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Original Article

Aspirin Prevents Colorectal Cancer by Normalizing EGFR Expression

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ABSTRACT

Background: Aspirin intake reduces the risk of colorectal cancer (CRC), but the molecular underpinnings remain elusive. Epidermal growth factor receptor (EGFR), which is overexpressed in about 80% of CRC cases, is implicated in the etiology of CRC. Here, we investigated whether aspirin can prevent CRC by normalizing EGFR expression.
Methods: Immunohistochemistry staining was performed on paraffin-embedded tissue sections from normal colon mucosa, adenomatous polyps from FAP patients who were classified as regular aspirin users or nonusers. The interplay between cyclooxygenase-2 (COX-2) and EGFR was studied in primary intestinal epithelial cells isolated from Apc^{Min} mice, immortalized normal human colon epithelial cells (HCECs) as well as murine embryonic fibroblasts (MEFs).
Results: Immunohistochemistry staining results established that EGFR overexpression is an early event in colorectal tumorigenesis, which can be greatly attenuated by regular use of aspirin. Importantly, EGFR and COX-2 were co-overexpressed and co-localized with each other in FAP patients. Further mechanistic studies revealed that COX-2 overexpression triggers the activation of the c-Jun-dependent transcription factor, activator protein-1 (AP-1), which binds to the *Egfr* promoter. Binding facilitates the cellular accumulation of EGFR and lowers the threshold required for pre-neoplastic cells to undergo transformation.
Conclusion: Aspirin might exert its chemopreventive activity against CRC, at least partially, by normalizing EGFR expression in gastrointestinal precancerous lesions.

1. Introduction

Colorectal cancer (CRC) represents the third leading cause of cancer-related death in the United States (Siegel et al., 2014). Consistent clinical trial data strongly suggest that regular use of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) lowers the lifetime risk of developing CRC (Algra and Rothwell, 2012; Arber et al., 2006; Chan and Lippman, 2011; Chan et al., 2007; Rothwell et al., 2010). Based on data from a variety of sources, COX-2 has long been suspected to be the primary target for aspirin and NSAID-mediated CRC chemoprevention (Chan and Lippman, 2011; Chulada et al., 2000; Markowitz, 2007). This idea is supported not only by compelling intervention trials with various COX-2 inhibitors (Algra and Rothwell, 2012; Mitchell et al., 1993), but also by the observed resistance to colorectal carcinogenesis in the absence of *ptgs-2* (gene coding for COX-2) in Apc^{Min} mice (Chan et al., 2007). However, COX-2 activation alone is insufficient to cause tumorigenesis, evidenced by the fact that COX-2 transgenic mice fail to develop tumors spontaneously (Oshima et al., 1996). Collectively, COX-2 might function as a tumor promoter rather than as an initiator, but the mechanism of action by which COX-2 drives tumorigenesis remains imperfectly understood.

The epidermal growth factor receptor (EGFR), a transmembrane receptor tyrosine kinase of the ErbB family, has recently been implicated in the etiology of CRC (Repetto et al., 2005; Roberts et al., 2002; Winder and Lenz, 2010). For example, the protein level of EGFR was elevated in up to 80% of CRC cases and is associated with clinical outcomes. Transfer of the Apc^{Min} allele onto a homozygous $Egfr^{wa2}$ background resulted in a 90% reduction in intestinal polyp number relative to Apc^{Min} mice carrying a wildtype *Egfr* allele. Notably, although the EGFR level is frequently elevated in up to 80% of CRC cases, the mechanism underlying such widespread overexpression remains elusive. Here, we demonstrated that COX-2 might drive intestinal tumorigenesis by up-regulating EGFR expression in familial adenomatous

Abbreviations: CRC, colorectal cancer; FAP, familial adenomatous polyposis; EGFR, epidermal growth factor receptor; COX-2, cyclooxygenase-2; PGs, prostaglandins.

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polyposis (FAP) patients, an effect that could be attenuated by regular aspirin use.

2. Materials and Methods

2.1. Clinical Study

2.1.1. Subjects

Familial adenomatous polyposis (FAP) patients were recruited by the Gastroenterology and Hepatology group at Mayo Clinic, Rochester, Minnesota. Through a protocol approved by the Mayo Clinic Rochester MN IRB, all subjects with FAP seen at Mayo Clinic Rochester MN between January 1990 through May and from whom polyp tissue was available were identified through a search of clinical diagnoses and pathology reports in the electronic medical record (EMR). The EMR of the identified FAP cases with available tissue ($n = 178$) was searched to identify those with a history of NSAID use at the time that the available polyp tissue was collected. Cigarette smoking and inflammatory bowel diseases are independent risk factors for CRC, while FAP patients suffering hypertension or cardiovascular diseases might take additional anticoagulant drugs other than aspirin. Thus, exclusion criteria also included cigarette smoking, inflammatory bowel diseases, hypertension, a history of cardiovascular disease, and pregnancy and subjects were not on any other pharmacological treatments. All clinical studies on human subjects or human materials were approved by the Mayo Clinic review board and the Zhengzhou University review board (#2014xjs28). Written, informed consent was required for entry of any patient into this study.

2.1.2. Study Design

FAP patients who reported taking two or more standard (325 mg) aspirin tablets per week within the previous 12 months were classified as regular aspirin users ($n = 25$) and those reporting consumption of less aspirin were classified as aspirin nonusers ($n = 25$) (Chan et al., 2007). Individuals in the healthy control group ($n = 25$) were normal subjects who underwent colonoscopy screening. The gender ratio in each group was approximately 1:1.

