The Adenomatous Polyposis Coli Tumor Suppressor Gene Regulates Expression of Cyclooxygenase-2 by a Mechanism That Involves Retinoic Acid

Mutations in the adenomatous polyposis coli (APC) gene result in uncontrolled proliferation of intestinal epithelial cells and are associated with the earliest stages of colorectal carcinogenesis. Cyclooxygenase-2 (COX-2) is elevated in human colorectal cancers and plays an important role in colorectal tumorigenesis; however, the mechanisms by which APC mutations result in increased COX-2 expression remain unclear. We utilized APC mutant zebrafish and human carcinoma cell lines to investigate how APC modulates COX-2 expression. We report that COX-2 is up-regulated in APC mutant zebrafish because of a deficiency in retinoic acid biosynthesis. Treatment of both APC mutant zebrafish and human carcinoma cell lines with retinoic acid significantly reduces COX-2 expression. Retinoic acid regulates COX-2 levels by a mechanism that involves participation of the transcription factor C/EBP-β. APC mutant zebrafish express higher levels of C/EBP-β than wild-type animals, and retinoic acid supplementation reduces C/EBP-β expression to basal levels. Both morpholino knockdown of C/EBP-β in APC mutant zebrafish and silencing of C/EBP-β using small interfering RNA in HT29 colon cancer cells robustly decrease COX-2 expression. Our findings support a sequence of events in which mutations in APC result in impaired retinoic acid biosynthesis, elevated levels of C/EBP-β, up-regulation of COX-2, increased prostaglandin E2 accumulation, and activation of Wnt target genes.

Cyclooxygenases (COXs)² are the rate-limiting enzymes in the conversion of arachidonic acid into prostaglandins (PGs). The COX enzyme family consists of COX-1, which is constitutively expressed and thought to be involved in the housekeeping functions of PGs, and COX-2, an inducible isoform of COX that is involved in many inflammatory reactions. COX-2 is overexpressed in 40% of human adenomas and 80% of adenocarcinomas relative to normal mucosa (1, 2). COX-2 is not detected in most normal tissues but is rapidly induced in response to mitogens, cytokines, and tumor promoters, leading to increased amounts of prostanoids in neoplastic and inflamed tissues (3, 4). Previous studies demonstrated a key role for COX-2 in colorectal tumorigenesis, as well as a strong link between COX-2/PGE2 signaling and expression of the adenomatous polyposis coli (APC) gene in intestinal neoplasia. Mutations in the tumor suppressor gene APC result in familial adenomatous polyposis, an inherited type of colon cancer characterized by the development of thousands of polyps in the colon. Deletion of the cox-2 gene in apc knock-out mice, a murine model of familial adenomatous polyposis, resulted in a marked reduction in the number and size of intestinal polyps (5). Moreover, inhibition of COX-2 activity by administration of traditional nonsteroidal anti-inflammatory drugs or selective COX-2 inhibitors has been shown to reduce tumor size and multiplicity in genetic and carcinogen-induced animal models of colon cancer (5–8). Finally, treatment of familial adenomatous polyposis patients with the nonsteroidal anti-inflammatory drug sulindac or the selective COX-2 inhibitor celecoxib was shown to diminish the number of existing colorectal adenomas relative to placebo (9, 10). These data point at a key role of the enzymatic activity of COX-2 in the pathogenesis of colorectal carcinomas.

Studies to define the molecular mechanisms controlled by APC have elucidated a model wherein APC is a negative regulator of β-catenin and the Wnt signaling pathway. In this model, APC serves as a scaffolding molecule that facilitates assembly of a multiprotein complex containing axin, glycogen synthase kinase-3β, and casein kinase 1ε/δ. This complex promotes the phosphorylation of β-catenin, resulting in its ubiquitin-mediated degradation. In cells lacking functional APC, β-catenin accumulates and translocates into the nucleus where it associates with the transcription factors TCF/LEF, leading to the expression of Wnt target genes and inducing a program of cell proliferation.

We recently reported that another essential function of APC is to regulate retinoic acid biosynthesis and promote colonocyte differentiation (11, 12). Retinoic acid is a biologically active lipid mediator involved in the control of cell fate and differentiation (13). In contrast to normal colon tissues, colon adenomas and carcinomas lack the ability to convert dietary retinol (vitamin A) into retinoic acid because they do not express ret-
COX-2 Expression Is Regulated by Retinoic Acid

EXPERIMENTAL PROCEDURES

Embryo Culture and Zebrafish Stocks—Wild-type and APC mutant Danio rerio (zebrafish) were maintained on a 14:10-h light/dark cycle. The characteristics of the truncated APC mutant zebrafish utilized in this study were described previously (17). Fertilized embryos were collected following natural spawnsings and were allowed to develop at 28.5 °C. Control and experimental embryos were raised in 0.003% phenylthiourea to inhibit pigment formation.

Whole Mount in Situ Hybridization—Zebrafish embryos were fixed in sucrose-buffered 4% paraformaldehyde, rinsed in phosphate-buffered saline, dehydrated in methanol, and stored at −20 °C. Riboprobes for COX-2 and C/EBP-β were generated by linearization of pCRII (Invitrogen) containing COX-2 or C/EBP-β cDNA followed by in vitro transcription with SP6 or T7 RNA polymerase (Roche Applied Science). The embryos were cleared in 70% glycerol/phosphate-buffered saline and photographed using an Olympus DP12 digital camera.

