methods

Materials. DMEM, Eagle's MEM (EMEM), RPMI 1640, and LipofectAMINE were from Invitrogen Corp. (Carlsbad, CA). Poly(deoxyinosinic-deoxycytidylic acid) [poly(dI-dC)], antisera to β -actin and platelet-derived growth factor (PDGF), normal mouse IgG, and *o*-nitrophenyl-D-galactopyranoside from Sigma-Aldrich Co. (St. Louis, MO). 2'-Amino-3'-methoxyflavone (PD 98059), SP 600125, and AG1478 were from EMD Biosciences, Inc. (San Diego, CA). Enzyme immunoassay reagents for PGE₂ assays were from Cayman Chemical Co. (Ann Arbor, MI). [³²P]CTP, [³²P]UTP, and [³²P]ATP were from Perkin-Elmer Life and Analytical Sciences (Wellesley, MA). Random priming kits were from Roche Applied Biosciences (Indianapolis, IN). Nitrocellulose membranes were from Schleicher & Schuell Bioscience (Keene, NH). Reagents for the luciferase assay were from Analytical Luminescence (San Diego, CA). The 18S rRNA cDNA was from Ambion, Inc. (Austin, TX). Antisera to COX-2, COX-1, c-Fos, c-Jun, phosphorylated c-Jun, NCoR1, histone deacetylase 3 (HDAC3), transducin β -like protein 1-related protein (TBLR1), UbcH5, and CBP/p300 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antisera to EGFR, phosphorylated EGFR, and kits to determine MAPK activities were from Cell Signaling Technology (Beverly, MA). Western blotting detection reagents [enhanced chemiluminescence (ECL)] were from Amersham Biosciences (Piscataway, NJ). Plasmid DNA was prepared using a kit from Promega Corp. (Madison, WI). Oligonucleotides were synthesized by Sigma

and Genosys Biotechnologies, Inc. (The Woodlands, TX). Small interfering RNA (siRNA) to green fluorescent protein (GFP) and TBLR1 was obtained from Dharmacon, Inc. (Lafayette, CO). Chromatin immunoprecipitation (ChIP) assay kits were purchased from Upstate (Lake Placid, NY).

Cell lines. CaSki, SiHa, C33A, and HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA). CaSki cells were maintained in RPMI 1640 with 25 μ mol/L HEPES supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% sodium pyruvate. C33A and SiHa cells were grown in EMEM with 2 mmol/L L-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g/L sodium bicarbonate and 0.1 mmol/L nonessential amino acids, 1.0 mmol/L sodium pyruvate, and 10% FBS. HEK293 cells were grown in DMEM supplemented with 10% FBS. HPV16 E6 and E7 oncoproteins were overexpressed in HEK293 cells and C33A cell lines. Stable E6 and E7 clones were selected using hygromycin. Reverse transcription-PCR analysis was done to confirm transgene expression. All cells were grown to 60% confluence in a 5% CO₂/water-saturated incubator at 37°C before being placed in serum-free medium for 24 h. Subsequently, treatments were carried out in serum-free medium.

PGE₂ production. Cells were plated in six-well dishes and grown to 60% confluence in growth medium. The amount of PGE₂ released by cells was measured by enzyme immunoassay. Production of PGE₂ was normalized to protein concentrations.

Western blotting. Cell lysates were prepared by treating cells with lysis buffer [150 mmol/L NaCl, 100 mmol/L Tris (pH 8.0), 1% Tween 20, 50 mmol/L diethyldithiocarbamate, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μ g/mL aprotinin, 10 μ g/mL trypsin inhibitor, 10 μ g/mL leupeptin]. Lysates were sonicated for 20 s on ice and centrifuged at 10,000 $\times g$ for 10 min to sediment the particulate material. The protein concentration of the supernatant was measured as described previously (17).

Protein (500 µg) from the supernatant was preabsorbed with 20 µL of normal goat IgG and 20 µL of rabbit IgG at 4°C; 20 µL of protein G Plus-agarose were then added. The mixture was then centrifuged at 3,000 × *g* for 5 min at 4°C. The pellet was discarded. Rabbit antiserum (20 µL) to COX-2 or β-actin was then added to the supernatant; the mixture was then incubated at 4°C on a rocker platform for 1 h. Protein A-agarose (20 µL) was then added, and the mixture was incubated on a rocker platform for 16 h at 4°C; the mixture was then centrifuged at 3,000 × *g* for 5 min at 4°C. The supernatant was discarded. After washing the pellet four times with radioimmunoprecipitation assay buffer, the pellet was resuspended and immunoblotting was then done.

SDS-PAGE was done under reducing conditions on 10% polyacrylamide gels. The resolved proteins were transferred onto nitrocellulose sheets. The nitrocellulose membrane was then incubated with primary antibodies. Secondary antibody to IgG conjugated to horseradish peroxidase was used. The blots were probed with the ECL Western blot detection system according to the manufacturer's instructions.

Northern blotting. Total cellular RNA was isolated from cell monolayers using a RNA isolation kit from Qiagen, Inc. (Valencia, CA). Total cellular RNA (10 µg) per lane was electrophoresed in a formaldehyde-containing 1.2% agarose gel and transferred to nylon-supported membranes. After baking, membranes were prehybridized overnight in a solution containing 50% formamide, 5× sodium chloride/sodium phosphate/EDTA buffer (SSPE), 5× Denhardt's solution, 0.1% SDS, and 100 µg/mL single-stranded salmon sperm DNA and then hybridized for 12 h at 42°C with radiolabeled cDNA probes for human COX-2 and 18S rRNA. COX-2 and 18S rRNA probes were labeled with [³²P]CTP by random priming. After hybridization,

membranes were washed twice for 20 min at room temperature in 2× SSPE and 0.1% SDS, twice for 20 min in the same solution at 55°C, and twice for 20 min in 0.1× SSPE and 0.1% SDS at 55°C. Washed membranes were then subjected to autoradiography.

Nuclear run-off assay. Cells (2.5 × 10⁵) were plated in four T150 dishes for each condition. Cells were grown in growth medium until ~60% confluent. Nuclei were isolated and stored in liquid nitrogen. For the transcription assay, nuclei (1.0 × 10⁷) were thawed and incubated in reaction buffer [10 mmol/L Tris (pH 8), 5 mmol/L MgCl₂, 0.3 mol/L KCl] containing 100 µCi of uridine 5'-[α-³²P]triphosphate and 1 mmol/L of unlabeled nucleotides. After 30 min, labeled nascent RNA transcripts were isolated. The human COX-2 and 18S rRNA cDNAs were immobilized onto nitrocellulose and prehybridized overnight in hybridization buffer. Hybridization was carried out at 42°C for 24 h using equal cpm/mL of labeled nascent RNA transcripts for each treatment group. The membranes were washed twice with 2× SSC buffer for 1 h at 55°C and then treated with 10 mg/mL RNase A in 2× SSC at 37°C for 30 min, dried, and autoradiographed.

Plasmids. The COX-2 promoter constructs (1,432/+59, 327/+59, 220/+59, 124/+59, 52/+59, KBM, ILM, CRM, and ILM) were a generous gift of Dr. Tadashi Tanabe (National Cardiovascular Research Institute, Osaka, Japan; ref. 40). HPV16 E6 and E7 expression vectors were from Dr. Craig Woodworth (Clarkson University, Potsdam, NY). The human COX-2 cDNA was generously provided by Dr. Stephen M. Prescott (Oklahoma Medical Research Foundation, Oklahoma City, OK). Extracellular signal-regulated kinase (ERK) expression vectors were provided by Dr. Melanie Cobb (Southwestern Medical Center, Dallas, TX). The expression vectors for c-Jun NH₂-terminal kinase (JNK) were a gift of Dr. Roger Davis (University of