2.1.3. Histology and Immunohistochemistry

Colonic mucosal biopsy samples were fixed in 10% formalin, embedded in paraffin, sectioned at 5 μm, and stained with haematoxylin and eosin (H&E) according to standard protocols. Immunohistochemistry staining for COX-2 (#12282, Cell Signaling Technology, Beverly, MA; dilution 1:200) or EGFR (#4267, Cell Signaling Technology, Beverly, MA; dilution 1:50) was performed using an ABC complex kit (PK-6100, Vector Laboratories, Burlingame, CA) following the manufacturer's instructions. Sections were counterstained with Harris's haematoxylin. For antibody-negative controls, the primary antibodies were substituted with normal rabbit serum. Colorectal cancer tissues known to highly express both COX-2 and EGFR served as positive controls. Immunohistochemistry staining intensity was quantified by calculating the integrated optical density (IOD, sum) using the Image Pro-Plus 7.0 software program (Media Cybernetics, Bethesda, MD).

2.2. Materials, Chemicals, and Reagents

Primary antibodies against Jun B, Fra1 and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other primary antibodies were purchased from Cell Signaling Technology (Beverly, MA). Full-length cDNAs for human *cox-2, Jun B, c-Jun* and *Fra1* were from Addgene Inc. (Cambridge, MA). All chemicals were obtained from Sigma-Aldrich (St Louis, MO) unless otherwise specified.

2.3. Cell lines and Transfection

Primary mouse intestinal epithelial cells and adenoma cells were isolated from C57BL/6-Apc^{Min} mice (Apc^{Min} mice) as reported (Zhang et al., 2010). In brief, intestinal polyps or normal adjacent small intestines were cut into 2–3 mm segments, transferred to a 50 mL tube, washed at least 5 times in 20 mL of cold Hank's buffered salt solution (HBSS) with vigorous shaking, and diced into <1 mm^3 pieces using a sharp scalpel blade, and incubated with enzyme solution (0.5 mg/mL dispase type I and 300 U/mL collagenase type IV in HBSS) at 37 °C for 30 min. After enzymatic dissociation, the obtained tissue pieces were transferred to a 50 mL tube with 20 mL Dulbecco's modified Eagle's medium (DMEM) containing 100 mL^{-1} penicillin and 100 mL^{-1} streptomycin. The mixtures were allowed to settle under gravity for 1 min, and all but a small amount at the bottom was carefully removed. This procedure was repeated 5 times. The mixtures were then washed 3 times with DMEM and the pellet was resuspended in epithelial cell medium containing equal volumes of DMEM and Ham's F12 medium with the following additives: 5 μg mL^{-1} insulin, 10 ng mL^{-1} epidermal growth factor, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM glutamine, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 0.2% D-glucose, and 2% FBS.

COX-2 wildtype (COX-2$^{+/+}$) and COX-2 knockout (COX-2$^{-/-}$) mouse embryonic fibroblasts (MEFs) were kind gifts from Drs. Jeff Reese and Sudhansu K. Dey (University of Kansas Medical Center). The cells were derived from COX-2 knockout mice supplied by Drs. Joseph E. Dinchuk and James M. Trzaskos (DuPont Merck Pharmaceutical Co.) The cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 2 mM L-glutamine.

All other cell lines used were obtained from the American Type Culture Collection (Manassas, VA) and maintained following ATCC instructions. All cells were cytogenetically tested and authenticated before being frozen. The passage number was routinely limited to approximately 20 and morphology monitored with each passage.

Transient and stable transfections were performed using jetPEI reagent (Qbiogen Inc., Montreal, Quebec, Canada) following the manufacturer's instructions. For stable transfection, cells were transiently transfected with either an empty vector (pcDNA3.1) or effector plasmid. After 24 h, G418 was added for selection of stable subclones. After 3 weeks, the individual clones obtained were ring-isolated and expanded in culture medium in the presence of G418. Expression of the protein of interest was verified by Western blot.

2.4. Determination of Prostaglandin Production

The measurement of prostaglandins in the cell culture medium was conducted using enzyme immunoassay kits from Cayman Chemical Company (Ann Arbor, MI). Briefly, cells (6×10^5) were plated in a 6-well-plate in the presence of 10% serum. When cells reached 50–60% confluence, 1 mL fresh medium with or without selected test agents was added and cells were further incubated for different time periods. The supernatant fractions were then collected for prostaglandin measurement following the manufacturer's instructions.

2.5. Electrophoretic Mobility-shift Assay

Nuclear protein extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Scientific, Waltham, MA) according to the manufacturer's protocol. The AP-1 DNA binding reaction was performed at room temperature for 30 min in a mixture containing 5 mg nuclear protein and 1 μL IR Dye 700 AP-1 Consensus Oligonucleotide (5′-CGCTTGATGACTCAGCCGGAA-3′; 3′-GCGAACTACTGA GTCGGCCTT-5′). The samples were then fractionated through a 5% polyacrylamide gel and gels were analyzed using the Odyssey® Infrared Imaging System (LI-COR® Biosciences, Lincoln, NE). For the super-shift gel assay, nuclear extracts were pre-incubated with 1 μg of the respective antibody for 20 min before adding the DNA.

2.6. RT-PCR Analysis

Total RNA was collected using the TriPure isolation reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's protocol. RT-PCR was performed on 1 μg of RNA using the One-Step SuperScript RT Platinum TaqRT-PCR kit (Invitrogen). The primer sequences used are as follows: EGFR forward: 5′-GTTATCCATC CCTGACTCTCATCT-3′; EGFR reverse: 5′-ACCTGACAGGCTCATTCTATAT CC-3′. PCR cycle number was 25 for *Egfr*, and 20 for *β-actin*. Products were analyzed by gel electrophoresis, ethidium bromide staining and a Digital Science IC440 camera system (Kodak, Rochester, NY).

