Published in final edited form as:

Clin Cancer Res. 2017 January 15; 23(2): 549–561. doi:10.1158/1078-0432.CCR-15-3140.

ADAM17 is a tumor promoter and therapeutic target in Western diet-associated colon cancer

Reba Mustafi^{#1}, Urszula Dougherty^{#1}, Devkumar Mustafi⁴, Fatma Ayaloglu-Butun¹, Michelle Fletcher¹, Sarbani Adhikari¹, Farhana Sadiq¹, Katherine Meckel¹, Haider I Haider¹, Abdurahman Khalil¹, Joel Pekow¹, Vani Konda¹, Loren Joseph², John Hart², Alessandro Fichera³, Yan Chun Li¹, and Marc Bissonnette^{1,‡}

¹Department of Medicine, University of Chicago, Chicago IL 60637

²Department of Pathology, University of Chicago, Chicago IL 60637

³Department of Surgery, University of Chicago, Chicago IL 60637

⁴Department of Radiology, University of Chicago, Chicago IL 60637

[#] These authors contributed equally to this work.

Abstract

Purpose—Epidermal growth factor receptors (EGFR) are required for tumor promotion by Western diet (WD). 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 expression of CXCR4 ligand, CXCL2, and ADAM17 in mice fed WD 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 effects of ADAM17/10 inhibitor INCB3619 on tumorigenesis. To assess 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, WD increased CXCL12 in stromal cells and TGF α 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

[‡]To whom correspondence should be addressed: Marc Bissonnette, M.D., The University of Chicago, Department of Medicine, KCBBD Building; Rm 9112, 900 East 57th Street, Chicago, IL 60637, Phone: (773) 795-0833, Fax: (773) 702-2281, mbbisonn@medicine.bsd.uchicago.edu.

Author contributions: MB designed the research; RM, UD, MF, FA-B, SA, HHH, AK and FS performed the experiments; DM, KM, LJ, JH and AF provided research reagents and technical assistance; VJK, JP, LJ, AF and YCL assisted in data analysis and manuscript revision; MB wrote the manuscript and was responsible for overall research design, data analyses and manuscript preparation.

The authors disclose that they have no potential conflict of interest

signals and colonic tumor development ($p<0.05$). Finally, ADAM17 was up-regulated >2.5-fold in human malignant colonocytes.

Conclusions—ADAM17 is a WD-inducible enzyme activated by CXCL12-CXCR4 signaling, suggesting the pathway: **Western diet->CXCL12->CXCR4->ADAM17->TGF α ->EGFR**. ADAM17 might serve as a druggable target in chemoprevention strategies.

Keywords

ADAM17; EGFR; colon cancer; Western diet; CXCL12

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 (WD) are implicated in their pathogenesis (2). WD are low in digestible fiber and rich in animal fats. Increasing epidemics of obesity and diabetes mellitus in the United States are linked to WD, and are also associated with increased colon cancer risk (3, 4). Disturbingly, the worldwide incidence of colon cancer is increasing as more countries adopt WD (1, 5). Thus, efforts to increase our understanding of mechanisms by which WD 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). WD promotes tumorigenesis in the AOM and Min mouse model (8-10). In prior studies using epidermal growth factor receptor (EGFR) inhibitors, we showed that 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 WD-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 transforming growth factor alpha (TGF α) 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 cancer EGFR is most frequently activated by increases in ligand abundance (16). In this regard, we showed that WD alone increased colonic mucosal TGF α , whereas TGF α and amphiregulin were up-regulated in colonic tumors (9).

ADAM enzymes (members of a disintegrin and metalloproteinase family) regulate cleavage of EGFR ligands from membrane-bound pro-ligands. TGF α and AREG release are controlled by ADAM17, which was shown to be up-regulated in human colon cancers (17). Since a WD increased TGF α , it was of interest to examine the effects of WD on ADAM17

expression. In many cancers, ADAM17 is also a convergence point downstream of G-protein coupled receptors (GPCR) (18) that can increase ADAM17 activity. In this regard, GPCR CXCR4 and its ligand CXCL12 are implicated in the progression of colon cancer (19). Using pharmacological inhibitors we asked if WD might alter CXCL12 *in vivo* and if CXCL12-CXCR4 signals could regulate ADAM17-EGFR signals in colon cancer cells *in vitro*. Marimastat is a broad-spectrum metalloprotease inhibitor that blocks ADAM17 activity, and AMD3100 is a potent CXCR4 inhibitor (20, 21). Here, using marimastat and AMD3100, we investigated the requirements for ADAM17 activity and CXCL12-CXCR4 signals in WD-promoted colonic EGFR signals.

In fibroblasts, ADAM17 and ADAM10 regulate release of different EGFR ligands (22). The availability of a dual ADAM17 and ADAM10 inhibitor INCB3619 allowed us to examine the roles of these enzymes in intestinal tumorigenesis in the Min mouse (23). This model requires EGFR signals for robust tumorigenesis and responds to WD with increased tumor burden (8, 24, 25). To isolate ADAM17 effects and dissect the cell-specific role of ADAM17 in colon cancer development, we examined the effect of deleting ADAM17 from colonic epithelial cells in the AOM model. Thus, in the current study we examined the role of ADAM17 in two different WD-promoted models of colon cancer. Using an ADAM17-specific inhibitor BMS566394, we also determined that CXCL12-CXCR4 signals activate EGFR via ADAM17 in colon cancer cells. Taken together, results of this study expand our understanding of CXCL12-CXCR4 circuit activating ADAM17 as a potential link between EGFR and WD in colon cancer.

