Epidermal Growth Factor Receptor Is Required for Colonic Tumor Promotion by Dietary Fat in the Azoxymethane/Dextran Sulfate Sodium Model: Roles of Transforming Growth Factor-α and PTGS2

Urszula Dougherty,[1] Dario Cerasi,[1] Ieva Taylor,[1] Masha Kocherginsky,[2] Ummuhan Tekin,[1] Shamiram Badal,[1] Lata Aluri,[1] Amikar Sehdev,[1] Sonia Cerda,[1] Reba Mustafi,[1] Jorge Delgado,[1] Loren Joseph,[4] Hongyan Zhu,[1] John Hart,[4] David Threadgill,[5] Alessandro Fichera,[3] and Marc Bissonnette[1]

Abstract

Purpose: Colon cancer is a major cause of cancer deaths. Dietary factors contribute substantially to the risk of this malignancy. Western-style diets promote development of azoxymethane-induced colon cancer. Although we showed that epidermal growth factor receptors (EGFR) controlled azoxymethane tumorigenesis in standard fat conditions, the role of EGFR in tumor promotion by high dietary fat has not been examined.

Experimental Design: A/J × C57BL6/J mice with wild-type *Egfr* (*Egfr*^{wt}) or loss-of-function waved-2 *Egfr* (*Egfr*^{wa2}) received azoxymethane followed by standard (5% fat) or western-style (20% fat) diet. As F_1 mice were resistant to azoxymethane, we treated mice with azoxymethane followed by one cycle of inflammation-inducing dextran sulfate sodium to induce tumorigenesis. Mice were sacrificed 12 weeks after dextran sulfate sodium. Tumors were graded for histology and assessed for EGFR ligands and proto-oncogenes by immunostaining, Western blotting, and real-time PCR.

Results: *Egfr*^{wt} mice gained significantly more weight and had exaggerated insulin resistance compared with *Egfr*^{wa2} mice on high-fat diet. Dietary fat promoted tumor incidence (71.2% versus 36.7%; $P < 0.05$) and cancer incidence (43.9% versus 16.7%; $P < 0.05$) only in *Egfr*^{wt} mice. The lipid-rich diet also significantly increased tumor and cancer multiplicity only in *Egfr*^{wt} mice. In tumors, dietary fat and *Egfr*^{wt} upregulated transforming growth factor-α, amphiregulin, CTNNB1, MYC, and CCND1, whereas PTGS2 was only increased in *Egfr*^{wt} mice and further upregulated by dietary fat. Notably, dietary fat increased transforming growth factor-α in normal colon.

Conclusions: EGFR is required for dietary fat-induced weight gain and tumor promotion. EGFR-dependent increases in receptor ligands and PTGS2 likely drive diet-related tumor promotion. (Clin Cancer Res 2009;15(22):6780–9)

Colon cancer is the second leading cause of cancer-related deaths in males and females in the United States (1). Germ-line mutations, such as those occurring in familial adenomatous polyposis syndrome and hereditary nonpolyposis colon cancer, cause hereditary forms of colon cancer. Environmental factors especially dietary constituents, however, are believed to play major roles in sporadic forms of this malignancy (2). The 20-fold differences in worldwide colon cancer incidence rates and rapidly changing incidence in immigrant populations support environmental exposure as a causal factor for colon cancer (3). Historically, for example, colon cancer rates were low in Japan; however, within two

Authors' Affiliations: Departments of [1]Medicine, [2]Health Studies, [3]Surgery, and [4]Pathology, University of Chicago, Chicago, Illinois and [5]Department of Genetics, North Carolina State University, Raleigh, North Carolina

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Requests for reprints: Marc Bissonnette, Department of Medicine, MC 4076, University of Chicago Hospitals and Clinics, 5841 South Maryland Avenue, Chicago, IL 60637. Phone: 773-702-8597; Fax: 773-702-2182; E-mail: mbissonn@medicine.bsd.uchicago.edu.

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Translational Relevance

Colon cancer is a leading cause of cancer-related deaths in the United States. Western-style diet is strongly linked to sporadic colon cancer. Epidermal growth factor receptor (EGFR) is also implicated in the genesis of colon cancer and the development of effective receptor inhibitors suggests future strategies to prevent this disease. We showed previously that EGFR regulates colonic tumorigenesis in the azoxymethane model of colon cancer. In the current study, we asked if EGFR controls tumor promotion by western-style diet. Dietary fat increases colonic secondary bile acids, enhances circulating insulin-like growth factor-I levels, and alters the enteric microbiome that might promote tumorigenesis by EGFR-independent mechanisms. We used a genetic approach with wild-type and *Egfr* loss-of-function waved-2 mutant mice to address this question. We studied a western-style diet that mimics the dietary fat composition of a large proportion of Americans. We showed that EGFR was required for tumor promotion by dietary fat in the azoxymethane/dextran sulfate sodium model of colon cancer. EGFR was also required for this diet to upregulate PTGS2. In addition, we showed that dietary fat increased transforming growth factor-α transcripts in normal colonic mucosa, reflecting a "field effect" that might contribute to the increased risk of colon cancer in obesity. These findings have important implications for chemoprevention strategies that target EGFR and PTGS2. There are several naturally occurring dietary substances with such dual inhibitory activities, including curcumin, green tea, and fish oil.

generations, the incidence of colon cancer among Japanese Americans approached rates for Caucasian Americans (4). Diets rich in animal fat and red meat and relatively deficient in fiber and micronutrients have been implicated in this increased risk in the industrialized western world (2).

Experimental animal models have been widely used to study the role of dietary factors in colonic carcinogenesis. Azoxymethane is a mutagen that methylates guanine bases resulting in activating mutations in K-*ras* and *CTNNBI*, which encodes β-catenin. The azoxymethane model mimics many features of sporadic human colon cancer, including promotion by dietary fat (5). Using this model, we showed that epidermal growth factor receptor (EGFR) plays an important role in colonic tumorigenesis (6, 7). To assess the role of EGFR in tumor promotion by dietary fat, we examined mice with wild-type *Egfr* ($Egfr^{wt}$) and waved-2 *Egfr* ($Egfr^{wa2}$) because *Egfr*-null mice are not viable (8). $Egfr^{wa2}$ possesses a naturally occurring hypomorphic mutation in the kinase domain that abrogates 90% of kinase activity *in vitro* (9). This mutation has been shown to attenuate intestinal tumorigenesis in *Apc* mutant *Min* mice, a model of familial adenomatous polyposis syndrome (10). We compared azoxymethane-induced tumorigenesis in $Egfr^{wt}$ and $Egfr^{wa2}$ mice fed standard rodent chow (5% fat) or a western-style high-fat diet (20% fat). A modification of this lipid-rich diet, which mimicked a westernized diet high in animal fat and low in calcium and vitamin D, has been shown to induce spontaneous colonic tumors in mice during long-term feeding (11). As these mice were resistant to azoxymethane alone, we modified the protocol to include azoxymethane followed by dextran sulfate sodium (DSS). DSS is a nonmutagenic agent that arrests crypt cell proliferation, leading to colonic crypt shortening and eventual mucosal ulcerations and inflammation (12). Many strains of mice resistant to azoxymethane alone are susceptible to the proinflammatory and tumor-promoting effects of azoxymethane/DSS (13). Although EGFR contributes to azoxymethane tumorigenesis, the role of this receptor in azoxymethane/DSS tumor promotion by dietary fat has not been examined. There are other potential tumor-promoting factors modulated by dietary fat that might drive tumor promotion independent of EGFR signals. These include increases in colonic luminal secondary bile acids and circulating insulin-like growth factors (14–16).

