ADAM17 is a Tumor Promoter and Therapeutic Target in Western Diet-associated Colon Cancer

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Abstract

Purpose: Epidermal growth factor receptors (EGFR) are required for tumor promotion by Western diet. The metalloprotease, ADAM17 activates EGFR by releasing pro-EGFR ligands. ADAM17 is regulated by G-protein-coupled receptors, including CXCR4. Here we investigated CXCR4–ADAM17 crosstalk and examined the role of ADAM17 in tumorigenesis.

Experimental Design: We used CXCR4 inhibitor, AMD3100 and ADAM17 inhibitor, BMS566394 to assess CXCR4–ADAM17 crosstalk in colon cancer cells. We compared the expression of CXCR4 ligand, CXCL2, and ADAM17 in mice fed Western diet versus standard diet. Separately, mice were treated with marimastat, a broad-spectrum ADAM17 inhibitor, or AMD3100 to assess EGFR activation by ADAM17 and CXCR4. Using Apc-mutant Min mice, we investigated the effects of ADAM17/10 inhibitor INCB3619 on tumorigenesis. To assess the effects of colonocyte ADAM17, mice with ADAM17 conditional deletion were treated with azoxymethane (AOM). ADAM17 expression was also compared in colonocytes from primary human colon cancers and adjacent mucosa.

Results: CXCL12 treatment activated colon cancer cell EGFR signals, and CXCR4 or ADAM17 blockade reduced this activation. In vivo, Western diet increased CXCL12 in stromal cells and TGFβ in colonocytes. Marimastat or AMD3100 caused >50% reduction in EGFR signals (P < 0.05). In Min mice, INCB3619 reduced EGFR signals in adenomas and inhibited intestinal tumor multiplicity (P < 0.05). In the AOM model, colonocyte ADAM17 deletion reduced EGFR signals and colonic tumor development (P < 0.05). Finally, ADAM17 was upregulated >2.5-fold in human malignant colonocytes.

Conclusions: ADAM17 is a Western diet-inducible enzyme activated by CXCL12–CXCR4 signaling, suggesting the pathway: Western diet→CXCL12→CXCR4→ADAM17→TGFβ→EGFR. ADAM17 might serve as a druggable target in chemoprevention strategies.

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Introduction

Colon cancer is a major cause of cancer-related morbidity and mortality in the Western world (1). Most colon cancers are sporadic, and Western diets are implicated in their pathogenesis (2). Western diets are low in digestible fiber and rich in animal fats. Increasing epidemics of obesity and diabetes mellitus in the United States are linked to Western diet, and are also associated with increased colon cancer risk (3, 4). Disturbingly, the worldwide incidence of colon cancer is increasing as more countries adopt Western diets (1, 5). Thus, efforts to increase our understanding of the mechanisms by which Western diets promote colon cancer are important to develop more effective strategies to prevent this disease.

Experimental models of colon cancer are widely employed to dissect diet-related influences in colonic tumorigenesis. Both the genetic Apc-mutant Min mouse and the carcinogen-induced azoxymethane (AOM) model of colon cancer are widely used to investigate the roles of dietary factors in tumor development (6, 7). Western diet promotes tumorigenesis in the AOM and Min mouse model (8–10). In prior studies, using EGFR inhibitors, we showed that the signals from these receptors were required for efficient AOM-induced tumor development in rodents (11, 12). We also established that this receptor was required for Western diet–induced tumor promotion in the AOM and azoxymethane/dextran sulfate sodium (AOM/DSS) models (9, 10).

EGFR is a member of the transmembrane ErbB tyrosine kinase receptor family that controls growth and differentiation of the colonic epithelium (13). EGFR is expressed on colonic epithelial cells and stromal cells including fibroblasts and endothelial cells (14, 15). This receptor is activated by numerous ligands, including TGFβ and amphiregulin (AREG) that are produced in the colon by epithelial cells and stromal cells. Upon ligand binding, EGFR homodimerizes or heterodimerizes with other ErbB members. Dimerization stimulates the intrinsic receptor tyrosine kinase activity (13). In colon
**Translational Relevance**

Western diets are implicated in sporadic colon cancer. Epidermal growth factor receptors (EGFR) are increased in human colon cancer and required for tumor promotion by Western diet in models of colon cancer. EGFR is activated by receptor ligands released from membrane-bound pro-ligands. ADAM17 and ADAM10 are major enzymes that regulate EGFR ligand release. While ADAM17 is increased in sporadic colon cancer, no studies have directly tested whether this enzyme promotes tumor development. Moreover, as ADAM17 controls the release of many substrates, its role in colon cancer development is not predictable. In this study, using both genetic and chemical models of colon cancer, we demonstrate for the first time that ADAM17, and possibly ADAM10, are tumor-promoting. This establishes ADAM17 as a potential therapeutic target in colon cancer prevention. Furthermore, we uncovered a potentially important pathway linking ADAM17 to Western diet promoted colon cancer involving CXCL12–CXCR4 as upstream signals activating ADAM17.

