Abstract

Purpose: Colonic carcinogenesis deranges growth-regulating epidermal growth factor receptors (EGFR). We previously showed that EGFR signals were up-regulated in human aberrant crypt foci (ACF), putative colon cancer precursors. The azoxymethane model of colon cancer recapitulates many aspects of human colon tumors. Recent studies indicate that flat dysplastic ACF with increased β-catenin are tumor precursors in this model. We asked, therefore, if EGFR signals are required for flat dysplastic ACF development and cancer progression.

Experimental Design: Rats received azoxymethane or saline, and standard chow or chow supplemented with gefitinib, an EGFR inhibitor, for 44 weeks. EGFR signals were quantified in normal colon, flat ACF, and tumors by computerized analysis of immunostains and Western blots. K-ras mutations were assessed by PCR and mRNA for egfr ligands by quantitative real-time PCR.

Results: EGFR inhibition with gefitinib decreased the incidence of flat dysplastic ACF from 66% to 36% and tumors from 71% to 22% (P < 0.05). This inhibitor also reduced the overexpressions of cyclin D1 and Cox-2 in flat ACF. Furthermore, in flat ACF, EGFR blockade decreased the up-regulation of c-Jun, FosB, phosphorylated active signal transducers and activators of transcription 3, and CCAAT/enhancer binding protein-β, potential regulators of cyclin D1 and Cox-2. In colon tumors, EGFR blockade significantly decreased angiogenesis, proliferation, and progression while also increasing apoptosis (P < 0.05). Gefitinib also inhibited the activations of extracellular signal regulated kinase, Src, and AKT pathways in tumors.

Conclusions: We have shown for the first time that EGFR promotes the development of flat dysplastic ACF and the progression of malignant colonic tumors. Furthermore, we have mechanistically identified several transcription factors and their targets as EGFR effectors in colonic carcinogenesis.

Colonic carcinogenesis is characterized by the accumulation of activating mutations in proto-oncogenes and inhibiting mutations in tumor suppressor genes. These mutations dysregulate pathways, including epidermal growth factor receptor (EGFR) signals that control cell growth, maturation, and cell death. Up-regulations of EGFRs and ligands have been described in many tumors, including colon cancers (1). Recently, we reported that EGFR signals were up-regulated in human aberrant crypt foci (ACF) identified in situ using image magnification chromoendoxy (2). ACF are the earliest identifiable lesions in experimental colonic carcinogenesis and dysplastic ACF are believed to be precursors of colon cancer (3).

EGFR (ErbB1) is a member of the ErbB family of receptor tyrosine kinases which also includes ErbB2, ErbB3, and ErbB4 (4). Ligand binding induces a conformational change, causing receptors to dimerize and activating the receptor's intrinsic tyrosine kinase. ErbB2 is unique in that it has no identified ligand, but is the preferred heterodimeric partner for other members. EGFR signals activate multiple pathways including Ras-Raf-MEK-ERK and phosphoinositide-3-kinase–AKT. Cyclin D1 and Cox-2 are key effectors of ERK and AKT pathways. Cyclin D1 is a G1 cell cycle regulator that is overexpressed in colon cancer (5). Cox-2 is an inducible enzyme that catalyzes the rate-limiting step in prostanox biosynthesis and is also up-regulated in large bowel tumors (6). The role of EGFR in
The azoxymethane model of rodent colon cancer recapitulates many of the clinical, histologic, and molecular features of human colon cancer. Azoxymethane is a mutagen that causes guanine methyl adduct formation leading to activating mutations in K-ras and β-catenin (7). K-ras encodes Ras, a GTP-regulated protein that controls extracellular signal–regulated kinases (ERK). K-ras mutations cause constitutive activation of Ras. We previously showed that wild-type Ras could also be activated by upstream signals, including ErbB2 overexpression in azoxymethane-induced colonic tumors (8). Activated wild-type Ras in turn stimulated ERK and increased Cox-2 in tumors. More recently, in azoxymethane-treated mice, we showed that EGFR signals were activated in apparently normal colon in the premalignant phase prior to tumor emergence (9). EGFR signals regulate proliferation and cell survival, and their widespread activation in premalignant colon is believed to contribute to colonic hyperproliferation and tumorigenesis. In azoxymethane-treated mice, gefitinib, an EGFR inhibitor, blocked receptor activation and inhibited ERK signaling in colonic mucosa, reducing epithelial crypt cell proliferation and limiting the development of premalignant precursors (9).