MATERIALS AND METHODS

See Supplemental Materials and Methods for additional information

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 Std diet (5% fat) or WD (20% fat) for 40 wks (9). Samples included distal colonic segments fixed in 10% formalin or frozen in OCT, as well as RNA and proteins from scrape-isolated colonic mucosa

Acute WD 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 μ moles marimastat per day per mouse in 50% PEG or 50% PEG alone (vehicle control), or 6.3 μ moles 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 hrs to recover following pump implantation and then fed a WD (9). Mice were sacrificed after 2 wks and left colon 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 BrdU 1ml/100g body wt 2 hrs before sacrifice as recommended by 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 Jackson Laboratories (#002020). Mice were genotyped as recommended by Jackson laboratories. At 6 wks age Min mice (males and females) were randomized and fed WD (n=20) or WD supplemented with INCB3619 (n=20; 860 mg/kg chow). INCB3619 was added as a powder mixed with sucrose and provided 120 mg/kg body wt/day. WD composition was previously described (9). Mice were sacrificed at 7 mo age and tumors >2 mm in size collected in 4 cm segments from duodenum, jejunum and ileum and entire colon as described (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 Jackson laboratories (Stock Number 009597) and genotyped as recommended. To confirm conditional ADAM17 deletion, we treated homozygous ADAM17LoxP mice with intra-rectal adeno-Cre or adeno EV (control) as described (28). After 7d 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 C57Bl6 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 (ADAM17^{+/+}) mice and littermate controls homozygous ADAM17 LoxP mice lacking villin-Cre transgene were treated with azoxymethane (AOM) 10 mg/kg-body wt weekly × 6 wks. Mice were then fed a WD (9). Tumor development was monitored by colonoscopy, and mice were sacrificed 24 wks after the first AOM injection (29). Colons were excised, and tumors graded histologically by a GI pathologist (JH). 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 3 times by gentle inversion and collected by gravity sedimentation and re-suspended in 10 ml chelating buffer (transport media plus 1 mM EDTA and 1 mM 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 $400 \times g$ for 5 min at 4°C, yielding loosely packed pellets from which proteins were solubilized in 2x 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 pre-treated for 2 hrs with 20 µg/ml C225 antibodies, 5 µM 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, 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 min, 37°C for 120 min, and 85°C for 5 min. 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 Supplemental Methods for further details.

Immunohistochemistry—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 hr, deparaffinized by 5 min washes $\times 3$ 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 10 mM 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 BrdU were quantified using the Fiji image software (National Institutes of Health) 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 min, 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 min and air dried at room temperature (RT). Sections were blocked with Super Block (AAA500, ScyTek Laboratories) for 30 min at RT followed by overnight incubation with Abcam primary anti-CXCL12 antibodies (1:35) at 4°C. See Supplemental Methods for further details.

In situ staining for TGF α transcripts—Circular left colon segments (12 microns) frozen in OCT were prepared with a cryostat, placed on Superfrost Plus slides, fixed in 4% paraformaldehyde in PBS for 20 min and washed twice with DEPC-PBS for 5 min. *In situ*

hybridization and detection of TGF α transcripts was performed using Exiqon miCURY LNA anti-sense oligonucleotide probes labeled 5'- and 3'- with digoxigenin (see Supplemental 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 previously described (9). Protein expression levels were expressed as fold of indicated protein in scrape-isolated tumor-free intestinal mucosa (Apc+/Min mouse), or colon mucosa from saline-treated genotype-matched ADAM17LoxP mice (mean $\pm$ SD). Separate aliquots were probed for β -actin.

Statistical Methods—Western blotting densitometry data were normalized as fold of vehicle-treated (cells) or normal mucosa (mice) and expressed as means $\pm$ SD. Differences among multiple groups were assessed with ANOVA, and differences between specific groups were then compared using unpaired Student's t-test. Reverse transcriptase 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^{-Ct} , 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 ≤ 0.05 were considered statistically significant.

RESULTS

Western diet up-regulates 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-TGF α 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-TGF α and K-Ras, as well as CXCL12 in the distal colonic mucosa (Figs 1C-D). *In situ* hybridization of TGF α mRNA showed that transcripts were predominantly expressed in colonic epithelial cells (Figs 1E-F), whereas CXCL12 was most abundant in stromal cells (Figs 1G-H). WD increased both TGF α (Figs 1B-D; E-F) and CXCL12 levels (Fig 1B-D; G-H).