Quantitative RT-PCR—We isolated RNA from embryos and cell lines using an RNasy kit (Qiagen) and then synthesized single-stranded cDNA from 1 μg of total RNA using Superscript III (Invitrogen). PCR was performed using a Roche Light Cycler instrument and software (version 3.5; Roche Applied Science). The sequences of PCR primers used were as follows: COX-2, forward, 5′-GGATGATGAGAGAATCTTCCAAACC-3′, and reverse, 3′-GGTGGAGCGATGTTGTCTT-5′; and C/EBP-β, forward, 5′-AAAGTAAACGGGCGACGAAACAC-3′, and reverse, 5′-TGAGTCAATTAAGCACCGACGCT-3′. We performed duplicate PCRs using the LightCycler FastStart DNA Master SYBR Green 1 kit (Roche Applied Science). The amplification conditions for COX-2 included 10 s of denaturation at 95 °C, 5 s of annealing at 61 °C, and 10 s of extension at 72 °C, for a total of 35 cycles. MMP9, and C/EBP-β were amplified using the same protocol except that annealing was set at 57 °C. A template-free negative control was included in each experiment. Quantitative RT-PCR primers and conditions for amplification of 28 S rRNA and human 18 S rRNA were described previously (11, 18).

Morpholino and RNA Microinjections—We obtained morpholino oligonucleotides from Gene Tools LLC. An APC splice-blocking morpholino (5′-TAGCATACTCTACCTGTCTCT-TCG-3′), an rh11 splice-blocking morpholino (5′-TCTGTCAGTGACTCACCCTTCTGTC-3′), a C/EBP-β translation blocking morpholino (5′-GATCTTTAACCCCAGCATTGC-G3-3′), and a control morpholino (5′-CCTCTACCTGTCTTACATTATA-3′) were dissolved in 1× Danieau buffer and adjusted to a final concentration of 1.0 mM. For microinjections, 1.0 nl of 1.0 mM morpholino was injected into wild-type embryos at the one- to four-cell stage.

Messenger RNA for a stabilized form of β-catenin (β-catenin S37A) was synthesized using mMessage mMachine (Ambion) according to the manufacturer’s instructions. This stabilized β-catenin mRNA was injected into embryos at the one-cell stage.

Retinoic Acid and Lithium Chloride Treatments—To investigate the role of retinoic acid in COX-2 expression in APC mutants and rh11 morphants, we incubated embryos with 900 nm all-trans-retinoic acid (Sigma) in Me2SO at 50% epiboly for 1 h. After washing with embryo water, we treated the embryos with 20 nm all-trans-retinoic acid at 30 and 54 hpf for 1 h and then repeated the washing step. To suppress C/EBP-β in APC mutants, embryos were incubated with 1 μM all-trans-retinoic acid for 2 h or with an equivalent amount of Me2SO as a control. To inhibit GSK-3β, we incubated embryos at 50% epiboly, 30 and 54 hpf with the indicated concentrations of LiCl (Sigma) dissolved in embryo water.

Histological Analysis—Following in situ hybridization, embryos were rinsed in phosphate-buffered saline and embedded in paraffin. Six-micron sections were cut using a Leica microtome. Sections were analyzed using a Zeiss Axiosvert 200 Microfire camera (Optronics). Images were captured on Pictureframe software.

Cell Culture and Drug Treatments—HEK293, HT29, HCT116, HaCaT, and SCC-9 cells were cultured as recommended by the American Type Culture Collection. HEK293, HCT116, HT29, and HaCaT cells were maintained in basal media in the absence of serum for 16 h prior to treatment with all-trans-retinoic acid or Me2SO as a control.

Overexpression, Gene Silencing, and Promoter Activity Assays—HCT116 and SCC-9 cells were seeded at a density of 150,000 cells/well and transfected the next day using Lipofectamine (Invitrogen), according to the instructions provided...
by the manufacturer. We used 1–2 μg of a stabilized form of β-catenin (β-catenin S37A), and we harvested the cells 48 h after the addition of DNA. We knocked down endogenous β-catenin expressed in HCT116, HT29, and SCC-9 cells using the same experimental approach except that Lipofectamine 2000 (Invitrogen) was used to transfect the siRNAs (final concentration = 50–100 nM).

To assess promoter activation, we transfected HEK293 cells with 2 μg of DNA combined with a Rous sarcoma virus- Renilla luciferase reporter plasmid for normalization purposes using Lipofectamine 2000 (Invitrogen) according to the instructions provided by the manufacturer. We seeded cells in 12-well plates at a density of 100,000 cells/well, performed transfections the next day, and harvested the cellular protein 24 h later. Luciferase activity was assessed using a dual luciferase assay system (Promega). The normalized values reported are the ratio between firefly and Renilla luciferase activities.

**Plasmids and Small Interfering RNAs—**The COX-2 promoter-luciferase constructs used in this study were described in detail previously (19, 20). The β-catenin S37A expression vector and dominant negative glycogen synthase kinase-3β (DN-GSK-3β) were kindly provided by Dr. Donald Ayer (University of Utah, Salt Lake City). The duplex targeting sequences used to silence expression of β-catenin were as follows: sense, 5'-CAGUUGUGGUAAUCUUTT-3’, and antisense, 5'-AAGAGCUAAACCACAACCUTT-3’. The sequences used to silence C/EBP-β expression were described previously (21), and the control siRNA sequences were as follows: sense, 5'-UUCUCCGAACGUUCACGU-3’, and antisense, 5'-ACGUGACGUUCGGAGAATT-3’.