2.7. Reporter Assays

The reporter plasmid, effector plasmid, and internal control plasmid (*Renilla* luciferase reporter, pRL-SV40) were transfected using jetPEI reagent (Qbiogen) into cells following the manufacturer's instructions. At 24 h after transfection, cells were disrupted and collected for luciferase activity assays using a Luciferase Assay System (Promega Corporation, Madison, WI). Luciferase activity was measured by luminometer (Monolight 2010, Analytical Luminescence Laboratory, Ann Arbor, MI). All firefly luciferase reporter activities were normalized for transfection efficiency by determining the ratio between firefly and *Renilla* luciferase activity. The Col-Luc plasmid DNA was used as the *AP-1 luciferase reporter* plasmid and the pER1-Luc plasmid was used for measuring *Egfr luciferase* reporter activity.

2.8. Western Blot Analysis

Protein samples (20 μg) were resolved by SDS-PAGE and transferred to Hybond C nitrocellulose membranes (Amersham Corporation, Arlington Heights, IL). After blocking with nonfat milk, the membranes were probed with primary antibodies (1:1000) overnight at 4 °C. The targeted protein bands were visualized by an enhanced chemiluminescence reagent (Amersham Corporation) after hybridization with a secondary antibody conjugated with horseradish peroxidase.

2.9. Statistical Analysis

Statistical analysis was performed using the Prism 5.0 statistical software package. The Tukey's t-test was used to compare data between two groups. One-way ANOVA and the Bonferroni correction were used to compare data between 3 or more groups. Pearson correlation was used to measure the strength of association between two variables. Values are expressed as means ± S.D. and a p value of <0.05 was considered statistically significant.

3. Results

3.1. COX-2 and EGFR are Positively Correlated in FAP Patients

We first analyzed the profile of EGFR expression in the colon during CRC progression. The transition of normal epithelial mucosa to polyps (any grossly visible protrusion from the mucosal surface of the colon) or adenomas (also known as adenomatous polyps) to adenocarcinomas is well-established in patients with familial adenomatous polyposis (FAP) (Tsao et al., 2004). Accordingly, we recruited FAP patients, subgrouped them based upon pathological disease stage, and determined their colonic EGFR expression by immunohistochemistry staining (Fig. 1A). EGFR staining was positive in 13 (72%) of 18 adenocarcinomas, 20 (69%) of the 29 adenomas, and 6 (67%) of the 9 polyp tissue samples. In contrast, only 1 (10%) of the 10 normal colorectal tissues stained positive for EGFR (Note: the lower panels of Fig. 1A are representative of data shown in the upper table and graph).

Histopathologically, the positive regions of EGFR immunostaining were mainly in colonic neoplastic tissue (such as polyps and adenomas), but less in normal colonic mucosa tissues (Fig. 1A). Consistent with previous findings by others (Eberhart et al., 1994), we observed COX-2 overexpression in those premalignant lesions, too. Interestingly, our data strongly suggested that EGFR and COX-2 were co-localized in the polyp tissues in FAP patients (Fig. 1B). Inspired by the findings above, we examined the relationship between COX-2 and EGFR expression in colonic adenomatous polyps from FAP patients. Results indicated that in 95% of the FAP cases (19/20) exhibiting COX-2 overexpression, EGFR was also highly expressed (Fig. 1C). Further quantification analysis indicated that the observed expression levels of COX-2 and EGFR were not independent of each other but were highly and positively correlated ($p = 0.0015$; $R = 0.7585$) in adenomatous polyps from FAP patients (Fig. 1D).

We then compared the expression of COX-2 and EGFR in FAP patients who were classified as regular aspirin users or nonusers (Fig. 1E, F). As expected, significantly decreased EGFR protein levels were observed in the adenomatous polyps of regular aspirin users, suggesting that aspirin exposure decreases the elevated EGFR levels in FAP patients. Interestingly, regular aspirin users also exhibited a significantly lower level of COX-2. Overall, these findings strongly indicated a functional role for COX-2 in the regulation of EGFR during gastrointestinal tumorigenesis.

3.2. Functional Relevance of COX-2 in the Regulation of EGFR

To study the interplay between COX-2 and EGFR in CRC, knowing the specific cell types that overexpress these proteins would be essential. In this regard, we found that both COX-2 and EGFR are primarily overexpressed and localized in the pre-malignant epithelial cells in adenomas from FAP patients (Fig. 1E). Accordingly, we isolated primary epithelial cells from mouse intestinal polyps and normal adjacent intestinal tissues of Apc^{Min} mice. Consistent with our observations from immunohistochemistry analysis, COX-2 and EGFR were highly expressed in intestinal adenoma epithelial cells, but not in normal intestinal epithelial cells (Fig. 2A). Furthermore, treatment with either aspirin or celecoxib, a well-known COX-2 inhibitor, lowered EGFR expression in intestinal adenoma epithelial cells (Fig. 2B).

To further characterize the role of COX-2 in the regulation of EGFR, we successfully established a COX-2 overexpressing stable sub-clone from an immortalized normal human colon epithelial cell line (HCEC). Forced expression of COX-2 resulted in an increase in the protein level of EGFR (Fig. 2C). Once again, treatment with aspirin or celecoxib lowered EGFR expression levels (Fig. 2D).

