Published in final edited form as:
Int J Cancer. 2013 March 1; 132(5): 1004–1012. doi:10.1002/ijc.27762.

Epigenetic inactivation of endothelin-2 (ET-2) and ET-3 in colon cancer

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Abstract

Endothelin-1 (ET-1) and its receptors are overexpressed in human cancers, but much less is known about the roles of ET-2 and ET-3 in cancer etiology. We sought to examine human and rat colon tumors for dysregulation of ET-2 and ET3 expression, and determine the underlying mechanisms. Human primary colon cancers and carcinogen-induced rat colon tumors were subjected to real-time RT-PCR, immunoblotting, and immunohistochemistry; *EDN2* and *EDN3* genes were examined by methylation-specific PCR, bisulfite sequencing, and pyrosequencing; and forced expression of ET-2 and ET-3 was conducted in human colon cancer cells followed by real-time cell migration and invasion assays. Rat and human colon tumors had markedly reduced expression of ET-2 and ET-3 mRNA and protein compared with matched controls. Mechanistic studies revealed hypermethylation of *EDN2* and *EDN3* genes in human primary colon cancers, and in a panel of human colon cancer cell lines. Forced expression of ET-2 and ET-3 attenuated significantly the migration and invasion of human colon cancer cells. We conclude that epigenetic inactivation of ET-2 and ET-3 occurs frequently in both rat and human colon cancers. Current therapeutic strategies target overexpressed members of the ET axis via small molecule inhibitors and receptor antagonists, but this work supports a complementary approach based on the re-expression of ET-2 and ET-3 as natural antagonists of ET-1 in colon cancer.

Keywords

Colorectal Cancer; DNA methylation; Endothelin Axis; Epigenetics

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Contributors RW was responsible for the carcinogenicity bioassay and molecular studies; WMD assisted with the carcinogenicity bioassays, necropsies, and cell culture; CVL and KAF performed histopathology and immunohistochemistry; JAG assistance with invasion/migration assays; HA provided TMAs; EH, DEW, and MRD were responsible for critical revision of the manuscript; RHD provided the study concept, and was responsible for data interpretation and drafting/editing of the manuscript.

INTRODUCTION

There is growing interest in the endothelin (ET) axis and its dysregulation in cancer development[1-3]. This "axis" comprises the 21-amino acid peptides ET-1, ET-2, and ET-3, the G-protein-coupled receptors ET_AR and ET_BR, and upstream processing enzymes such as endothelin converting enzyme 1[1-3]. ET-1 is by far the best characterized isoform, being a potent vasoconstrictor with key roles in normal physiology and in various pathophysiological conditions[4,5]. ET-2 and ET-3 differ from ET-1 by two and six amino acids, respectively, and undergo similar processing of the precursor peptides (preproETs). Overexpression of ET-1, ET_AR, and ET_BR has been documented in various human cancers, but much less is known about the roles of ET-2 and ET-3.

Whereas ET-1 is expressed in many tissue types, ET-2 is restricted mainly to the gastrointestinal tract, sex organs, and pituitary gland. ET-3 is more ubiquitous, and has been detected in brain, kidney, lung, spleen, stomach, and intestine. In human breast cancer cells, ET-2 acts as a hypoxia-induced autocrine survival factor with a potential role in tumor invasion[6,7]. ET-3 was reported to be decreased in cervical cancer compared with normal cervical epithelial cells[8], and loss of ET-3 gene (*EDN3*) expression was observed in human breast cancer[9].

Given this background information, we sought to examine the roles of ET-2 and ET-3 in colon cancer development. In addition to studies of primary human colon cancers and colon cancer cell lines, a preclinical rat model was used to examine both early and late stages of colon carcinogenesis. Mechanistic work focused on epigenetic inactivation of *EDN2* and *EDN3*, coupled with experiments involving forced expression of ET-2 and ET-3 in human colon cancer cells.

MATERIALS AND METHODS

Source of human and rat colon tumors

Thirty primary human colon cancers and patient-matched controls were provided under an IRB-approved protocol by Steven F. Moss and Lelia Simao (Rhode Island Hospital, Providence, RI). The tumors were characterized as late-stage adenocarcinomas and were the primary source for the molecular studies. In a separate but parallel arm of the investigation, immunohistochemical analyses of ET-2 and ET-3 were performed on tissue slides of human normal colon (ab4327, Abcam) and on human colon cancer tissue microarrays (TMAs).[10] Rat tumors and adjacent normal looking tissues were from a 1-year study[10] in which male F344 rats were treated with 2-amino-1-methyl-6-phenylimidazo[4,5- *b*]pyridine, a heterocyclic amine from cooked meat. An interim sacrifice was included in order to assess early molecular changes before tumor onset. Specifically, 24 h after the last dose of carcinogen, rats (5-6/group) were euthanized, the colon was removed and opened longitudinally, and the mucosa was scraped and frozen in liquid nitrogen. The study received prior approval from the Institutional Animal Care and Use Committee.