ADAM17 mediates EGFR transactivation induced by CXCL12 in colon cancer cells

Since colonic mucosal CXCL12 was increased by Western diet (Fig 1) and shown to transactivate EGFR in other cancer cells (34), we asked if CXCL12 could activate EGFR in colon cancer cells. We pre-treated 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. EGF receptor 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 BMS566934, a specific ADAM17 inhibitor (35). As shown in Fig 2C, BMS566934 suppressed EGFR signals induced by CXCL12 as assessed by reductions in phospho-EGFR, pErbB2, pAKT and pERK. These results are quantified in Fig 2D. pEGFR and pErbB2 were greatest at 1 min, while pAKT and pERK increases were greatest at 10 min, consistent with the latter being down-stream effectors of EGFR. We obtained similar results in HCT116 cells (Supplemental Fig S1). As expected, AMD3100, a CXCR4 inhibitor, also reduced EGFR activation by CXCL12 (as assessed by reductions in pErbB2, pERK and pAKT) in HT29 cells (Fig 2E-F). A signaling diagram showing colon cancer cell EGFR transactivation by CXCL12-CXCR4 is shown in Fig S2.

EGFR signals require metalloprotease activity and CXCR4 signals in WD fed mice

Marimastat—Since ADAM17 and several EGFR components were increased in colonic mucosa by WD, it was of interest to assess the role of ADAM17 in these diet effects. As specific ADAM17 inhibitors were not commercially available, we chose marimastat, 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 Supplemental Fig S3, marimastat significantly inhibited EGFR signals in colonic mucosa, with greater than 50% reductions in phospho-active EGFR (pEGFR), pErbB2 and pERK compared to WD alone. Thus, EGFR signaling in the colonic mucosa in WD fed mice requires metalloprotease activity. These results implicate ADAM17 and possibly ADAM10 as they are major ADAMs responsible for regulating EGFR ligand release (22).

AMD3100—Since CXCL12 was increased by WD (Fig 1B-D, G,H) and can transactivate EGFR in colon cancer cells (Fig 2), we examined the effects of blocking CXCL12 signals in WD-fed mice using AMD3100, an inhibitor of G-protein coupled receptor CXCR4 (Fig 3A). As shown in Fig 3B-C, AMD3100 significantly reduced phospho-active EGFR and down-stream 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 BrdU incorporation in the colonic mucosa. As shown in Fig 3D-E, AMD3100 significantly reduced BrdU incorporation in colonic crypts from $12.8 \pm 6.7\%$ to $6.3 \pm 2.9\%$, (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* (Supplemental Fig S4A). INCB3619 supplementation *in vivo* (120 mg/kg body wt/day) also inhibited intestinal mucosal ADAM17 activity, decreasing $V_{\max}$ by 50% (Supplemental 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 WD supplemented with INCB3619 grew normally, with growth curve closely paralleling mice fed a WD 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 WD plus INCB3619-treated group, compared to 50% in WD 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, $n=20$ WD alone, and $n=20$ WD+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 up-regulated in Min tumors as shown in Fig 5A and quantified in Fig 5B. Compared to levels in mice on WD alone, INCB3619 significantly reduced activation levels of several of these proteins in adenomas and adjacent normal-appearing intestinal mucosa (Fig 5A-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-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 ± 10.3 to 13.3 ± 3.0 ($*p<0.001$; $n=10$ adenomas from mice on WD and $n=11$ adenomas in mice on WD plus INCB3619). Proliferation in normal-appearing small intestinal mucosa was also significantly reduced by INCB3619 (Supplemental Fig S5). Since 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 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-TNF α was also increased in small intestine by INCB3619, consistent with the predicted effect of inhibited cleavage of pro-TNF α (Supplemental Fig S7).

Although Min mice have substantially fewer colonic tumors compared to small intestinal tumors, we also examined the effects of INCB3619 on colonic crypt cell proliferation and tumors. INCB3619 reduced colonic crypt cell proliferation to $16.7 \pm 1.7\%$, compared to $24.1 \pm 3.3\%$ for WD alone as assessed by Ki67 staining ($n=10$ mice per group, $p<0.005$). There were 25 colonic tumors in 10 mice in the WD 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 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 WD 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 floxed 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. Colons from control (no AOM treatment) homozygous ADAM17LoxP mice and villin-Cre-homozygous ADAM17LoxP mice (ADAM17^{-/-}) were harvested, and colonocytes and stromal cells prepared as described in the 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 (ADAM17^{+/-}) and control homozygous ADAM17LoxP mice (no Cre) with AOM or saline (AOM vehicle) weekly $\times$ 6 wks. Animals were sacrificed after 28 wks 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 Supplemental Fig S8. In contrast, ADAM17 was detected only in stromal cells of tumors from villin-Cre-homozygous ADAM17LoxP mice (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 ADAM17LoxP mice ($n = 32$) versus 2 tumors per mouse in villin-Cre-homozygous ADAM17LoxP mice ($n = 29$). Deletion of colonocyte ADAM17 also reduced tumor cell proliferation (Fig 6D) and tumor size (Fig 6E). We next examined the effects of colonocyte ADAM17 deletion on EGFR and Notch1 signals. As shown in Fig 6F and quantified in Fig 6G, EGFR signals (pErbB2, pERK) and Hes1 were significantly reduced in tumors from mice with ADAM17^{-/-}, compared to tumors from mice with ADAM17LoxP.