Recently, flat-appearing variants of ACF were described that were dysplastic and exhibited increased β-catenin accumulation (10, 11). These flat ACF are believed to be precursors of colonic tumors based on their dysplastic features and their temporal relationship to tumors, with flat ACF incidences increasing prior to tumor development and declining as tumors emerged. The roles of EGFR in the development of these putative malignant precursors and their possible progression to cancer, however, have not been examined. In the current study using gefitinib, an EGFR inhibitor, we addressed these important questions. We chose the rat model for these studies because rats develop larger flat ACF and tumors compared with mice in response to this carcinogen (10, 11). Prior studies in multiple intestinal neoplasia (Min) mice, which possess apc germ line mutations, showed that EGFR signals limited the number but not the growth rate of intestinal adenomas (12). In the Min model, tumors develop predominantly in the small intestine and do not progress to cancers. It was of interest, therefore, to determine whether EGFR controls not only premalignant events, but also large bowel tumor growth and histologic progression to carcinomas in the azoxymethane model. In addition, we asked if EGFR controlled proliferation, cell survival, and angiogenesis because the roles of this receptor in regulating these growth characteristics have not been directly elucidated in colonic tumors.

In the current study, we also examined tumors for several transcriptional regulators overexpressed in human colon cancers. The transcription factors activator protein 1 (AP-1), signal transducers and activators of transcription 3 (STAT3), and CAAT/enhancer binding protein-β (C/EBPβ) are up-regulated in colonic tumors (5, 13, 14). EGFR blockade in this model provided us an ideal opportunity to directly assess the roles of these transcription factors as EGFR effectors in this malignancy. AP-1 is composed of homodimeric complexes of Jun family proteins, or heterodimers of Jun and Fos members, and is activated by EGFR in several noncolonic cell types (15). STAT3 also mediates EGFR signaling in other cell types (16). A third transcription factor, C/EBPβ is activated by EGFR in other cells and is required for ras-dependent transformation in another model of epithelial cancer (17, 18). For this analysis, we investigated the effects of tumorigenesis and EGFR blockade on each of these transcription factors to assess their potential roles as EGFR effectors in the development of colon cancers. Our findings and their implications for colon cancer chemoprevention are the subject of this report.

Materials and Methods

Materials
Male Fisher-344 rats were purchased from The Jackson Laboratory. AIN-76A rodent chow diets were purchased from Harlan Teklad Laboratories. Azoxymethane was obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository, Midwest Research. Gefitinib was generously provided by AstraZeneca. DNA and RNA extractions were done using the Qiagen DNasey kit and RNase lipid extraction kit, respectively (Qiagen). BstN1 and Bgl restriction enzymes were obtained from New England Biolabs. RNA later RNA storage solution, and DNA-free DNaseI kit were purchased from Ambion. TRizol RNA/DNA/protein isolation reagent was obtained from Life Technologies. RiboGreen RNA quantitation reagent and kit were purchased from Molecular Probes. Custom PCR primers were obtained from Integrated DNA Technologies, Inc. Other PCR reagents, including Moloney murine leukemia virus reverse transcriptase, random hexamers, and SYBR Green were purchased from Applied Biosystems. HotStarTaq DNA polymerase was supplied by Qiagen. SuperScript III Platinum Two-Step qRT-PCR kit was obtained from Invitrogen. SuperFrost Plus slides were purchased from Fisher Scientific. Polyclonal antibodies to phosphorylated (active) EGFR (SC-12351), phosphorlyated (active) ErbB2 (SC-12352R), C/EBPβ antibodies (SC-150), phosphorylated (active) C/EBPβ (SC-1699R), phosphorylated (active) ERK-1 and ERK-2 (SC-7383) antibodies, anti–c-Jun (sc-1694), anti–c-Jun antibodies, anti–Cyclin D1 (sc-718), and pan-ErbB2 (SC-284) antibodies were obtained from Santa Cruz Biotechnology. Polyclonal pan-ERK, pan-AKT antibodies, and pSTAT3 antibodies were obtained from Cell Signaling Technology. Monoclonal antibodies against Ki-67 (clone SP1) were obtained from Neomarkers. Monoclonal β-actin antibodies were obtained from Sigma-Aldrich Corp. Rat monoclonal anti-nestin and mouse monoclonal anti–β-catenin antibodies were obtained from BD Pharmingen. Rabbit anti–Cox-2 antibodies were purchased from Cayman Chemicals. Anti–caspase-3 antibodies (CP229A) were purchased from Biocare Medical. Polyclonal pan-EGFR antibodies were obtained from Upstate Biotechnology. RC-DC protein assay was from Bio-Rad Labs. Kodak supplied the X-Omat AR film. Polyvinylidene difluoride membranes (Immobilon-P) were purchased from Millipore, Inc. Unless otherwise noted, all other reagents were of the highest quality available and were obtained from Sigma-Aldrich.