RNA extraction and real-time PCR

Frozen samples of tumor and normal tissue were thawed and the mRNA was extracted using the RNeasy kit (Qiagen, Valencia, CA). RNA (2 μg) was reverse-transcribed in 20 μl using the SuperScript^R III first-strand synthesis system for RT-PCR (Invitrogen). *EDN2* and *EDN3* mRNA levels were measured by qPCR and normalized to *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*. Forty cycles of PCR (95°C/10s, 58°C/10s, 72°C/10s) were run on a LightCycler 480 II system (Roche, Indianapolis), in a 20-μl total reaction

volume containing cDNAs, SYBR Green I dye (Roche), and primers. The amount of specific mRNA was quantified by determining the point at which the fluorescence accumulation entered the exponential phase (C_t), and the C_t ratio of the target gene to *GAPDH* was calculated for each sample. At least three separate experiments were performed for each sample.

Protein expression

Proteins (20 μg) were subjected to SDS-PAGE and immunoblotted according to reported methods[10]. Primary monoclonal mouse antibodies were to ET-2 (1:500 dilution, Novus Biologicals, H00001907-MO1) and ET-3 (1:500 dilution, Novus, H00001908-MO1). Proteins were visualized by Western Lightning chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston MA, USA) and quantified using an Alpha Innotech Image System (Alpha Innotech, San Leandro, CA). For immunohistochemistry studies, tissues were sectioned at 4-5 μm and processed in a Dako Autostainer. Polyclonal rabbit anti-ET-2 (Atlas, Sweden, Lot# 27432) was used at 1:50 dilution (human tissues) or 1:25 dilution (rat tissues). Polyclonal rabbit anti-ET3 (Phoenix Pharmaceuticals, Cat# H-023-17) was used at 1:200 dilution. Primary antibodies were applied for 0.5 h at room temperature. Dako Universal negative rabbit (N1699) served as a negative control. MaxPoly-One Polymer HRP Rabbit Detection solution (MaxVision Biosciences) was applied at room temperature, followed by Nova Red (Vector Laboratory SK-4800) as the chromagen and Dako hematoxylin (S3302) as the counterstain.

DNA methylation assays

Genomic DNA (0.5 μg) was bisulphite-modified using the EZ DNA methylation kit (Zymo Research Corp, Orange County, CA) and eluted in 20 μl Tris-Buffer (10 mM). Quantitative methylation-specific PCR (MSP) was performed with primers that specifically recognized unmethylated or methylated *EDN2* and *EDN*3 promoter sequence after bisulphite conversion. Primer sequences were designed by MethPrimer software. Forty cycles of PCR were run on a LC480 LightCycler (Roche) in a 10-μl reaction containing bisulphite-treated DNA, SYBR Green I dye (Roche), and primer set. PCR conditions were 95 °C /10s, 58 °C / 10s, and 72 °C /10s. DNA was quantified by determining the point at which the fluorescence accumulation entered the exponential phase (Ct), and the Ct ratio of target to β-actin was calculated for each sample. At least two independent experiments were performed per sample. For pyrosequencing, primers were designed with PyroMark Software SW2.0 (Qiagen), and targeted *EDN2* (−198 to −60) or *EDN*3 (−250 to −77). Forty-five cycles of PCR were run on a GeneAmp system 9700 (Applied Biosystems) in a 20-μl reaction containing PyroMark PCR Master Mix (Qiagen), bisulphite-treated DNA, and *EDN2* or *EDN3* primer set. After confirming the correct amplicon size, PCR products and sequencing primers were sent to the Protein and Nucleic Acid Facility (Stanford University) for pyrosequencing on a PyroMark Q24 system.

Cell culture, ET overexpression, and invasion/migration assays

Human colorectal cancer lines Caco2, HCT116, HT29, SW48 and SW480 were obtained from American Type Culture Collection (ATCC, Manassas, VA). Caco2 cells were maintained in MEM medium (ATCC) with 20% heat-inactivated fetal bovine serum (FBS, Hyclone Laboratories). Other cell lines were maintained in McCoy's 5A medium (Invitrogen) supplemented with 10% FBS, 100 units/ ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2. Cells typically were seeded at a density of 1×10^5 in a six-well plate format. 5′-Aza-2′-deoxycytidine (Sigma-Aldrich) was added to a final concentration of 10 μM, whereas controls were treated with the equivalent volume of PBS. Cells were harvested on day 4 for DNA methylation analyses, and on day 5 for quantification of mRNA expression. In ET-2 and ET-3 overexpression experiments, human

EDN2 and *EDN3 cDNA* clones were obtained from OriGene Technologies (Rockville, MD). Cells (3×10^5) were seeded in 6-well plates overnight. The media was aspirated and replaced with fresh antibiotic free-transfection media containing 6 μl lipofectmine2000 and 0.5 μg *EDN2* or *EDN3* plasmid DNA, or 0.5 μg XL5 empty vector (OriGene). At 72 h after transfection, cells were harvested for protein analyses and cell migration/invasion assays. The latter were conducted in real time using the xCELLigence System (Roche). Colon cancer cells were transfected with *EDN2, EDN3,* or empty vector XL5, and harvested at 72 h. Cells (0.2×10^6) in 100 μl serum-free media were loaded onto the upper chamber of CIM plates coated with or without 2.5% matrigel (BD Bioscience, San Diego, CA), to assess invasion and migration, respectively. The lower chamber was filled with MEM medium containing 20% FBS. Measurements were taken at intervals of 10 min for at least 40 h.