**Western Blotting—**We obtained extracts from zebrafish embryos in 1× reporter lysis buffer (Promega) with the aid of a Dounce homogenizer. The same buffer was used to harvest mammalian cells after individual treatments. Lysates were frozen at −80 °C and then centrifuged at 12,000 × g for 2 min to remove the insoluble components. We assessed protein concentration in the supernatants (using the BCA protein assay, Pierce) and then subjected 20–60 μg of protein to electrophoresis on 7.5% denaturing polyacrylamide gels. After electrotransfer to polyvinylidene difluoride membranes (PolyScreen), we stained the transferred proteins using Ponceau S solution for 2 min and then blocked the remaining protein-binding sites with 5% nonfat dry milk in Tris-buffered saline. The primary antibodies used throughout this study were obtained from Cayman Chemical (anti-COX-2), Cell Signaling (anti-β-catenin), and ICN Biomedicals (anti-actin). We then probed the membranes with horseradish peroxidase-labeled secondary antibody (BIOSOURCE) and used a chemiluminescence detection system (Western Lightning) to reveal the presence of immunoreactive proteins.

**RESULTS**

**COX-2 Is Up-regulated in APC Mutant Zebrafish—**Up-regulation of COX-2 following loss of the APC tumor suppressor gene appears required for the initiation of colon adenomas and carcinomas in mice and humans. It has been reported recently that zebrafish heterozygous for a truncating APC mutation spontaneously develop highly proliferative intestinal, hepatic, and pancreatic neoplasias (22). Because this model recapitulated key features characteristic of digestive tract malignancies, we examined the connection between homozygous loss of APC and COX-2 overexpression using zebrafish embryos homozygous for mutant APC. We first compared COX-2 expression in APC mutants to that of wild-type zebrafish at the same stage of development. We performed quantitative RT-PCR using total RNA isolated from wild-type and APC mutant zebrafish embryos at 72 hpf. We found that COX-2 mRNA levels were 9-fold higher in the homozygous APC mutant zebrafish (APC<sup>−/−</sup>) compared with wild-type 72 hpf zebrafish (Fig. 1A).

We saw a similar induction of COX-2 mRNA in embryos wherein APC was knocked down using an antisense morpholino construct (APC MO). Immunoblot analyses of APC mutant embryos revealed that the COX-2 protein was also elevated in homozygous APC mutant embryos compared with wild-type animals (Fig. 1B).

COX-2 is normally expressed in the carotid arteries, the vasculature of the pharyngeal arches, and in the vascular endothelium of the intestine at 96 hpf (23). Whole mount in situ hybridization of COX-2 in wild-type embryos confirmed this expression pattern (Fig. 1C). In contrast, APC mutant zebrafish embryos expressed remarkably high levels of COX-2 mRNA in additional tissues and organs, including the fin buds, the pancreas, the intestine, and the skin (Fig. 1C). The hybridization studies were conducted at the same time and using the same developing solutions to rule out artifactual differences related to technical manipulations. Histological cross-sectional analyses performed after whole mount in situ hybridization revealed that COX-2 was expressed in the stromal compartment of the intestine and in the epithelial layer of the skin (Fig. 1D).

**COX-2 Regulation Is Independent of β-Catenin—**Two recent independent studies reported that PGE<sub>2</sub>, one of the bioactive products of COX-2, activates components of the Wnt signaling pathway, thus providing solid evidence for a direct link between these pathways (24). Based on these observations, we reasoned that the factor(s) accounting for up-regulation of COX-2 in the absence of functional APC must operate upstream of Wnt signaling. However, previous studies have reported that COX-2 was up-regulated by nuclear β-catenin accumulation in colon cancer cell lines, thereby suggesting it as a target downstream of Wnt signaling. We investigated this incongruity using several experimental approaches first in zebrafish by injection of an mRNA encoding a variant of β-catenin, S37A, that is resistant to proteasomal degradation (25). Functional expression of β-catenin was confirmed by Western blotting. We found that overexpression of β-catenin had no effect on endogenous COX-2 mRNA expression levels (Fig. 2A, solid bars). In contrast, we observed robust up-regulation of mRNA for matrix metalloproteinase 9 (MMP9), a gene that is transcriptionally regulated by β-catenin (27) (Fig. 2A, open bars).

In complementary experiments, we increased β-catenin stability and enhanced its nuclear import by inhibiting the activity of endogenous glycogen synthase kinase-3β (GSK-3β) (28). We used both a dominant negative approach (DN-GSK-3β; Fig. 2A) and pharmacological inhibition with LiCl (29). We found that although inhibition of GSK-3β effectively induced β-catenin...

...expression in APC mutants to that of wild-type zebrafish at the same stage of development. We performed quantitative RT-PCR using total RNA isolated from wild-type and APC mutant zebrafish embryos at 72 hpf. We found that COX-2 mRNA levels were 9-fold higher in the homozygous APC mutant zebrafish (APC<sup>−/−</sup>) compared with wild-type 72 hpf zebrafish (Fig. 1A).

We saw a similar induction of COX-2 mRNA in embryos wherein APC was knocked down using an antisense morpholino construct (APC MO). Immunoblot analyses of APC mutant embryos revealed that the COX-2 protein was also elevated in homozygous APC mutant embryos compared with wild-type animals (Fig. 1B).