Methods

Experimental animal protocol. The experimental animal protocol is summarized in Supplementary Fig. S1. Male Fisher-344 rats were maintained on AIN-76A rodent chow diet in approved specific pathogen–free facilities with 12 h light at 22°C to 25°C and humidity at 25% to 35%. All animal procedures were approved by the University of Chicago Institutional Animal Care and Use Committee and followed NIH-approved guidelines. Rats were treated with azoxymethane (1.p. 20 mg/kg body weight, 80 rats) weekly for 2 weeks, or with saline (azoxymethane vehicle, 40 rats). Two weeks later, each of the groups were divided equally and fed AIN-76A chow, or AIN-76A chow supplemented with gefitinib (10 mg/kg body weight). This dose of gefitinib approximates the dose used in humans to achieve EGFR inhibition in vivo and higher doses result in diarrhea, skin rash, and weight loss (19). Gefitinib is 100 times more selective for EGFR than other ErB receptors and exhibits an even higher selectivity (lower
Western blotting. Proteins were extracted in SDS-containing Laemmli buffer, quantified by RC-DC assay, and subjected to Western blotting as described (8). Blots were incubated overnight at 4°C with specific primary antibodies followed by 1 h of incubation with appropriate peroxidase-coupled secondary antibodies that were detected by enhanced chemiluminescence using X-OMAT film. Xerograms were digitized with an Epson scanner and band intensity quantified using IP Lab Gel (Scanalytics). For changes in EGFR signals in left colonic segments (field effects), Western blotting band intensities of activated kinases were normalized to total kinase. These ratios were then expressed as fold increases of control colons from rats treated with saline (azoxymethane vehicle) and receiving unsupplemented chow. For EGFR signals in tumors, values were first normalized to β-actin expression. Then EGFR effector values in tumors from azoxymethane + gefitinib–treated animals were expressed as the percentage of effector signals in tumors from azoxymethane-treated animals. The latter were normalized to 100%.

**R-καs mutation analysis.** DNA from ACF was purified using the Qiagen DynaEasy kit following the recommendations of the manufacturer. Primer-mediated RFLP assays were used to detect mutations in R-καs codon 12 and codon 13, as previously described (8). Mismatched 5’ primers created restriction sites in wild-type R-καs that were abolished by G to A mutations in codons 12 (BstN1) or 13 (BglI).

**Real-time PCR.** Total RNA was extracted using RNeasy Lipid Tissue Mini Kit (Qiagen). Colonic tissue was cryosectioned into 20-μm-thick sections and suspended in diethyl pyrocarbonate-water in a 1.5 ml tube. Samples were collected by centrifugation and homogenized by Polytron in 0.5 ml of QIAzol. Samples were loaded onto the RNA-binding spin column, washed, digested with DNase I, and eluted in 30 μl of elution buffer. RNA samples were tested by Agilent chip for RNA purity and quantified by Ribogreen. RNA (250 ng) was reverse-transcribed into cDNA using SuperScript III Platinum Two-Step qRT-PCR kit (Invitrogen) in 2 μl total volume. Incubation conditions were 25°C for 10 min, 42°C for 50 min, and 85°C for 5 min. Samples were then incubated with RNase H at 37°C for 20 min. The resulting first-strand complementary DNA (cDNA) was used as a template for quantitative PCR in triplicate using SYBR Green QPCR Master Mix kit (Stratagene). Oligonucleotide PCR primer pairs were designed to cross intron-exon boundaries from published rat sequences in the GenBank database using Primer3 (23). Primer sequences are provided in Supplementary Table S1. Reverse-transcribed cDNA (1 μl after 1.8 dilution) and appropriate primers were mixed with SYBR Green dye 1 master mixture in a 25 μl volume. Reactions were initially heated to 95°C for 5 min followed by 40 cycles: denaturation at 95°C for 10 s, and then combined annealing and extension steps at 60°C for 30 s. The last cycle was followed by a 7-min extension at 72°C, and thermal denaturing profile to identify the Tm. PCR amplification was verified by melting curve and electrophoretic analysis of the PCR products on 3% agarose gel. Negative controls (no reverse transcriptase and no template) were included and yielded no products. The data were analyzed using the complementary Ct method (ΔCt), and mRNA abundance normalized to β2-microglobulin and expressed as fold control.