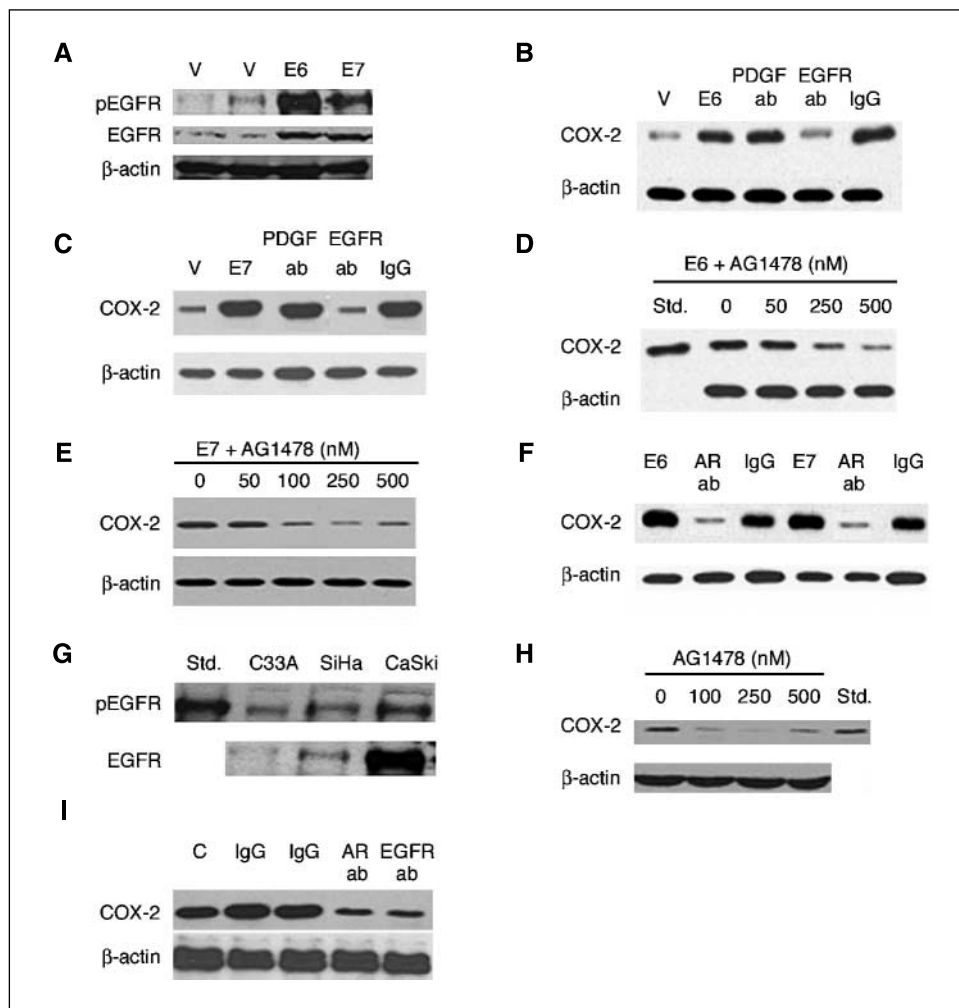
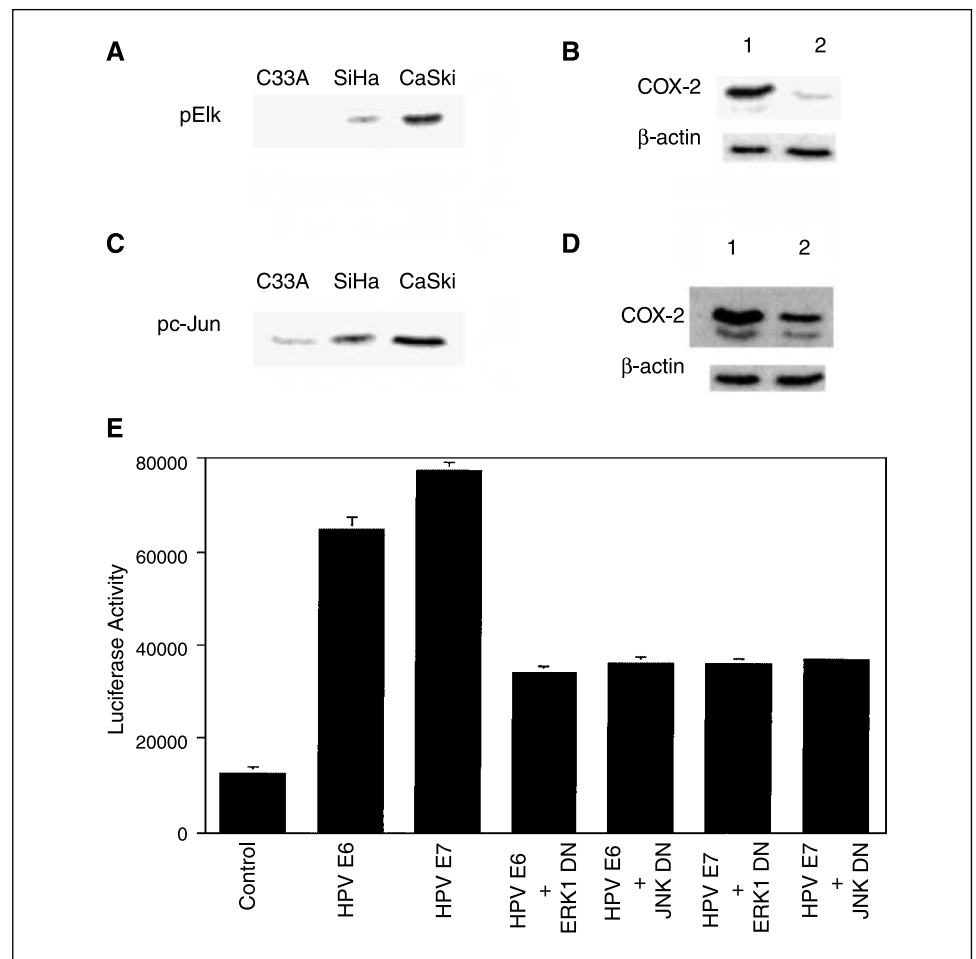


Figure 2. HPV16 E6- and E7-mediated induction of COX-2 is dependent on EGFR activation. A, C33A cells were stably transfected with empty vector (V), HPV16 E6 (E6), or HPV16 E7 (E7). Cell lysate protein (500 µg) was subjected to immunoprecipitation with antibodies to phosphotyrosine, EGFR, or β-actin. The immunoprecipitates were then subjected to Western blotting. The blots were probed with antibodies to phosphorylated EGFR (pEGFR), EGFR, and β-actin. B and C, C33A cells stably transfected with empty vector, HPV E6 (B), or HPV E7 (C) were grown in medium with or without the indicated antibody (ab) treatment for 12 h. D and E, C33A cells stably transfected with HPV E6 (D) or HPV E7 (E) were treated with 0 to 500 nmol/L of AG1478 for 12 h. F, C33A cells stably transfected with HPV E6 or HPV E7 were grown in medium with antibody to amphiregulin (AR) or IgG for 12 h. B to F, cell lysate protein (100 µg) was subjected to 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. The immunoblots were sequentially probed with antibodies specific for COX-2 or β-actin. G, levels of phosphorylated EGFR and EGFR were compared in CaSki, SiHa, and C33A cells. Immunoblot analysis was carried out as described above. H, CaSki cells were treated with 0 to 500 nmol/L of AG1478 for 12 h. Subsequently, cell lysate protein (500 µg) was subjected to immunoprecipitation with antibodies to COX-2 or β-actin and the immunoprecipitates were subjected to Western blotting. I, CaSki cells were grown in medium with or without the indicated antibodies for 12 h. Subsequently, analysis was carried out as in (H).

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Figure 3. ERK1/2 and JNK activities are important for HPV16 E6- and E7-mediated induction of COX-2 expression. The activities of ERK1/2 (A) and JNK (C) were measured. B, CaSki cells were treated with vehicle (lane 1) or 100 $\mu\text{mol/L}$ PD 98059 (lane 2) for 8 h. D, CaSki cells were treated with vehicle (lane 1) or 1 $\mu\text{mol/L}$ SP 600125 (lane 2) for 8 h. B and D, following the indicated treatments, 500 μg of cell lysate protein were immunoprecipitated with antibodies to COX-2 or β -actin. The immunoprecipitates were then loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. Immunoblots were probed separately for COX-2 and β -actin. E, C33A cells were transfected with 0.9 μg of a human COX-2 5'-untranslated region construct ligated to luciferase (-327/+59) and 0.2 μg of *pSV β gal*. HPV E6 and HPV E7, cells that received 0.45 μg of expression vector for HPV16 E6 or E7, respectively. HPV E6 + ERK1 DN and HPV E6 + JNK DN, cells that received 0.45 μg of expression vectors for HPV16 E6 and ERK1 or JNK dominant-negative forms, respectively. HPV E7 + ERK1 DN and HPV E7 + JNK DN, cells that received 0.45 μg of expression vectors for HPV16 E7 and ERK1 or JNK dominant negatives, respectively. The total amount of DNA in each reaction was kept constant at 2 μg by using corresponding empty expression vector. Twenty-four hours after transfection, the cells were lysed and reporter activities were measured. Luciferase activity represents data that have been normalized to β -galactosidase activity. Columns, mean ($n = 6$); bars, SD.