Next, we determined whether deficiency of COX-2 could influence the level of EGFR in murine embryonic fibroblasts (MEFs). Results clearly indicated that compared with wildtype MEFs ($COX\text{-}2^{+/+}$; Fig. 2E), MEFs with *cox-2* gene deficiency ($COX\text{-}2^{-/-}$) have a much lower EGFR level. Furthermore, typical COX-2 activators, including inflammatory cytokine interleukin-1 beta (IL-1β) and bacterial lipopolysaccharide (LPS), dramatically boosted both COX-2 and EGFR protein expression in COX-2 wildtype cells compared to knockout cells (Fig. 2F). These data indicate that manipulating COX-2 activity dramatically affects the protein level of EGFR.

3.3. COX-2 Modulates EGFR Transcription Through Activator Protein-1 (AP-1)

Generally, increased EGFR expression is either due to increased biogenesis, decreased degradation, or both. We hypothesized that COX-2 might modulate EGFR at the transcriptional level by activating the *Egfr* promoter. This idea was supported by results showing that both *Egfr* mRNA (Fig. 3A, left panel) and promoter activity (Fig. 3A, right panel) were dramatically reduced in the absence of *cox-2* gene expression. Aspirin was previously reported as an inhibitor of the activity of activator protein-1 (AP-1), one of the potential transcription factors for *Egfr* (Dong et al., 1997; Johnson et al., 2000; Zenz et al., 2003). We then hypothesized that COX-2 might present an unrecognized signaling