To begin to elucidate potential EGFR effectors that might mediate tumor promotion by dietary fat, we examined several proto-oncogenes, including CTNNB1, MYC, CCND1 (cyclin D1), and PTGS2 (cyclooxygenase-2) that are known to play important roles in colonic tumorigenesis. MYC, CCND1, and PTGS2 are transcriptional targets of CTNNB1 (17–21). Recent studies have shown that dietary fat enhances expression of these proto-oncogenes in colonic carcinogenesis (22–24). Furthermore, EGFR regulates tyrosine phosphorylation and nuclear localization of CTNNB1 as well as MYC expression (25, 26). We have shown, moreover, that EGFR regulates CCND1 and PTGS2 levels in the azoxymethane model under standard dietary fat conditions (6, 7). In the current study, we show that CTNNB1, MYC, and CCND1 upregulations by dietary fat are amplified by EGFR signals. In contrast, diet-related increases in PTGS2 require EGFR signals. To identify potential upstream effectors of EGFR induced by dietary fat, we also examined the influence of diet on transforming growth factor-α (TGF-α) and amphiregulin, two EGFR ligands that are increased in colonic tumorigenesis (6, 7).

Materials and Methods

Materials

C57BL6/J $Egfr^{wt/wa2}$ mice were interbred with A/J $Egfr^{wt/wa2}$ mice to generate the F_1 hybrid C57BL6/J × A/J experimental group. Formulated high-fat diet was based on western-style diet that contained 20% fat as described (11). A standard fat diet was also formulated that contained 5% fat with the additional calories provided by cornstarch. Harlan Teklad Laboratories prepared these diets and also supplied AIN-76A rodent chow. The specific dietary components are provided in Supplementary Table S1. Azoxymethane was obtained from Midwest Research, the National Cancer Institute Chemical Carcinogen Reference Standard Repository. Superfrost Plus slides were purchased from Fisher Scientific. Polyclonal antibodies to CCND1 and monoclonal antibodies to MYC (clone 9E10) and vascular endothelial growth factor were obtained from Santa Cruz Biotechnology. Monoclonal anti-CTNNB1 antibodies were obtained from BD Pharmingen. Rabbit polyclonal anti-PTGS2 antibodies were purchased from Cayman Chemical. Monoclonal β-actin antibodies were purchased from Sigma-Aldrich. DNeasy kit and RNeasy lipid extraction kit and HotStarTaq DNA polymerase were obtained from Qiagen. *Fok*I restriction enzyme was purchased from New England Biolabs. RNAlater RNA storage solution and DNA-free DNase I kit were purchased from Ambion. Trizol RNA/DNA/protein isolation reagent was obtained from Life Technologies. RiboGreen reagent for

RNA quantitation was purchased from Molecular Probes. Custom PCR primers were obtained from Integrated DNA Technologies. Other PCR reagents, including Moloney murine leukemia virus reverse transcriptase, random hexamers, and SYBR Green were purchased from Applied Biosystems. SuperScript III Platinum Two-Step qRT-PCR kit was obtained from Invitrogen. Electrophoretic-grade acrylamide, bisacrylamide, Tris, SDS, prestained molecular weight markers, and RC-DC protein assay were from Bio-Rad Laboratories. Kodak supplied the X-OMAT AR film. Polyvinylidene fluoride membranes (Immobilon-P) were purchased from Millipore. Unless otherwise noted, all other reagents were of the highest quality available and were obtained from Sigma-Aldrich.

Methods

Egfr *genotyping*. The *Egfr*^{wa2} point mutation is a T-to-G transversion (valine-to-glycine) that creates a recognition site for the restriction enzyme *Fok*I (GGATG). To genotype this locus, we PCR-amplified genomic sequences and digested products with *Fok* that were separated on 2% agarose containing 100-bp DNA markers (The Jackson Laboratory protocol). Primers are in intron 19 and exon 20 of mouse *Egfr*, respectively, and amplify 326-bp fragments for *Egfr*^{wt} and *Egfr*^{wa2} (27). *Fok*I cuts the *Egfr*^{wa2} sequence (GGATG) but not the *Egfr*^{wt} sequence (TGATG) to generate a doublet of 166 and 160 bp.

Experimental animal protocol. Mice were treated with azoxymethane (7.5 mg intraperitoneally/kg body weight) or saline (azoxymethane vehicle) weekly × 6 weeks and maintained on AIN-76A diet. Two weeks after the last azoxymethane treatment, animals were started on standard or high-fat diets. The high-fat diet is based on a diet formulation that approximates dietary amounts consumed in western-style diets with increased animal fat and lower levels of vitamin D_3 and calcium (11). The diet compositions are shown in Supplementary Table S1. Chow was replaced weekly and remaining chow was weighed to estimate food intake. Animals were weighed weekly.

As animals in the first cohort sacrificed did not develop tumors, we modified the protocol by giving azoxymethane/DSS. Mice were switched to AIN-76A chow for 2 weeks and then treated with azoxymethane (7.5 mg/kg body weight) weekly × 2 weeks. One week after the second azoxymethane injection, mice received 2.5% DSS in the drinking water for 5 days. Control animals received intraperitoneal saline (azoxymethane vehicle) and were provided tap water (DSS control) for drinking. Two weeks after completing DSS or vehicle, animals were restarted on standard or high-fat diets. Twelve weeks after DSS administration, mice were anesthetized and colons were excised. Perirenal and mesenteric fat were collected to estimate visceral fat stores. Colons were cleared of feces and opened longitudinally. Tumors were harvested, fixed in 10% buffered formalin, and embedded in paraffin. A small portion of tumors was flash-frozen in liquid nitrogen for RNA and proteins. Tumors were classified according to histologic grade by an expert gastrointestinal pathologist (J.H.) following consensus criteria (28). A 1 cm left colonic segment (distal margin 1 cm above the anus) that was cleared of any tumor was scraped and the mucosa flash was frozen for protein or RNA. The remaining colons were fixed-flat in 10% formalin for immunostaining or in 70% ethanol to preserve proteins for Western blotting.

Blood glucose and serum insulin levels

Blood samples from nonfasted mice were obtained at the time of sacrifice and serum was separated from clotted blood. Glucose levels were measured using an Abbott Laboratory blood glucose monitoring system. Insulin levels were measured by EIA using an insulin assay with a standard insulin curve from 0 to 6.9 ng/mL following the manufacturer's directions (Alpco).