**Materials and Methods**

For details, see Supplemental Materials and Methods.

**Experimental animals**

Archived colonic mucosal tissue for chronic diet studies. All animal studies were approved by the Animal Care and Use Committee at the University of Chicago and are fully compliant with the NIH guidelines for humane use of animals. Archived samples were available from prior studies taken from control mice fed standard diet (5% fat) or Western diet (20% fat) for 40 weeks (9). Samples included distal colonic segments fixed in 10% formalin or frozen in optimal cutting temperature (OCT) medium as well as RNA and proteins from scrape-isolated colonic mucosa.

**Acute Western diet studies**

Marimastat and AMD3100 experiments. We used A/J mice for acute dietary studies as we planned to study colon tumor development in the AOM model that required an AOM-susceptible mouse. Male A/J mice were implanted with Alzet pumps delivering 1.1 μmoles marimastat per day per mouse in 50% polyethylene glycol (PEG) or 50% PEG alone (vehicle control), or 0.126 μmoles AMD3100 per mouse per day or water (vehicle control). There were 10 mice in each group. Marimastat is a broad-spectrum metalloprotease inhibitor, and AMD3100 is a CXCR4 inhibitor (21, 26). Mice were allowed 48 hours to recover after pump implantation and then fed a Western diet (9). Mice were sacrificed after 2 weeks and left colonic mucosa scrape-isolated and flash frozen. Circular segments (3-mm length) were frozen in OCT or fixed in 10% buffered formalin and embedded in paraffin for immunostaining. For AMD3100 studies, mice were injected with bromodeoxyuridine (BrdUrd) 1 mL/100 g bodyweight 2 hours before sacrifice as recommended by the manufacturer.

**Apc-mutant Min mouse studies.** Apc-mutant Min mice on C57Bl6/J background, containing a truncating mutation in Apc codon 850, were obtained from The Jackson Laboratory (#002020). Mice were genotyped as recommended by The Jackson laboratory. At 6 weeks of age, Min mice (males and females) were randomized and fed Western diet (n = 20) or Western diet supplemented with INCB3619 (n = 20; 860 mg/kg chow). INCB3619 was added as a powder mixed with sucrose and provided 120 mg/kg bodyweight/day. Western diet composition was described previously (9). Mice were sacrificed at 7 months of age and tumors >2 mm in size were collected in 4-cm segments from duodenum, jejunum and ileum, and entire colon as described previously (27). Tumors were collected for proteins and RNA, and segments fixed in formalin for immunostaining. Control proteins and RNA were prepared by scrape isolation from tumor-free colonic and intestinal mucosa.

ADAM17 LoxP studies

Acute adeno-Cre ADAM17 LoxP studies. Mice with LoxP sequences flanking ADAM17 exon 2 were obtained from The Jackson Laboratory (stock number 009597) and genotyped as recommended. To confirm conditional ADAM17 deletion, we treated homozygous ADAM17loxP mice with intrarectal adeno-Cre or adeno EV
(control) as described previously (28). After 7 days, mice were sacrificed and distal colon segments frozen in OCT and stained for ADAM17.

Chronic villin-Cre ADAM17 LoxP AOM tumor studies. Homozygous ADAM17Loxp mice originally on a C57B6 background were interbred 10 generations to AOM-susceptible A/J background and mated to villin-Cre–expressing mice that were also interbred 10 generations to A/J background. Experimental villin-Cre – homozygous ADAM17 LoxP (ADAM17ΔΔ) mice and littermate controls homozygous ADAM17 LoxP mice lacking villin-Cre transgene were treated with AOM 10 mg/kg bodyweight weekly for 6 weeks. Mice were then fed a Western diet (9). Tumor development was monitored by colonoscopy, and mice were sacrificed 24 weeks after the first AOM injection (29). Colonies were excised, and tumors graded histologically by a gastrointestinal pathologist (J. Hart). AOM-induced tumors, and scrape-isolated control mucosa from left colon of genotype-matched ADAM17Loxp mice treated with saline (AOM vehicle), were fixed in 10% formalin or snap frozen in OCT or flash frozen for RNA and protein extraction.