Massachusetts, Worcester, MA). The Ras constructs were gifts from Dr. Geoffrey Cooper (Harvard University, Cambridge, MA). The expression vectors for Raf-1 and kinase-deficient Raf-1 were obtained from Dr. Ulf Rapp (University of Wurzburg, Wurzburg, Germany). *pSV β gal* was obtained from Promega. Expression vectors for NCoR and SMRT were from Open Biosystems, Inc. (Huntsville, AL).

Transient transfection assays. Cells were seeded at a density of 5×10^4 per well in six-well dishes and grown to 50% to 60% confluence. For each well, 2 μg of plasmid DNA were introduced into cells using 8 μg LipofectAMINE as per the manufacturer's instructions. After 7 h of incubation, the medium was replaced with basal medium. The activities of luciferase and β -galactosidase were measured in cellular extract.

Oligonucleotides. Oligonucleotides containing different COX-2 promoter sites were synthesized as follows: CRE, 5'-AAACAGTCATTTTCGTCACATGGGCTTG-3' (sense) and 5'-CAAGCCCATGTGACGAAATGACTGTTT-3' (antisense); mutant CRE, 5'-AAACAGTCATTTAATCACATGGGCTTG-3' (sense) and 5'-CAAGCCCATGTGATTAATGACTGTTT-3' (antisense); nuclear factor- κ B (NF- κ B), 5'-AGGAGAGTGGGGACTACCCCTCTG-3' (sense) and 5'-AGAGGGGTAGTCCCCTCTCT-3' (antisense); and nuclear factor-interleukin-6 (NF-IL-6), 5'-CCCACGGGCTTACGCAATT-3' (sense) and 5'-AATTGCGTAAGCCCGGTGGG-3' (antisense). All oligonucleotides were synthesized by Genosys Biotechnologies.

Electrophoretic mobility shift assay. Cells were harvested and nuclear extracts were prepared. For binding studies, oligonucleotides containing different response elements in the COX-2 promoter were used. The complementary oligonucleotides were annealed in 20 mmol/L Tris (pH 7.6), 50 mmol/L NaCl, 10 mmol/L MgCl₂, and 1 mmol/L DTT. The annealed oligonucleotide was phosphorylated at the 5'-end with [γ -³²P]ATP and T4 polynucleotide kinase. The binding reaction was done by incubating 5 μg of

nuclear protein in 20 mmol/L HEPES (pH 7.9), 10% glycerol, 300 μg bovine serum albumin, and 1 μg poly(dI-dC) in a final volume of 10 μL for 10 min at 25°C. The labeled oligonucleotide was added to the reaction mixture and allowed to incubate for an additional 20 min at 25°C. The samples were electrophoresed on a 4% nondenaturing polyacrylamide gel. The gel was then dried and subjected to autoradiography at 80°C.

ChIP assay. ChIP assay was done with a kit (Upstate) according to the manufacturer's instructions. Cells (1×10^6) were cross-linked in a 1% formaldehyde solution for 10 min at 37°C. Cells were then lysed in 200 μL of SDS buffer and sonicated to generate 200- to 1,000-bp DNA fragments. After centrifugation, the cleared supernatant was diluted 10-fold with ChIP buffer and incubated with 1.5 μg of the indicated antibody at 4°C. Immune complexes were precipitated, washed, and eluted as recommended. DNA-protein cross-links were reversed by heating at 65°C for 4 h, and the DNA fragments were purified and dissolved in 50 μL water. Each sample (10 μL) was used as a template for PCR amplification. COX-2 oligonucleotide sequences for PCR primers were 5'-AAAGCTATGTATGTATGTGCTGCAT-3' and 5'-AACCAGAGAGAACCTTCTCTTTTAT-3'. This primer set encompasses the COX-2 promoter segment from nucleotide -7 to -621, which includes the CRE binding site. PCR was done at 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s for 30 cycles. The PCR products generated from the ChIP template were sequenced, and the identity of the COX-2 promoter was confirmed.

Statistics. Comparisons between groups were made by the Student's *t* test. A difference between groups of $P < 0.05$ was considered significant.

Results

HPV16 E6 and E7 induce COX-2 transcription. Initially, we compared levels of PGE₂ production in two HPV16-expressing

cervical cancer cell lines (CaSki and SiHa) versus a cervical cancer cell line uninfected with HPV (C33A; ref. 41). As shown in Fig. 1A, both CaSki and SiHa cell lines produced more PGE₂ than the C33A cell line. To determine whether these differences in PGE₂ biosynthesis potentially reflected differences in COX-2 expression, immunoblot analysis was carried out. Consistent with the observed differences in PGE₂ production, CaSki and SiHa cell lines expressed higher levels of COX-2 protein than C33A cells (Fig. 1B). Similarly, levels of COX-2 mRNA were highest in CaSki cells and lowest in C33A cells (Fig. 1C). Nuclear runoffs were done to compare rates of COX-2 transcription among the three cell lines. Rates of COX-2 transcription were highest in CaSki cells and lowest in C33A cells (Fig. 1D). Collectively, these results suggested the possibility that HPV16 oncoproteins activated COX-2 transcription, leading to higher levels of PGE₂ production in HPV16-infected cell lines (CaSki and SiHa) than in the cell line (C33A) that was uninfected by HPV. To further evaluate this possibility, we investigated whether overexpressing either HPV16 E6 or E7 induced COX-2. Overexpression of either HPV16 E6 or E7 caused a marked increase in levels of COX-2 in both C33A (Fig. 1E) and HEK293 cells (Fig. 1F). By contrast, levels of COX-1 were unaffected (data not shown).

Defining the signaling mechanism by which HPV16 E6 and E7 induce COX-2. We next attempted to define the signal transduction pathway by which HPV16 E6 and E7 stimulated COX-2 transcription. Activation of receptor tyrosine kinases, including the EGFR, can stimulate COX-2 transcription (16, 17). Hence, we investigated whether overexpressing HPV16 E6 or E7 increased EGFR tyrosine kinase activity in C33A cells. Overexpressing either viral oncoprotein induced EGFR, EGFR tyrosine kinase activity, and COX-2 expression (Fig. 2A–C). Experiments were next done to determine whether EGFR activation was causally linked to COX-2 induction. AG1478, an inhibitor of EGFR tyrosine kinase, suppressed the induction of COX-2 in C33A cells that overexpressed either HPV16 E6 or E7 (Fig. 2D and E). Because EGFR can be activated by either extracellular or intracellular mechanisms, we investigated whether an antibody to the EGFR ligand-binding site suppressed HPV16 E6- and E7-mediated induction of COX-2. As shown in Fig. 2B and C, HPV16 E6- and E7-mediated induction of COX-2 was suppressed by this neutralizing antibody. In contrast, control antibodies (IgG and anti-PDGF) did not suppress levels of COX-2. Importantly, EGFR can be activated by several different ligands. With this in mind, experiments were done to identify the EGFR ligand that was responsible for HPV16 E6- and E7-mediated induction of COX-2. Notably, an antibody to amphiregulin, an EGFR ligand, blocked HPV16 E6- and E7-mediated induction of COX-2 (Fig. 2F). In this context, we note that amphiregulin release was increased ~2-fold in both HPV16 E6- and E7-overexpressing C33A cell lines (data not shown). Levels of EGFR tyrosine kinase activity were also measured in CaSki, SiHa, and C33A cells. Consistent with the observed differences in COX-2 protein levels (Fig. 1B), CaSki and SiHa cell lines expressed higher levels of EGFR and phosphorylated EGFR than C33A cells (Fig. 2G). In further support of the role for activation of EGFR signaling driving COX-2 expression, treatment with AG1478 (Fig. 2H) or antibodies to EGFR or amphiregulin suppressed levels of COX-2 in CaSki cells (Fig. 2I).