Real-time PCR

Frozen colonic mucosa or tumors were thawed and RNA extracted using RNeasy Lipid Tissue Mini kit. Samples were homogenized with a Polytron and loaded onto a RNA-binding spin column, washed, digested with DNase I, and eluted in 30 μL 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 in 20 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 cDNA was used as template for quantitative PCR in triplicate using SYBR Green QPCR Master Mix kit. Oligonucleotide PCR primer pairs were designed to cross intron-exon boundaries from published mouse sequences in the GenBank database using Primer3 (29). The TGF-α primers were forward 5′-TGGGCACTTGTTGAAGTGAG-3′ and reverse 5′-TGCTAGCGCTGGGTATCC-3′. The amphiregulin primers were forward 5′-GCTATT-GGCATCGGCATC-3′ and reverse 5′-ACAGTCCCGTTTTCTTGTCG-3′. Reverse transcribed cDNA (1 μL of 1:8 dilution) and primers were mixed with SYBR Green dye I master mixture in 25 μL. Reactants 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 step 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) yielded no products. The data were analyzed using the comparative ΔΔCt method, and mRNA abundance was normalized to β-actin mRNA and expressed as fold-control (30).

Immunohistochemistry

Sections (5 μm) of formalin-fixed, paraffin-embedded colonic tissue (normal colons or tumors) were cut and mounted on Vectabond-coated Superfrost Plus slides. The slides were heated to 60°C for 1 h, deparaffinized by three washes of 5 min each in xylene, hydrated in a graded series of ethanol washes, and rinsed with distilled water. Epitope retrievals were achieved by microwave heating for 15 min in 0.01 mol/L citrate buffer (pH 6; CTNNB1) or in a steamer with Tris-EDTA (pH 9;

Table 1. Effects of *Egfr* genotype and diet on glucose, insulin, body weight, and visceral fat/body weight

Egfr genotype	*n*	Diet	Glucose (mg/100 mL)	Insulin (ng/mL)	Body weight (g)	Visceral fat/body weight
Egfr$^{wt/}$	14	Standard fat*	121 ± 11	2.3 ± 0.8	35 ± 3	0.05 ± 0.03
	13	High fat†	242 ± 23‡	5.2 ± 1.6‡	45 ± 6‡	0.09 ± 0.01‡
Egfr^{wa2}	7	Standard fat	117 ± 16	2.2 ± 1.9	28 ± 3	0.03 ± 0.02
	7	High fat	117 ± 5	3.9 ± 1.7§	30 ± 3	0.06 ± 0.01§

NOTE: Indicated parameters were measured at the time of sacrifice.
*Standard fat (5%).
†High fat (20%).
$^{\ddagger}P < 0.05$, compared with *Egfr*^{wt} on standard fat diet.
$^{\S}P < 0.05$ compared with *Egfr*^{wa2} on standard fat diet.

Table 2. Effects of *Egfr* genotype and diet on tumor incidence and multiplicity

Genotype	Diet	Mice total (n = 152)	Tumor incidence				Tumor multiplicity			
			Mice with tumor	Tumor incidence (%)	Mice with cancer	Cancer incidence (%)	Total tumors	Average n tumors/ mouse	Total cancers	Average n cancers/ mouse
$Egfr^{wt/}$	Standard fat*	60	22	36.7	10	16.7	53	0.9	18	0.3
	High fat†	66	47	71.2‡	29	43.9‡	133	2.0‡	62	0.9‡
$Egfr^{wa2}$	Standard fat	11	5	45.4	1	9.1	8	0.7	1	0.09
	High fat	15	7	46.7	2	13.3	9	0.6	2	0.13

*Standard fat (5%).
†High fat (20%).
‡$P < 0.002$, compared with standard fat conditions (based on logistic regression models for incidence and negative binomial regression models for multiplicity; each model contained genotype, diet, and genotype × diet interaction).

CCND1). The antigen retrieval step was omitted for MYC staining. Frozen sections were used for PTGS2 staining and the peroxidase-blocking step was omitted. Following epitope retrieval, sections were washed three times for 2 min each in TBS-0.1% Tween 20. The endogenous peroxidase activity was quenched by incubation for 15 min in methanol/H_2O_2 solution (0.5%) protected from light. Sections were washed three times in TBS-0.1% Tween 20 for 2 min each and nonspecific binding was saturated using Protein Block (DAKO) for 20 min. The sections were incubated with primary antibody for 24 h at room temperature (1:150 dilution for CTNNB1, 1:25 dilution for MYC, 1:50 dilution for CCND1, and 1:100 dilution for PTGS2). After three TBS-0.1% Tween 20 washes, the slides were incubated at room temperature for 30 min with 1:200 dilution of biotinylated secondary antibodies. Antigen-antibody complexes were detected using a horseradish peroxidase–labeled DAKO EnVision+ System (DAKO LSAB+ System) and 3,3′-diaminobenzidine as substrate. After washing with distilled water, the slides were counterstained with Gill's III hematoxylin, rinsed with water, dehydrated in ethanol, and cleared with xylene. Tumors of comparable stage were used for immunostaining comparisons. For negative controls, primary antibodies were omitted or sections were incubated with isotype matched nonimmune antibodies. Control sections showed no specific staining.

Western blotting

Proteins were extracted in SDS-containing Laemmli buffer, quantified by RC-DC protein assay, and subjected to Western blotting as described (31). Briefly, proteins were separated by SDS-PAGE on 4% to 20% resolving polyacrylamide gradient gels and electroblotted to polyvinylidene fluoride membranes. Blots were incubated overnight at 4°C with specific primary antibodies followed by 1 h incubation with appropriate peroxidase-coupled secondary antibodies that were detected by enhanced chemiluminescence using X-OMAT film. Xerograms were digitized using an Epson scanner and band intensity quantified using UN-SCAN-IT gel 5.3 software (Silk Scientific). Protein expression levels in tumors were expressed as fold of control colonic mucosa (mean ± SD) matched for diet and *Egfr* genotype. Separate aliquots were probed for β-actin to assess loading and expression levels were normalized to β-actin levels. Protein lysates from tumors and colonic mucosa with equal protein abundance as assessed by RC-DC assays also showed comparable Western blotting β-actin levels. Tumors of comparable stage were used for Western blotting comparisons.