Statistical analyses

Data were plotted as mean±SE and compared using Student's *t*-test, or by Anova for group comparisons, unless stated otherwise. In the figures, significant outcomes were shown as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

RESULTS

ET-2 and ET-3 expression is reduced in colon cancer

Human primary colon cancers and patient-matched controls were immunoblotted for preproETs (Fig. 1A). Colon tumors had markedly lower levels of preproET-2 and preproET-3, whereas no such loss of preproET-1 was detected. Based on these initial findings, *EDN2* and *EDN3* mRNA levels were determined by qPCR and normalized to *GAPDH*. Human primary colon cancers had significantly reduced expression of *EDN2* and *EDN3* compared with patient-matched controls (Fig. 1B). Specifically, *EDN2* expression had a relative value of 4.27±1.38 in controls compared with 0.37±0.11 in the colon tumors (mean±SE, n = 30, $P < 0.01$), *i.e.*, an 11-fold reduction overall. In the case of *EDN3*, the corresponding values were 8.42±1.89 *versus* 1.04±0.38 (mean±SE, n = 30, $P < 0.001$), *i.e.*, 8-fold lower expression in the colon tumors.

Loss of preproET-2 and preproET-3 expression also was detected in carcinogen-induced colon tumors compared with adjacent normal-looking tissue (Fig. 1C), and in normal-looking colonic mucosa obtained several weeks before the onset of frank tumors (Fig. 1D). The relative expression of *Edn2* mRNA in rat colon tumors was 0.02±0.01 (Fig. 1E, left panel, black bar), ~20-fold lower than in normal-looking tissue adjacent to tumor (0.40±0.06, $P < 0.001$), and colonic mucosa from untreated controls (0.43±0.06, $P < 0.001$). No significant difference was detected for *Edn2* expression when comparing normal colon from untreated rats and normal-looking tissue adjacent to tumors (Fig. 1E, left panel, white *versus* grey bar, $P>0.05$). In contrast, *Edn3* expression was significantly lower not only in colon tumors compared with adjacent normal-looking tissue (Fig. 1E, right panel, black *versus* grey bars, $P<0.01$), but also in adjacent normal-looking tissue compared with colonic mucosa from untreated rats (Fig. 1E, right panel, grey *versus* white bars, $P <0.001$). The corresponding relative mRNA expression levels were as follows: 0.26±0.03 (colon tumors) < 0.45±0.05 (adjacent normal-looking tissue) < 0.88±0.09 (control colonic mucosa).

Loss of ET-2 and ET-3 was confirmed by immunohistochemistry

Immunostaining for ET-2 (Fig. 2A) revealed intense expression in the *propria mucosae* of normal human colon tissue, mainly in macrophages, whereas neurons of the autonomous nerve plexus in the *tunica muscularis* were moderately stained and enterocytes of the *lamina epithelialis* were lightly stained. In human colon cancer TMAs, normal-looking areas stained positive in macrophages, whereas adenocarcinoma regions had little or no detectable ET-2.

In rat normal colon, neurons of the autonomous nerve plexus and segments of the epithelial brush border were strongly stained for ET-2, whereas epithelial cells *per se* were faintly stained. Colon tumors in the rat were largely ET-2 negative, or exhibited faint background staining.

In the case of ET-3 (Fig. 2B), both human and rat normal colonic epithelium stained strongly, most notably in the basal and apical enterocytes within colonic crypts. ET-3 also was detected in enteric ganglion cells from both human and rat. In human colon cancer TMAs, most cores were negative, except a few that exhibited cytoplasmic punctate ET-3 staining. In the rat, sections with tumor (T) adjacent to normal (N) tissue typically revealed positive staining for ET-3 only in the latter regions (Fig. 2B, lower right, 40x magnification).

Hypermethylation of *EDN2* and *EDN3* in human primary colon cancers

A search using MethPrimer software confirmed previous findings[9] of two regions with high CpG density in *EDN3*, and revealed a putative CpG island in *EDN2*, located at position -5 to +142 relative to the translation initiation site (Fig. 3A). Using primers that flanked these regions, methylation-specific PCR (MSP) detected a 30-fold increase in the methylation of *EDN2* in human primary colon cancers compared with patient-matched controls (Fig. 3B, left panel, 19.80±4.9 *versus* 0.64±0.08, n = 30, $P < 0.001$). MSP also revealed a ~2-fold increase in *EDN3* methylation in cancers *versus* controls (Fig. 3B, right panel, 2.49±0.31 *versus* 1.40±0.15, n = 30, $P < 0.001$).