To assess ADAM17 changes in human colon cancers, we isolated colonocytes and stromal cells from colon cancers and adjacent normal-appearing mucosa. As shown in Supplemental Fig S9, ADAM17 appeared to be increased in malignant colonocytes compared to adjacent non-transformed colonocytes ($p = 0.06$, $n = 4$ tumors). Interestingly, three of the 4 patients summarized in the right panel of Supplemental Fig S9 had diabetes mellitus and/or hyperlipidemia - two metabolic disorders frequently associated with Western style diets. In an additional 6 intact human cancers we found that ADAM17 expression was 3.1 ± 1.8 -fold higher in tumors compared to adjacent mucosa ($p < 0.05$, $n = 6$). These results are consistent with prior findings showing increased ADAM17 immunostaining in tumors with staining predominantly in malignant colonocytes (17).

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 WD. Furthermore, we showed that pharmacological inhibition of ADAM17 and ADAM10 and genetic deletion of colonocyte ADAM17 suppress WD-promoted tumor development, establishing an oncogenic role for ADAM17, and possibly ADAM10, in WD promoted colon cancer. Other preclinical studies showed that ADAM17 haplo-insufficiency 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 WD increased ADAM17, colonocyte TGF α and stromal cell CXCL12 in the colonic mucosa (Fig 1). Furthermore, CXCL12 activated EGFR signals in HT29 cells and HCT116 cells, prototypes of chromosomal- unstable and microsatellite- unstable colon cancer cells, respectively (Fig 2 and 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 S2, Fig 3). These studies uncover a potentially important role for ADAM17 and CXCL12-CXCR4 signals in WD-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 suggests that AMD3100 might exert anti-tumor 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).

Since ADAM17 and ADAM10 control release of EGFR ligands (22), it was of interest to examine the anti-tumor effects of INCB3619, an inhibitor that blocks both these metalloproteases. In prior studies, INCB3619 demonstrated anti-growth effects in several tumor xenograft models (45). We confirmed that INCB3619 inhibited recombinant ADAM17 *in vitro* and intestinal mucosal ADAM17 *in vivo* (Fig S4). INCB3619 also reduced intestinal tumor burden in WD 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 metalloproteases such as marimastat were poorly tolerated because of debilitating peri-articular inflammation and tendonitis (26). In contrast, INCB3619 was well tolerated (45). A newer generation ADAM17/ADAM10 inhibitor INCB7839 was also well tolerated in a phase 1 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 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 α . Blocking TNF α and Notch signaling may contribute to the anti-tumor effects of INCB3619. Pro-TNF α was more abundant in Min adenomas from mice supplemented with INCB3619, consistent with reduced pro-TNF α cleavage by ADAM17 (supplemental Fig S7). INCB3619 also suppressed Notch1 signaling as assessed by decreased Hes1 (Fig 5C-D). ADAM10 is the major regulator of Notch signaling in intestinal stem cells under non-stress conditions (36). In preliminary studies, we found that Alcian blue staining was increased by INCB3619 in Apc+/Min mouse small intestine, consistent with ADAM10 blockade (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 colonic epithelial cells significantly reduced tumor development (Fig 6). These studies established an important oncogenic role for colonocyte ADAM17 in a WD-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 colonic tumors, but did decrease pErbB2, pERK, 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 α and Notch1 (52). Our human studies emphasize the potential oncogenic importance of increased ADAM17 signaling in malignant colonocytes as this enzyme was up-regulated in transformed human colonocytes (Fig S9) in agreement with prior studies in intact tumors (17).

In the current study we focused on WD effects on tumor development as our prior studies showed that EGFR was required for tumor promotion under WD, but not Std diet conditions (9). Our initial observations, however, were carried out in hybrid C57Bl6 $\times$ 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 Std 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 WD 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 Fig S2. These studies support further research targeting colonic ADAM17 in individuals at increased risk for colon cancer development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We gratefully acknowledge the generous gift of ADAM17 inhibitor BMS566394 from Bristol Myers Squibb (Bristol-Myers Squibb Company, Princeton, NJ) provided by Dr. Bruce Walcheck (University of Minnesota) and INCB3619 from the Incyte Corporation (Wilmington, DE). We also thank Dr. T-C He for providing the adenovirus and adeno-Cre virus.

Grant Support: This work was supported by the National Institutes of Health Grants [CA164124 to M.B., CA180087 to Y.C.L]; P30DK42086 (Digestive Disease Research Core Center); and the Samuel Freedman GI Cancer Laboratory Fund at the University of Chicago.

Abbreviations used in this paper

ADAM17	<u>a</u> <u>dis</u> integrin and <u>met</u> alloproteinase17
ACTB	beta actin
AMD3100	CXCR4 inhibitor
AOM	azoxymethane
AREG	amphiregulin
CCND1	cyclin D1
CXCL12	(Sdf1 α), chemokine (C-X-C motif) ligand 12
CXCR4	chemokine (C-X-C motif) receptor 4
DSS	dextran sulfate sodium
EGFR	epidermal growth factor receptor
EV	empty vector
pEGFR	phospho-active EGFR
pErbB2	phospho-active ErbB2
Sdf1α (CXCL12)	stromal-derived factor-1alpha