Statistical methods

Continuous data (glucose, insulin, weight, and fat ratio) were summarized as mean ± SD and compared between groups using Student's t test. Analyses for all values summarized in Table 1 were log-transformed. Differences in Western blotting protein expression were compared by unpaired Student's t test. Real-time PCR samples were run in triplicate, and Ct values were averaged. Untransformed Ct values were compared between groups using saturated ANOVA models with genotype, diet, and tissue type (tumor or normal mucosa) effects and their interactions (30). Relative abundance, expressed as $2^{-\Delta\Delta Ct}$, was calculated by exponentiating the estimated differences in Ct between individual groups. Tumor incidence was defined as the proportion of mice with at least one tumor. Tumor multiplicity was defined as the average number of tumors in a given group. Nonparametric trend test was used to test for trends in tumor and cancer multiplicity across $Egfr^{wt/wt}$, $Egfr^{wt/wa2}$, and $Egfr^{wa2/wa2}$ genotypes. Because, in general, $Egfr^{wa2}$ behaves as a recessive allele (10), $Egfr^{wt/wt}$ and $Egfr^{wt/wa2}$ genotypes were combined in subsequent analyses. Tumor incidence was compared between groups using logistic regression. Tumor multiplicity was compared between groups using negative binomial regression (32). Estimates and P values reported in Table 2 are based on the corresponding saturated regression models with genotype, diet, and genotype × diet interaction. All statistical analyses were done using SAS version 9.1 or Stata version 10. P values < 0.05 were considered statistically significant.

Results

Effects of EGFR signals and dietary fat on colonic tumorigenesis. We studied F_1 progeny derived from interbreeding $Egfr^{wt/wa2}$ C57BL6/J and $Egfr^{wt/wa2}$ A/J mice for these experiments to provide an A/J background for increased azoxymethane susceptibility and a C57BL6/J background for greater hybrid vigor because A/J $Egfr^{wa2/wa2}$ mice tolerated azoxymethane poorly. We controlled for hybrid genetic background by using F_1 littermates for the experimental groups. Mice were treated with six weekly injections of azoxymethane or saline and begun on experimental diets 2 weeks after the last azoxymethane injection. The high-fat diet is based on a formulation that approximates dietary amounts consumed in western-style diets with increased animal fat and lower levels of vitamin D_3 and calcium (11). Growth rates were comparable in mice homozygous and heterozygous for $Egfr^{wt}$. We, therefore, combined these groups for growth analyses. Compared with mice fed the standard fat diet, $Egfr^{wt}$ mice, but not in $Egfr^{wa2}$ mice, gained significantly more weight on the high-fat diet (Fig. 1). Chow consumption was increased but comparable in $Egfr^{wt}$ and $Egfr^{wa2}$ mice on the high-fat diet. F_1 mice, however, were resistant to azoxymethane as no aberrant crypt foci, microadenomas, or tumors developed up to 1 year after carcinogen treatment in the first 50 mice sacrificed regardless of genotype or diet. Colons were prepared as Swiss rolls and multiple sections were extensively examined. Presumably, this reflected the relative azoxymethane resistance of the C57BL6/J parental strain.

To enhance tumorigenesis, the remaining azoxymethane-treated mice received a modified protocol involving azoxymethane/DSS administration (13). The azoxymethane/DSS treatment protocol is summarized in Supplementary Fig. S1. Five staggered cohorts of mice initially treated with azoxymethane were available for azoxymethane/DSS treatment. We ensured that the 1 year interval between azoxymethane treatment and azoxymethane/DSS protocol was identical for each of the groups. Mice were switched to AIN-76A chow for 2 weeks and then treated with azoxymethane (7.5 mg/kg body weight) weekly × 2 weeks to prevent confounding effects of azoxymethane and experimental diets. One week after the second azoxymethane injection, mice received 2.5% DSS in the drinking water for 5 days. Control animals received intraperitoneal saline (azoxymethane vehicle) and were provided tap water (DSS control) for drinking. The azoxymethane and DSS treatments were well tolerated with no unexpected deaths. DSS induced mild clinical colitis as manifested by ~5% weight loss and loose stools that were positive for occult blood. Two weeks after completing DSS or vehicle, animals were restarted on standard or high-fat diets to prevent confounding DSS inflammation with effects of experimental diets. Twelve weeks after DSS administration, mice were sacrificed.

The high-fat diet increased visceral fat in both genotypes, but weight gain was greater in the $Egfr^{wt}$ group. Serum insulin was increased in both $Egfr^{wa2}$ and $Egfr^{wt}$ mice, but levels were higher in the $Egfr^{wt}$ group and blood glucose was only elevated in the $Egfr^{wt}$ group, suggesting greater insulin resistance in the latter group (Table 1). There were no tumors in the dietary control groups treated with saline and given only water (no DSS). We examined the effects of *Egfr* genotype on tumorigenesis. As summarized in Supplementary Table S2, tumor incidence was 0.57 in the $Egfr^{wt/wt}$ group, 0.52 in the $Egfr^{wt/wa2}$ group, and 0.46 in the $Egfr^{wa2/wa2}$ group ($P = 0.62$, Fisher's exact test). Cancer incidences were 0.35, 0.27, and 0.12 ($P = 0.08$, Fisher's exact test), respectively. Tumor multiplicities in these groups were 1.7, 1.3, and 0.7 and cancer multiplicities were 0.8, 0.5, and 0.1, respectively. These decreases in tumor and cancer multiplicities across genotypes $Egfr^{wt/wt} > Egfr^{wt/wa2} > Egfr^{wa2/wa2}$ were statistically significant by nonparametric trend test ($P = 0.05$ and 0.01 for tumor and cancer multiplicity, respectively). Because the Wa2 mutation functions as a recessive allele (10), we compared the effects of $Egfr^{wt}$ [$Egfr^{wt/} = Egfr^{wt/wt} + Egfr^{wt/wa2}$] to $Egfr^{wa2/wa2}$ on tumorigenesis. Cancer incidence was significantly higher in the combined $Egfr^{wt/}$ group compared with the $Egfr^{wa2/wa2}$ group (31% versus 11.5%; $P = 0.05$, Fisher's exact test). Tumor incidence was also higher in the $Egfr^{wt/}$ group compared with the $Egfr^{wa2/wa2}$ group (55% versus 46%), although the difference was not statistically significant ($P = 0.52$). Tumor multiplicity (1.5 versus 0.7) and cancer multiplicity (0.6 versus 0.1) were also significantly higher in $Egfr^{wt/}$ groups compared with the $Egfr^{wa2/wa2}$ group ($P = 0.02$ and 0.01, respectively; negative binomial regression). Thus, homozygous $Egfr^{wa2}$ mutations inhibited tumor progression to cancers, with significantly lower cancer incidence and cancer multiplicity compared with $Egfr^{wt/}$ mice.

We next examined the interaction of *Egfr* genotype and diet as summarized in Table 2. A high-fat diet significantly increased tumor incidence from 36.7% to 71.2% ($P < 0.001$) and cancer incidence from 16.7% to 43.9% ($P = 0.002$, logistic regression) in the $Egfr^{wt}$ group. As also shown in Table 2, high dietary fat significantly increased tumor multiplicity from 0.9 to 2.0 ($P = 0.001$) and cancer multiplicity from 0.3 to 0.9 in the $Egfr^{wt}$ group ($P = 0.002$). In contrast, tumor incidence and tumor multiplicity were comparable in $Egfr^{wa2/wa2}$ mice fed standard versus high-fat diet (Table 2). Although the interaction between diet and genotype did not reach statistical significance in these regression models ($P = 0.11$ and 0.15 for tumor incidence and multiplicity), models fitted separately within each genotype confirmed highly significant increases in tumor and cancer incidence and multiplicity induced by the high-fat diet in the $Egfr^{wt}$ group ($P < 0.002$ in all four models) but not in $Egfr^{wa2/wa2}$ mice. Additionally, the relatively small sample size in the $Egfr^{wa2/wa2}$ group potentially limited our ability to detect a diet × genotype interaction. Thus, these results suggest that dietary fat significantly increased tumor incidence and promoted tumor progression only in $Egfr^{wt}$ animals.