Isolation of colonocytes and stromal cells from colonic mucosa

We isolated colonocytes and stromal cells following previously published methods with minor modifications (30, 31). Briefly, colons were removed and mucosa scrape-isolated and minced with razor blade into 2-mm pieces. Fragments were collected in tubes containing 6-ml sterile ice-cold transport media, 50 IU/mL penicillin, 50 µg/mL streptomycin, and 0.5 mg/mL gentamycin. Tissue was washed three times by gentle inversion and collected by gravity sedimentation and resuspended in 10-ml chelating buffer (transport media plus 1 mmol/L EDTA and 1 mmol/L EGTA). Tissue was incubated on a shaker overnight at 4°C to release colonocytes into the supernatant. The pellet was washed three times with 3-ml ice-cold PBS, releasing residual colonocytes, and colonocyte-containing supernatants were combined. Epithelial cell fractions (supernatants) and stromal cell fractions (pellets) were centrifuged at 400 x g for 5 minutes at 4°C, yielding loosely packed pellets from which proteins were solubilized in 2 x Laemmli buffer.

Cell culture and proliferation

HCT116 and HT29 human colon cancer cells were obtained within 6 months from ATCC and authenticated by ATCC using short tandem repeat DNA fingerprinting. Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂, 95% air under conditions recommended by ATCC. For EGFR transactivation experiments, preconfluent cells were pretreated for 2 hours with 20 µg/mL C225 antibodies, 5 µmol/L BMS566394, 10 ng/mL AMD3100, or PBS (vehicle) followed by 50 ng/mL CXCL12 or vehicle. Positive controls included cells treated with 10 ng/mL EGF. At indicated times, cells were broken and lysates probed for proteins by Western blotting.

Real-time PCR

RNA was extracted from snap-frozen tissue using RNeasy Lipid Tissue Mini Kit. Samples were homogenized in a bullet blender and loaded onto an RNA-binding spin column, washed, and digested with DNase I and eluted in 30-µl elution buffer. Samples were examined by Agilent chip for RNA purity and quantified by Ribogreen. RNA (100 ng) was reverse transcribed into cDNA using a high capacity reverse transcription kit in 20-µl total volume. Incubation conditions were 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes. The resulting first-strand complementary DNA (cDNA) was used as a template for quantitative PCR in triplicate using Fast SYBR Green Master Mix Kit. See Supplementary Methods for further details.

IHC

Five-micron sections of formalin-fixed paraffin-embedded tissue were cut and mounted on Vectabond-coated Superfrost Plus slides. Slides were heated to 60°C for 1 hour, deparaffinized by 5-minute washes three times in xylene, hydrated in a graded series of ethanol washes, and rinsed with distilled water. Epitope retrievals were achieved by microwave heating for 15 minutes in 10 mmol/L Tris-EDTA buffer, pH 9. For Ki67 staining, sections were incubated in Ki67 antibodies (clone SP6) overnight at 4°C at 1:500 final dilution (cat. # RM-9106-S0, Fisher Scientific). Ki67 and BrdUrd were quantified using the Fiji image software (NIH, Bethesda, MD) with ImmunoRatio plug-in (32). Alcian blue staining was performed on 5-µm paraffin sections as recommended by the manufacturer. After deparaffinization and rehydration, sections were incubated with Alcian Blue solution for 30 minutes, rinsed in water, and counterstained with Nuclear Fast Red. For CXCL12 staining, circular colonic segments (10 µm) in OCT were fixed with cold acetone (−20°C) for 15 minutes and air dried at room temperature. Sections were blocked with Super Block (AAA500, ScyTek Laboratories) for 30 minutes at room temperature followed by overnight incubation with Abcam primary anti-CXCL12 antibodies (1:35) at 4°C. See Supplementary Methods for further details.

In situ staining for TGFβ transcripts

Circular left colon segments (12-µm) frozen in OCT were prepared with a cryostat, placed on Superfrost Plus slides, fixed in 4% paraformaldehyde in PBS for 20 minutes, and washed twice with DEPC-PBS for 5 minutes. In situ hybridization and detection of TGFβ transcripts was performed using Etxion miCURY LNA anti-sense oligonucleotide probes labeled 5’- and 3’- with digoxygenin (see Supplementary Methods for further details).

Western blotting

Proteins were extracted in SDS-containing Laemmli buffer, quantified by RC-DC protein assay, and subjected to Western blotting as described previously (9). Protein expression levels were expressed as fold of indicated protein in scrape-isolated tumor-free intestinal mucosa (Apc+/Min mouse), or colonic mucosa from saline-treated genotype-matched ADAM17Loxp mice (mean ± SD). Separate aliquots were probed for β-actin.