COX-2 is normally expressed in the carotid arteries, the vasculature of the pharyngeal arches, and in the vascular endothelium of the intestine at 96 hpf (23). Whole mount in situ hybridization of COX-2 in wild-type embryos confirmed this expression pattern (Fig. 1C). In contrast, APC mutant zebrafish embryos expressed remarkably high levels of COX-2 mRNA in additional tissues and organs, including the fin buds, the pancreas, the intestine, and the skin (Fig. 1C). The hybridization studies were conducted at the same time and using the same developing solutions to rule out artifactual differences related to technical manipulations. Histological cross-sectional analyses performed after whole mount in situ hybridization revealed that COX-2 was expressed in the stromal compartment of the intestine and in the epithelial layer of the skin (Fig. 1D).

**COX-2 Regulation Is Independent of β-Catenin—**Two recent independent studies reported that PGE<sub>2</sub>, one of the bioactive products of COX-2, activates components of the Wnt signaling pathway, thus providing solid evidence for a direct link between these pathways (24). Based on these observations, we reasoned that the factor(s) accounting for up-regulation of COX-2 in the absence of functional APC must operate upstream of Wnt signaling. However, previous studies have reported that COX-2 was up-regulated by nuclear β-catenin accumulation in colon cancer cell lines, thereby suggesting it as a target downstream of Wnt signaling. We investigated this incongruity using several experimental approaches first in zebrafish by injection of an mRNA encoding a variant of β-catenin, S37A, that is resistant to proteasomal degradation (25). Functional expression of β-catenin was confirmed by Western blotting. We found that overexpression of β-catenin had no effect on endogenous COX-2 mRNA expression levels (Fig. 2A, solid bars). In contrast, we observed robust up-regulation of mRNA for matrix metalloproteinase 9 (MMP9), a gene that is transcriptionally regulated by β-catenin (27) (Fig. 2A, open bars).

In complementary experiments, we increased β-catenin stability and enhanced its nuclear import by inhibiting the activity of endogenous glycogen synthase kinase-3β (GSK-3β) (28). We used both a dominant negative approach (DN-GSK-3β; Fig. 2A) and pharmacological inhibition with LiCl (29). We found that although inhibition of GSK-3β effectively induced β-catenin...
and MMP9 (Fig. 2, A and B), these treatments failed to alter COX-2 expression levels (Fig. 2, A and B).

To investigate if the observed lack of regulation of COX-2 by β-catenin was peculiar to zebrafish embryos, we used similar approaches in human cell lines. First, we transfected the stable β-catenin construct (S37A) into HCT116 colon carcinoma cells and SCC-9 squamous cell carcinoma cells and found no changes in the expression levels of endogenous COX-2 (Fig. 2C). Second, we silenced endogenous expression of β-catenin using siRNA in HCT116 and SCC-9 cells. We found that although this treatment successfully knocked down β-catenin protein levels, it had no effect on COX-2 expression levels (Fig. 2D). Overexpression of β-catenin also failed to activate a reporter construct wherein we fused 1.8 kb of the COX-2 promoter region to luciferase (data not shown). We concluded that the regulation of COX-2 is not subject to control by β-catenin in human cancer cells or in APC mutant zebrafish embryos.

COX-2 Expression Is Regulated by Retinoic Acid

COX-2 Expression Is Suppressed by Retinoic Acid—We recently reported a previously unrecognized function of APC as a factor that controls retinoic acid biosynthesis through the retinoil dehydrogenase enzymes in human colon cancer cell lines and in zebrafish embryos (11, 12, 14). Here we hypothesized that APC may regulate COX-2 expression in a retinoic acid-dependent fashion. To test this, we asked if retinoic acid

FIGURE 1. COX-2 is up-regulated in APC mutant zebrafish. A, quantitative RT-PCR with primers specific for COX-2 was performed on cDNAs from wild-type (WT), APC morphant, and APC mutant zebrafish embryos (72 hpf). Fold increase for each transcript was determined by comparing the number of COX-2 transcripts per μg of total RNA in both APC morpholino-injected embryos and in APC mutant embryos with the number of COX-2 transcripts per μg of total RNA in control morpholino-injected embryos. B, immunoblot analysis of COX-2 protein in extracts from wild-type and APC mutant embryos at 54 and 72 hpf showing overexpression of COX-2 in 72-hpf embryos. Staining with Ponceau S served as a control for sample loading. C, whole mount in situ hybridization with a probe specific for COX-2 in wild-type and APC mutant embryos. D, histological cross-sections of 72-hpf embryos following whole mount in situ hybridization. COX-2 was expressed in the stromal layer of the intestine and in the epithelial layer of the skin. * denotes statistical significance with a p value of <0.05 as determined by a Student’s t test.
COX-2 Expression Is Regulated by Retinoic Acid

We measured luciferase expression in HEK293 cells as well as the colon cancer cell line HCT116 following transfection with a reporter construct representing 1.8 kb of the COX-2 promoter fused to a luciferase reporter gene (19). In the case of HCT116 cells, we co-transfected the cells with a retinoic acid receptor-α expression construct necessary to attain cellular responsiveness to the ligand. Twenty four hours after transfection, we treated the cells with 5.0 μM retinoic acid for 6 h and measured subsequent luciferase activity. We found that this treatment significantly suppressed COX-2 promoter activity levels in both cell types (Fig. 4A). These findings were supported by examination of endogenous COX-2 expression in two cell lines, HT29 and HaCaT, which express relatively high amounts of COX-2. Treatment of either HT29 or HaCaT cells maintained in basal medium for 16 h, with vehicle, 1.0 μM or 2.0 μM retinoic acid in Me₃SO for 4 h caused a dose-dependent decrease in COX-2 at both the mRNA and protein levels (Fig. 4, B and C).