Effects of EGFR signals and dietary fat on proto-oncogene effector signals. To begin to uncover EGFR-dependent pathways that mediate effects of dietary fat on tumor promotion, we examined expression levels of several proto-oncogenes implicated in colonic carcinogenesis. As assessed by Western blotting, CTNNB1 was significantly upregulated in tumors compared with controls in $Egfr^{wt}$ animals. Dietary fat further increased CTNNB1 expression levels in tumors (Fig. 2, *top*). Significant increases in tumor CTNNB1 were also observed in $Egfr^{wa2}$ mice on high dietary fat. Note that fold increases in CTNNB1 in tumors were higher $Egfr^{wa2}$ mice compared with $Egfr^{wt}$ mice because the normalizing control mucosal levels were lower in the $Egfr^{wa2}$ mice. CTNNB1 levels, however, were higher in tumors from $Egfr^{wt}$ compared with $Egfr^{wa2}$ mice. We immunostained tumors and found that CTNNB1 was expressed predominantly in colonocytes (Fig. 2, *top*). In agreement with Western blotting results,

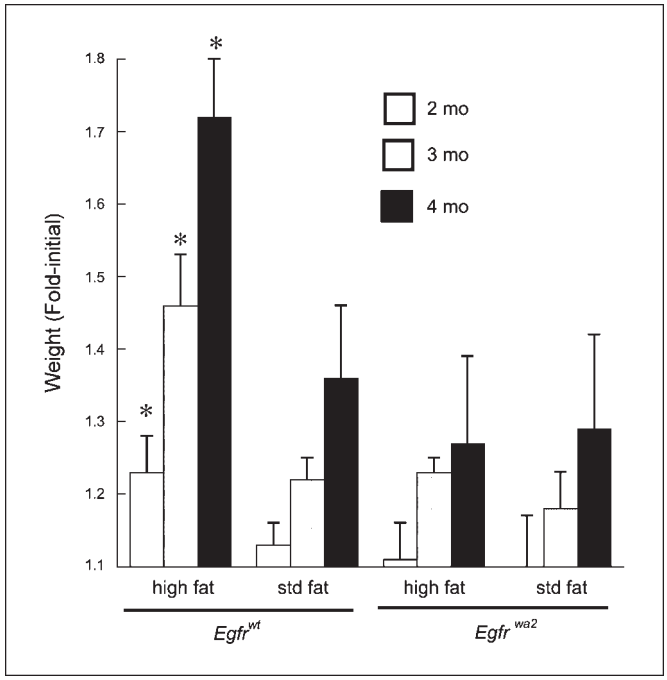

Fig. 1. $Egfr^{wa2}$ mutation prevents dietary fat-induced weight gain. Following azoxymethane treatment, mice were started on standard (*Std*; 5% fat) or high-fat diet (20% fat) and weighed weekly. Monthly average weights for the indicated genotype and diet normalized to the first month weight. Within 4 months of diet initiation, weights were stable. Saline-treated control groups, matched for genotype and diet, gained slightly more weight than azoxymethane-treated animals but then closely paralleled azoxymethane-treated groups for the remainder of the study with no significant differences. *, $P < 0.05$, compared with age-matched $Egfr^{wt}$ animals on standard fat diet.