Statistical analysis

Western blotting densitometry data were normalized as fold of vehicle-treated (cells) or normal mucosa (mice) and expressed as means ± SD. Differences among multiple groups were assessed with ANOVA, and differences between specific groups were then compared using unpaired Student’s t test. Repeated transciptase reactions were run in duplicate and assayed in triplicate, and Ct values were averaged. Untransformed Ct values were compared between groups (33). Relative abundance, expressed as 2−ΔΔCt, was calculated by exponentiating the estimated differences in Ct and significance between groups calculated by Student’s t test. Tumor incidence was defined as the percent of mice with at least one tumor and compared using Fisher exact test. Tumor
multiplicity was defined as the average number of tumors per tumor-bearing mouse and compared using Mann–Whitney U test. For all statistical analyses, $P$ values $\leq 0.05$ were considered statistically significant.

**Results**

Western diet upregulates CXCL12 and EGFR signaling components

We previously investigated the effects of Western diet on colon cancer development (9). For the current study, we used archived samples from the control arm of the prior study to dissect effects of diet alone on EGFR signals. We examined transcript and protein expression levels of ADAM17, pro-TGFα, and K-Ras in colonic mucosa from control mice. In addition, we assessed the effects of diet on CXCL12, a cytokine implicated in colon cancer, which can activate EGFR in other cancers. The protocol is shown in Fig. 1A. Western diet significantly increased transcripts (Fig. 1B) and protein levels of ADAM17, pro-TGFα, and K-Ras, as well as CXCL12 in the distal colon mucosa (Fig. 1C and D). In situ hybridization of TGFα mRNA showed that transcripts were predominantly expressed in colonic epithelial cells (Fig. 1E and F), whereas CXCL12 was most abundant in stromal cells (Fig. 1G and H). Western diets increased both TGFα (Fig. 1B–F) and CXCL12 levels (Fig. 1B–D, G, and H).

ADAM17 mediates EGF transactivation induced by CXCL12 in colon cancer cells

As colonic mucosal CXCL12 was increased by Western diet (Fig. 1) and shown to transactivate EGFR in other cancer cells (34), we asked whether CXCL12 could activate EGFR in colon cancer cells. We pretreated HT29 colon cancer cells with vehicle or EGFR-neutralizing C225 antibodies that block receptor signals by inhibiting ligand binding. Cells were then treated with CXCL12 or vehicle. As shown in Fig. 2A, CXCL12 caused time-dependent increases in phospho-active EGFR (pEGFR), pErbB2, a heterodimeric partner of EGFR, and CXCL12 increased ERK and AKT activation. EGFR transactivation by CXCL12 required EGFR ligand binding as assessed by suppression with anti-EGFR–specific C225 antibodies. These results are quantified in Fig. 2B. To assess whether EGFR transactivation by CXCL12 required ADAM17, we pretreated cells with BMS566394, a specific ADAM17 inhibitor (35). As shown in Fig. 2C, BMS566394 suppressed EGFR signals induced by CXCL12 as assessed by reductions in Western diet alone group ($\text{mean} \pm \text{SD}$, 20 Western diet alone, and 20 AMD3100–treated group) compared with 50% in Western diet alone group ($\text{mean} \pm \text{SD}$, 20 Western diet alone, and 20 AMD3100–treated group). As shown in Fig. 2D, INCB3619 significantly reduced activation levels of several of these proteins in adenomas and adjacent normal-appearing intestinal mucosa (Fig. 5A and B). EGFR effectors c-Myc, cyclin D1 and Cox-2 and Notch1 effector Hes1 were also increased in tumors, and INCB3619 significantly reduced these proteins in Min adenomas (Fig. 5C and D). To determine the biological consequences

**Marinamastat.** As ADAM17 and several EGFR components were increased in colonic mucosa by Western diet, it was of interest to assess the role of ADAM17 in these diet effects. As specific ADAM17 inhibitors were not commercially available, we chose marinamastat, a broad-spectrum metalloprotease inhibitor that blocks ADAM17 (26), to assess a metalloprotease requirement for EGFR signals in mice fed a Western diet. As shown in Supplementary Fig. S3, marinamastat significantly inhibited EGFR signals in colonic mucosa, with greater than 50% reductions in phospho-active EGFR (pEGFR), pErbB2, and pERK compared with Western diet alone. Thus, EGFR signaling in the colonic mucosa in Western diet–fed mice requires metalloprotease activity. These results implicate ADAM17 and possibly ADAM10 as major ADAMs responsible for regulating EGFR ligand release (22).

**AMD3100.** As CXCL12 was increased by Western diet (Fig. 1B–D, G, and H) and can transactivate EGFR in colon cancer cells (Fig. 2), we examined the effects of blocking CXCL12 signals in Western diet–fed mice using AMD3100, an inhibitor of G-protein–coupled receptor CXCR4 (Fig. 3A). As shown in Fig. 3B and C, AMD3100 significantly reduced phospho-active EGFR and downstream effectors, indicating that EGFR signals are positively modulated by CXCR4 signals under Western diet conditions. To assess the biological consequences of CXCR4 blockade, we measured BrdUrd incorporation in the colonic mucosa. As shown in Fig. 3D and E, AMD3100 significantly reduced BrdUrd incorporation in colonic crypts from 12.8% ± 6.7% to 6.3% ± 2.9% (Fig. 3F, $n = 4$; $P < 0.05$).