Retinoic Acid Suppresses COX-2 by Regulating C/EBP-β—We reported previously that altered expression of COX-2 in colon cancer occurs at the transcriptional level (19). Several studies have demonstrated that CCAAT/enhancer-binding protein elements (C/EBP) in the promoter are necessary for a variety of agents to induce COX-2 expression (3, 30). First, the human COX-2 promoter contains multiple regulatory elements, including two functional cis-acting C/EBP sites. Second, the murine cox-2 promoter harbors C/EBP and ATF/CREB transcription factor-binding sites that have been identified as regulators of basal COX-2 expression in a

deficiency and COX-2 up-regulation were causally related. First, we treated developing APC mutant zebrafish embryos with exogenous retinoic acid and then determined the level of COX-2 mRNA. We found that this treatment significantly reduced COX-2 mRNA transcript levels in APC mutant embryos (Fig. 3A) and that this reduction included decreased expression of COX-2 in the intestinal stroma (Fig. 3B). To investigate this issue further, we knocked down expression of the retinol dehydrogenase, rdh11, in zebrafish and then examined COX-2 expression. We found that retinoic acid deficiency alone resulted in the up-regulation of COX-2 using both in situ hybridization with a specific COX-2 probe and quantitative RT-PCR (Fig. 3, C and D). Confirming that this was a retinoic acid-specific effect, treatment of rdh11 morphant embryos with exogenous retinoic acid reduced the levels of COX-2 (Fig. 3D).

To extend the above findings in mammalian systems, we used human colon cancer cells and tested the effect of retinoic acid on the expression of COX-2 at the transcriptional level.

**FIGURE 3.** COX-2 expression is suppressed by retinoic acid in zebrafish. A, quantitative RT-PCR with primers specific for COX-2 was performed on cDNAs obtained from wild-type (WT) embryos, APC mutant embryos treated with Me₃SO, or APC mutant embryos treated with retinoic acid (RA). Fold change was determined by comparing the number of COX-2 transcripts in APC mutant embryos treated with Me₃SO (DMSO) or RA to the number of COX-2 transcripts in wild-type embryos. Each sample was first normalized to 28S RNA. B, treatment of APC mutant embryos with exogenous RA partially rescued abnormally COX-2 up-regulation in the gut stroma. C, whole mount in situ hybridization with a probe specific for COX-2 indicated that embryos (24 and 72 hpf) injected with a rdh11 morpholino had increased levels of COX-2 compared with embryos injected with a control morpholino. D, quantitative RT-PCR with primers specific for COX-2 was performed on cDNAs obtained from embryos injected with control morpholino or rdh11 morpholino in the absence and presence of exogenous RA. Fold increase for each transcript was determined by comparing the number of COX-2 transcripts per μg of total RNA in rdh11 morpholino-injected embryos treated with Me₃SO or RA to the number of COX-2 transcripts per μg of RNA in control morpholino-injected embryos. Treatment of rdh11 morphant embryos with RA partially rescued expression of COX-2 compared with vehicle-treated rdh11 morphants. *, denotes statistical significance with a p value <0.05 as determined by a Student’s t test.

**FIGURE 4.** COX-2 expression is suppressed by retinoic acid in human cells. A, HEK293 cells and HCT116 cells expressing the retinoic acid receptor-α were transfected with a COX-2 reporter vector and normalization vector Rous sarcoma virus-Renilla luciferase. After 24 h, cells were treated with retinoic acid for 6 h. Data were analyzed as described in the legend of Fig. 3A. B, quantitative RT-PCR with primers specific for COX-2 was performed on cDNAs from HT29 and HaCaT cells treated with vehicle (Me₃SO) or retinoic acid for 4 h. Each sample was first normalized to β-actin. Fold decrease was determined by comparing the number of COX-2 transcripts in retinoic acid-treated cells with the number of COX-2 transcripts in Me₃SO-treated cells. C, HT29 and HaCaT cells were maintained in basal medium for 16 h before treatment with vehicle, 1.0 μM or 2.0 μM retinoic acid. After 4 h, cells were harvested in lysis buffer and analyzed by immunoblotting, *, denotes statistical significance with a p value <0.05 as determined by a Student’s t test.
The above observations combined with our findings suggested that C/EBP-β may play a role as an intermediate in retinoic acid-mediated suppression of COX-2 expression. To test this, we quantified C/EBP-β transcript levels in APC mutant zebrafish and found that they were up-regulated (Fig. 5, A and B). To test whether COX-2 levels depended on C/EBP-β, we designed two translation-blocking morpholinos (C/EBP-β MO) targeting nonoverlapping regions 5’ to the ATG start of the C/EBP-β mRNA. Injection of both C/EBP-β morpholinos resulted in decreased C/EBP-β protein levels (Fig. 5C and data not shown); injection of a control morpholino was without effect. Importantly, APC mutant embryos injected with a C/EBP-β morpholino expressed much lower levels of COX-2 mRNA (2.8-fold decrease) compared with APC mutant zebrafish (Fig. 5C). By using whole mount in situ hybridization, we determined that the largest decrease in COX-2 expression occurred primarily in the intestinal stroma (Fig. 5D). These results strongly suggested that C/EBP-β plays a key role in the regulation of COX-2 expression in APC mutant zebrafish.