**Statistical Methods**

Data were expressed as means ± SD. Differences in Western blotting protein expression, computer-assisted image area analyses, or fold changes in real-time PCR were compared by unpaired Student’s t test. Differences in ACF, tumor incidences or K-ras mutations were compared by Fisher exact test. P < 0.05 values were considered statistically significant.

**Results**

Male Fisher-344 rats were treated with azoxymethane or saline, and provided with AIN-76A chow or chow supplemented with gefitinib as summarized in the experimental protocol
Azoxyymethane and gefitinib were well tolerated, with no premature deaths. Animals in all groups grew at comparable rates throughout the study and the group mean weights differed by <10% at sacrifice. The hair coats of gefitinib-supplemented rats, however, were coarser—consistent with an in vivo reduction in EGFR signals (24). We first assessed EGFR signals, including levels of phospho-active EGFR, pErbB2, pERK, and pAKT in left colonic segments with normal-appearing mucosa from azoxymethane-treated and control rats. In carcinogen-treated animals, these signals were up-regulated, as shown in Fig. 1. Gefitinib significantly inhibited the activations of EGFR and ErbB2 and downstream effectors ERK and AKT (Fig. 1). The expression levels of β-catenin and cyclin D1 were also increased in normal-appearing left colons from azoxymethane-treated rats. Gefitinib increased the cyclin D1, but not β-catenin (see Supplementary Table S2). To mechanistically clarify activators of EGFR, we examined the expression of EGFR ligands. In the group given azoxymethane without gefitinib supplementation, transforming growth factor-α and amphiregulin were significantly increased to 2.5 ± 0.4-fold and 3.2 ± 0.9-fold of control (P < 0.05), respectively, whereas epidermal growth factor, heparin-binding epidermal growth factor, β-cellulin, and epi- regulin were not elevated (data not shown). In azoxymethane-treated animals, increases in transforming growth factor-α and amphiregulin would be expected to contribute to the observed up-regulated EGFR signals. Numerous biochemical and molecular abnormalities have been described in normal-appearing colonic mucosa adjacent to colon cancers that are referred to as “field cancerization effects” (25). It is presumed that carcinogen-related or tumor-derived factors have induced these effects. In addition to increases in EGFR signals in normal-appearing colonic mucosa in the current report, we and others have previously shown crypt cell hyperproliferation and cyclin D1 up-regulation in crypts adjacent to neoplastic lesions (26, 27). These field cancerization effects are believed to reflect, and in some cases, contribute to colonic neoplastic transformation. Thus, widespread increases in EGFR ligands might drive diffuse crypt cell hyperproliferation and apoptotic resistance, two important characteristics of premalignancy that predispose to malignant transformation.

There were no flat ACF in the groups receiving saline (azoxyymethane vehicle) alone or saline plus gefitinib. Azoxyymethane induced flat-appearing ACF in 66% of rats, whereas gefitinib significantly reduced the incidence of flat ACF to 36% (P < 0.05; Table 1). These ACF were uniformly dysplastic and showed increased β-catenin accumulation in agreement with earlier reports (Fig. 2, top; ref. 10). Because azoxymethane-induced tumors overexpress cyclin D1 and Cox-2 (8), and flat dysplastic ACF are thought to be tumor precursors, we examined cyclin D1 and Cox-2 expression in these lesions. They were both up-regulated at this stage (Fig. 2D and F, bottom). Thus, although some EGFR signals, including ErbB2, ERK, and AKT were increased in normal-appearing mucosa in azoxymethane-treated rats (Fig. 1), progression to flat dysplastic ACF was accompanied by additional abnormalities, including further increases in β-catenin, cyclin D1, and Cox-2 (Fig. 2). It should be noted that EGFRs are expressed on colonocytes and stromal cells (28, 29). Furthermore, stromal fibroblasts and macrophage cells are believed to be important sources of Cox-2 in premalignant colonic lesions (30, 31). Gefitinib inhibited cyclin D1 and Cox-2 up-regulations in flat ACF (Fig. 2E and G, bottom), identifying these proteins as EGFR effectors in colonic carcinogenesis. ACF can develop despite EGFR blockade, albeit at a lower incidence. Thus, up-regulations of cyclin D1 and Cox-2 might promote flat ACF development, but are not absolutely essential for their formation. It appears, however, that EGFR signals are required to promote efficient malignant transformation.