Transient transfections were done to define the signal transduction pathway downstream of EGFR that was responsible for HPV16 E6- and E7-mediated induction of COX-2. Overexpressing either Ras or Raf stimulated COX-2 promoter activity

(data not shown). Overexpressing dominant-negative forms of either Ras or Raf kinase abrogated HPV16 E6- and E7-mediated stimulation of COX-2 promoter activity (Supplementary Fig. S1). Ras signaling can alter gene expression by activating distinct MAPKs (42). It was important, therefore, to investigate whether increased MAPK activity contributed to HPV16 E6- and E7-mediated induction of COX-2. Initially, we compared MAPK activities in the two HPV16-expressing cervical cancer cell lines (CaSki and SiHa) versus a cervical cancer cell line uninfected with

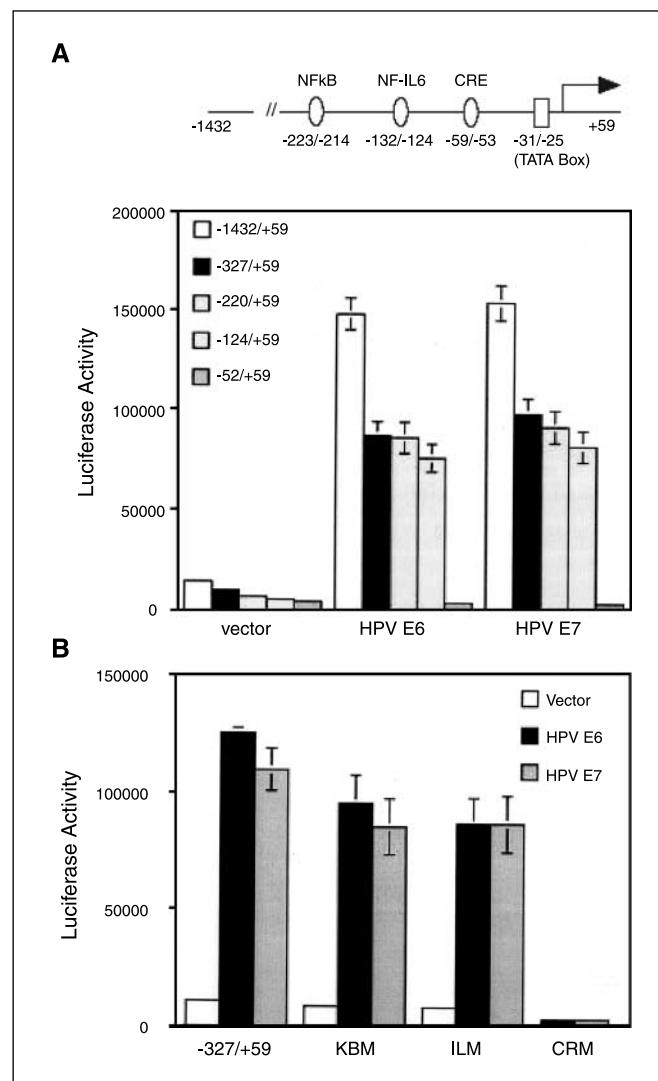


Figure 4. Localization of region of COX-2 promoter that mediates the effects of HPV16 E6 and E7 oncoproteins. *A*, schematic of the human COX-2 promoter. C33A cells were transfected with 0.9 μ g of a series of human COX-2 promoter-luciferase constructs (–1,432/+59, –327/+59, –220/+59, –124/+59, and –52/+59). HPV E6 and HPV E7, cells that also received 0.9 μ g of expression vector for HPV16 E6 and E7 oncoproteins. *B*, C33A cells were transfected with 0.9 μ g of a series of human COX-2 promoter-luciferase (–327/+59, KBM, ILM, and CRM). KBM, –327/+59 COX-2 promoter construct in which the NF- κ B site was mutagenized; ILM, –327/+59 COX-2 promoter construct in which the NF-IL-6 site was mutagenized; CRM, –327/+59 COX-2 promoter construct in which the CRE was mutagenized. Black columns, cells that also received HPV16 E6; gray columns, cells that also received HPV16 E7 oncoprotein. *A* and *B*, all cells received 0.2 μ g of pSV β gal. The total amount of DNA in each reaction was kept constant at 2 μ g by using empty vector. Reporter activities were measured in cellular extract 24 h following transfection. Luciferase activity represents data that have been normalized to β -galactosidase activity. Columns, mean ($n = 6$); bars, SD.

HPV (C33A). Consistent with the observed differences in COX-2 expression (Fig. 1B) and PGE₂ production (Fig. 1A), CaSki and SiHa cell lines expressed higher levels of ERK1/2 and JNK activity than C33A cells (Fig. 3A and C). Pharmacologic and genetic approaches were next used to further evaluate the link between MAPK activity and COX-2 expression in HPV16 E6- and E7-expressing cells. In the first experiment, we used PD 98059, a specific inhibitor of MAPK kinase, which prevents activation of ERK1/2. Treatment with PD 98059 suppressed COX-2 expression in CaSki cells (Fig. 3B); SP 600125, a JNK inhibitor, also suppressed levels of COX-2 (Fig. 3D). To further investigate the importance of MAPK in mediating the induction of COX-2, a series of transient transfections were done. Consistent with the pharmacologic findings, overexpressing dominant negatives for ERK1 or JNK suppressed HPV16 E6- or E7-mediated activation of the COX-2 promoter (Fig. 3E).

The cAMP-responsive element, AP-1, and NCoR are involved in HPV16 E6- and E7-mediated induction of COX-2 transcription. Overexpressing either HPV16 E6 or E7 caused a significant increase in COX-2 promoter activity with all COX-2 promoter deletion constructs, except the -52/+59 construct (Fig. 4A), suggesting that the CRE site may be responsible for mediating the effects of the oncoproteins. To further evaluate this possibility, transient transfections were done using COX-2 promoter constructs in which different elements had been mutated. As shown in Fig. 4B, mutagenizing the CRE site caused a loss of responsiveness to HPV16 E6 and E7. In contrast, mutating either the NF- κ B or NF-IL-6 sites had no effect on HPV16 E6- or E7-mediated activation of the COX-2 promoter. Electrophoretic mobility shift assays (EMSA) were next done to identify the transcription factor responsible for HPV16 E6- and E7-mediated induction of COX-2. Overexpressing either HPV16 E6 or E7 led to increased binding of nuclear protein to the CRE site of the COX-2 promoter (Supplementary Fig. S2A). This increase in binding to the COX-2 CRE was competed by incubating nuclear extract from HPV16 E6- or E7-overexpressing cells with a 100-fold excess of unlabeled CRE or AP-1 probes (Supplementary Fig. S2B and C). Binding was not competed when nuclear extracts were incubated with a 100-fold excess of unlabeled CEBP- α , NF- κ B, or mutant CRE probes. Supershifts were done to determine the composition of the nuclear binding protein complex that recognized the CRE. As shown in Supplementary Fig. S2D and E, both phosphorylated c-Jun and c-Fos were present in the complex in HPV16 E6-overexpressing C33A cells. Phosphorylated c-Jun and c-Fos were also present in the complex in HPV16 E7-overexpressing C33A cells (data not shown). Consistent with the observed differences in COX-2 expression (Fig. 1B) and transcription (Fig. 1D), higher levels of nuclear protein binding to the COX-2 CRE were found in HPV16-infected CaSki and SiHa cells versus uninfected C33A cells (Supplementary Fig. S2F). Supershifts were done using nuclear protein from CaSki cells. Consistent with the findings in HPV16 E6- and E7-overexpressing C33A cells, phosphorylated c-Jun and c-Fos were identified in the binding complex (Supplementary Fig. S2G).

To further examine the mechanism by which HPV16 E6 or E7 stimulate COX-2 transcription, ChIP assays were done. Protein-DNA complexes were immunoprecipitated with different antibodies, and bound DNA fragments were recovered and subjected to semiquantitative PCR with oligonucleotides specific for the COX-2 promoter. The binding of unphosphorylated c-Jun to the COX-2 promoter was not altered in C33A cells that overexpressed HPV16 E6 or E7 (Fig. 5A). In contrast, overexpression of HPV16 E6 or E7 led to

increased binding of c-Fos and CBP/p300, a coactivator of AP-1-mediated transcription. Recently, NCoR was found to serve as a checkpoint that controls signal-dependent exchange of c-Jun/co-repressor complexes for c-Jun/c-Fos/coactivator complexes (43). Hence, we also investigated whether overexpression of HPV16 E6 or E7 modulated NCoR binding to the COX-2 CRE. Notably, NCoR binding was dismissed in association with increased binding of c-Fos and CBP/p300 (Fig. 5A). In contrast, binding of the highly related silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) was unaffected (data not shown). A similar strategy was used to compare CaSki and SiHa cells, the two HPV16-expressing cervical cancer cell lines, with C33A cells, the cervical cancer cell line that is uninfected by HPV. Notably, the highest levels of binding of c-Fos and CBP/p300 were detected in CaSki cells, with the lowest levels of binding of each of these proteins found in C33A cells (Fig. 5A). The opposite binding pattern was observed for NCoR. More specifically, the highest level of NCoR binding was found in C33A cells and the lowest level of binding in CaSki cells (Fig. 5A). Consistent with the repressive effects of NCoR, COX-2 promoter activity was lowest in C33A cells and highest in CaSki cells (Fig. 5B, a). Notably, overexpressing NCoR but not SMRT caused a marked reduction in COX-2 promoter activity in CaSki cells (Fig. 5B, b) and blocked HPV16 E6- and E7-mediated activation of the COX-2 promoter in C33A cells (Fig. 5B, c and d). Collectively, the increased binding of AP-1 and CBP/p300 and reduced binding of NCoR to the COX-2 CRE in HPV16 E6- and E7-expressing cervical cancer cell lines (CaSki and SiHa) versus the uninfected C33A cell line corresponded to the differences in COX-2 transcription (Fig. 1D) and COX-2 promoter activity (Fig. 5B, a) that were observed.