Hypermethylation of *EDN2* and *EDN3* in human colon cancer cells

A panel of human colon cancer cell lines was examined by qPCR for *EDN2* mRNA expression, normalized to *GAPDH*. After treatment with the DNA demethylating agent 5-aza-2′-deoxycytidine (5-aza), *EDN2* expression was increased 7-fold in Caco-2 cells, 46-fold in HCT116 cells, 24-fold in HT29 cells, 23-fold in SW48 cells, and 6.5-fold in SW480 cells (Fig. 4A). Four days after treatment with 5-aza, MSP analyses revealed a shift from high to low *EDN2* methylation status (Fig. 4B,C). In HCT116 cells, for example, 5-aza treatment decreased by ~7-fold the *EDN2* methylated PCR product (MSP-M) and increased by 15-fold the corresponding unmethylated signal (MSP-U). Similar trends were observed for *EDN3* in the panel of five colon cancer cell lines (data not shown). In Caco-2 cells, for example, *EDN3* mRNA expression was increased 2-fold after 5-aza treatment, there was a corresponding loss of the methylated product in MSP analyses ($P < 0.01$), and a 2.8-fold increase was detected for the unmethylated product ($P < 0.001$).

Bisulfite sequencing in human colon cancer cells treated with and without 5-aza provided initial confirmation of the predicted methylation sites in *EDN2* and *EDN3* (data not shown), and pyrosequencing subsequently was used to generate quantitative data. In the case of *EDN2*, six separate CpG sites were shown to be hypermethylated in human colon cancer cells (Fig. 5A, grey bars). At site CpG1 for example, 100% methylation was reduced to 60% methylation after 5-aza treatment ($P < 0.001$, compare grey bar *versus* solid black bar). Similar results were obtained for seven CpG sites in *EDN3* (Fig. 5B). For example, at site CpG1, 85% methylation was reduced to <50% methylation by 5-aza ($P < 0.001$).

Inhibition of migration/Invasion by ET-2 and ET-3 overexpression

Human colon cancer cells were transiently transfected with constructs expressing ET-2 or ET-3. Empty vector XL5 served as the control. Immunoblotting confirmed that preproET-2 and preproET-3 levels were increased relative to the vector control at 72-h post-transfection (Fig. 6A). Forced expression of ET-2 or ET-3 inhibited cell migration significantly, relative to vector control (Fig. 6B, inset). Cell invasion also was inhibited significantly by ET-2

overexpression ($P < 0.05$), whereas ET-3 was less effective under the same assay conditions (Fig. 6C). Results shown here were from real-time monitoring assays in Caco-2 cells, but similar findings were obtained in HCT116 colon cancer cell lines (data not presented).

DISCUSSION

Despite the growing interest in the ET axis and its role in cancer,[1-3] most attention to date has focused on ET-1 and its receptors, with surprisingly little information on ET-2 and ET-3. In fact, there is a significant gap in our general understanding of the lesser studied ET isoforms and their functions in normal physiology and pathophysiology, especially in the case of ET-2. As noted in the introduction, ET-2 differs by only two amino acids from ET-1, has the same affinity as ET-1 for the receptors ET_AR and ET_BR, and may co-exist in the same tissue compartments. However, there is converging evidence for a distinct ET-2 pathway, and this "forgotten isoform"[11] may have critical roles in ovary development, immunology, the cardiovascular system, and cancer.

This investigation has provided the first evidence for the involvement of ET-2 in colon cancer development. In marked contrast to ET-1,[1,2,12,13] ET-2 and ET-3 expression was low or undetectable in both rat and human colon cancers, suggesting a possible tumor suppressor function in the colon. Importantly, in the rat model, loss of ET-2 and ET-3 was detected in colonic mucosa several weeks before the onset of frank tumors. This suggests that reduced expression of ET-2 and ET-3 might provide an early biomarker of cancer development in the colon.

Interestingly, epigenetic inactivation of ET-3 recently was identified in human breast cancer.[9] We have extended these observations by showing hypermethylation of both *EDN2* and *EDN3* in human primary colon cancers, and in a panel of widely used human colon cancer cell lines. Although there was variation in the constitutive levels of *EDN2* and *EDN3* among the cell lines examined, in all cases treatment with 5-aza produced a significant increase in the corresponding gene expression. One potentially important observation was that, in any given cell line, the relative mRNA expression following 5-aza treatment was consistently higher for ET-2 than for ET-3 (Fig. 4A and data not shown). Moreover, in human primary colon cancers, MSP analyses revealed a 30-fold increase in ET-2 after 5-aza treatment, compared with only a 2-fold increase in ET-3. One interpretation is that ET-2 is the more critically silenced factor in the colon, and that its re-expression effectively antagonizes dysregulated ET-1. It is noteworthy that in normal colon of both rat and human, ET-2 was restricted largely to proprial macrophages and enteric ganglion cells, with mild expression in the epithelium and brush border (Fig. 2A). Similar findings were reported in the mouse;[14] ET-2 was detected at the bottom of villi in the duodenum, ileum and jejunum, but the pattern was reversed in crypts of the colon and rectum.