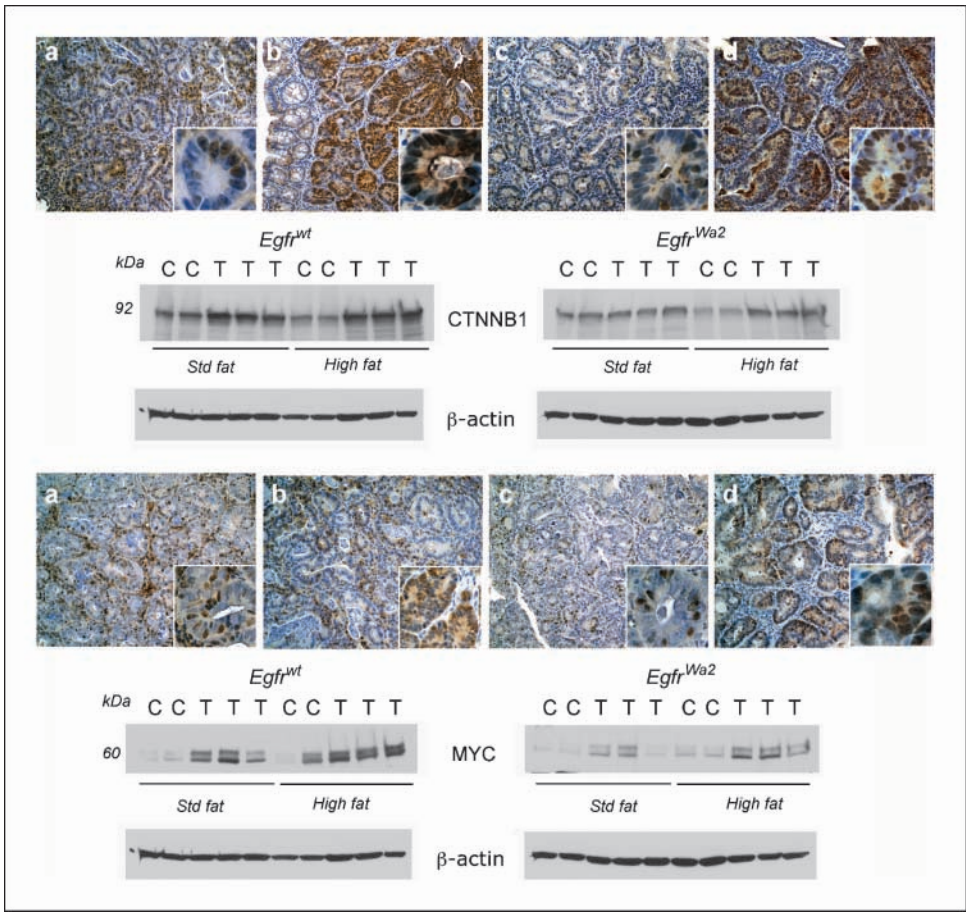

Fig. 2. CTNNB1 and MYC expression levels in colonic tumors are controlled by *Egfr* genotype and diet. Colonic tumors were immunostained and Western blotted as described in Materials and Methods. Representative tumors from each group. *Top,* CTNNB1 immunohistochemistry. *a, Egfr*^{wt}, standard fat; *b, Egfr*^{wt}, high fat; *c, Egfr*^{wa2}, standard fat; *d, Egfr*^{wa2}, high fat. Images are ×20 and insets are ×100. CTNNB1 Western blot. Proteins from colonic tumors (*T*) and control colons (*C*) from animals on standard fat or high-fat diets were Western blotted for CTNNB1. Densitometry units were expressed as fold control matched for *Egfr* genotype and diet. In *Egfr*^{wt} animals, CTNNB1 levels were significantly higher in tumors compared with control under both standard fat (1.4 ± 0.1-fold; $P < 0.05$) and high-fat (1.6 ± 0.2-fold; $P < 0.05$) conditions. In animals with *Egfr*^{wa2} on a high-fat diet, CTNNB1 was 3.8 ± 0.8-fold higher in tumors compared with control ($P < 0.05$). *Bottom,* MYC immunohistochemistry. *a, Egfr*^{wt}, standard fat; *b, Egfr*^{wt}, high fat; *c, Egfr*^{wa2}, standard fat; *d, Egfr*^{wa2}, high fat. Images are ×20 and insets are ×100. MYC Western blot. In *Egfr*^{wt} animals under standard fat and high-fat conditions, MYC in tumors was 8.7 ± 1.9-fold ($P < 0.05$) and 2.5 ± 0.5-fold ($P < 0.05$) of control, respectively. In tumors from *Egfr*^{wa2} animals, MYC was significantly increased 3.8 ± 0.8-fold control ($P < 0.05$) in high-fat conditions. Note that CTNNB1 and MYC levels were controlled by *Egfr* genotype and dietary fat. Under high-fat conditions, CTNNB1 and MYC were expressed predominantly in malignant epithelial cells with both cytoplasmic and nuclear distributions. Fold increases in CTNNB1 and MYC were higher in tumors from *Egfr*^{wa2} mice on high-fat compared with *Egfr*^{wt} mice because the normalizing control mucosal levels were lower. Expression levels of these proto-oncogenes, however, were higher in tumors from *Egfr*^{wt} mice compared with *Egfr*^{wa2} mice.

CTNNB1 staining levels were higher in tumors from *Egfr*^{wt} animals compared with *Egfr*^{wa2} animals on a standard fat diet. Dietary fat further increased tumor CTNNB1 staining levels in *Egfr*^{wt} and *Egfr*^{wa2} animals.

We next examined MYC expression. As in the case of CTNNB1, MYC tumor levels were higher in *Egfr*^{wt} animals compared *Egfr*^{wa2} animals under standard fat conditions (Fig. 2, *bottom*). High dietary fat increased MYC expression in tumors regardless of *Egfr* genotype and levels were greater in tumors from *Egfr*^{wt} compared with *Egfr*^{wa2} animals. As assessed by immunostaining, MYC expression appeared to be relatively restricted to colonocytes in tumors from *Egfr*^{wa2} animals. This suggests that MYC expression might be more dependent on EGFR signals in stromal cells compared with epithelial cells. In *Egfr*^{wt} animals, MYC was expressed in stromal cells and malignant colonocytes in low-fat conditions, whereas under high-fat conditions MYC was predominantly in colonocytes (Fig. 2, *bottom* compare *inset A* with *inset B*). Thus, dietary fat increased CTNNB1 and MYC in tumors regardless of *Egfr* genotype and the presence of *Egfr*^{wt} enhanced these increases (Fig. 2). Furthermore, *Egfr* genotype modulated the effects of dietary fat on cell-specific MYC expression.

CCND1 was also increased in tumors compared with control mucosa in *Egfr*^{wt} and *Egfr*^{wa2} mice, with the highest levels occurring in *Egfr*^{wt} mice under high-fat conditions (Fig. 3, *top*). As assessed by immunostaining, CCND1 was predominantly nuclear and localized to epithelial cells in agreement with azoxymethane studies (6, 7).

In contrast to CTNNB1, MYC, and CCND1, PTGS2 was almost undetectable in tumors from *Egfr*^{wa2} mice fed standard or high dietary fat as assessed by Western blotting (Fig. 3, *bottom*). PTGS2 upregulation required *Egfr*^{wt} and was strongly influenced by dietary fat. PTGS2 was increased in 7 of 8 tumors from *Egfr*^{wt} animals on high-fat diet compared with only 1 of 7 tumors from *Egfr*^{wt} animals on a standard fat diet (Supplementary Table S3; $P < 0.05$). In agreement with Western blotting

results, PTGS2 staining levels were greater in tumors from *Egfr*^{wt} animals on high dietary fat compared with a standard fat diet (Fig. 3, *bottom*, compare *B* with *A*). PTGS2 was expressed predominantly in tumor stromal cells, with lower levels in malignant colonocytes. Dietary fat also increased tumor vascular endothelial growth factor and was higher in *Egfr*^{wt} mice (data not shown).

Effects of EGFR signals and dietary fat on EGFR ligand expression. Upregulated EGFR signals can be driven by gene amplification, activating mutations and increased ligand or receptor abundance. In colonic carcinogenesis, increases in ligand abundance are very important. The effect of dietary fat on these ligands, however, has not been examined. As shown in Table 3, in normal mucosa, there was a significant interaction between diet and genotype in regulating TGF-α expression ($P = 0.01$): high-fat diet significantly increased TGF-α expression in the *EGFR*$^{wt/}$ mice ($2^{-\Delta\Delta Ct} = 2.8$; $P = 0.009$) but not in the EGFR$^{wt2/wt2}$ group ($2^{-\Delta\Delta Ct} = 0.96$; $P = 0.89$). Diet had no significant effect on amphiregulin levels in normal mucosa regardless of genotype (data not shown). TGF-α and amphiregulin transcripts were significantly increased in tumors compared with normal colonic mucosa matched for *Egfr* genotype and diet. Increases ranged from 4.9- to 46-fold of normal mucosa (Table 3). In tumors, there was a significant genotype × diet interaction for TGF-α ($P = 0.045$): high-fat diet increased tumor TGF-α levels in both EGFR$^{wt/}$ mice ($2^{-\Delta\Delta Ct} = 10.0$; $P < 0.0001$) and EGFR$^{wa2/wa2}$ mice ($2^{-\Delta\Delta Ct} = 3.6$; $P = 0.001$), but the increase in the EGFR$^{wt/}$ mice was much greater. Thus, there appears to be an important interaction between diet and *Egfr* genotype that regulates TGF-α abundance. In contrast, the increase in tumor amphiregulin due to high-fat diet was smaller across genotypes ($2^{-\Delta\Delta Ct} = 1.9$; $P = 0.05$) and there was not a diet × genotype interaction.