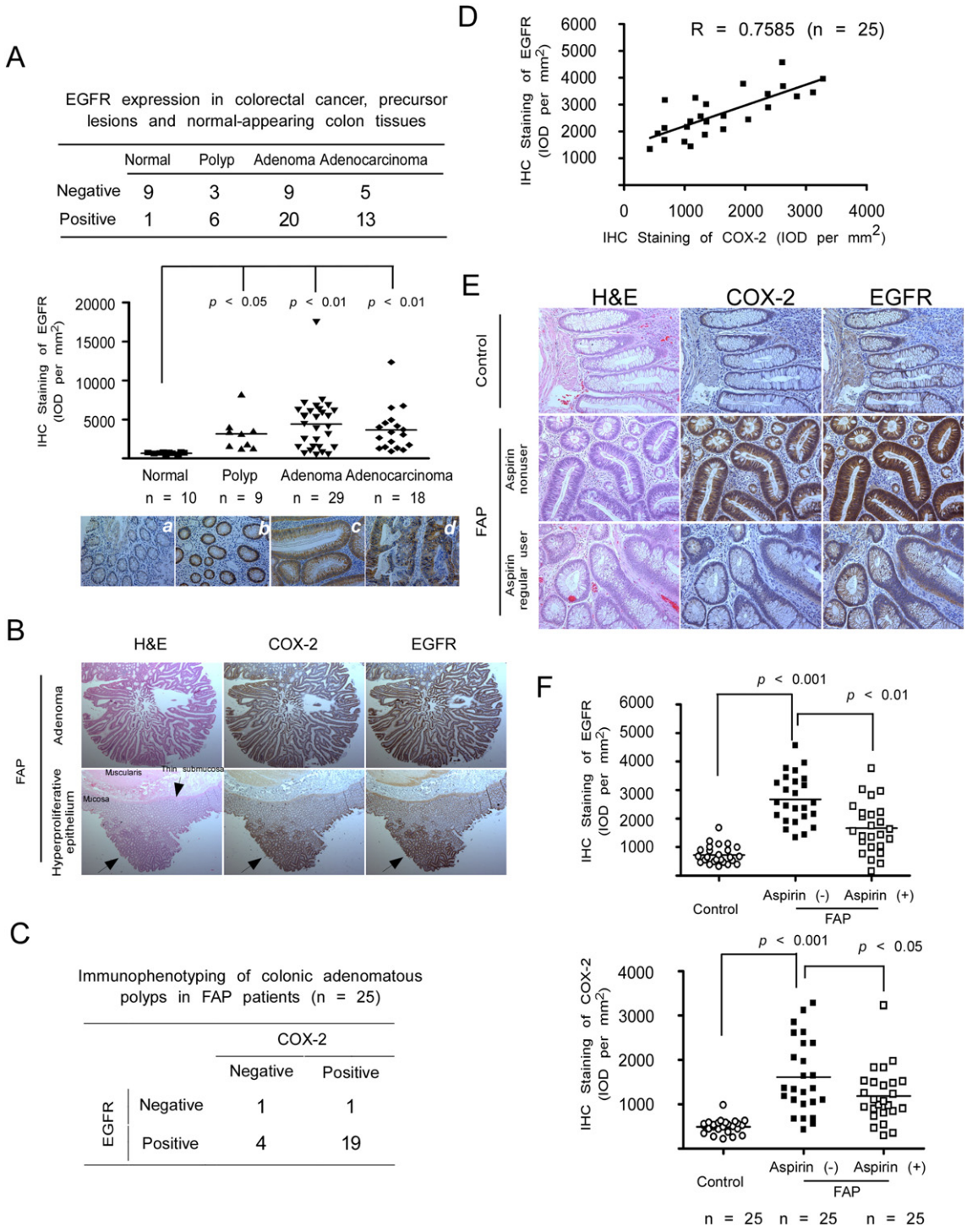

Fig. 1. Expression of COX-2 and EGFR is positively correlated in FAP patients. (A) EGFR is up-regulated during CRC progression. Immunohistochemistry of normal colonic epithelial mucosa (A*a*), polyps (A*b*), adenomas (A*c*), and adenocarcinomas (A*d*). Normal colonic epithelial mucosa was isolated from healthy individuals who underwent routine colonoscopy screening, whereas colonic neoplastic tissues were from FAP patients. Original magnification: 200×. (B) COX-2 and EGFR are co-overexpressed in colonic adenomatous polyps in FAP patients. Original magnification: 40×. (C) Immunophenotyping of colonic adenomatous polyps shows negative or positive staining for COX-2 and EGFR. Immunostaining intensities are defined in Materials and methods. FAP patients (aspirin nonusers), n = 25; double-positive cases, n = 19. (D) COX-2 and EGFR are correlated in colonic adenomatous polyps from FAP patients. FAP patients (aspirin nonusers), n = 25. The positive correlation value (R = 0.7585) indicates a strong linear relationship. Data were analyzed using Prism 5.0 statistical software. (E) and (F) Effects of regular aspirin use on EGFR and COX-2 expression in FAP patients. FAP patients who reported taking two or more standard (325 mg) aspirin tablets per week were classified as regular aspirin users and those taking less aspirin were defined as aspirin nonusers. Original magnification: 200×. Note: IOD (integrated optical density) units.

pathway leading to AP-1 activation, which functionally couples with *Egfr* transcription. Our data indicated that AP-1 activity (Fig. 3B, left panel) as well as AP-1 DNA binding activity (Fig. 3B, right panel) was markedly attenuated in the absence of *cox-2* gene expression. Potential AP-1 components were identified by Western blot (Fig. 3C) and electrophoretic mobility-super shift assay (Fig. 3D). In this regard, overexpression of Jun B and c-Jun increased *Egfr* promoter activity as well as EGFR protein level (Fig. 3E). Moreover, we established that both the *Egfr* promoter and AP-1 activity were suppressed by celecoxib treatment (Fig. 3F).