As in the case of ACF, there were no tumors in the groups receiving saline alone or saline plus gefitinib. In the azoxymethane group, 27 of 38 animals developed tumors, compared with only 8 of 37 animals in the azoxymethane + gefitinib-treated group. Thus, gefitinib significantly decreased tumor incidence from 71.1% to 21.6% (P < 0.05; Table 1). The inhibitory effects of gefitinib on tumor incidence and histologic progression were paralleled by 40% reduction in tumor size from 6.4 ± 0.6 mm in the azoxymethane group to 3.9 ± 0.6 mm in the azoxymethane + gefitinib group.
0.6 mm in the azoxymethane plus gefitinib-treated animals ($P < 0.05$; Table 1).

Because K-ras mutations occur in the azoxymethane model and could activate ERK and increase Cox-2 independent of EGFR signals (8), we examined ACF for these mutations. In 11 ACF from the azoxymethane group, there were three K-ras mutations in codon 12 (27.3%). In an equal number of ACF from the azoxymethane + gefitinib-treated group, we detected only one K-ras mutation in codon 12 (9.1%), a ras mutation incidence that was not significantly different from the azoxymethane alone group ($P = 0.25$, Fisher exact test). Thus, the majority of premalignant lesions possess wild-type Ras that are regulated by EGFR signals.

We next assessed EGFR signals in azoxymethane tumors. As in the case of up-regulated EGFR signals in premalignant colonic mucosa that reflected field effects ErbB2, Src, ERK, and AKT were activated in tumors (Fig. 3A). Cyclin D1 and Cox-2 were also up-regulated in these tumors (Fig. 3B). These signals were inhibited in tumors from animals supplemented with gefitinib, consistent with their roles as EGFR effectors (Fig. 3). We postulate that decreases in growth-promoting EGFR signals contributed to the reduction in size of these tumors. In summary, our results indicate that EGFR signals promote colonic malignant transformation at the flat dysplastic ACF stage, and increase tumor growth and enhance tumor progression from adenomas to cancers.

To begin to identify EGFR mechanisms regulating malignant transformation, we examined azoxymethane tumors for changes in several transcription factors, including AP-1, STAT3, and C/EBP which are up-regulated in colon cancer and are shown to be EGFR effectors in other cell types (5, 13–17). It is important to emphasize, however, that effector roles are cell

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>Flat ACF incidence (%)</th>
<th>Tumor incidence (%)</th>
<th>Tumor size (mm)</th>
<th>Adenomas (%)</th>
<th>Carcinomas (%)</th>
</tr>
</thead>
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<td>Azoxymethane</td>
<td>38</td>
<td>66</td>
<td>71.1</td>
<td>6.4 ± 0.6</td>
<td>24 (55%)</td>
<td>20 (45%)</td>
</tr>
<tr>
<td>Azoxymethane plus gefitinib</td>
<td>37</td>
<td>36*</td>
<td>21.6*</td>
<td>3.9 ± 0.6*</td>
<td>10 (77%)</td>
<td>3 (23%)*</td>
</tr>
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* $P < 0.05$, compared with azoxymethane alone.