TGFα	transforming growth factor alpha
TNFα	tumor necrosis factor alpha

REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin.* 2011; 61:69–90. [PubMed: 21296855]
2. Weisburger JH. Dietary fat and risk of chronic disease: mechanistic insights from experimental studies. *J Am Diet Assoc.* 1997; 97:S16–23. [PubMed: 9216563]
3. La Vecchia C, Negri E, Decarli A, Franceschi S. Diabetes mellitus and colorectal cancer risk. *Cancer Epidemiol Biomarkers Prev.* 1997; 6:1007–10. [PubMed: 9419395]
4. Frezza EE, Wachtel MS, Chiriva-Internati M. Influence of obesity on the risk of developing colon cancer. *Gut.* 2006; 55:285–91. [PubMed: 16239255]
5. Sung JJ, Lau JY, Goh KL, Leung WK. Asia Pacific Working Group on Colorectal C. Increasing incidence of colorectal cancer in Asia: implications for screening. *Lancet Oncol.* 2005; 6:871–6. [PubMed: 16257795]
6. Kwong LN, Dove WF. APC and its modifiers in colon cancer. *Adv Exp Med Biol.* 2009; 656:85–106. [PubMed: 19928355]
7. Takahashi M, Wakabayashi K. Gene mutations and altered gene expression in azoxymethane-induced colon carcinogenesis in rodents. *Cancer Sci.* 2004; 95:475–80. [PubMed: 15182426]
8. Wasan HS, Novelli M, Bee J, Bodmer WF. Dietary fat influences on polyp phenotype in multiple intestinal neoplasia mice. *Proc Natl Acad Sci U S A.* 1997; 94:3308–13. [PubMed: 9096389]
9. Dougherty U, Cerasi D, Taylor I, et al. 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. *Clin Cancer Res.* 2009; 15:6780–9. [PubMed: 19903783]
10. Zhu H, Dougherty U, Robinson V, et al. EGFR signals downregulate tumor suppressors miR-143 and miR-145 in Western diet-promoted murine colon cancer: role of G1 regulators. *Mol Cancer Res.* 2011; 9:960–75. [PubMed: 21653642]
11. Fichera A, Little N, Jagadeeswaran S, et al. EGFR signaling is required for microadenoma formation in the mouse azoxymethane model of colonic carcinogenesis. *Cancer Res.* 2007; 67:827–35. [PubMed: 17234795]
12. Dougherty U, Sehdev A, Cerda S, et al. Epidermal growth factor receptor controls flat dysplastic aberrant crypt foci development and colon cancer progression in the rat azoxymethane model. *Clin Cancer Res.* 2008; 14:2253–62. [PubMed: 18413814]
13. Prenzel N, Fischer OM, Streit S, Hart S, Ullrich A. The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. *Endocr Relat Cancer.* 2001; 8:11–31. [PubMed: 11350724]
14. Kuwai T, Nakamura T, Sasaki T, et al. Phosphorylated epidermal growth factor receptor on tumor-associated endothelial cells is a primary target for therapy with tyrosine kinase inhibitors. *Neoplasia.* 2008; 10:489–500. [PubMed: 18472966]
15. Yoo J, Rodriguez Perez CE, Nie W, Edwards RA, Sinnott-Smith J, Rozengurt E. TNF- α induces upregulation of EGFR expression and signaling in human colonic myofibroblasts. *Am J Physiol Gastrointest Liver Physiol.* 2012; 302:G805–14. [PubMed: 22301110]
16. Barber TD, Vogelstein B, Kinzler KW, Velculescu VE. Somatic mutations of EGFR in colorectal cancers and glioblastomas. *N Engl J Med.* 2004; 351:2883. [PubMed: 15625347]
17. Blanchot-Jossic F, Jarry A, Masson D, et al. Up-regulated expression of ADAM17 in human colon carcinoma: co-expression with EGFR in neoplastic and endothelial cells. *J Pathol.* 2005; 207:156–63. [PubMed: 16041691]
18. Fischer OM, Hart S, Gschwind A, Ullrich A. EGFR signal transactivation in cancer cells. *Biochem Soc Trans.* 2003; 31:1203–8. [PubMed: 14641026]