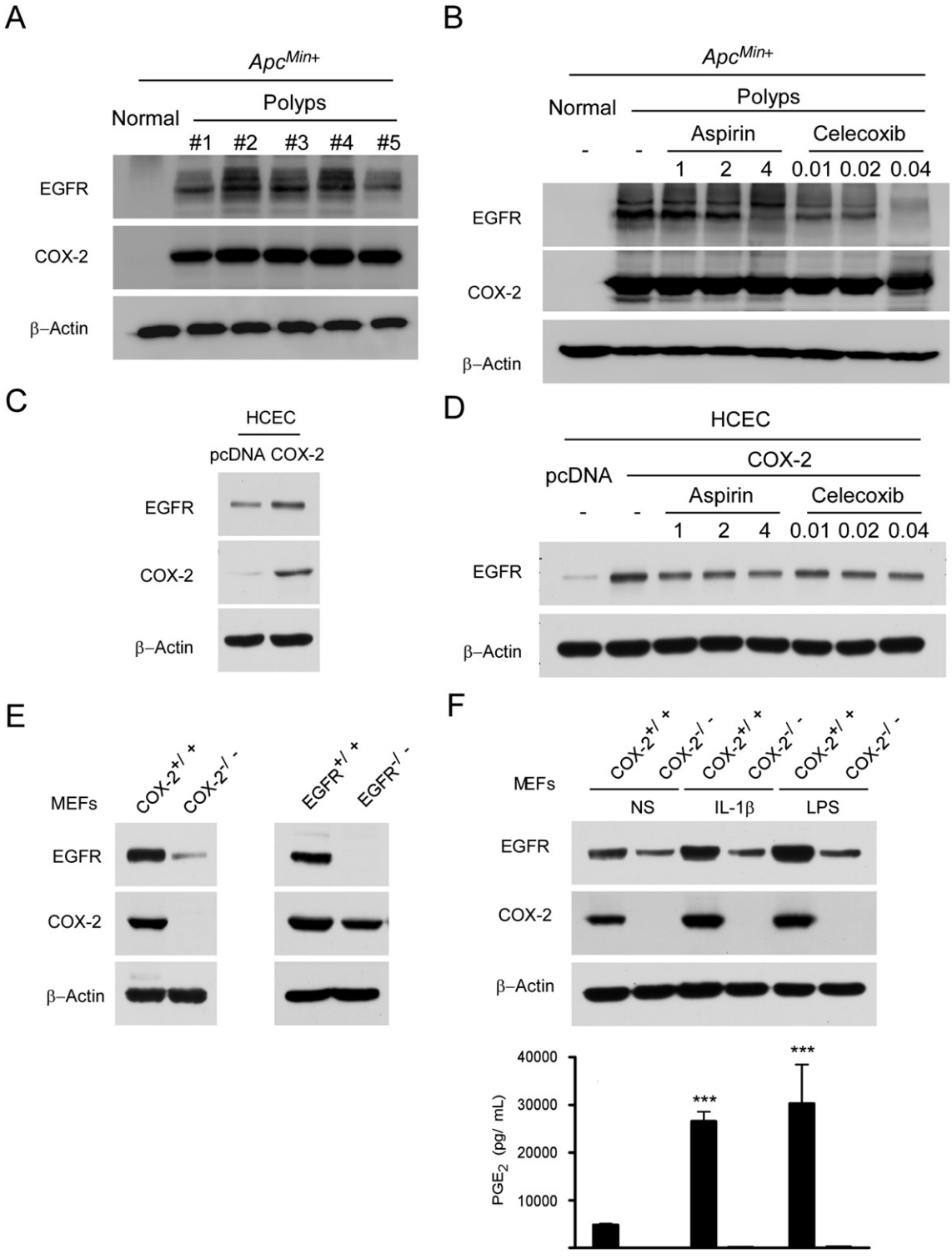

Fig. 2. Functional relevance of COX-2 in the regulation of EGFR. (A) and (B) Primary epithelial cells were isolated from mouse intestinal polyps and normal adjacent intestinal tissues of *Apc*^{Min} mice and treated with aspirin (0, 1, 2, or 4 mM) or celecoxib (0, 0.01, 0.02, or 0.04 mM) for 72 h. After treatment, cells were disrupted and immunoblotted with either anti-COX-2 or anti-EGFR. Data is representative of 3 similar experiments. (C) and (D) Stable transfection of exogenous COX-2 results in EGFR up-regulation in an immortalized normal human colon epithelial cell line (HCEC), an effect that could be attenuated by aspirin and celecoxib treatment. At 50–60% confluence, HCEC cells were transiently transfected with either an empty vector (pcDNA3.1) or a COX-2 plasmid (pcDNA3-Flag-COX-2). After 24 h, G418 (600 ng/mL) was added for the selection of stable subclones. After 3 weeks, the stable clones obtained were treated with aspirin (0, 1, 2, or 4 mM) or celecoxib (0, 0.01, 0.02, or 0.04 mM) for 72 h. Data are representative of 3 similar experiments. (E) GENETIC deficiency of *cox-2* is associated with a lower level of EGFR in murine embryonic fibroblasts (MEFs). Data are representative of 3 similar experiments. (F) MEFs were incubated with normal saline (NS), IL-1β (10 ng/mL) or LPS (1 ng/mL). After treatment for 72 h, cells were disrupted and immunoblotted with either anti-COX-2 or anti-EGFR (upper panels). Supernatant fractions were collected for prostaglandin E2 (PGE_2) measurement (lower panel). Data are presented as mean values ± S.D. (n = 4) of a representative experiment that was repeated 3 times with similar results. The asterisks (***) indicate a significant ($p < 0.001$) difference compared to normal saline-treated COX-$2^{+/+}$ MEFs (lower panel).