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**Fig. 2.** Gefitinib inhibits cyclin D1 and Cox-2 up-regulation in flat dysplastic ACF. Top: A, conventional ACF (white arrows) and flat-appearing ACF (white arrowheads) in situ. B, flat ACF stained with H&E showing dysplastic crypts. C, flat dysplastic ACF stained for p-catenin. Bottom: D, cyclin D1 (azoxymethane alone). E, cyclin D1 (azoxymethane + gefitinib). F, Cox-2 (azoxymethane alone). G, Cox-2 (azoxymethane + gefitinib). Note that cyclin D1 and Cox-2 were increased in epithelial cells, and to a lesser extent, in stromal cells compared with adjacent crypts. Representative of three flat ACF in each group. Nonimmune antibodies gave no staining.
context—specific and their mediation of EGFR signals in colonic cancer have not been established. AP-1 is a dimeric transcription factor that includes c-Jun and FosB (5). We showed increases in c-Jun and FosB in colonic tumors as assessed by Western blotting (Fig. 3B). To identify the cell of origin, we stained tumors for FosB and c-Jun as shown in Fig. 4. These proteins were overexpressed in stromal and malignant cells and localized to the nuclei, consistent with their roles as active transcription factors (compare Fig. 4A with B and Fig. 4D with E). Quantitative computer-assisted image analysis showed that nuclear FosB was increased 16.3 ± 5.5-fold of control (P < 0.001) and nuclear c-Jun was increased 9.8 ± 2.5-fold of control (P < 0.001). Gefitinib reduced the overexpressions of FosB and c-Jun to 2.3 ± 0.5-fold and 3.8 ± 1.4-fold of control (P < 0.05) as assessed by immunostaining (compare Fig. 4C with B and Fig. 4F with E), respectively. These results were consistent with our Western blotting studies (Fig. 3B).

STAT3, a member of the signal transducers and activators of transcription family, is also activated in colon cancer (13). We showed in this study that STAT3 activation is regulated by EGFR signals (Fig. 3A). In noncolonic cells, STAT3 was also shown to be activated by EGFR and could mediate cyclin D1 induction (16, 32). Although we showed that EGFR coregulates STAT3 and cyclin D1 in colonic tumors (Fig. 3A and B), further investigations will be required to determine whether STAT3, a member of the signal transducers and activators of transcription (STAT) family, is involved in EGFR-mediated proliferation, stromal cell survival, and angiogenesis. Whether these effects of EGFR blockade on malignant cells and stromal cells, including endothelial cells, reflect loss of autocrine or paracrine EGFR signals will require further study.

The transcription factor C/EBPβ is also activated by EGFR signals in noncolonic cells and is required for ras-dependent transformation in another epithelial cancer model (17, 18). As shown in Fig. 4H, and as quantified by computer-assisted imaging, total C/EBPβ was up-regulated 8.4 ± 0.9-fold of control (P < 0.001) in tumors and was expressed predominantly in the nuclei of stromal and epithelial cells. Gefitinib reduced this up-regulation to 2.4 ± 0.7-fold of control (P < 0.05). The inhibitory effects of gefitinib on C/EBPβ expression seem to be greater in epithelial cells than stromal cells, perhaps suggesting that EGFR-independent signals might drive C/EBPβ expression in stromal cells (compare Fig. 4H with I). Because this transcription factor is activated by phosphorylation (33), we examined the level of phospho-active C/EBPβ. In azoxymethane tumors, phospho-active C/EBPβ was increased and gefitinib inhibited this phosphorylation (Fig. 3B). This is the first report to our knowledge demonstrating that these transcription factors are EGFR effectors in colonic carcinogenesis.

important hallmark of neoplasia include dysregulated proliferation, enhanced cell survival, and increased angiogenesis (34). As assessed by Ki67 staining, proliferation was significantly increased in azoxymethane tumors (42 ± 7%, P < 0.05) compared with control crypts (15 ± 4%), and gefitinib inhibited this hyperproliferation (23 ± 6%, P < 0.05; Fig. 5, top). EGFR blockade also significantly increased apoptosis from 0.2 ± 0.1% to 2.5 ± 0.8% (P < 0.05; Fig. 5, middle). Intriguingly, the apoptotic cells were predominantly in the tumor stroma, as assessed by increased staining for cleaved caspase-3 (Fig. 5, middle, see black arrows). Neoangiogenesis could be identified by nestin-1 expression in human colon cancers (35). In the current study, we also showed that blood vessels increased in tumors and gefitinib significantly inhibited tumor angiogenesis (Fig. 5, bottom). These gefitinib-induced decreases in tumor vasculature paralleled reductions in tumor size and histologic progression (Table 1). Our findings indicate that EGFR promotes malignant colonocyte hyperproliferation, stromal cell survival, and angiogenesis. Whether these effects of EGFR blockade on malignant cells and stromal cells, including endothelial cells, reflect loss of autocrine or paracrine EGFR signals will require further study.