Discussion

Diet is believed to play a key role in sporadic colonic tumorigenesis. Western-style dietary fat has been shown to upregulate several key proto-oncogenes in experimental colonic tumorigenesis including CTNNB1, CCND1, and PTGS2 that are regulated by multiple signaling pathways (23, 33). Although

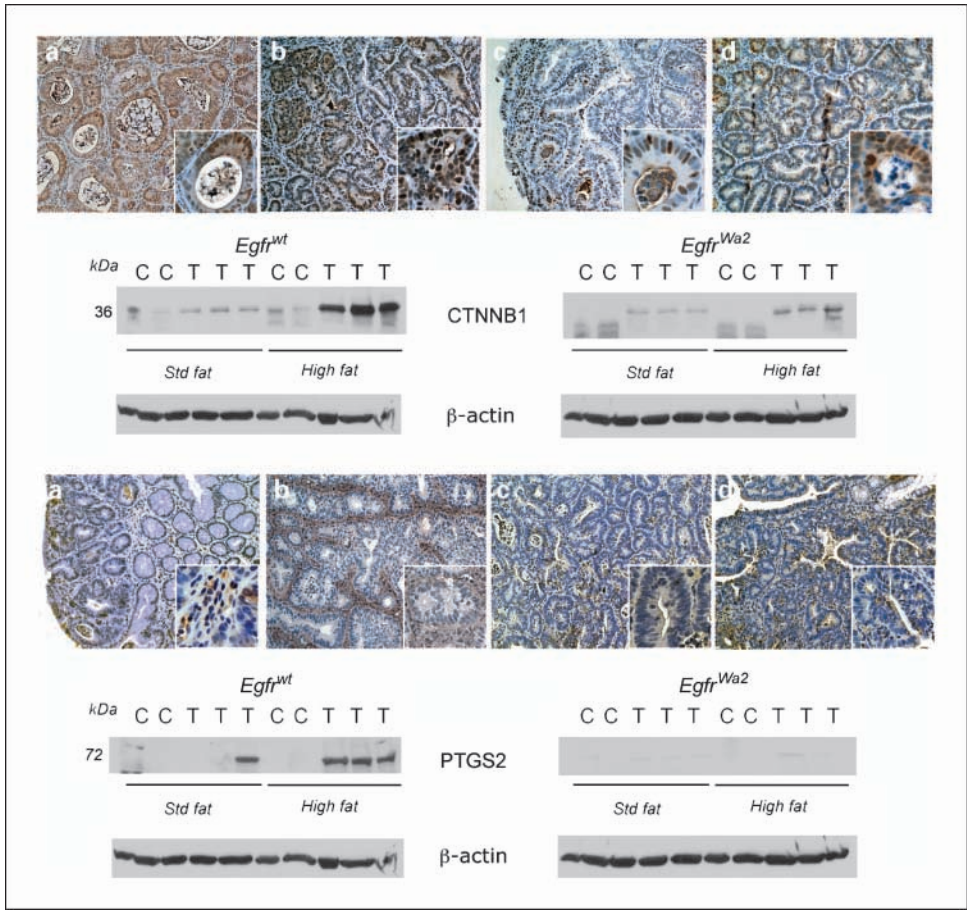

Fig. 3. CCND1 and PTGS2 expression levels in colonic tumors are controlled by *Egfr* genotype and diet. Colonic tumors were immunostained and Western blotted as described in Materials and Methods. Representative tumors from each group. *Top,* CCND1 immunohistochemistry. *a, Egfr*^{wt}, standard fat; *b, Egfr*^{wt}, high fat; *c, Egfr*^{wa2}, standard fat; *d, Egfr*^{wa2}, high fat. Images are ×20 and insets are ×100. CCND1 Western blot. Proteins from colonic tumors and control colons from animals on standard fat or high-fat diets were Western blotted for CCND1. Densitometry units were expressed as fold control matched for *Egfr* genotype and diet. CCND1 levels were significantly higher in tumors compared with controls under high-fat conditions in both *Egfr*^{wt} animals (5.5 ± 1.4-fold control; $P < 0.05$) and *Egfr*^{wa2} animals (10.0 ± 0.5.4-fold control; $P < 0.05$). Note that whereas fold increase in tumor CCND1 was higher in *Egfr*^{wa2} mice on high-fat compared with *Egfr*^{wt} mice because the normalizing control mucosal levels were lower, CCND1 expression levels were much higher in tumors from *Egfr*^{wt} mice. *Bottom,* PTGS2 immunohistochemistry. *a, Egfr*^{wt}, standard fat; *b, Egfr*^{wt}, high fat; *c, Egfr*^{wa2}, standard fat; *d, Egfr*^{wa2}, high fat. Images are ×20 and insets are ×100. PTGS2 was increased in *Egfr*^{wt} animals on a high-fat diet and predominantly expressed in stromal cells (*bottom*, compare *b* with *a*). PTGS2 Western blot. In *Egfr*^{wt} animals, PTGS2 levels were significantly higher in tumors compared with control under high-fat conditions (21.8 ± 2.8-fold; $P < 0.005$).

Table 3. Effects of diet and genotype on EGFR ligands in normal mucosa and tumors

Genotype	Diet	Normal mucosa TGF-α		Tumor TGF-α		Tumor vs normal TGF-α		Tumor vs normal amphiregulin	
		$2^{-\Delta\Delta Ct}$	*P*	$2^{-\Delta\Delta Ct}$	*P*	$2^{-\Delta\Delta Ct}$	*P*	$2^{-\Delta\Delta Ct}$	*P*
Egfr $^{wt/}$	Standard fat*	1	-	1	-	12.9	<0.0001	8.8	<0.0001
	High fat†	2.8	0.009	10.0	<0.0001	46.1	<0.0001	29.9	<0.0001
Egfr^{wa2}	Standard fat	1	-	1	-	4.9	<0.0001	11.8	<0.0001
	High fat	0.9	0.9	3.6	0.001	18.0	<0.0001	25.5	<0.0001

NOTE: *Egfr* transcript levels were measured by real-time PCR as described in Materials and Methods. Fold changes ($2^{-\Delta\Delta Ct}$) comparing TGF-α expression between diets in each genotype in normal mucosa and tumors ($n = 6$ in each group). The estimates were obtained by exponentiating the estimated differences in Ct levels based on separate two-way ANOVA models within tissue type, diet, genotype, and their interactions (30). Fold-changes ($2^{-\Delta\Delta Ct}$) comparing TGF-α expression and amphiregulin expression in tumor versus normal mucosa in each genotype and diet group were obtained by exponentiating the estimated differences in Ct levels based on a three-way ANOVA model with diet, genotype, tissue type (tumor versus normal), and their interactions (30). *P* values were obtained post hoc based on the saturated model.
*Standard fat (5%).
†High fat (20%).

growing lines of evidence from human and experimental animal studies support an etiologic role for EGFR in colonic carcinogenesis, dietary fat could potentially circumvent the need for this receptor. In the current report, we show that this growth factor receptor is required for promotion of azoxymethane/DSS-induced colonic tumors by a western-style diet. In *Egfr*^{wt} mice, a western-style high-fat diet significantly increased weight gain and visceral fat as well as blood glucose and insulin levels. These metabolic derangements were accompanied by increased colonic tumor burden and tumor progression compared with a standard fat diet. In contrast, increased dietary fat did not enhance weight gain or tumor promotion in *Egfr*^{wa2} mice. When data from the dietary groups were aggregated to assess the contribution of *Egfr* genotype to tumorigenesis, we found that cancer incidence and multiplicity were significantly higher in *Egfr*^{wt} animals compared with *Egfr*^{wa2} mice. High dietary fat strongly promoted tumor development, increasing both tumor and cancer incidence in *Egfr*^{wt} but not *Egfr*^{wa2} animals.