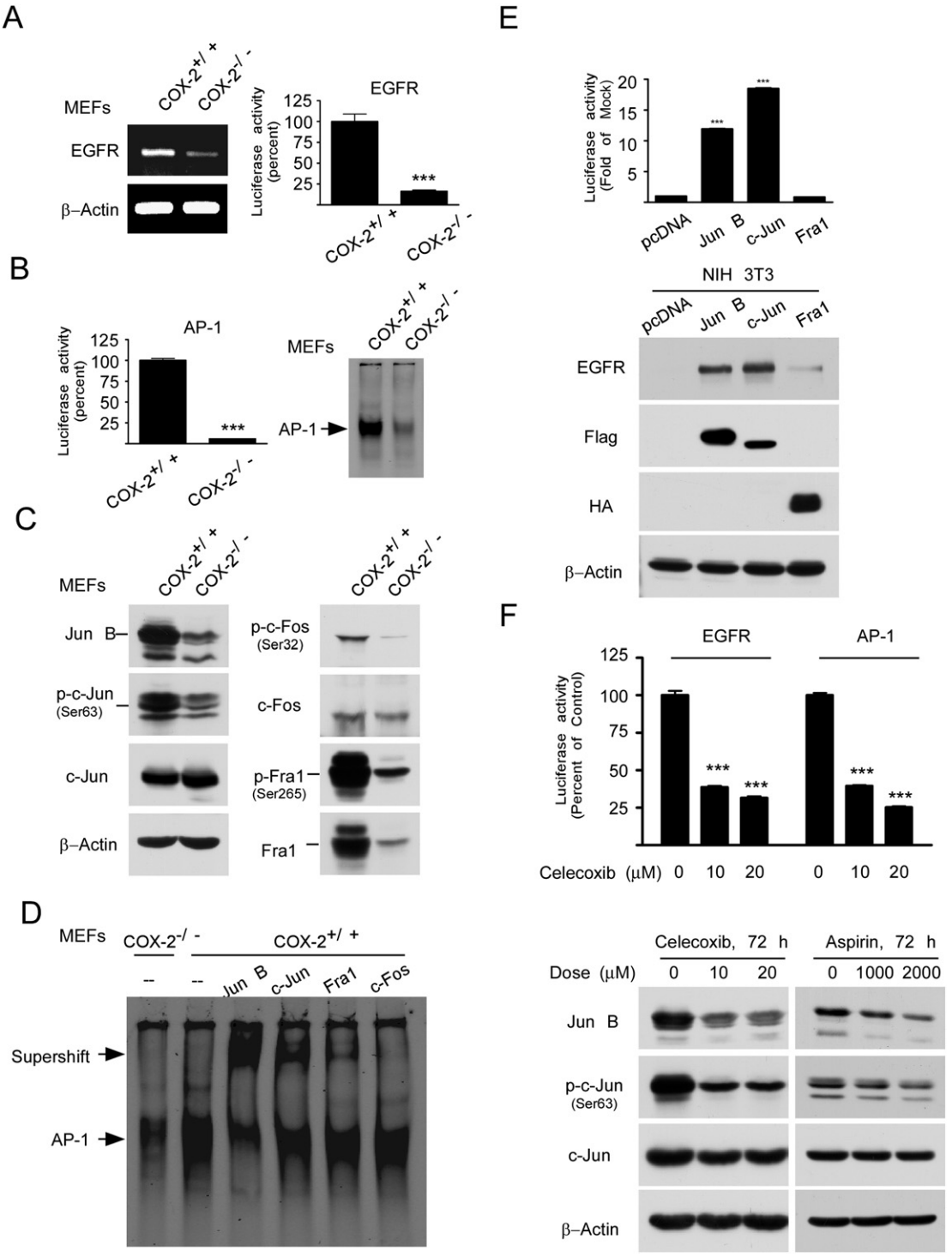

Fig. 3. COX-2 modulates EGFR transcription through AP-1. (A) Effects of *cox-2* gene deficiency on EGFR transcriptional activation in MEFs. (Left) RT-PCR analysis of *Egfr* mRNA levels. β-Actin served as a loading control. (Right) *Egfr* promoter activity. (B) Effects of *cox-2* gene deficiency on AP-1 activation. (Left) AP-1 activity. (Right) AP-1 DNA binding activity. (C) Identification of potential AP-1 components by Western blot analysis. (D) AP-1 component candidates are identified by super-shift gel assay. (E) Effects of transient transfection of respective AP-1 components on *Egfr* promoter activity and EGFR expression in NIH3T3 cells. Data are presented as mean values ± S.D. (n = 4) and the asterisks (***) indicate a significant ($p < 0.001$) increase compared to the Mock-transfected group. (F) COX-2 inhibition represses *Egfr* promoter activity as well as AP-1 activation. The data are expressed as percent activity relative to untreated control. The asterisks (***) indicate a significant ($p < 0.001$) decrease compared to untreated control MEFs.

3.4. COX-2 Amplifies EGFR Signaling to Facilitate Neoplastic Cell Transformation

Based on the findings above, we determined whether COX-2 activation could facilitate neoplastic cell transformation by amplifying EGFR signaling. JB6 cells, a promotion sensitive (P+) mouse epidermal cell line, has enabled the study of genetic susceptibility to promotion of cell transformation, and thus provides a unique cell model to characterize activated COX-2 in pre-neoplastic cells (Dong et al., 1994). We found that compared with parent cells, stable sub-clones overexpressing COX-2 were significantly more readily transformed in the presence of EGF (Fig. 4A, B). We also evaluated the effects of a pro-inflammatory microenvironment on cell transformation by treating cells with NS, IL-1β or LPS, and observed

increased transformation in the presence of COX-2 activation (Fig. 4C). Further studies suggested that with EGF stimulation, EGFR downstream signaling cascades were substantially amplified in the presence of COX-2 forced expression (Fig. 4D). Additional results indicated that the contribution of COX-2 to transformation was markedly attenuated by treatment with either aspirin or celecoxib (Fig. 4E), at a clinically achievable serum concentration.

4. Discussion

Although widely accepted as a prognostic biomarker in advanced CRC, our findings in this study clearly showed that EGFR protein expression was dramatically elevated in colonic adenomatous polyps in FAP patients, even at a very early stage. More importantly, a mechanistic study indicated that the widespread overexpression of EGFR might occur as a consequence of COX-2 activation, a common pathological event in various gastrointestinal precancerous lesions. Coupled with the fact that the absence of *Egfr* reduced intestinal polyps by 90% in Apc^{Min} mice (Roberts et al., 2002), these results indicated that COX-2 might drive colon tumorigenesis, at least in partly, through up-regulation of EGFR, which phenotypically facilitates neoplastic cell transformation in precancerous lesions. As such, EGFR might be a novel target for CRC chemoprevention.

Previously, PGE_2, the major COX-2-derivated PG, was reported to be capable of transactivating the EGFR kinase cascade in colon cancer cells (Pai et al., 2002), whereas activation of EGFR could conversely