Discussion

Our laboratory has previously shown that EGFR signals are up-regulated in human ACF, the earliest microscopically detectable premalignant lesion in the colon (2). More recently, we showed that EGFR signals promote premalignant progression in azoxymethane-treated mice (9). In the current study, we extended these investigations to address three important questions. (a) Are EGFR signals required for the formation of flat dysplastic ACF that were recently recognized as precursors.
of colon cancers? (b) Do EGFR signals promote the growth of established tumors and their histologic progression to cancer? In this regard, in the Min mouse model, EGFR seems to play a role in tumor initiation (12). (c) What EGFR effectors might mediate these events? We have chosen the azoxymethane rat model because rats treated with this carcinogen develop larger flat ACF and tumors than mice. In this report, we have shown for the first time that EGFR signals promote flat dysplastic ACF development and colonic tumor growth and progression to cancer. EGFR signals induced tumor-enhancing changes in cell proliferation and cell death, as well as blood vessel formation that were inhibited by gefitinib. In addition, this study identified c-Jun, FosB, STAT3, and C/EBPβ as EGFR effectors in colon cancer.

EGFR is up-regulated in many cancers including breast, lung, head and neck, and central nervous system cancers (36). Growing lines of evidence have also emphasized the importance of EGFR signals in colonic carcinogenesis (1, 2, 9). Signals from this receptor are known to regulate many biological processes, including cell survival, growth, differentiation, motility, and angiogenesis (4). In prior azoxymethane studies in mice, we showed generalized abnormalities in several EGFR effectors, including increased ErbB2 and ERK activations in normal-appearing premalignant colonic mucosa (9). We also showed in azoxymethane-treated rats that conventional (elevated) ACF were hyperproliferative and overexpressed cyclin D1 (26). More recent evidence suggests that flat-appearing ACF, with dysplastic features and increased β-catenin accumulation, are authentic precursors of colonic tumors (10, 11). In agreement with this postulation, in the current study, gefitinib inhibited the incidence of flat ACF and concomitantly reduced tumor development (Table 1).

In flat dysplastic ACF, we also showed that cyclin D1 and Cox-2 were increased and that their up-regulation required EGFR signals (Fig. 2). Although EGFR signals activated ERK and increased Fos proteins transiently in isolated cells (immediate

Fig. 4. Gefitinib inhibits up-regulations of FosB, c-Jun, and C/EBPβ in azoxymethane tumors. Normal mucosa (Control) or tumors from the indicated groups were stained for FosB, c-Jun, or C/EBPβ. Top (FosB): A, normal mucosa; B, azoxymethane tumor; C, azoxymethane + gefitinib tumor. Middle (c-Jun): D, normal mucosa; E, azoxymethane tumor; F, azoxymethane + gefitinib tumor. Bottom (C/EBPβ): G, normal mucosa; H, azoxymethane tumor; I, azoxymethane + gefitinib tumor. Compared with normal mucosa, FosB, c-Jun, and C/EBPβ were increased in stromal and epithelial cells in azoxymethane tumors and gefitinib inhibited these increases. Representative of three tumors in each group. In saline-treated animals, gefitinib did not alter the expression levels of FosB, c-Jun, or C/EBPβ in normal mucosa. Staining with isotype-matched nonimmune serum was negative.
early gene responses), sustained ERK activation leads to the stabilization of Jun and Fos members (37). We believe that this may be an important mechanism to up-regulate AP-1 signals and perhaps cyclin D1 and Cox-2 in colonic tumors because ERK signals were sustained in this malignant condition (Fig. 3). In addition to AP-1, our studies have shown for the first time that STAT3 and C/EBPβ are controlled by EGFR in colonic tumors (Figs. 3 and 4). Although these transcription factors have been implicated in regulating cyclin D1 or Cox-2 expression in other cell types (32, 38–41), our studies emphasize the importance of elucidating their signaling pathways in vivo in a model of colonic carcinogenesis. STAT3, for example, is only activated in colonic tumors in vivo, but not in isolated colon cancer cells (13). We have begun to investigate the mechanisms driving the up-regulation of these important signal transduction elements in earlier stages of colonic carcinogenesis. Thus far, we have shown that FosB, c-Jun, C/EBPβ, and pSTAT3 are up-regulated as early as in the flat dysplastic ACF stage, and are inhibited by gefitinib (see Supplementary Fig. S2). Taken together, our findings indicate that these transcription factors are EGFR effectors in colonic tumorigenesis and suggest that they might mediate EGFR control of cyclin D1 and Cox-2.