ADAM17/10 inhibitor INCB3619 suppresses ADAM17 activity and reduces tumor development in ApC-mutant Min mice

We next employed INCB3619, an inhibitor with greater specificity, to block EGFR ligand release. INCB3619 is orally bioavailable and highly specific for ADAM17 and ADAM10, which regulate release of EGFR ligands (22, 23). In initial experiments, we confirmed that INCB3619 blocked recombinant ADAM17 activity in vitro (Supplementary Fig. S4A). INCB3619 supplementation in vivo (120 mg/kg bodyweight/day) also inhibited intestinal mucosal ADAM17 activity, increasing $V_{\text{max}}$ by 50% (Supplementary Fig. S4B). To dissect the role of ADAM17/10 on tumor growth, we next examined the effects of INCB3619 on intestinal tumorigenesis in the ApC-mutant Min mouse. Mice on Western diet supplemented with INCB3619 grew normally, with growth curve closely paralleling mice fed a Western diet alone (Fig. 4A). As shown in Fig. 4B, INCB3619 did not alter tumor incidence in the small intestine, but it appeared to reduce tumors in the colon, with 25% colonic tumor incidence in the Western diet plus INCB3619–treated group, compared with 50% in Western diet alone group ($P = 0.07$). In the small intestine, INCB3619 significantly reduced median tumor multiplicity from 67.0 ± 11.0 in the Western diet group to 38.5 ± 5.8 in the group given INCB3619 (Fig. 4C, $n = 20$ Western diet alone, and $n = 20$ Western diet +INCB3619; $P < 0.01$). As shown in Fig. 4D, INCB3619 also decreased small intestinal tumor size from 3.3 ± 0.6 mm to 2.4 ± 0.2 mm ($P < 0.05$).

In agreement with prior studies (25), EGFR signals were upregulated in Min tumors as shown in Fig. 5A and quantified in Fig. 5B. Compared with levels in mice on Western diet alone, INCB3619 significantly reduced activation levels of several of these proteins in adenomas and adjacent normal-appearing intestinal mucosa (Fig. 5A and B). EGFR effectors c-Myc, cyclin D1 and Cox-2 and Notch1 effector Hes1 were also increased in tumors, and INCB3619 significantly reduced these proteins in Min adenomas (Fig. 5C and D). To determine the biological consequences
of these signaling changes, we assessed the effects of INCB3619 on cell proliferation in Min adenomas. As shown in Fig. 5E and quantified in Fig. 5F, INCB3619 significantly reduced Ki67 staining in adenomas from $58.1 \pm 10.3$ to $13.3 \pm 3.0$ ($^\ast, P < 0.001; n = 10$ adenomas from mice on Western diet and $n = 11$ adenomas in mice on Western diet plus INCB3619). Proliferation in normal-
appearing small intestinal mucosa was also significantly reduced by INCB3619 (Supplementary Fig. S5). As Notch signals in the intestine are controlled by ADAM10 in wild-type mice (36), we examined the effect of INCB3619 on goblet cells in Min mouse small intestine, as a read out of Notch activity. As shown in Supplementary Fig. S6, goblet cells were increased by INCB3619 consistent with blockade of Notch signals. Notch signals have been shown to contribute to intestinal tumorigenesis in the Min mouse (37). Pro-TNFα was also increased in small intestine by INCB3619, consistent with the predicted effect of inhibited cleavage of pro-TNFα (Supplementary Fig. S7).

Although Min mice have substantially fewer colonic tumors compared with small intestinal tumors, we also examined the effects of INCB3619 on colonic crypt cell proliferation and tumors. INCB3619 reduced colonic crypt cell proliferation to 16.7% of control, compared with 24.1% of Western diet alone as assessed by Ki67 staining (n = 10 mice per group, P < 0.005). There were 25 colonic tumors in 10 mice in the Western diet alone group (N = 20 mice) and 9 colonic tumors in 5 mice in the INCB3619 supplemented group (N = 20 mice). Tumor incidence was, therefore, 50% in unsupplemented group and 25% in the INCB3619 supplemented group (P = 0.07, Fisher test).
exact test). Tumor multiplicities were 2.5 and 1.8 tumors per tumor-bearing mouse, respectively ($P = 0.23$, Mann–Whitney $U$ test). The average colonic tumor size was 2.9 mm in the Western diet group and 2.2 mm in the INCB3619-supplemented group ($P < 0.05$).