We did not perform a comprehensive screening of all putative DNA methylation sites for the genes of interest, but focused on six CpGs in *EDN2* and seven CpGs in *EDN3*. Computational analyses identified these as likely sites for DNA methylation, which was confirmed by both bisulfite sequencing and pyrosequencing. The results for *EDN3* corroborate and extend prior studies of epigenetic silencing in human breast cancer,[9] whereas the DNA methylation data reported here for *EDN2* are novel.

Based on these observations, we hypothesized that ET-2 and ET-3 might be silenced early in cancer development so as to circumvent competition with ET-1 for its receptors, thereby avoiding "mixed messages" in the ET axis. In support of this idea, forced expression of ET-2 and ET-3 inhibited migration and/or invasion of human colon cancer cells *in vitro* (Fig. 6), whereas ET-1 had the opposite effect (data not shown). Monitoring assays in real

time suggested a greater impact of ET-2 than ET-3 on cell invasion, and of ET-3 compared with ET-2 on cell migration. However, this was not always consistent among the colon cancer cell lines tested, and was dependent to some extent on the efficiency of transient transfection. We prescreened the colon cancer cell lines for expression of ET_AR and ET_BR (qPCR data not shown), and on this basis selected Caco2 and HCT116 cells for invasion/migration assays. It would be interesting to repeat the studies in HT29 and SW48 cells, since these lines had low or undetectable ET_AR and ET_BR levels yet responded to 5-aza treatment with strong re-expression of ET-2 and ET-3. This might provide insights in other cases, such as breast cancer, where ET-2 is a reported macrophage chemoattractant with a possible role in tumor cell invasion.[15,16]

We did not formally investigate the competition among ET-1, ET-2, and ET-3 for ET_AR and ET_BR receptors under the present conditions, but it is interesting to speculate on ligand *versus* receptor "silencing" as a driving mechanism in the disease context. Pao *et al.* examined the promoter methylation status of the gene coding for ET_BR (*EDNRB*) and noted that colon normal tissue had levels of methylation that occasionally exceeded those in cancers of the bladder and prostate.[17] These authors also examined a panel of human colon cancers, observing that 4/8 tumors had regions of the *EDNRB* promoter with methylation status exceeding 50%.[17] Might ET_BR downregulation in the colon serve as a trigger for reduced expression of selected ligands, such as ET-2 or ET-3? This seems to be unlikely, at least in the rat model, where ET_AR and ET_BR levels were consistently overexpressed, not silenced, in the colon tumors (R. Wang *et al.*, unpublished results). Since loss of ET-2 and ET-3 expression occurred before the onset of frank tumors, we conclude that ET-2 and ET-3 silencing was not a driver for subsequent downregulation of ET_A or ET_B receptors during rat colon carcinogenesis. A more likely driver of ET_AR and ET_BR expression was active ET-1 peptide, being consistently elevated in rat colon tumors compared with normal colonic mucosa (R. Wang *et al.*, manuscript in preparation). Further work is in progress to clarify these relationships and their effects on cancer cell migration and invasion.

In summary, the present work has shed new light on two largely forgotten isoforms in the ET axis, namely ET-2 and ET-3, and their roles in cancer etiology. Epigenetic silencing of *EDN2* and *EDN3* was clearly implicated in the loss of ET-2 and ET-3 expression in both human and rat colon tumors. The rat colon carcinogenesis model provided insights into the timing of epigenetic silencing, with evidence for loss of ET-2 and ET-3 several weeks before the onset of frank tumors. This raises the possibility that diminished expression of ET-2 and ET-3 might serve as an early biomarker of colon cancer risk. Mechanistic studies supported an inhibitory role of ET-2 and ET-3 on colon cancer cell migration and invasion, but further work is needed to clarify the molecular details and applicability of these findings to other cancers characterized by dysregulation of the ET axis. Nevertheless, this investigation has opened an avenue for potential new anticancer therapies targeting the re-expression of silenced members of the ET axis. This would provide for an alternative or complementary strategy to the small molecule inhibitors and receptor antagonists that are currently undergoing clinical evaluation.[1,2]

Acknowledgments

We thank Carmen P. Wong for technical assistance with pyrosequencing. Steven F. Moss and Lelia Simao (Rhode Island Hospital, Providence, RI) kindly provided the panel of primary human colon cancers and patient-matched controls. Zhiqiang Yang assisted with statistical analyses. Cell invasion and migration assays were conducted in the Cell Imaging and Analysis Core of the Environmental Health Sciences Center at Oregon State University.