We next investigated the participation of retinoic acid as a modulator of C/EBP-β levels in APC mutant zebrafish. We treated APC mutant embryos with retinoic acid or vehicle (Me2SO) and then determined the levels of C/EBP-β mRNA by quantitative RT-PCR. We found that treatment of embryos with exogenous retinoic acid significantly decreased C/EBP-β transcript levels in APC mutant embryos (Fig. 5E). These combined results demonstrate that the ability of retinoic acid to down-regulate COX-2 expression in APC mutant zebrafish is mediated by C/EBP-β.

**APC Suppresses COX-2 Expression through a Mechanism That Involves C/EBP-β**—To further explore the hypothesis that APC regulates COX-2 by retinoic acid-mediated suppression of C/EBP-β, we utilized an inducible expression system consisting of HT29 human colon carcinoma cells stably transfected with a zinc-inducible metallothionein promoter fused to wild-type APC CDNA (HT29-APC) (35). An HT29 cell line containing a zinc-inducible lacZ gene was used as negative control. Treatment of HT29-APC cells with 100 μM zinc decreased the levels of both COX-2 and C/EBP-β protein compared with zinc-treated HT29-LacZ cells (Fig. 6A). To demonstrate that C/EBP-β controls COX-2 expression under similar experimental conditions, we silenced its expression in HT29 cells and found that this treatment decreased COX-2 protein levels (Fig. 6B). These combined results indicate that APC suppression of COX-2 is mediated by C/EBP-β.
COX-2 Expression Is Regulated by Retinoic Acid

**A**

|               | LacZ | APC |
|---------------|------|-----|
| COX-2         |      |     |
| C/EBP-β       |      |     |
| β-actin       |      |     |

+ + + ZnCl₂

**B**

|               | Control | SCR | C/EBP-β | siRNA |
|---------------|---------|-----|---------|-------|
| COX-2         |         |     |         |       |
| C/EBP-β       |         |     |         |       |
| β-actin       |         |     |         |       |

**FIGURE 6. COX-2 is regulated by C/EBP-β in human cells.** A, HT29 APC-inducible and LacZ-inducible cells were treated for 24 h with 100 μM ZnCl₂ or with vehicle. The cellular proteins were harvested and analyzed for COX-2, C/EBP-β, and β-actin by immunoblotting. B, HT29 cells were transfected with C/EBP-β or scrambled (SCR) siRNAs. After 48 h, the cellular proteins were harvested and analyzed for COX-2, C/EBP-β, and β-actin protein levels by immunoblotting. The control lane depicts results obtained using cells transfected with no siRNA.

**DISCUSSION**

The finding that PGE₂ activates Wnt signaling and results in the accumulation of β-catenin in the nucleus has shed new insights into the role of the inflammatory process in activated Wnt signaling during colorectal cancer. This key observation of PGE₂ signaling through the EP2 receptor as a mechanism that enhances downstream signaling events, but the mechanistic details were not addressed (36). In the present study we investigated the mechanisms that mediate up-regulation of COX-2, the enzyme that catalyzes the production of PGE₂, following functional inactivation of APC. We used both mammalian cells and zebrafish embryos that are homozygous-deficient for APC. We found that COX-2 was up-regulated in these embryos, a finding that recapitulates the increased COX-2 expression previously reported in human and mouse tissues carrying APC mutations. We reasoned that if PGE₂ directly impinges on the Wnt pathway targeted by mutant APC (36), then COX-2 also must be controlled by downstream targets of APC. We previously demonstrated that one of the functions of APC is to control the expression of rdh11, an enzyme that defines the level of cellular retinoic acid (14). APC mutant zebrafish are deficient in rdh11, and knockdown of retinol dehydrogenase in vivo phenocopies APC deficiency. Both knockdown phenotypes are rescued by treatment with exogenous retinoic acid (12). The defect in retinoic acid biosynthesis in APC mutant zebrafish led us to examine the possibility that decreased levels of retinoic acid are responsible for the up-regulation of COX-2 observed in APC mutant zebrafish. In support of this hypothesis, we found that treatment of APC mutant embryos with exogenous retinoic acid restored COX-2 expression to basal levels. There was a significant decrease in the aberrant COX-2 up-regulation observed in the stromal layer of the gut following treatment with retinoic acid. In addition, COX-2 was increased in rdh11 morphants, which are deficient in retinoic acid. Treatment of rdh11 morphants with retinoic acid also suppressed the up-regulation of COX-2, further confirming its role in the regulation of COX-2. Retinoic acid also suppresses COX-2 expression in mammalian cells, suggesting that this regulatory mechanism is evolutionarily conserved.

Retinoic acid has been reported to suppress expression of COX-2 (37–40). Here we showed that the mechanism of retinoic acid-mediated regulation of COX-2 is indirect and involves participation of the transcription factor C/EBP-β, which is itself regulated by retinoic acid (33). First, we found that C/EBP-β was up-regulated in APC mutant zebrafish. Second, silencing C/EBP-β expression significantly decreased COX-2 transcript levels and nearly abolished COX-2 up-regulation in the stromal layer of the gut. Finally, expression of functionally active APC decreased expression of both C/EBP-β and COX-2. These results supported a role for C/EBP-β as an intermediate in the regulation of COX-2 by APC in zebrafish, in agreement with studies in human cells (this study and see Refs. 31, 32, and 41).