Luminal factors, including secondary bile acids, have been implicated in diet-induced tumor promotion (14, 34). High-fat diets increase colonic excretion of secondary bile acids that can activate EGFR in colorectal cancer cells (14, 35). In prior studies, we showed that dietary supplementation with cholic acid, the predominant primary bile acid, enhanced tumorigenesis in the azoxymethane model (36). Systemic factors, such as circulating insulin and insulin-like growth factors, are also increased by high-fat diets and linked to an elevated risk of colon cancer (16). In this regard, blood sugars and serum insulin levels were higher in *Egfr*^{wt} compared with *Egfr*^{wa2} mice on the high-fat diet, indicating that EGFR contributes to hyperglycemia and insulin resistance in this model.

CTNNB1 is an integral part of the cytoskeleton as well as an important transcription factor in colonic tumorigenesis. CTNNB1 is upregulated and activated in most colon cancers and controls several key tumor-promoting genes including MYC, CCND1, and PTGS2 (17, 20, 37). Prior studies showed that EGFR is an upstream regulator of CTNNB1, inducing CTNNB1 deacetylation and nuclear localization in colon cancer cells (26). Other studies have shown that western-style diets also increased CTNNB1 in premalignant colonic mucosa (23). In the current study, we showed that both dietary fat and EGFR controlled CTNNB1 expression in tumors. Thus, EGFR signals and dietary fat control CTNNB1 expression in premalignant and malignant colonocytes.

The proto-oncogene MYC is regulated by CTNNB1 and EGFR (17, 26). MYC was required for adenoma formation in the *Apc* mutant Min mouse (38). In prior studies, we showed that MYC was increased in both azoxymethane and azoxymethane/DSS models of experimental colonic tumorigenesis (7, 39). In the current study, dietary fat and EGFR controlled MYC expression. MYC levels were highest in tumors from *Egfr*^{wt} animals on a high-fat diet, the group with the greatest tumor burden. In *Egfr*^{wt} mice, dietary fat appeared to differentially increase MYC in tumor epithelial cells compared with stromal cells. The cell context specificity of this diet-induced and EGFR-dependent effect will require further study.

The proto-oncogene CCND1 controls G_1-S cell cycle progression and is increased in human and experimental models of colon cancer (19, 31). We showed that CCND1 is controlled by EGFR under standard fat conditions in azoxymethane colonic tumorigenesis (6, 7). In experimental colon cancer, high dietary fat upregulated colonic mucosal CCND1 (23, 40). Whether this increase required EGFR signals, however, has not been addressed. In the current study, we showed that EGFR and dietary fat controlled CCND1 expression in the azoxymethane/DSS model. Dietary fat enhanced tumor CCND1 expression more in *Egfr*^{wt} than in *Egfr*^{wa2} mice. In mutant mice, although dietary fat increased CTNNB1, MYC, and CCND1, it failed to enhance tumorigenesis. In this regard, threshold levels for *Apc* (and presumably β-catenin) and CCND1 have been reported for adenoma formation in the *Apc* mutant Min mouse (41, 42). In addition to lower amplitudes of these proto-oncogenes, reduced tumorigenesis in *Egfr*^{wa2} mice might reflect insufficiency of other tumor-promoting signals, such as PTGS2.

The proto-oncogene PTGS2 is the rate-limiting enzyme for prostanoid biosynthesis. PTGS2 is upregulated in human and experimental models of colon cancer (21, 31). Pharmacologic or genetic inhibition of PTGS2 inhibited experimental tumorigenesis, showing its critical role in intestinal neoplasia (43, 44). Western-style dietary fat has been shown to increase PTGS2 in azoxymethane tumorigenesis (33). In prior azoxymethane rat studies, we showed that activated Ras controlled

PTGS2 expression (31). In the current study, we showed that dietary fat strongly enhanced PTGS2 expression in tumors from *Egfr*^{wt} but not *Egfr*^{wa2} mice. These results indicate that PTGS2 is tightly controlled by EGFR. PTGS2 was predominantly expressed in stromal cells in agreement with findings in *Apc* mutant Min mice and azoxymethane/DSS-treated *Egfr*^{wt} mice (45, 46). Prior studies have shown that activated K-Ras and CTNNB1 are both required to induce PTGS2 in colon cancer cells (20). EGFR is an upstream activator of K-Ras, which is known to stabilize PTGS2 mRNA (47). Thus, our studies suggest that the pathway involving EGFR, K-Ras and PTGS2 plays a key role in tumor promotion by western-style diet.

Because EGFR signals in colonic carcinogenesis are frequently driven by upregulated ligands for this receptor, we measured TGF-α and amphiregulin transcript abundance. In prior studies, we observed increases in these ligands in the azoxymethane model (6, 7). Our finding that transcript levels of TGF-α and amphiregulin in tumors were controlled by both dietary fat and *Egfr* genotype explains, in part, tumor promotion by dietary fat. It is intriguing that increased dietary lipids upregulated TGF-α expression even in normal colonic mucosa (without carcinogen induction). Although the mechanisms by which dietary fat enhances EGFR ligand expression will require further study, it is known that insulin-like growth factors and secondary bile acids that are increased by dietary fat can enhance EGFR ligand release (48, 49). Thus, the diet-related risk of colon cancer could derive, in part, by a generalized "field effect" reflected by increases in EGFR ligands that expand mutant colonic crypt stem cells.

The potential tumor-promoting roles of secondary bile acids and metabolic derangements induced by western-style diets are also incompletely understood. High-fat diets increase colonic secondary bile acids and also predispose to metabolic syndromes with increased insulin resistance and upregulated insulin-like growth factor-I that can transactivate EGFR (15, 16). *Egfr*^{wt} mice on a high-fat diet had elevated blood glucose and increased serum insulin levels consistent with a metabolic syndrome. Further studies will be required to determine whether EGFR enhances tumor promotion by dietary fat via systemic effects on metabolism in addition to local receptor signals in the colon. Selective deletion of *Egfr* from colonocytes using floxed *Egfr* mice could be used to dissect colonocyte versus systemic EGFR effects. Dietary interventions, moreover, with nutrient constituents that reduce EGFR and/or PTGS2 levels might provide novel chemopreventive strategies to inhibit the increased risk of colon cancer associated with obesity or diabetes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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