Grant Sponsor: US National Institutes of Health grants CA090890, CA65525, CA122906, CA122959, CA80176, and ES00210

Abbreviations

ACTB	human β-actin gene
5-aza	5-aza-2′-deoxycytidine
ET	endothelin
EDN, Edn	endothelin gene (human, rat)
GADPH, Gapdh	*glyceraldehyde-3-phosphate dehydrogenase* gene (human, rat)
MSP	methylation-specific PCR
qPCR	quantitative real-time polymerase chain reaction
TMA	tissue microarray

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Novelty and Impact

The endothelin axis is dysregulated in cancer development. Most studies to date have focused on ET-1 overexpression and the use of small molecule inhibitors or receptor antagonists. We now report that rat and human colon cancers exhibit epigenetic inactivation of ET-2 and ET-3, and that their forced re-expression inhibited migration/invasion of human colon cancer cells. These findings suggest a new therapeutic strategy based on the activation of natural antagonists of ET-1 in the colon.

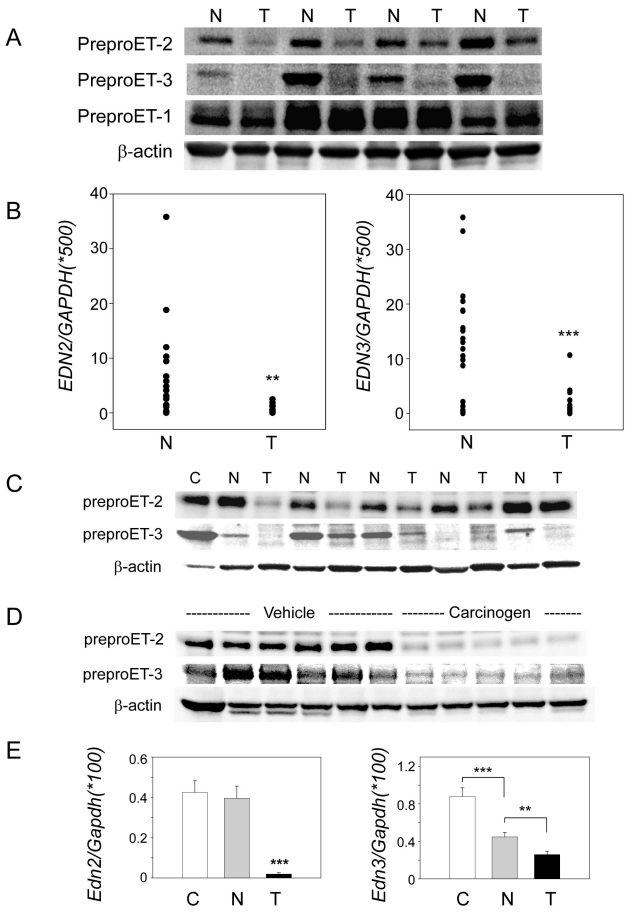

Figure 1.

Reduced expression of ET-2 and ET-3 in colon cancer. (*A*) Human colon cancers and patient-matched controls were immunoblotted for ET-1, ET-2, and ET-3 precursor peptides; loading control, β-actin. T, tumor; N, normal-looking control. (*B*) *EDN2* and *EDN3* mRNA expression was determined by qPCR, normalized to *GAPDH; **P<0.01, ***P<0.001*, n=30. (*C*) Male F344 rats were treated with a heterocyclic amine carcinogen or with vehicle, as reported.[10] Colon tumors (T) and adjacent normal-looking tissue (N) were immunoblotted for ET-2 and ET-3 precursor peptides. A lane was included for colonic mucosa from rats given no carcinogen or vehicle (controls, C). (*D*) Normal-looking colonic mucosa was obtained after completing the carcinogen treatment at 14 weeks and immunoblotted for ET-2 and ET-3 precursor peptides; n=5 rats (carcinogen treatment) or n=6 rats (vehicle controls). (*E*) *Edn2* and *Edn3* mRNA levels normalized to *Gapdh;* open bar (n=8), grey bar (n=24); black bar (n=24); mean ± SE, ***P<0.01, ***P<0.001*. For clarification of *EDN*, *Edn*, *GAPDH*, *Gapdh* and other terminology, see abbreviations.

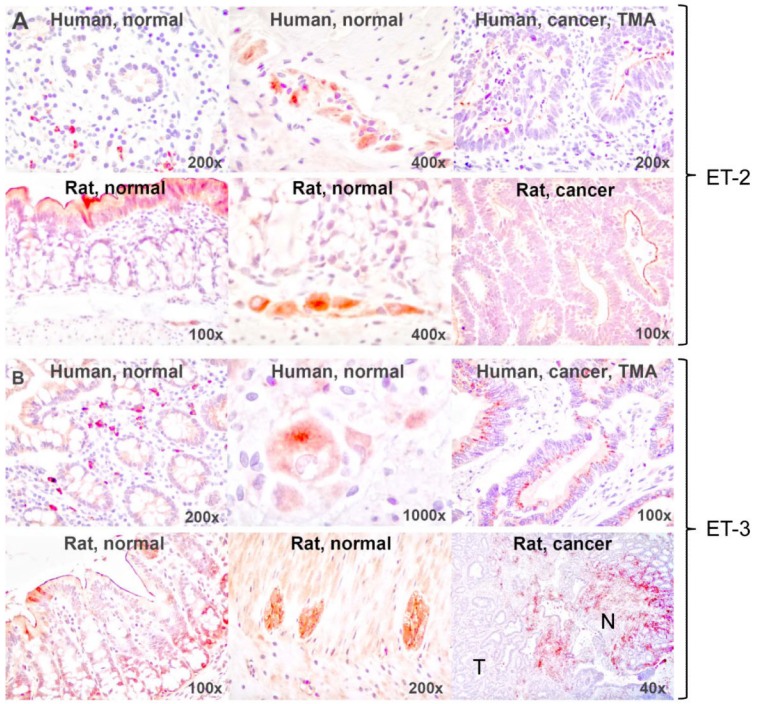

Figure 2.