**Colonocyte ADAM17 deletion suppresses AOM-induced colonic tumorigenesis**

The INCB3619 studies established that concomitant inhibition of ADAM17 and ADAM10 reduced Min tumorigenesis. To directly test the role of ADAM17 in tumor development and isolate epithelial cell–specific contribution, we deleted ADAM17 from colonocytes by interbreeding mice with an ADAM17 exon 2 flanked by LoxP sequences to transgenic mice expressing Cre-recombinase under a villin promoter (38, 39). Both the villin-Cre transgenic mice and the ADAM17 flanked mice were first intercrossed 10 generations with A/J mice to achieve an AOM-susceptible A/J background (C57Bl6 mice are relatively resistant to AOM). We examined the ability of villin-Cre to delete ADAM17 from colonocytes. Colonies from control (no AOM treatment) homozygous ADAM17LoxP mice and villin-Cre-homozygous ADAM17LoxP mice (ADAM17ΔΔ) were harvested, and colonocytes and stromal cells prepared as described in the Materials and Methods. ADAM17 was detected by Western blotting in fractions from the indicated mice in Fig. 6A. Stromal cells were detected by vimentin (VIM) expression and colonocytes by cytokeratin 20 (CK20) expression. Note that ADAM17 was readily detected in stromal cells of both genotypes. In contrast, ADAM17 was absent in colonocytes from mice expressing villin-Cre. These results confirmed that ADAM17 was deleted from crypt epithelial cells by villin-Cre recombinase.

To assess the role of colonocyte ADAM17 in colonic tumor development, we treated villin-Cre-homozygous ADAM17LoxP mice (ADAM17ΔΔ) and control homozygous ADAM17LoxP mice (no Cre) with AOM or saline (AOM vehicle) weekly for 6 weeks. Animals were sacrificed after 28 weeks when screening colonoscopy revealed the presence of tumors. ADAM17 was abundant in all cell types in tumors from homozygous ADAM17LoxP mice as shown by ADAM17 immunostaining in Supplementary Fig. S8. In contrast, ADAM17 was detected only in stromal cells of tumors from villin-Cre-homozygous ADAM17LoxP mice (Supplementary Fig. S8). Deletion of ADAM17 from colonocytes significantly reduced the incidence of tumors (adenomas + cancers) as well as cancers alone (Fig. 6B). ADAM17 deletion from colonocytes also significantly suppressed tumor multiplicity ($P < 0.05$) as shown in Fig. 6C, from a median of 5 tumors/mouse in homozygous...
INCB3619 suppresses EGFR signals and proliferation in adenomas from Apc-mutant Min mice. Mice were treated as described in the Materials and Methods. At 7 months of age, mice were sacrificed, and intestinal adenomas harvested. Diet-matched adjacent normal-appearing intestinal mucosa was scrape-isolated and proteins solubilized in Laemmli buffer. A, Western blots of indicated EGFR signals. B, Densitometry. Values represent 6–8 mice per group [*, *P < 0.05; **, *P < 0.005, phospho-protein levels in adenomas from mice on Western diet alone (tumor), compared with adjacent normal-appearing mucosa (NI); †, †P < 0.05, phospho-protein levels in normal-appearing mucosa in group receiving INCB3619 (NI + INCB), compared with levels in intestinal mucosa from mice on Western diet alone (NI); ‡, ‡P < 0.05, ‡‡P < 0.005, phospho-protein levels in adenomas from mice on Western diet alone (tumor)]; C, Western blots of indicated EGFR and Notch1 effectors. D, Densitometry [*, *P < 0.05; **, *P < 0.005, compared with levels in normal-appearing intestinal mucosa; †, †P < 0.05, compared with levels in adenomas from mice on Western diet alone; n = 6–8 mice per group]. E, Ki67 immunostaining in representative adenoma from mouse on Western diet alone (left) and adenoma from mouse on Western diet + INCB3619 (right). F, Quantitation of Ki67 staining (*, P < 0.001 compared with Western diet alone, n = 10 adenomas from mice on Western diet alone, n = 11 adenomas from mice on Western diet + INCB3619. Each adenoma is representative of a different mouse).
Figure 6.
Colonocyte ADAM17 deletion suppresses EGFR signals and reduces colonic tumor development in AOM-treated mice. Villin-Cre and ADAM17 LoxP/– mice were interbred to an AOM-susceptible A/J background. Villin-Cre-homozygous ADAM17LoxP (ADAM17ΔΔ) and homozygous ADAM17LoxP mice were then treated with AOM or vehicle (saline). Two weeks after the sixth AOM injection, mice were begun on a Western diet (WD, 20%) as described in the Materials and Methods. Twenty-eight weeks after the first AOM treatment, mice were sacrificed and tumors harvested. A, Cell-specific ADAM17 expression. Colonocytes and stromal cells were isolated from scraped mucosa from left colon of villin-Cre–/–, homozygous ADAM17LoxP (villin-Cre–/) and homozygous ADAM17LoxP mice (villin-Cre–/) as described in the Materials and Methods. (Mice in A received no AOM treatment). The indicated cell fractions were probed by Western blotting for ADAM17, vimentin (VIM, stromal cell marker) and cytokeratin20 (CK20, colonocyte marker). Note that in villin-Cre–/–, homozygous ADAM17LoxP mice (ADAM17ΔΔ), ADAM17 was deleted from colonocytes, but not from stromal cells. B, Colon tumor incidence. Tumors were classified histologically as adenomas or carcinomas (†, P < 0.05 compared with control homozygous ADAM17LoxP mice; n = 32 homozygous ADAM17LoxP mice and n = 29 ADAM17ΔΔ mice). C, Colon tumor multiplicity. The median number of tumors was significantly lower in ADAM17ΔΔ group compared with the homozygous ADAM17LoxP group (2.0 vs. 5.0 tumors per mouse-bearing mouse, respectively; †, P < 0.05, Mann–Whitney U test). D, Ki67 staining (*, P < 0.05, n = 14 tumors in each genotype). E, Tumor size (†, P < 0.05; n = 35 tumors from homzygous ADAM17LoxP mice and n = 12 tumors from ADAM17ΔΔ mice). F, EGFR and Notch1 signals in AOM tumors (T) and genotype-matched scrape-isolated left colon mucosa from vehicle-treated mice (N). Blots are representative of 6 tumors per group. G, Quantitative densitometry (*, P < 0.05 compared with homozygous ADAM17LoxP mucosa; †, P < 0.05 compared with adenomas in the homozygous ADAM17LoxP group).