The localization of COX-2 to the stromal compartment of the intestine in APC mutant zebrafish is consistent with previous studies of COX-2 expression in mouse and human intestinal adenomas (5, 42–46). However, previous reports indicate that rdh1 and rdh1l enzymes are expressed in the gut epithelium (12). Given that COX-2 levels appear to depend upon retinoic acid production, this suggests retinoic acid as a potential mediator for cross-talk between the intestinal stroma and intestinal epithelium. Alternatively, there may be as yet unidentified stromal retinol dehydrogenase enzymes that would allow for regulation of stromal C/EBP-β and COX-2 by locally synthesized retinoic acid.

Our studies also evaluated the possibility that mutations in APC cause up-regulation of COX-2 through a β-catenin-dependent pathway. However, our evidence and that of Haertel-Wiesmann et al. (47) suggest that β-catenin does not transcriptionally regulate COX-2 expression in zebrafish or in human cancer cell lines. First, we showed that overexpression of β-catenin in zebrafish failed to up-regulate COX-2 but efficiently up-regulated expression of MMP9, a known β-catenin target gene. Second, stabilization of β-catenin by inhibition of GSK-3β failed to up-regulate COX-2 in zebrafish but robustly up-regulated MMP9 levels. Third, β-catenin did not activate the COX-2 promoter in HEK293 cells. Finally, we observed no alteration in COX-2 expression by stabilization of β-catenin or knockdown of β-catenin in HT116 cells or SCC-9 cells, in
agreement with studies by Araki et al. (25) and Haertel-Wiesmann et al. (47). The inability of stabilized β-catenin to alter COX-2 expression was observed by other investigators in C57MG mouse mammary epithelial cells in which overexpression of transcriptionally active β-catenin-Lef-1 complexes failed to induce COX-2 (47). The converse approach, knocking down β-catenin levels using antisense oligonucleotides transfection, had no effect on COX-2 expression. Araki and co-workers (25) previously reported that co-expression of mutant K-ras was necessary for β-catenin to up-regulate COX-2 expression in a human hepatocellular carcinoma cell line suggesting the participation of other regulatory mechanisms in this system.

In summary, we have elucidated a mechanism by which COX-2 levels are transcriptionally regulated and that involves the concerted actions of APC, retinoic acid, and C/EBP-β using in vivo model systems and mammalian cell strategies. Our data support a novel model wherein defective retinoic acid biosynthesis allows for the up-regulation of C/EBP-β leading to elevated COX-2 (and presumably PGE₂) levels following loss of APC. Accumulation of products of COX-2 strengthens Wnt signaling and inappropriately activates target genes (Fig. 7). We have expanded our previous findings that retinoic acid deficiency is one of the consequences of altered APC function to have expanded our previous findings that retinoic acid deficiency is one of the consequences of altered APC function to