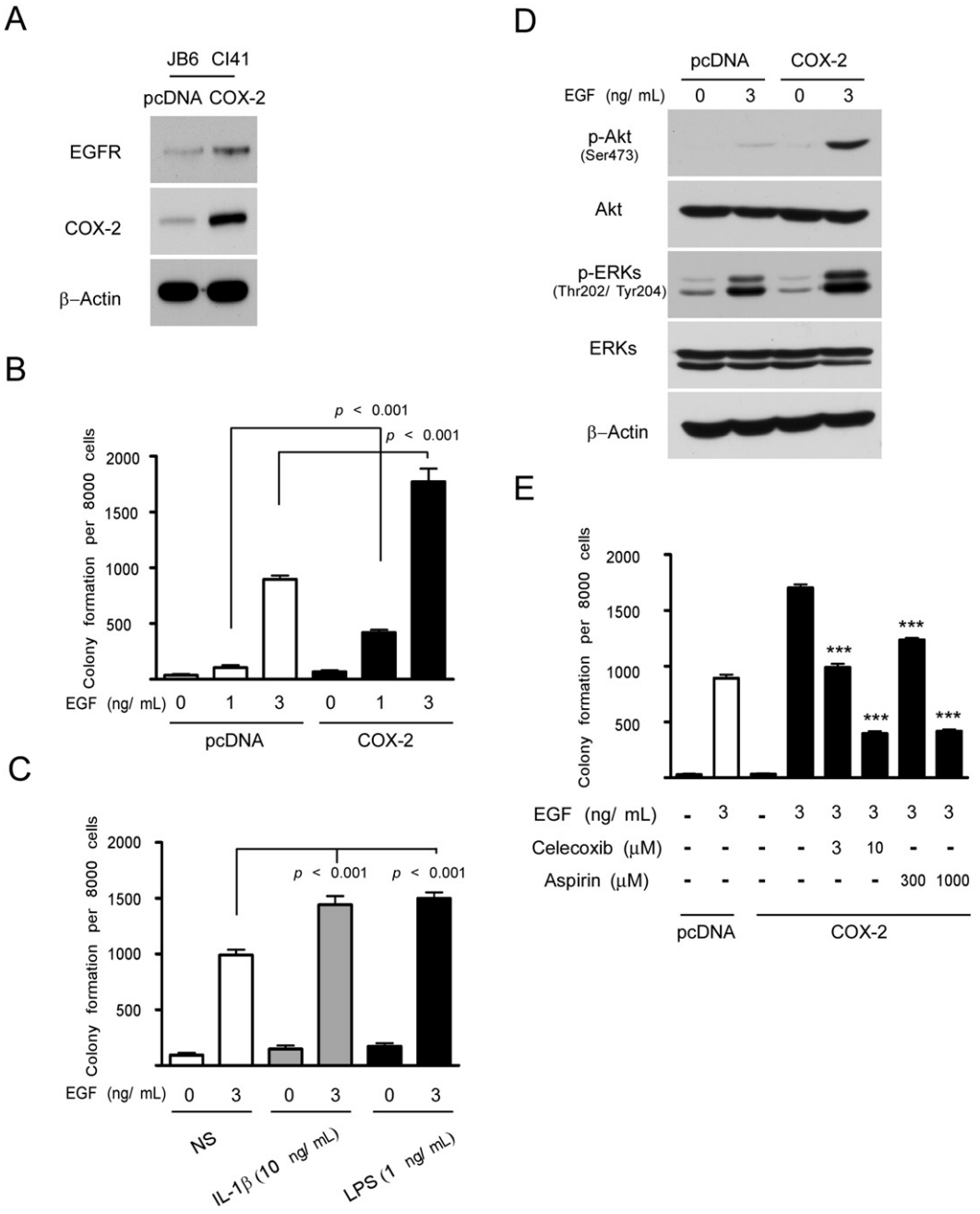

Fig. 4. COX-2 activation facilitates neoplastic cell transformation. (A) Stable transfection of exogenous COX-2 results in EGFR up-regulation in a promotion sensitive (P+) mouse epidermal cell line (JB6). (B) Effects of forced COX-2 expression on cell transformation. (C) Effects of typical COX-2 activators on cell transformation. (D) The presence of COX-2 amplifies the EGFR signal transduction cascade. (E) COX-2-mediated stimulation of cell transformation is decreased by NSAIDs. Data are presented as mean values ± S.D. (n = 12) and the asterisks (***) indicate a significant ($p < 0.001$) decrease in NSAID-treated cells overexpressing COX-2 compared to the EGF-treated COX-2 overexpressing cells.

stimulate COX-2 biosynthesis (Coffey et al., 1997). Taken together, a positive feedback loop could possibly exist between COX-2 and EGFR in intestinal tumorigenesis. In this regard, FAP patients whose colonic polyps express high levels of both COX-2 and EGFR might be the most likely to benefit from a combination anti-COX-2/EGFR therapy (Torrance et al., 2000).

COX-2 is known to exert its biological function through its derivate prostaglandins. If this is the case, identifying the specific prostaglandins that act downstream of COX-2 would be essential for understanding how COX-2 activation enhances EGFR expression. The most direct experimental approach is by examining PGs' biosynthesis upon COX-2 inhibition. However, the biosynthesis of all five of the major bioactive PGs' in primary intestinal adenoma epithelial cells was suppressed by aspirin or celecoxib treatment (Supplementary Fig. 1). An exception is the effect of aspirin on PGI_2 production. The interpretation of such phenomenon is still incomplete. Thus, further studies examining susceptibility to intestinal polyps in mice with targeted deletions in specific prostaglandin synthases and receptors are needed.

Knowing the specific cell type that expresses COX-2 would be essential for understanding how COX-2 promotes CRC progression. However, no consensus exists at present as to which cell types within a colon tumor specifically express COX-2. Some groups, including us, found that COX-2 is primarily expressed in the epithelial cells of colon adenomas and sporadic human colon cancers (Sano et al., 1995). Another group has reported COX-2 expression in the stromal compartment of polyps from Apc^{Min} mice (Oshima et al., 1996). We are uncertain whether these differences in COX-2 localization are due to experimental artifacts or simply because of inherent variability within the sample. Within a given tumor, COX-2 may also be expressed in more than one cell type (Masferrer et al., 2000). The interpretation of this phenomenon is still pending.

Although our findings are interesting, several questions remain unanswered. For example, one caveat is that the doses of aspirin and celecoxib used in in vitro studies are higher than their clinically relevant concentration. Although aspirin exposure decreases the elevated EGFR levels in FAP patients, in the majority of individuals who were aspirin users or non-users, the levels of EGFR were still overlapping. This is consistent with the outcome of CRC chemoprevention in which aspirin benefits most but not all of the overall population. Another issue is that our sample size is small, and data collection as well as the final conclusion might be limited only to colonic adenoma in FAP patients. Although COX-2 and EGFR were co-localized in colon adenocarcinomas from FAP patients, a functional association between them was not observed (Supplementary Fig. 2A, B). Moreover, knockdown of COX-2 didn't or only weakly affected the levels of EGFR in 4 colon cancer cell lines (Supplementary Fig. 2C). All of these findings suggested that colon cancer cells have already escaped from COX-2 dependence (Lev-Ari et al., 2007), but the mechanism of action remains unclear.

In summary, this study revealed a previously unknown functional association between COX-2 and EGFR during colorectal carcinogenesis, and provided an explanation as to how aspirin intake can lower the risk of CRC in FAP patients.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2015.03.019.

Conflicts of Interest

The authors disclose no conflicts of interest.

Author Contributions

Zigang Dong designed and supervised the experiments; Haitao Li and Ann M. Bode prepared the manuscript; Haitao Li, Feng Zhu, Lei Wang, Naomi Oi, Kangdong Liu, Xiang Li and Yang Fu performed experiments; Lisa A. Boardman and Paul J. Limburg recruited volunteers and performed clinical studies.

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