19. Akishima-Fukasawa Y, Nakanishi Y, Ino Y, Moriya Y, Kanai Y, Hirohashi S. Prognostic significance of CXCL12 expression in patients with colorectal carcinoma. *Am J Clin Pathol*. 2009; 132:202–10. [PubMed: 19605814]
20. de Meijer VE, Le HD, Meisel JA, Sharma AK, Popov Y, Puder M. Tumor necrosis factor alpha-converting enzyme inhibition reverses hepatic steatosis and improves insulin sensitivity markers and surgical outcome in mice. *PLoS One*. 2011; 6:e25587. [PubMed: 21980496]
21. Rosenkilde MM, Gerlach LO, Jakobsen JS, Skerlj RT, Bridger GJ, Schwartz TW. Molecular mechanism of AMD3100 antagonism in the CXCR4 receptor: transfer of binding site to the CXCR3 receptor. *J Biol Chem*. 2004; 279:3033–41. [PubMed: 14585837]
22. Sahin U, Weskamp G, Kelly K, et al. Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands. *J Cell Biol*. 2004; 164:769–79. [PubMed: 14993236]
23. Liu X, Fridman JS, Wang Q, et al. Selective inhibition of ADAM metalloproteases blocks HER-2 extracellular domain (ECD) cleavage and potentiates the anti-tumor effects of trastuzumab. *Cancer Biol Ther*. 2006; 5:648–56. [PubMed: 16627988]
24. Roberts RB, Min L, Washington MK, et al. Importance of epidermal growth factor receptor signaling in establishment of adenomas and maintenance of carcinomas during intestinal tumorigenesis. *Proc Natl Acad Sci U S A*. 2002; 99:1521–6. [PubMed: 11818567]
25. Moran AE, Hunt DH, Javid SH, Redston M, Carothers AM, Bertagnolli MM. Apc deficiency is associated with increased Egfr activity in the intestinal enterocytes and adenomas of C57BL/6J-Min/+ mice. *J Biol Chem*. 2004; 279:43261–72. [PubMed: 15294912]
26. Rasmussen HS, McCann PP. Matrix metalloproteinase inhibition as a novel anticancer strategy: a review with special focus on batimastat and marimastat. *Pharmacol Ther*. 1997; 75:69–75. [PubMed: 9364582]
27. Moser AR, Pitot HC, Dove WF. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science*. 1990; 247:322–4. [PubMed: 2296722]
28. Hung KE, Maricevich MA, Richard LG, et al. Development of a mouse model for sporadic and metastatic colon tumors and its use in assessing drug treatment. *Proc Natl Acad Sci U S A*. 2010; 107:1565–70. [PubMed: 20080688]
29. Dougherty U, Mustafi R, Wang Y, et al. American ginseng suppresses Western diet-promoted tumorigenesis in model of inflammation-associated colon cancer: role of EGFR. *BMC Complement Altern Med*. 2011; 11:111. [PubMed: 22070864]
30. Seidelin JB, Horn T, Nielsen OH. Simple and efficient method for isolation and cultivation of endoscopically obtained human colonocytes. *Am J Physiol Gastrointest Liver Physiol*. 2003; 285:G1122–8. [PubMed: 14613919]
31. Khalil H, Nie W, Edwards RA, Yoo J. Isolation of primary myofibroblasts from mouse and human colon tissue. *J Vis Exp*. 2013
32. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods*. 2012; 9:676–82. [PubMed: 22743772]
33. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{−(Delta Delta C(T))} Method. *Methods*. 2001; 25:402–8. [PubMed: 11846609]
34. Porcile C, Bajetto A, Barbieri F, et al. Stromal cell-derived factor-1alpha (SDF-1alpha/CXCL12) stimulates ovarian cancer cell growth through the EGF receptor transactivation. *Exp Cell Res*. 2005; 308:241–53. [PubMed: 15921680]
35. Romee R, Foley B, Lenvik T, et al. NK cell CD16 surface expression and function is regulated by a disintegrin and metalloprotease-17 (ADAM17). *Blood*. 2013; 121:3599–608. [PubMed: 23487023]
36. Tsai YH, VanDussen KL, Sawey ET, et al. ADAM10 regulates Notch function in intestinal stem cells of mice. *Gastroenterology*. 2014; 147:822–34. e13. [PubMed: 25038433]
37. Fre S, Pallavi SK, Huyghe M, et al. Notch and Wnt signals cooperatively control cell proliferation and tumorigenesis in the intestine. *Proc Natl Acad Sci U S A*. 2009; 106:6309–14. [PubMed: 19251639]
38. Horiuchi K, Kimura T, Miyamoto T, et al. Cutting edge: TNF-alpha-converting enzyme (TACE/ADAM17) inactivation in mouse myeloid cells prevents lethality from endotoxin shock. *J Immunol*. 2007; 179:2686–9. [PubMed: 17709479]