Discussion
In the current study, we demonstrated that the CXCL12–CXCR4 axis activated ADAM17–EGFR signals in vitro in colon cancer cells and in vivo in colonic mucosa of mice fed a Western diet. Furthermore, we showed that pharmacologic inhibition of ADAM17 and ADAM10 and genetic deletion of colonocyte ADAM17 suppress Western diet–promoted tumor development, establishing an oncogenic role for ADAM17, and possibly ADAM10, in Western diet–promoted colon cancer. Other preclinical studies showed that ADAM17 haploinsufficiency alters energy metabolism and protects mice against obesity and diabetes, suggesting a further benefit for targeting this...
protease (40, 41). ADAM17 and ADAM10 regulate cleavage of many substrates, including several pro-EGFR ligands (22). While the roles of other ADAM17 and ADAM10 substrates remain uncertain with respect to tumor development, growing evidence indicates that EGFR ligands contribute importantly to colon cancer development.

In the current study, we also demonstrated that a Western diet increased ADAM17, colonocyte TGF\(\alpha\), and stromal cell CXCL12 in the colonic mucosa (Fig. 1). Furthermore, CXCL12 activated EGFR signals in HT29 cells and HCT116 cells, prototypes of chromosomal- and microsatellite-unstable colon cancer cells, respectively (Fig. 2 and Supplementary Fig. S1). EGFR transactivation required ligand binding to EGF receptors and was mediated by ADAM17. As expected, AMD3100 suppressed EGFR activation by CXCL12 in colon cancer cells (Fig. 2). ADAM10 has also been implicated in EGFR transactivation by CXCR4 in other cancers (42), but we have not investigated this pathway in colon cancer cells. Mechanisms by which CXCR4 activates ADAM17 and possibly ADAM10 remain largely unknown (43).

Broad-spectrum metalloprotease inhibitor marimastat and CXCR4 inhibitor AMD3100 reduced colonic EGFR signals (Fig. 3 and Supplementary Fig. S2). These studies uncover a potentially important role for ADAM17 and CXCL12–CXCR4 signals in Western diet–promoted EGFR activation in the colon. We did not investigate potential antitumor effects of AMD3100 in cancer models, but our studies showing reductions in EGFR transactivation and colonic proliferation suggest that AMD3100 might exert antitumor effects in these models. AMD3100 was also shown to inhibit experimental colitis (44). A clinical trial is currently in progress that will test AMD3100 antitumor efficacy in advanced human cancers (Clinical Trials NCT02179970).