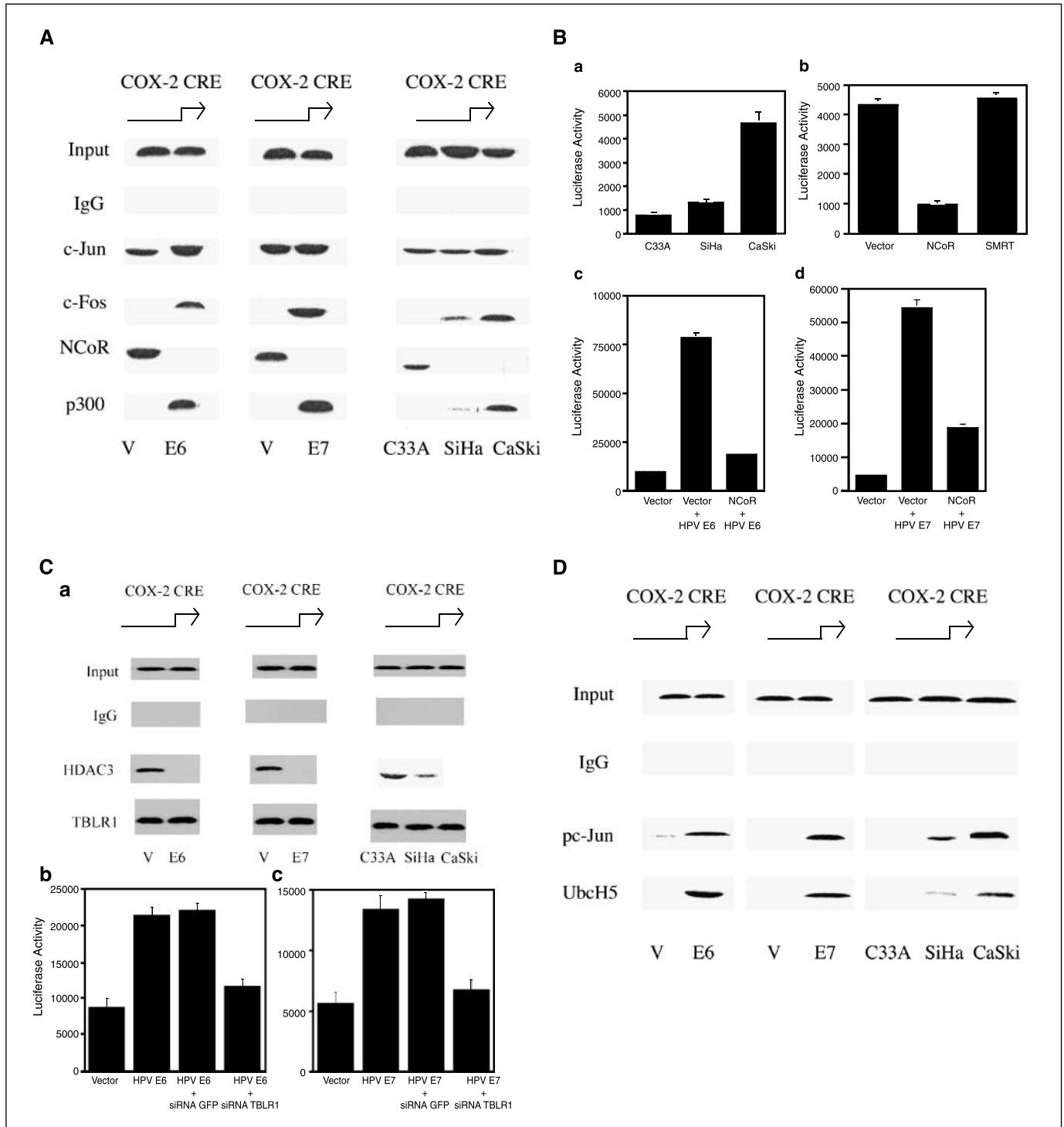
Earlier studies of AP-1-dependent gene regulation indicated an essential role of c-Jun NH₂-terminal phosphorylation for TBLR1-dependent recruitment of the proteolytic machinery required for NCoR and HDAC3 dismissal and coactivator exchange (43, 44). Hence, we investigated if HDAC3 and TBLR1 were bound to the COX-2 promoter. As shown in Fig. 5C, a, overexpression of HPV16 E6 and E7 oncoproteins led to loss of HDAC3 binding to the COX-2 promoter. On the other hand, no difference in binding of TBLR1 to the COX-2 promoter was observed on overexpression of HPV16 oncoproteins (Fig. 5C, a). Nonetheless, TBLR1 was found to be functionally important for HPV16 oncoprotein-mediated stimulation of COX-2 transcription (Fig. 5C, b and c). More specifically, siRNA to TBLR1 blocked HPV16 E6- and E7-mediated induction of COX-2 promoter activity. Signal-dependent phosphorylation of c-Jun is also known to cause TBLR1-dependent recruitment of the ubiquitin-conjugating enzyme UbcH5, leading to proteolytic removal of the NCoR-HDAC3 complex (43, 44). To determine whether HPV16 oncoprotein-mediated activation of JNK resulted in recruitment of phosphorylated c-Jun and UbcH5 to the COX-2 promoter, ChIP assays were done. As shown in Fig. 5D, overexpression of HPV16 E6 and E7 led to enhanced binding of both phosphorylated c-Jun and UbcH5 to the COX-2 promoter. Similarly, binding of phosphorylated c-Jun and UbcH5 to the COX-2 promoter was enhanced in HPV16 E6- and E7-expressing CaSki and SiHa cells compared with the uninfected C33A cell line (Fig. 5D).

Discussion

COX-2 is overexpressed in HPV-related diseases, including cervical cancer (18, 19, 38, 39). In the current study, we show that HPV16 E6 and E7 oncoproteins induced COX-2 gene expression. The induction of COX-2 by HPV16 oncoproteins was

mediated by activation of the EGFR→Ras→MAPK→AP-1 pathway. Several observations support a critical role for EGFR in HPV16 oncoprotein-mediated induction of COX-2. Overexpression of E6 or E7 in C33A cells stimulated the expression and phosphorylation of EGFR while inducing COX-2. Treatment with AG1478, an inhibitor of EGFR tyrosine kinase, or antibody blockade of the ligand-binding site of EGFR abrogated HPV16 oncoprotein-mediated induction of COX-2. Importantly, both AG1478 and the neutralizing antibody to

EGFR also suppressed levels of COX-2 in HPV16-infected CaSki cells. EGFR can be activated via either intracellular or extracellular mechanisms (45–47). The fact that a neutralizing antibody to EGFR blocked HPV16 oncoprotein-mediated induction of COX-2 suggested an extracellular process. Consistent with this notion, HPV16 oncoproteins stimulated the release of amphiregulin, a ligand of the EGFR. More importantly, an antibody to amphiregulin blocked the induction of COX-2 by HPV16 oncoproteins in C33A cells and



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suppressed the expression of COX-2 in CaSki cells. Notably, these findings are consistent with previous evidence that activation of EGFR signaling can drive *COX-2* transcription and PG biosynthesis (16, 47). Additional experiments were carried out to define the signal transduction pathway downstream of EGFR that mediated the induction of COX-2. Consistent with prior studies (14, 17), the Ras pathway was implicated. Dominant negatives for Ras and Raf-1 blocked HPV16 E6- and E7-mediated activation of the *COX-2* promoter. Several lines of evidence suggest that HPV16 induced COX-2 via activation of JNK and ERK MAPKs. First, the activities of ERK1/2 and JNK were higher in HPV16 E6- and E7-expressing cervical cancer cells (CaSki and SiHa) compared with the uninfected cervical cancer cell line (C33A). Second, inhibitors of MAPK kinase and JNK suppressed levels of COX-2 in CaSki cells (Fig. 3B and D). Third, overexpression of dominant negatives for ERK1 and JNK suppressed the induction of *COX-2* promoter activity by HPV16 E6 and E7 (Fig. 3E). Previously, activation of JNK was linked to increased *EGFR* transcription (48). It is reasonable to speculate, therefore, that increased JNK activity contributes to the overexpression of both EGFR and COX-2 in HPV16 E6- and E7-expressing cervical cancer cells.