39. Madison BB, Dunbar L, Qiao XT, Braunstein K, Braunstein E, Gumucio DL. Cis elements of the villin gene control expression in restricted domains of the vertical (crypt) and horizontal (duodenum, cecum) axes of the intestine. *J Biol Chem.* 2002; 277:33275–83. [PubMed: 12065599]
40. Serino M, Menghini R, Fiorentino L, et al. Mice heterozygous for tumor necrosis factor- α converting enzyme are protected from obesity-induced insulin resistance and diabetes. *Diabetes.* 2007; 56:2541–6. [PubMed: 17646208]
41. Gelling RW, Yan W, Al-Noori S, et al. Deficiency of TNF α converting enzyme (TACE/ADAM17) causes a lean, hypermetabolic phenotype in mice. *Endocrinology.* 2008; 149:6053–64. [PubMed: 18687778]
42. Kasina S, Scherle PA, Hall CL, Macoska JA. ADAM-mediated amphiregulin shedding and EGFR transactivation. *Cell Prolif.* 2009; 42:799–812. [PubMed: 19735466]
43. Blobel CP. ADAMs: key components in EGFR signalling and development. *Nat Rev Mol Cell Biol.* 2005; 6:32–43. [PubMed: 15688065]
44. Xia XM, Wang FY, Zhou J, Hu KF, Li SW, Zou BB. CXCR4 antagonist AMD3100 modulates claudin expression and intestinal barrier function in experimental colitis. *PLoS One.* 2011; 6:e27282. [PubMed: 22073304]
45. Fridman JS, Caulder E, Hansbury M, et al. Selective inhibition of ADAM metalloproteases as a novel approach for modulating ErbB pathways in cancer. *Clin Cancer Res.* 2007; 13:1892–902. [PubMed: 17363546]
46. Infante J, Burris H, Lewis N, et al. A multicenter phase Ib study of the safety, pharmacokinetics, biological activity and clinical efficacy of INCB7839, a potent and selective inhibitor of ADAM10 and ADAM17. *Breast Cancer Research and Treatment.* 2007; 106:S269. (2007) 106 S1:S269.
47. Newton R, Bradley E, Levy R, et al. Clinical benefit of INCB7839, a potent and selective ADAM inhibitor, in combination with trastuzumab in patients with metastatic HER2+ breast cancer. *J Clin Oncol.* 2010; 28 suppl abstract 3025.
48. Wang R, Ye X, Bhattacharya R, et al. A Disintegrin and Metalloproteinase Domain 17 Regulates Colorectal Cancer Stem Cells and Chemosensitivity Via Notch1 Signaling. *Stem Cells Transl Med.* 2016; 5:331–8. [PubMed: 26744411]
49. Lu J, Ye X, Fan F, et al. Endothelial cells promote the colorectal cancer stem cell phenotype through a soluble form of Jagged-1. *Cancer Cell.* 2013; 23:171–85. [PubMed: 23375636]
50. Feng Y, Tsai YH, Xiao W, et al. Loss of ADAM17-Mediated Tumor Necrosis Factor Alpha Signaling in Intestinal Cells Attenuates Mucosal Atrophy in a Mouse Model of Parenteral Nutrition. *Mol Cell Biol.* 2015; 35:3604–21. [PubMed: 26283731]
51. Shimoda M, Horiuchi K, Sasaki A, et al. Epithelial Cell-Derived a Disintegrin and Metalloproteinase-17 Confers Resistance to Colonic Inflammation Through EGFR Activation. *EBioMedicine.* 2016; 5:114–24. [PubMed: 27077118]
52. Rios-Doria J, Sabol D, Chesebrough J, et al. A Monoclonal Antibody to ADAM17 Inhibits Tumor Growth by Inhibiting EGFR and Non-EGFR-Mediated Pathways. *Mol Cancer Ther.* 2015; 14:1637–49. [PubMed: 25948294]

Translational Relevance

Western diets (WD) are implicated in sporadic colon cancer. Epidermal growth factor receptors (EGFR) are increased in human colon cancer and required for tumor promotion by WD 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 WD-promoted colon cancer involving CXCL12-CXCR4 as up-stream signals activating ADAM17.

Fig. 1. Effects of Western diet on CXCL12 and EGFR signaling components in colonic mucosa Flash frozen colonic mucosal tissues from control mice fed standard or Western diet (WD) for 40 wks were used from a prior study (9). Tissues were extracted for proteins and RNA, and real time PCR and Western blotting were performed. **A.** Experimental protocol. **B.** CXCL12, ADAM17, TGFα and K-Ras transcripts (*p<0.05, †p<0.005, ‡p<0.001, compared to levels in mice fed Std diet; n = 4 mice for each diet). **C.** Representative Western blots for indicated proteins. **D.** Expression levels for indicated proteins (*p<0.05, ‡p<0.001 compared to levels in Std diet fed mice; n=4 mice for each diet). **E, F** *in situ* TGFα mRNA (white arrows) under Std diet (**E**) and WD conditions (**F**), 10x. The relative fluorescence intensity of TGFα mRNA was 2.8 ± 1.4 in WD compared to Std diet (p<0.05). **G, H** CXCL12 immunostaining (white arrows), 10x. Colon sections in OCT from mice on standard diet (**G**) or Western diet (**H**) were stained for CXCL12. Note CXCL12 is predominantly expressed in stromal cells in the pericryptal mucosa and submucosa and increased in WD-fed mice. *In situ* TGFα mRNA hybridization and CXCL12 immunostained sections are representative of three mice in each dietary group.

Fig. 2. EGFR activation by CXCL12 requires EGF receptor ligand binding and is mediated by ADAM17 and inhibited by AMD3100

A. anti-EGFR antibody blocks EGF receptor activation by CXCL12. HT29 cells were pretreated for 2 hrs with PBS (vehicle, Ctl) or 20 µg/ml C225, an anti-EGFR antibody. Cells were then stimulated with CXCL12 (50 ng/ml) for the indicated times (1, 5, 10 min) and cell lysates probed for phospho-EGFR (pEGFR), pErbB2, pAKT and pERK, as well as for panEGFR, panErbB2, panAKT and panERK and β-actin as loading control. Separate wells were treated with vehicle or EGF (10 ng/ml) as a positive control. Note that C225 antibodies suppressed EGFR signals, indicating that EGFR activation by CXCL12 involves an EGF receptor ligand-dependent mechanism. **B.** Quantified phospho-active EGFR signals in cells pretreated with vehicle or C225 normalized to vehicle-treated (control) cells. **C.** ADAM17 mediates the effect of CXCL12 on EGFR activation. Cells were pretreated for 2 hrs with vehicle (–) or 5 µM BMS566394 (+, BMS), an ADAM17-specific inhibitor, and then stimulated with 50 ng/ml CXCL12 for the indicated times (1, 5, 10 min). Cell lysates were probed for the indicated phospho-active proteins (EGFR cascade). MWS: Lane containing molecular weight standards. Note that BMS566394 inhibits CXCL12-induced EGFR signals, indicating that ADAM17 mediates EGFR transactivation by CXCL12. **D.** Indicated phospho-protein signals in cells pretreated with BMS566394 normalized to vehicle-treated (control) cells. **E.** AMD3100 inhibits EGFR activation by CXCL12 as assessed by pErbB2, pERK and pAKT. HT29 cells were pretreated with 10 ng/ml AMD3100 or PBS (vehicle) for