Immunostaining of ET-2 and ET-3 in human and rat colon tissues. (*A*) ET-2 expression: In human normal colon, ET-2 was strongly stained in macrophages of the *propria mucosae*, whereas neurons of the enteric plexus were moderately stained. Human colon adenocarcinomas in tissue microarrays (TMAs) were negative. In rat normal colon, neurons and segments of the brush border were positive for ET-2, whereas the luminal epithelium was weakly stained. Rat colon tumors were largely ET-2 negative, or sometimes had faint background staining. (*B*) ET-3 expression: Staining for ET-3 was intense in epithelial cells and neurons of both human and rat normal colon. In human colon cancer TMAs, most cores were negative, except a few that exhibited cytoplasmic punctate ET-3 staining. In rat colon tumors (T), neoplastic epithelial cells were negative for ET-3, whereas adjacent normal tissue (N) and portions of the tumor stroma were positive. Nova red/hematoxylin.

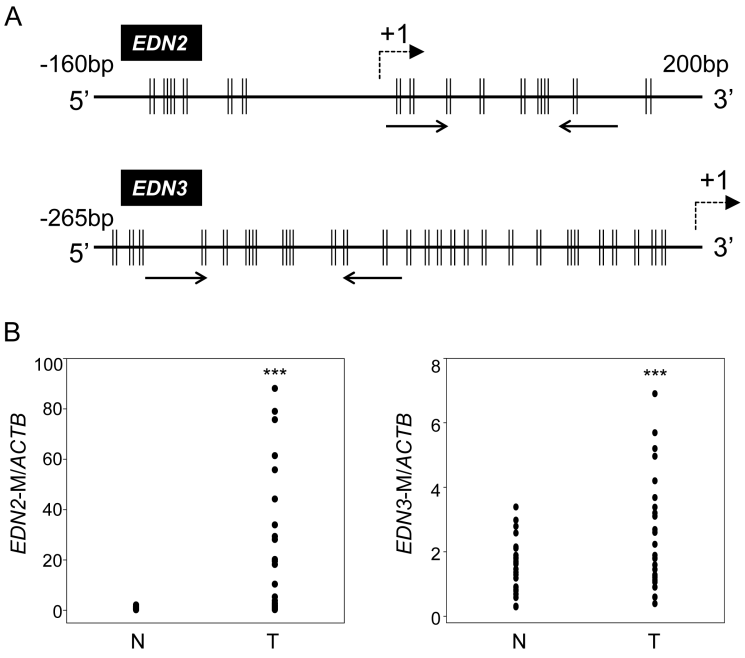

Figure 3.

Hypermethylation of *EDN2* and *EDN3* in human primary colon cancers. (*A*) Schematic representation of *EDN2* and *EDN3* genes in the vicinity of the translation initiation site at +1 (dashed arrow); horizontal solid arrows indicate hybridization sites of methylation-specific PCR (MSP) primers. Vertical lines depict CpG dinucleotides. (*B*) PCR products of methylated *EDN2* and methylated *EDN3* were analyzed by quantitative MSP, normalized to the β-actin promoter (*ACTB*). T, human primary colon tumor (adenocarcinoma); N, patient-matched control. Permutation tests on paired data for both *EDN2* and *EDN3* showed a significance difference for tumor *versus* control, ***$P<0.001$, n=30.