**COX-2 Expression Is Regulated by Retinoic Acid**

2. Sinicrope, F. A., Lemoine, M., Xu, L., Lynch, P. M., Cleary, K. R., Shen, Y., and Frazier, M. L. (1999) *Gastroenterology* **117**, 350–358
3. Smith, W. L., DeWitt, D. L., and Garavito, R. M. (2000) *Annu. Rev. Biochem.* **69**, 145–182
4. Subbaramaiah, K., Telang, N., Ramonetti, J. T., Araki, R., Devito, B., Weksler, B. B., and Dannenberg, A. J. (1996) *Cancer Res.* **56**, 4424–4429
5. Oshima, M., Dinchuk, J. E., Kargman, S. L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J. M., Evans, J. F., and Taketo, M. M. (1996) *Cell* **87**, 803–809
6. Kawamori, T., Rao, C. V., Seibert, K., and Reddy, B. S. (1998) *Cancer Res.* **58**, 409–412
7. Jacoby, R. F., Seibert, K., Cole, C. E., Kelloff, G., and Lubet, R. A. (2000) *Cancer Res.* **60**, 5040–5044
8. Mahmoud, N. N., Booibol, S. K., Dannenberg, A. J., Mestre, J. R., Bilinsky, R. T., Martucci, C., Newmark, H. L., Chadburn, A., and Bertagnolli, M. M. (1998) *Carcinogenesis* **19**, 87–91
9. Giardiello, F. M., Hamilton, S. R., Krush, A. J., Piantadosi, S., Hylind, L. M., Celano, P., Booker, S. V., Robinson, C. R., and Offerhaus, G. J. (1993) *N. Engl. J. Med.* **328**, 1313–1316
10. Steinbach, G., Lynch, P. M., Phillips, R. K., Wallace, M. H., Haw, E., Gordon, G. B., Wakabayashi, N., Saunders, B., Shen, Y., Fujimura, T., Su, L. K., and Levin, B. (2000) *N. Engl. J. Med.* **342**, 1946–1952
11. Jette, C., Peterson, P. W., Sandoval, I. T., Manos, E. J., Hadley, E., Ireland, C. M., and Jones, D. A. (2004) *J. Biol. Chem.* **279**, 34397–34405
12. Nadauld, L. D., Sandoval, I. T., Chidester, S., Yost, H. J., and Jones, D. A. (2004) *J. Biol. Chem.* **279**, 51581–51589
13. Duester, G. (2000) *Eur. J. Biochem.* **267**, 4315–4324
14. Nadauld, L. D., Shelton, D. N., Chidester, S., Yost, H. J., and Jones, D. A. (2005) *J. Biol. Chem.* **280**, 30490–30495
15. Castellone, M. D., Teramoto, H., Williams, B. O., Druey, K. M., and Gutkind, J. S. (2005) *Science* **310**, 1504–1510
16. Shao, J., Jung, C., Liu, C., and Sheng, H. (2005) *J. Biol. Chem.* **280**, 26565–26572
17. Hsi, L. C., Angerman-Stewart, J., and Eling, T. E. (1999) *Carcinogenesis* **20**, 2045–2049
18. Delaunay, F., Thisse, C., Marchand, O., Laudet, V., and Thisse, B. (2000) *Science* **289**, 297–300
19. Kutchera, W., Jones, D. A., Matsunami, N., Groden, J., McIntyre, T. M., Zimmerman, G. A., White, R. L., and Prescott, S. M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 4816–4820
20. Meade, E. A., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. (1999) *J. Biol. Chem.* **274**, 8328–8334
21. Heckman, C. A., Wheeler, M. A., and Boxer, L. M. (2003) *Oncogene* **22**, 7891–7899
22. Hamas, J. F., Hurlstone, A., van der Velden, Y., Begthel, H., van den Born, M., Offerhaus, G. J., and Clevers, H. C. (2006) *EMBO Rep.* **7**, 444–449
23. Grosser, T., Ysuf, S., Cheskes, E., Pack, M. A., and FitzGerald, G. A. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 8418–8423
24. Buchanan, F. G., and DuBois, R. N. (2006) *Cancer Cell* **9**, 6–8
25. Araki, Y., Okamuro, S., Hashin, S. P., Nakagami, M., He, P., Shiokai, M., Miura, K., and Harris, C. C. (2003) *Cancer Res.* **63**, 728–734
26. Orford, K., Crockett, C., Jensen, J. P., Weissman, A. M., and Byers, S. W. (1997) *J. Biol. Chem.* **272**, 24735–24738
27. Tamamura, Y., Otani, T., Kanatani, N., Koyama, E., Kitagaki, J., Komori, K., Yamada, Y., Costantini, F., Wakisaka, S., Pacifici, M., Iwamoto, M., and Enomoto-Iwamoto, M. (2005) *J. Biol. Chem.* **280**, 19185–19195
28. Yost, C., Torres, M., Miller, J. R., Huang, E., Kimelman, D., and Moon, R. T. (1996) *EMBO J.* **15**, 1443–1454
29. Stambolic, V., Ruel, L., and Woodgett, J. R. (1996) *Curr. Biol.* **6**, 1664–1668
30. Chen, J. J., Huang, W. C., and Chen, C. C. (2005) *Mol. Biol. Cell* **16**, 5579–5591
31. Wardlaw, S. A., Zhang, N., and Belinsky, S. A. (2002) *Mol. Pharmacol.* **62**, 326–333
32. Kim, Y., and Fischer, S. M. (1998) *J. Biol. Chem.* **273**, 27686–27694
33. Schwarz, E. J., Reginato, M., Shao, D., Krakow, S. L., and Lazar, M. A. (1997) *Mol. Cell. Biol.* **17**, 1552–1561

**REFERENCES**

1. Eberhart, C. E., Coffey, R. J., Radhika, A., Giardiello, F. M., Ferrenbach, S., and DuBois, R. N. (1994) *Gastroenterology* **107**, 1183–1188

**FIGURE 7. Model of COX-2 regulation by APC.** COX-2 is regulated by APC through a mechanism that involves retinoic acid-mediated suppression of C/EBP-β.
COX-2 Expression Is Regulated by Retinoic Acid

34. Elizondo, G., Corchero, J., Sterneck, E., and Gonzalez, F. J. (2000) J. Biol. Chem. 275, 39747–39753
35. Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7950–7954
36. Clevers, H. (2006) N. Engl. J. Med. 354, 761–763
37. Mestre, J. R., Subbaramaiah, K., Sacks, P. G., Schantz, S. P., Tanabe, T., Inoue, H., and Dannenberg, A. J. (1997) Cancer Res. 57, 1081–1085
38. Shattuck-Brandt, R. L., Varilek, G. W., Radhika, A., Yang, F., Washington, M. K., and DuBois, R. N. (2000) Gastroenterology 118, 337–345
39. Li, M., Song, S., Lippman, S. M., Zhang, X. K., Liu, X., Lotan, R., and Xu, X. C. (2002) Oncogene 21, 411–418
40. Subbaramaiah, K., Cole, P. A., and Dannenberg, A. J. (2002) Cancer Res. 62, 2522–2530
41. Shao, J., Sheng, H., Inoue, H., Morrow, J. D., and DuBois, R. N. (2000) J. Biol. Chem. 275, 33951–33956
42. Sonoshita, M., Takaku, K., Oshima, M., Sugihara, K., and Taketo, M. M. (2002) Cancer Res. 62, 6846–6849
43. Shattuck-Brandt, R. L., Lamps, L. W., Heppner Goss, K. J., DuBois, R. N., and Matrisian, L. M. (1999) Mol. Carcinog 24, 177–187
44. Hull, M. A., Booth, J. K., Tisbury, A., Scott, N., Bonifer, C., Markham, A. F., and Coletta, P. L. (1999) Br. J. Cancer 79, 1399–1405
45. Haertel-Wiesmann, M., Liang, Y., Fantl, W. J., and Williams, L. T. (2000) J. Biol. Chem. 275, 32046–32051