2 hrs, and cells were then stimulated with 100 ng/ml CXCL12 or vehicle. At the indicated times (1, 5, 10 min), cell lysates were probed for pErbB2, pERK and pAKT. **F.** Phospho-active protein signals in cells pretreated with vehicle or AMD3100 normalized to signals in vehicle-treated cells. Results in Figs 2A-F are representative of two independent platings with comparable findings.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 3. CXC4 inhibitor AMD3100 suppresses EGFR signals and proliferation in colonic mucosa of mice fed Western diet

Mice were implanted with Alzet pumps and received vehicle or AMD3100 (5 mg/kg body weight/day) and were fed a Western diet. After 2 wks, mice were sacrificed and left colons were harvested. Indicated proteins were measured by Western blotting. **A.** Treatment protocol. **B.** EGFR signals. Western blots of indicated proteins from scrape-isolated mucosa in left colon from mice on WD alone or WD + AMD3100 (+). **C.** Quantitative expression levels (* $p < 0.001$, † $p < 0.005$ compared to WD alone, $n = 4$ mice in each group). **D.** BrdU incorporation in vehicle-treated mouse. **E.** BrdU incorporation in AMD3100-treated mouse. **F.** BrdU quantitation (* $p < 0.05$, compared to BrdU in WD alone; $n = 4$ mice per condition).

Fig. 4. ADAM17 inhibitor INCB3619 suppresses tumor development in *Apc* mutant *Min* mice
A. Effect of INCB3619 on mouse growth. (* $p < 0.05$ compared to WD alone). **B.** Effects of INCB3619 on small intestine and colon tumor incidence. **C.** INCB3619 suppresses small intestinal tumor multiplicity (n=20 mice on WD and 20 mice on WD+INCB3619; * $p < 0.01$, Mann-Whitney-U test). **D.** INCB3619 suppresses small intestinal tumor growth (mean $\pm$ SD, $p < 0.05$).

Fig. 5. INCB3619 suppresses EGFR signals and proliferation in adenomas from *Apc* mutant *Min* mice

Mice were treated as described in the Methods. At 7-mo 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 WD alone (Tumor), compared to adjacent normal-appearing mucosa (NI); †p<0.05, phospho-protein levels in normal-appearing mucosa in group receiving INCB3619 (NI + INCB), compared to levels in intestinal mucosa from mice on WD alone (NI); ‡p<0.05, ‡‡p<0.005, phospho-protein levels in adenomas from mice on INCB3619 (Tumor + INCB), compared to adenomas from mice on WD alone (Tumor)]. **C.** Western blots of indicated EGFR and Notch1 effectors. **D.** Densitometry [*p<0.05, **p<0.005, compared to levels in normal-appearing intestinal mucosa; †p<0.05, compared to levels in adenomas from mice on WD alone; n= 6-8 mice per group]. **E.** Ki67 immunostaining in representative adenoma from mouse on WD alone (*left panel*) and adenoma from mouse on WD + INCB3619 (*right panel*). **F.** Quantitation of Ki67 staining (*p<0.001 compared to WD alone, n=10 adenomas from mice on WD alone, n=11 adenomas from mice on WD + INCB3619. Each adenoma is representative of a different mouse).

Fig. 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 ADAM17^{LoxP} (ADAM17^{ΔΔ}) and homozygous ADAM17^{LoxP} mice were then treated with AOM or vehicle (saline). Two weeks after the 6th AOM injection, mice were begun on a Western diet (WD, 20%) as described in the Methods. Twenty-eight wks 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 ADAM17^{LoxP} (villin-Cre⁺) and homozygous ADAM17^{LoxP} mice (villin-Cre⁻) as described in the Methods. (Mice in Fig 6A 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 ADAM17^{LoxP} mice (ADAM17^{ΔΔ}), 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 to control homozygous ADAM17^{LoxP} mice; n=32 homozygous ADAM17^{LoxP} mice and n=29 ADAM17^{ΔΔ} mice). **C**. Colon tumor multiplicity. The median number of tumors was significantly lower in ADAM17^{ΔΔ} group compared to the homozygous ADAM17^{LoxP} group (2.0 vs. 5.0 tumors per tumor-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 homozygous ADAM17^{LoxP} mice and n=12 tumors from ADAM17^{ΔΔ} 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 to homozygous ADAM17^{LoxP} mucosa; †p<0.05 compared to adenomas in the homozygous ADAM17^{LoxP} group).