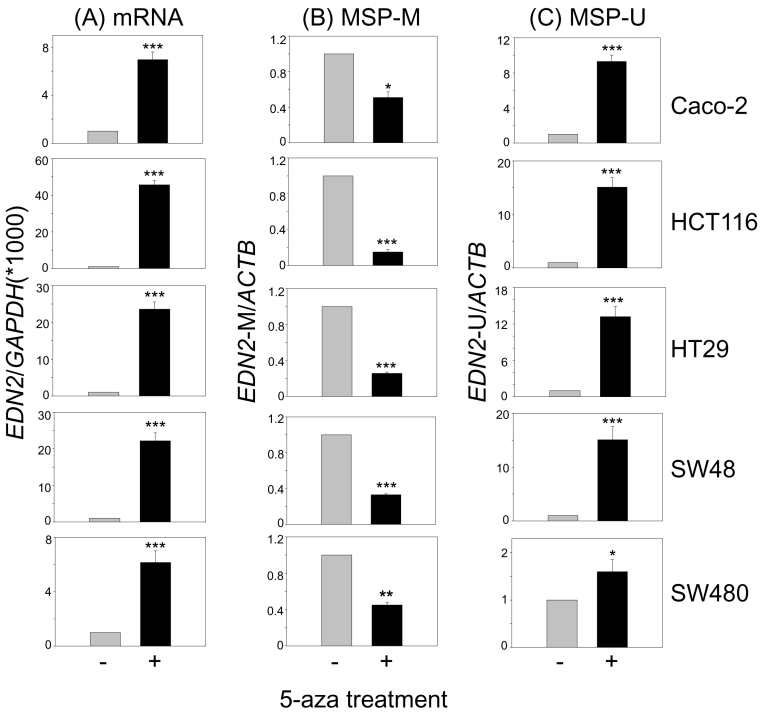

Figure 4.

EDN2 hypermethylation in human colon cancer cells. (*A*) Messenger RNA levels were measured by qPCR and normalized to *GAPDH*, on day 5 after starting 5-aza-2′-deoxycytidine (5-aza) treatment. (*B*) Methylated-M and (*C*) unmethylated-U DNA status was assessed via bisulfite conversion-based MSP, normalized to *ACTB* at day 4. Mean ± SE, **P<0.05, **P<0.01, ***P<0.001.* Similar findings were obtained for *EDN3* (not shown).

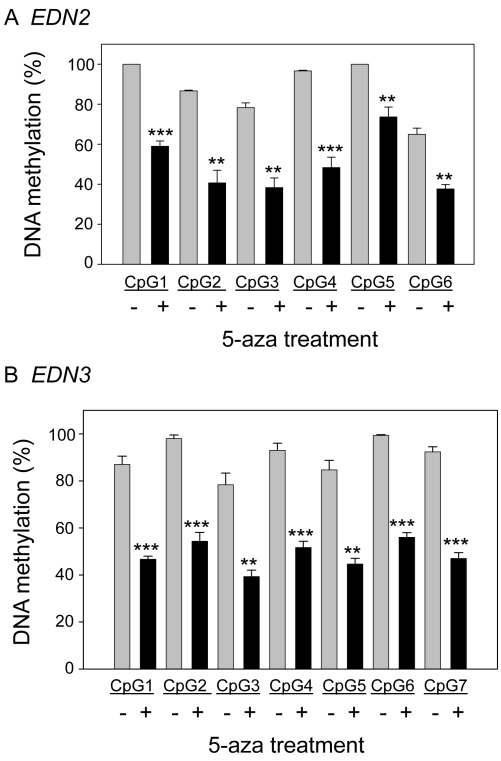

Figure 5.

Quantitative DNA methylation analyses of *EDN2 and EDN3*. Percent DNA methylation in six CpGs of the *EDN2* gene (*A*) and seven CpGs of the *EDN3* gene (*B*) was measured by pyrosequencing, four days after human HCT116 colon cancer cells were treated with and without 5-aza-2′-deoxycytidine (5-aza). Mean±SE, ****P<0.01*, ****P<0.001*, from three independent experiments.

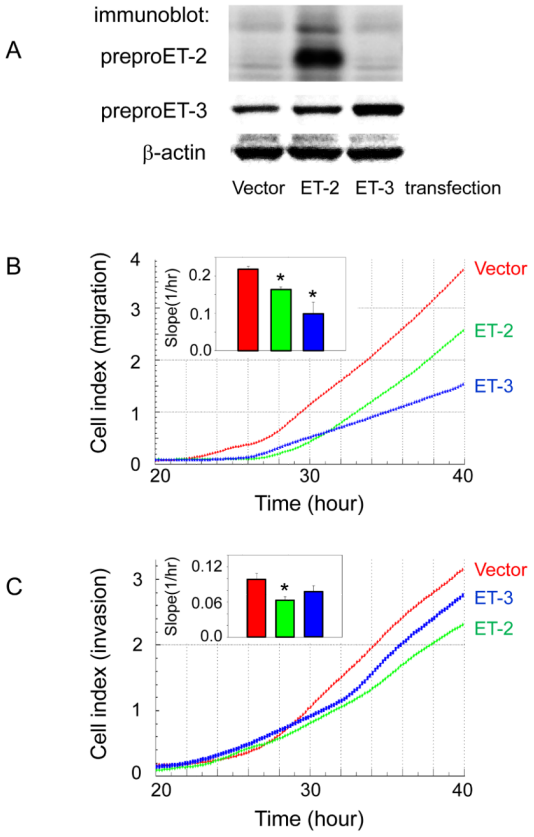

Figure 6.

Reduced cell migration and invasion by forced expression of ET-2 and ET-3. (*A*) Human colon cancer cells were transiently transfected with constructs expressing ET-2 or ET-3 (or with empty vector XL5), and 72 h later cells were transferred to CIM plates for real-time monitoring of (*B*) cell migration and (*C*) cell invasion. Data bars, mean±SE (n=3); *P<0.05, Anova followed by Tukey's *post hoc* test.