We also report that induction of *COX-2* promoter activity by HPV16 E6 and E7 oncoproteins was mediated through an AP-1-binding site located 53 nucleotides upstream of the transcriptional start site (Fig. 4). EMSAs showed increased binding of phosphorylated c-Jun and c-Fos, components of the AP-1 transcription factor complex, to the CRE of the *COX-2* promoter in HPV16 E6- or E7-expressing cervical cancer cells (Supplementary Fig. S2). This result was confirmed by ChIP analysis (Fig. 5). The functional importance of AP-1 was established because HPV16 E6- and E7-mediated activation of the *COX-2* promoter was suppressed by mutagenizing the CRE (Fig. 4B). Collectively, these findings are consistent with previous evidence that stimulation of MAPKs results in AP-1-dependent induction of *COX-2* transcription (16, 17).

Coactivators and corepressors are important for AP-1-mediated regulation of gene transcription (43). Previously, the coactivator CBP/p300 was found to be very important for AP-1-mediated

induction of COX-2 expression (49, 50). Consistent with this notion, ChIP assays revealed greater association between CBP/p300 and the *COX-2* promoter in HPV16 oncoprotein-expressing cells (Fig. 5). Under basal conditions, the NCoR corepressor complex has been found to impose an active block of exchange of c-Jun for c-Jun/c-Fos heterodimers (43). This checkpoint function of NCoR is relieved by signal-dependent phosphorylation of c-Jun, which directs removal of NCoR-HDAC3 complexes through recruitment of a specific ubiquitination complex leading to proteasome degradation (43). In this study, we show for the first time that NCoR acts as a transcriptional checkpoint for AP-1-mediated stimulation of *COX-2* transcription. This conclusion is based on several findings. ChIP assays indicated that E6 and E7 oncoproteins inhibited the binding of the NCoR-HDAC3 corepressor complex while inducing the recruitment of phosphorylated c-Jun, c-Fos, UbcH5, and CBP/p300 to the *COX-2* promoter. Consistent with NCoR functioning as a corepressor, we observed an inverse relationship between the extent of NCoR interaction with the *COX-2* promoter (Fig. 5) and levels of *COX-2* transcription (Fig. 1D). High levels of interaction between NCoR and the *COX-2* promoter were observed in the HPV16-uninfected C33A cell line in which *COX-2* transcription was negligible. Overexpression of HPV16 E6 or E7 in C33A cells led to a marked decrease in NCoR interaction with the *COX-2* promoter (Fig. 5A) and a reciprocal increase in *COX-2* expression (Fig. 1E). Furthermore, the interaction between NCoR and the *COX-2* promoter was negligible in HPV16-infected CaSki cells in which *COX-2* transcription was robust. Overexpression of NCoR also suppressed HPV16 E6- or E7-mediated activation of the *COX-2* promoter (Fig. 5B, C and D). The finding that NCoR inhibits COX-2 expression under basal conditions may prove to be very important for explaining why COX-2 is ordinarily not detected in normal epithelial cells. In addition to providing evidence that NCoR inhibits COX-2 expression, this study shows for the first time that HPV oncoproteins modulate the function of the corepressor NCoR. Our findings in combination with previous reports (43, 44) strongly suggest a model whereby activation of JNK stimulated the phosphorylation of c-Jun, leading, in turn, to UbcH5-mediated ubiquitination and degradation

Figure 5. COX-2 transcription is regulated by HPV16 E6 and E7 oncoproteins via a corepressor/coactivator exchange. A, ChIP assays were done. A comparison was made between C33A cells that overexpressed vector, HPV16 E6, and HPV16 E7. Additionally, comparisons were made among C33A, SiHa, and CaSki cancer cell lines. Chromatin fragments were immunoprecipitated with the indicated antibodies, and the *COX-2* promoter region was amplified by PCR. DNA sequencing was carried out, and the PCR product was confirmed to be the *COX-2* promoter. We were unable to detect the COX-2 promoter (-7 to -621) when normal IgG was used or antibody was omitted from the immunoprecipitation step. B, a, C33A, SiHa, and CaSki cells were transfected with 0.9 μ g of human *COX-2* promoter-luciferase (-327/+59). Vector, cells that also received 0.9 μ g of vector; NCoR, cells that also received 0.9 μ g of expression vector for NCoR; SMRT, cells that also received 0.9 μ g of expression vector for SMRT. c, C33A cells were transfected with 0.9 μ g of human *COX-2* promoter-luciferase (-327/+59). Vector, cells that received 0.9 μ g of vector alone; Vector + HPV E6, cells that received 0.45 μ g of vector and 0.45 μ g of expression vector for HPV16 E6; NCoR + HPV E6, cells that received 0.45 μ g of expression vector for NCoR and 0.45 μ g of expression vector for HPV16 E6. d, C33A cells were transfected with 0.9 μ g of human *COX-2* promoter-luciferase (-327/+59). Vector, cells that received 0.9 μ g of empty vector alone; Vector + HPV E7, cells that received 0.45 μ g of vector and 0.45 μ g of expression vector for HPV16 E7; NCoR + HPV E7, cells that received 0.45 μ g of expression vector for NCoR and 0.45 μ g of expression vector for HPV16 E7. a to d, all cells received 0.2 μ g of pSV β gal. The total amount of DNA in each reaction was kept constant at 2 μ g by using empty vector. Reporter activities were measured in cellular extract 24 h following transfection. Luciferase activity represents data that have been normalized to β -galactosidase activity. Columns, mean (n = 6); bars, SD. C, a, ChIP assays were done. A comparison was made between C33A cells that overexpressed vector, HPV16 E6, and HPV16 E7. Additionally, comparisons were made among C33A, SiHa, and CaSki cancer cell lines. Chromatin fragments were immunoprecipitated with the indicated antibodies, and the *COX-2* promoter region was amplified by PCR. DNA sequencing was carried out, and the PCR product was confirmed to be the *COX-2* promoter. We were unable to detect the COX-2 promoter (-7 to -621) when normal IgG was used or antibody was omitted from the immunoprecipitation step. b and c, C33A cells were transfected with 0.9 μ g of human *COX-2* promoter-luciferase (-327/+59). Vector, cells that received 0.9 μ g of empty vector alone. b, HPV E6, cells that received 0.45 μ g of vector and 0.45 μ g of expression vector for HPV16 E6; HPV E6 + siRNA GFP, cells that received 0.45 μ g of expression vector for HPV16 E6 and 0.45 μ g of siRNA to GFP; HPV E6 + siRNA TBLR1, cells that received 0.45 μ g of expression vector for HPV16 E6 and 0.45 μ g of siRNA to TBLR1. c, HPV E7, cells that received 0.45 μ g of vector and 0.45 μ g of expression vector for HPV16 E7; HPV E7 + siRNA GFP, cells that received 0.45 μ g of HPV16 E7 expression vector and 0.45 μ g of siRNA to GFP; HPV E7 + siRNA TBLR1, cells that received 0.45 μ g of HPV16 E7 expression vector and 0.45 μ g of siRNA to TBLR1. b and c, all cells received 0.2 μ g of pSV β gal. Reporter activities were measured in cellular extract 24 h following transfection. Luciferase activity represents data that have been normalized to β -galactosidase activity. Columns, mean (n = 6); bars, SD. D, ChIP assays were done. A comparison was made between C33A cells that overexpressed vector, HPV16 E6, and HPV16 E7. Additionally, comparisons were made among C33A, SiHa, and CaSki cancer cell lines. Chromatin fragments were immunoprecipitated with the indicated antibodies, and the *COX-2* promoter region was amplified by PCR. DNA sequencing was carried out, and the PCR product was confirmed to be the *COX-2* promoter. We were unable to detect the COX-2 promoter (-7 to -621) when normal IgG was used or antibody was omitted from the immunoprecipitation step.