As ADAM17 and ADAM10 control release of EGFR ligands (22), it was of interest to examine the antitumor effects of INCB3619, an inhibitor that blocks both these metalloproteases. In prior studies, INCB3619 demonstrated antigrowth effects in several tumor xenograft models (45). We confirmed that INCB3619 inhibited recombinant ADAM17 in vitro and intestinal mucosal ADAM17 in vivo (Supplementary Fig. S4). INCB3619 also reduced intestinal tumor burden in Western diet–fed Min mice (Fig. 4) and suppressed EGFR downstream signals in tumors and adjacent normal-appearing intestinal mucosa (Fig. 5). Interestingly, INCB3619 reduced pEGFR levels in normal appearing mucosa, but not in tumors, suggesting that the inhibitor may suppress tumor initiation more than tumor progression. Supporting this hypothesis regarding an effect on tumor initiation, INCB3619 also suppressed intestinal and colonic epithelial cell proliferation.

In the clinic, broad-spectrum metalloprotease inhibitors, such as marimastat, were poorly tolerated because of debilitating periarthritis and tendonitis (26). In contrast, INCB3619 was well tolerated (45). A newer generation ADAM17/ADAM10 inhibitor INCB7839 was also well tolerated in a phase I trial (46). In another clinical trial, administration of this analogue, along with trastuzumab to patients with advanced HER2-positive breast cancers, was well tolerated and induced responses in 50% of the evaluable patients (47). Ongoing development of more selective agents to inhibit ADAM17 and possibly ADAM10 suggest, therefore, that this may be a useful strategy in individuals at increased risk for colon cancer.

ADAM17 has more than 40 identified substrates, including Notch1 and Notch ligands and TNF\(\alpha\). Blocking TNF\(\alpha\) and Notch signaling may contribute to the antitumor effects of INCB3619. Pro-TNF\(\alpha\) was more abundant in Min adenomas from mice supplemented with INCB3619, consistent with reduced pro-TNF\(\alpha\) cleavage by ADAM17 (Supplementary Fig. S7). INCB3619 also suppressed Notch1 signaling as assessed by decreased Hes1 (Fig. 5C and D). ADAM10 is the major regulator of Notch signaling in intestinal stem cells under non-stress conditions (36). In preliminary studies, we found that Alacian blue staining was increased by INCB3619 in Apc\(-\)/Min mouse small intestine, consistent with ADAM10 blockade (Supplementary Fig. S6). In primary human colon cancers and colon cancer cells, however, ADAM17 has also been shown to regulate Notch signaling by paracrine and autocrine mechanisms involving release of Notch ligands (48, 49).

Mice with colonocyte ADAM17 deletion have a normal phenotype in the absence of stress, but develop more severe DSS-induced colitis (50, 51). To dissect the role of colonocyte ADAM17 in colon cancer development, we showed that ADAM17 deletion in colon epithelial cells significantly reduced tumor development (Fig. 6). These studies established an important oncogenic role for colonocyte ADAM17 in a Western diet–promoted model of colon cancer. Despite colonocyte ADAM17 deletion, however, tumors did emerge, albeit with lower efficiency. This might reflect persistence of ADAM17 in stromal cells, releasing stromal-derived EGFR ligands or other ADAM17 substrates that can activate colonocytes by a paracrine mechanism.

In this regard, as in the case of INCB3619 studies, deletion of colonocyte ADAM17 did not significantly reduce pEGFR in colon tumors, but did decrease p\(\alpha\)B2, p\(\alpha\)K, and Hes1, suggesting contributions from other ADAM17-sensitive pathways such as Notch1. It is possible that INCB3619 inhibited aberrant Notch signaling by blocking ADAM10, whereas colonocyte ADAM17 deletion inhibited release of Notch ligands such as Jagged1 or Jagged2. In this regard, in addition to EGFR-dependent growth-inhibitory effects in colon cancer cells, studies with potent anti-ADAM17 antibodies have uncovered EGFR-independent growth-inhibitory effects consistent with contributions from TNF\(\alpha\) and Notch1 (52). Our human studies emphasize the potential oncogenic importance of increased ADAM17 signaling in malignant colonocytes as this enzyme was upregulated in transformed human colonocytes (Supplementary Fig. S9) in agreement with prior studies in intact tumors (17).

In the current study, we focused on Western diet effects on tumor development as our prior studies showed that EGFR was required for tumor promotion under Western diet, but not standard diet conditions (9). Our initial observations, however, were carried out in hybrid C57BL6 × A/J mice that are relatively resistant to AOM. It is possible that colonocyte ADAM17 deletion in AOM-susceptible A/J mice might also suppress tumor development under standard diet conditions. This could suggest an even wider role for ADAM17 blockade in colon cancer prevention.

In conclusion, we have identified a CXCL12–CXCR4 circuit that activates ADAM17, linking Western diet to colonic EGFR signals, and we have shown that ADAM17 plays an important role in tumor development in the AOM and Min mouse models of colon cancer. A schema summarizing these findings is shown in Supplementary Fig. S2. These studies support further research targeting colonic ADAM17 in individuals at increased risk for colon cancer development.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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