Although cyclin D1 and Cox-2 are targets of adenomatous polyposis coli β-catenin signals in colon cancer (42–44), our studies emphasize the importance of EGFR signals in their expression. In this study, we showed that EGFR blockade inhibited up-regulated cyclin D1 in normal-appearing colons in azoxymethane-treated animals despite persistent elevations in β-catenin. In agreement with this, other investigations have shown the importance of growth factor pathways involving ERK and AKT in controlling cyclin D1 and Cox-2 expression (8, 45, 46). Several studies have also suggested that cyclin D1 and Cox-2 are not immediate downstream targets of β-catenin (47, 48). Epidermal growth factor stimulates Cox-2 in HCA-7 rectal cancer cells (49), and in preliminary studies, we found
that egfr expression was associated with increased cyclin d1 andcox-2 in human colon cancer and vascular fibroblasts (data not shown). recent xenograft studies also indicate that egfr regulates cyclin d1 andcox-2 in human colon cancer (50). our study is the first report that describes egfr regulation of cyclin d1 andcox-2 in an in vitro model of colon cancer. thus, it seems likely that wnt-β-catenin and egfr signals must collaborate to drive colon tumorogenesis. studies in the min mouse model of intestinal tumorogenesis also support this contention as egfr loss of function mutations, or egfr pharmacologic inhibitors decreased the incidence of small bowel adenomas (12). differences in egfr requirements for tumor progression in the min mouse versus azoxymethane rat model presumably reflect differences in species and/or models. egfr and β-catenin share interacting pathways that might be differentially modulated depending, for example, on whether the β-catenin regulator adenomatous polyposis coli is wild-type (azoxymethane model) or mutant (min mouse; refs. 51, 52).

egfr is known to regulate tumor proliferation, apoptosis, and angiogenesis (53, 54). in agreement with this observation, we have shown in colon tumors that egfr blockade reduced hyperproliferation, increased apoptosis, and inhibited tumor angiogenesis (fig. 5). reductions in erk, src, and akt activities and decreases in cyclin d1 andcox-2 up-regulations would be expected to contribute to the antiproliferative and proapoptotic effects of gefitinib (figs. 1–3, and 5). studies in noncolonic tissues have indicated that the antiangiogenic effects of gefitinib included not only direct inhibitory effects on egfr expressing endothelial cells, but also decreased the production of tumor-derived proangiogenic factors, including transforming growth factor-α (55, 56). in agreement with prior studies (9), we showed that proangiogenic transforming growth factor-α was up-regulated in the colon of azoxymethane-treated rats. egfr effector signals are also required for stromal cell survival as gefitinib enhanced stromal cell apoptosis (fig. 51).

taken together, our findings underscore the role of egfr to control malignant cell proliferation and stromal cell survival. efforts are under way in our laboratory to dissect the autocrine and paracrine egfr signals in epithelial and stromal cells that mediate tumor promotion.

in summary, we have shown for the first time that egfr signals promote the development of flat dysplastic acf. furthermore, these signals control cox-2 and cyclin d1 expression in these premalignant lesions. egfr signals also mediate tumor growth and progression to cancer in the azoxymethane rat model by mechanisms involving increased proliferation, decreased apoptosis, and enhanced angiogenesis. in addition, we have shown that c-jun, fosb, c/ebpα, and stat3 are egfr effectors in colon carcinogenesis. their potential regulatory roles in cyclin d1 andcox-2 overexpression will require additional studies. thus, egfr signals play key roles in the premalignant and malignant phases of colon carcinogenesis. germane to these observations, metastatic human colon tumors, with up-regulated egfr ligands and wild-type k-ras, are significantly more sensitive to anti-egfr therapies than tumors without these characteristics (57). these are also the egfr signaling characteristics in our model. because there is no curative therapy for resectable colon cancers, efforts in chemoprevention including the development of more effective oral egfr inhibitors are warranted. in this regard, several naturally occurring dietary constituents, including curcumin and green tea, possess anti-egfr activity that have been linked to their potential chemopreventive efficacy in colon cancer (58, 59).

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