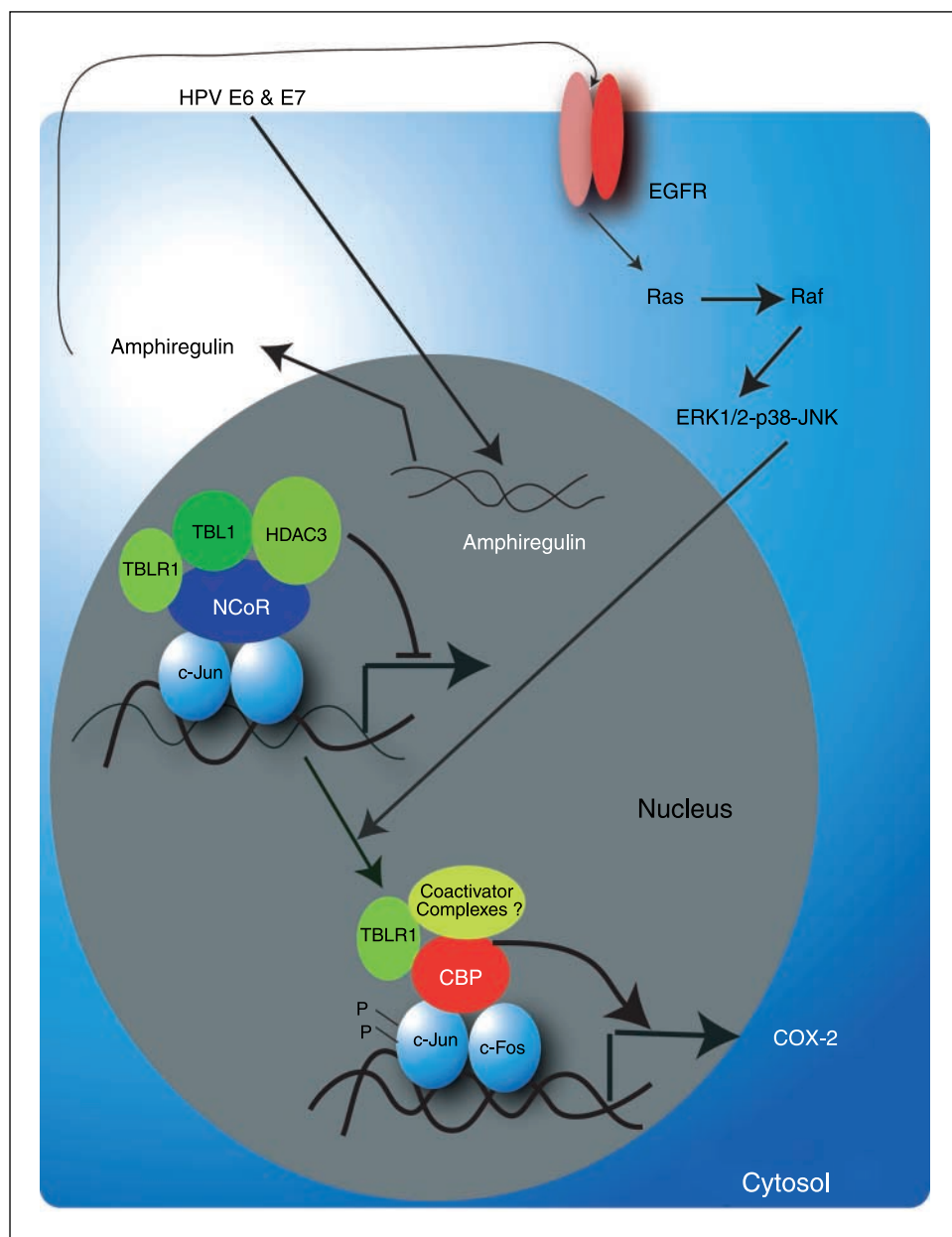


Figure 6. Schematic of pathway by which HPV16 oncoproteins regulate COX-2 expression. HPV16 E6 and E7 oncoproteins stimulate production of amphiregulin and thereby activate EGFR→Ras→MAPK signaling. This results, in turn, in phosphorylation of c-Jun, leading to TBLR1-dependent degradation of the NCoR/HDAC3 corepressor complex and recruitment of the coactivator CBP/p300 and phosphorylated c-Jun/c-Fos heterodimer to the COX-2 promoter. This corepressor/coactivator exchange triggered by HPV oncoproteins leads to enhanced COX-2 transcription.

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of the NCoR-HDAC3 corepressor complex and recruitment of the coactivator complex consisting of phosphorylated c-Jun-c-Fos-CBP/p300 (Fig. 6). Thus, HPV16 E6 and E7 oncoproteins stimulated COX-2 transcription by inducing a corepressor/coactivator exchange. In addition to HPV16 E6 and E7, other HPV oncoproteins may have similar effects. For example, HPV E5 expression can cause a ligand-dependent increase in EGFR phosphorylation (51, 52) and therefore may also induce COX-2 transcription.

The results of this study provide other potentially significant insights. We have shown that HPV16 E6 and E7 oncoproteins stimulated COX-2 transcription via the EGFR→Ras→MAPK→AP-1 pathway. Several of these effects would be predicted to support sustained HPV infection. For example, as mentioned above, COX-2-derived PGs can stimulate cell proliferation and angiogenesis while inhibiting apoptosis and immune surveillance (16, 33, 34, 37). In fact, short-term treatment with the COX-2 inhibitor celecoxib caused a decrease in markers of both cell proliferation and

angiogenesis in cervical cancer patients (53). Activation of EGFR is well recognized to promote mitogenesis and angiogenesis (45). Several investigators have shown significant cross-talk between COX-2 and EGFR in other contexts (46, 47). Hence, overexpression of HPV16 E6 or E7 might initiate a positive feedback loop whereby activation of EGFR results in enhanced expression of COX-2 and increased synthesis of PGs, leading, in turn, to a further increase in EGFR activity. Collectively, these HPV16 oncoprotein-induced changes would be predicted to support a sustained HPV infection and promote carcinogenesis.

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References

1. Munoz N, Bosch FX, Castellsague X, et al. Against which human papillomavirus types shall we vaccinate and screen? The international perspective. *Int J Cancer* 2004;111:278–85.
2. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2006. *CA Cancer J Clin* 2006;56:106–30.
3. Munoz N, Bosch FX, de Sanjose S, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med* 2003;348:518–27.
4. zur Hausen H. Papillomavirus and cancer: from basic studies to clinical application. *Nat Rev Cancer* 2002;2:342–50.
5. Munger K, Phelps WC, Bubb V, Howley PM, Schlegel R. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *J Virol* 1989;63:4417–21.
6. Werness BA, Levine AJ, Howley PM. Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science* 1990;248:76–9.
7. Dyson N, Howley PM, Munger K, Harlow E. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* 1989;243:934–7.
8. Veldman T, Horikawa I, Barrett JC, Schlegel R. Transcriptional activation of the telomerase hTERT gene by human papillomavirus type 16 E6 oncoprotein. *J Virol* 2001;75:4467–72.
9. Jones DL, Alani RM, Munger K. The human papillomavirus E7 oncoprotein can uncouple cellular differentiation and proliferation in human keratinocytes by abrogating p21Cip1-mediated inhibition of cdk2. *Genes Dev* 1997;11:2101–11.
10. Lowy DR, Schiller JT. Prophylactic human papillomavirus vaccines. *J Clin Invest* 2006;116:1167–73.
11. Smith WL, DeWitt DL. Biochemistry of prostaglandin endoperoxide H synthase-1 and synthase-2 and their differential susceptibility to nonsteroidal anti-inflammatory drugs. *Semin Nephrol* 1995;15:179–94.
12. DuBois RN, Awad J, Morrow J, Roberts LJ, Bishop PR. Regulation of eicosanoid production and mitogenesis in rat intestinal epithelial cells by transforming growth factor- α and phorbol ester. *J Clin Invest* 1994;93:493–8.
13. Inoue H, Yokoyama C, Hara S, Tone Y, Tanabe T. Transcriptional regulation of human prostaglandin endoperoxide synthase-2 gene by lipopolysaccharide and phorbol ester in vascular endothelial cells. Involvement of both nuclear factor for interleukin-6 expression site and cAMP response element. *J Biol Chem* 1995;270:24965–71.
14. Subbaramaiah K, Telang N, Ramonetti JT, et al. Transcription of cyclooxygenase-2 is enhanced in transformed mammary epithelial cells. *Cancer Res* 1996;56:4424–9.
15. Smith WL, DeWitt DL, Garavito RM. Cyclooxygenases: structural, cellular, and molecular biology. *Annu Rev Biochem* 2000;69:145–82.
16. Subbaramaiah K, Dannenberg AJ. Cyclooxygenase 2: a molecular target for cancer prevention and treatment. *Trends Pharmacol Sci* 2003;24:96–102.
17. Subbaramaiah K, Norton L, Gerald W, Dannenberg AJ. Cyclooxygenase-2 is overexpressed in HER-2/neu-positive breast cancer: evidence for involvement of AP-1 and PEA3. *J Biol Chem* 2002;277:18649–57.
18. Ryu HS, Chang KH, Yang HW, Kim MS, Kwon HC, Oh KS. High cyclooxygenase-2 expression in stage IB cervical cancer with lymph node metastasis or parametrial invasion. *Gynecol Oncol* 2000;76:320–5.
19. Kulkarni S, Rader JS, Zhang F, et al. Cyclooxygenase-2 is overexpressed in human cervical cancer. *Clin Cancer Res* 2001;7:429–34.
20. Liu CH, Chang SH, Narko K, et al. Overexpression of cyclooxygenase-2 is sufficient to induce tumorigenesis in transgenic mice. *J Biol Chem* 2001;276:18563–9.
21. Neufang G, Furstenberger G, Heidt M, Marks F, Muller-Decker K. Abnormal differentiation of epidermis in transgenic mice constitutively expressing cyclooxygenase-2 in skin. *Proc Natl Acad Sci U S A* 2001;98:7629–34.
22. Muller-Decker K, Furstenberger G, Annan N, et al. Preinvasive duct-derived neoplasms in pancreas of keratin 5-promoter cyclooxygenase-2 transgenic mice. *Gastroenterology* 2006;130:2165–78.
23. Oshima M, Dinchuk JE, Kargman SL, et al. Suppression of intestinal polyposis in Apc Δ 716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell* 1996;87:803–9.
24. Tiano HF, Loftin CD, Akunda J, et al. Deficiency of either cyclooxygenase (COX)-1 or COX-2 alters epidermal differentiation and reduces mouse skin tumorigenesis. *Cancer Res* 2002;62:3395–401.
25. Howe LR, Chang SH, Tolle KC, et al. HER2/neu-induced mammary tumorigenesis and angiogenesis are reduced in cyclooxygenase-2 knockout mice. *Cancer Res* 2005;65:10113–9.
26. Kawamori T, Rao CV, Seibert K, Reddy BS. Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. *Cancer Res* 1998;58:409–12.
27. Fischer SM, Lo HH, Gordon GB, et al. Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, and indomethacin against ultraviolet light-induced skin carcinogenesis. *Mol Carcinog* 1999;25:231–40.
28. Sheng H, Shao J, Kirkland SC, et al. Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. *J Clin Invest* 1997;99:2254–9.
29. Howe LR, Subbaramaiah K, Patel J, et al. Celecoxib, a selective cyclooxygenase 2 inhibitor protects against human epidermal growth factor receptor 2 (HER-2)/neu-induced breast cancer. *Cancer Res* 2002;62:5405–7.
30. Steinbach G, Lynch PM, Phillips RK, et al. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N Engl J Med* 2000;342:1946–52.
31. Bertagnolli MM, Eagle CJ, Zaubler AG, et al. Celecoxib for the prevention of sporadic colorectal adenomas. *N Engl J Med* 2006;355:873–84.
32. Farley JH, Truong V, Goo E, Uyehara C, Belnap C, Larsen WI. A randomized double-blind placebo-controlled phase II trial of the cyclooxygenase-2 inhibitor Celecoxib in the treatment of cervical dysplasia. *Gynecol Oncol* 2006;103:425–30.
33. Sheng H, Shao J, Washington MK, DuBois RN. Prostaglandin E2 increases growth and motility of colorectal carcinoma cells. *J Biol Chem* 2001;276:18075–81.
34. Tsujii M, Kawano S, Tsuji S, Sawaoka H, Hori M, DuBois RN. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell* 1998;93:705–16.
35. Tsujii M, Kawano S, DuBois RN. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. *Proc Natl Acad Sci U S A* 1997;94:3336–40.
36. Dohadwala M, Luo J, Zhu L, et al. Non-small cell lung cancer cyclooxygenase-2-dependent invasion is mediated by CD44. *J Biol Chem* 2001;276:20809–12.
37. Sheng H, Shao J, Morrow JD, Beauchamp RD, DuBois RN. Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells. *Cancer Res* 1998;58:362–6.
38. Golijanin D, Tan JY, Kazior A, et al. Cyclooxygenase-2 and microsomal prostaglandin E synthase-1 are overexpressed in squamous cell carcinoma of the penis. *Clin Cancer Res* 2004;10:1024–31.
39. Wu R, Abramson AL, Shikowitz M, Dannenberg AJ, Steinberg BM. Epidermal growth factor-induced cyclooxygenase-2 expression is mediated through phosphatidylinositol-3 kinase, not mitogen-activated protein/extracellular signal-regulated kinase kinase, in recurrent respiratory papillomas. *Clin Cancer Res* 2005;11:6155–61.
40. Inoue H, Yokoyama C, Hara S, Tone Y, Tanabe T. Transcriptional regulation of human prostaglandin-endoperoxide synthase-2 gene by lipopolysaccharide and phorbol ester in vascular endothelial cells. Involvement of both nuclear factor for interleukin-6 expression site and cAMP response element. *J Biol Chem* 1995;270:24965–71.
41. Brown J, Higo H, McKalip A, Herman B. Human papillomavirus (HPV) 16 E6 sensitizes cells to actinolytic-induced apoptosis: role of p53, ICE-like proteases and the mitochondrial permeability transition. *J Cell Biochem* 1997;66:245–55.
42. Vojtek AB, Der CJ. Increasing complexity of Ras signaling pathway. *J Biol Chem* 1998;273:19925–8.
43. Ogawa S, Lozach J, Jepsen K, et al. A nuclear receptor corepressor transcriptional checkpoint controlling activator protein 1-dependent gene networks required for macrophage activation. *Proc Natl Acad Sci U S A* 2004;101:14461–6.
44. Perissi V, Aggarwal A, Glass CK, Rose DW, Rosenfeld MG. A corepressor/coactivator exchange complex required for transcriptional activation by nuclear receptors and other regulated transcription factors. *Cell* 2004;116:511–26.
45. Grunwald V, Hidalgo M. Developing inhibitors of epidermal growth factor receptor for cancer treatment. *J Natl Cancer Inst* 2003;95:851–67.
46. Buchanan FG, Wang D, Bargiacchi F, DuBois RN. Prostaglandin E2 regulates cell migration via the intracellular activation of the epidermal growth factor receptor. *J Biol Chem* 2003;278:35451–7.
47. Moraitis D, Du B, De Lorenzo MS, et al. Levels of cyclooxygenase-2 are increased in the oral mucosa of smokers: evidence for the role of epidermal growth factor receptor and its ligands. *Cancer Res* 2005;65:664–70.
48. Mialon A, Sankinen M, Soderstrom H, et al. DNA topoisomerase I is a cofactor for c-Jun in the regulation of epidermal growth factor receptor expression and cancer cell proliferation. *Mol Cell Biol* 2005;25:5040–51.
49. Subbaramaiah K, Lin DT, Hart JC, Dannenberg AJ. Peroxisome proliferator-activated receptor γ ligands suppress the transcriptional activation of cyclooxygenase-2. Evidence for involvement of activator protein-1 and CREB-binding protein/p300. *J Biol Chem* 2001;276:12440–8.
50. Liu Y, Borchert GL, Phang JM. Polyoma enhancer activator 3, an ets transcription factor, mediates the induction of cyclooxygenase-2 by nitric oxide in colorectal cancer cells. *J Biol Chem* 2004;279:18694–700.
51. Straight SW, Hinkle PM, Jewers RJ, McCance DJ. The E5 oncoprotein of human papillomavirus type 16 transforms fibroblasts and effects the downregulation of the epidermal growth factor receptor in keratinocytes. *J Virol* 1993;67:4521–32.
52. Fehrmann F, Laimins LA. Human papillomaviruses: targeting differentiating epithelial cells for malignant transformation. *Oncogene* 2003;22:5201–7.
53. Ferrandina G, Ranelletti FO, Legge F, et al. Celecoxib modulates the expression of cyclooxygenase-2, ki67, apoptosis-related marker, and microvessel density in human cervical cancer: a pilot study. *Clin Cancer Res* 2003;9:4324–31.