FXR silencing in human colon cancer by DNA methylation and KRAS signaling

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COLON CANCER IS THE THIRD most common cancer and the third leading cause of cancer-related deaths in the US. Two major risk factors for colon cancer development are high-fat and/or low-fiber diet (15, 19, 41). Bile acids (BAs) are amphiphilic molecules essential for digestion and absorption of fats. High-fat diet increases BA load in the intestine whereas low dietary fiber prolongs gastrointestinal transit time, thus collectively increasing the level and time of BA exposure (2, 33). Although BAs are essential for lipid absorption, high concentration of BAs is linked to increased colon tumorigenesis (2, 17, 27). Indeed, patients with colorectal cancer increased fecal BA excretion (11, 43).

Farnesoid X receptor (FXR) is a ligand-activated transcription factor belonging to the nuclear receptor superfamily. FXR is highly expressed in liver and intestine and BAs are its endogenous ligands (28). FXR critically regulates the homeostasis of BAs, including BA synthesis, transport, and intestinal reabsorption, as well as the free intracellular concentration of BAs to prevent their accumulation to cytotoxic levels (9, 12, 26, 39, 47). FXR deficiency in mice promotes the development of intestinal tumors (21, 25), implicating FXR as a tumor suppressor. Furthermore, overexpression of a constitutively active form of FXR decreases tumor size in mouse xenograft models (25). The mechanism of tumor-suppressor effects of FXR is not defined but may be mediated through the protection of colonic epithelium from inflammation and BA toxicity by upregulating intracellular BA binding proteins and efflux transporters while downregulating influx transporters and de novo synthesis of BAs (8). However, FXR also has antitumorigenic functions independent of its regulation of BA homeostasis (25). For example, FXR deficiency increases susceptibility to colon cancer development by increasing epithelial permeability to bacteria, promoting WNT/β-catenin signaling, increasing intestinal inflammation, and protecting against genotoxic compounds (8, 13, 25, 44).

Studies, on small samples sizes, have shown that FXR was silenced in later stages of colon cancer, implicating FXR as a marker of tumor malignancy (6, 16). Although polymorphisms...
within the FXR gene have been associated with decreased function in intrahepatic cholestasis of pregnancy (ICP) (23), no clinically known mutations exist within the FXR gene to explain decreased FXR expression or function in human colon cancer. The present study was conducted to identify the mechanism(s) of FXR silencing during colon cancer development in humans and 2) the mechanism(s) of FXR silencing. The results of this study suggest a potential therapeutic strategy for preventing and/or inhibiting colon cancer promotion by suppressing colon cancer-associated FXR silencing or activation of remnant FXR in surrounding healthy tissues.

MATERIALS AND METHODS

Human colon cancer samples. Immunohistochemistry (IHC) analysis was done on paraffin-embedded normal colon tissue, polyps, and adenocarcinomas obtained with IRB approval from University of Texas MD Anderson Cancer Center [MDACC; Protocol Numbers ID99-296(38) and LAB09-0373] and University of Arizona Gastrointestinal SPORE (P50 CA95060; protocol no. 10-696-01). Tissue analysis was done on paraffin-embedded normal colon tissue, polyps, and adenocarcinomas obtained with IRB approval from University of Texas MD Anderson Cancer Center [MDACC; Protocol Numbers ID99-296(38) and LAB09-0373] and University of Arizona Gastrointestinal SPORE (P50 CA95060; protocol no. 10-696-01). Tissue cores consisted of “real normal” (right and left tissues from patients with no history of cancer; n = 47), polyps with “matched normal” (n = 32), and adenocarcinomas stage I (n = 43), II (n = 39), III (n = 68), and IV (n = 9) with matched normal (n = 114). Standard IHC was performed by use of an automated IHC Leica Bond-Max system (Leica Microsystems, Buffalo Grove, IL). Anti-FXR mouse monoclonal antibody (PP-A9033A, R&D Biosystems) was used and labeling was detected by use of a Bond Polymer Refined Detection Kit (DS9800, Leica Microsystems). Staining was quantified by the following equation:

\[
(N3 + 0) + (N2 + 2) + (N1 + 1)/N = \text{Labeling Index (LI)}.
\]

N = total number of nuclei analyzed per sample;
N3 = number of nuclei labeled at intensity 3;
N2 = number of nuclei labeled at intensity 2;
N1 = number nuclei labeled at intensity 1

β-Catenin IHC analysis of polyps (n = 9) and adenocarcinomas (n = 2) was done with anti-β-catenin mouse monoclonal antibody (PA0083, Leica Microsystems) and the same detection system listed above. All IHC slides were determined positive or negative for nuclear FXR and β-catenin labeling and validated by a gastrointestinal pathologist (D. Maru).

cDNA from normal and cancerous colons (n = 5–18) was obtained from OriGene Technologies (Rockville, MD; http://www.origene.com; HCRTS01). mRNA levels of FXR, organic solute transporter (OST)α, and OSTβ3 were measured and normalized to hβ-actin.

TCGA analysis. Clinical patient data, NR1H4 (FXR gene name) expression, NR1H4 promoter methylation, microsatellite instability (MSI), V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) mutation, hypermutated tumor status, and CpG island methylator phenotype (CIMP) were downloaded from published Cancer Genome Atlas (TCGA) Network work (4) and reanalyzed for correlation analysis. Full methylation of the FXR CpG island is designated by β-value > 0.6.

Cell line study. FXR expression was studied in a panel of colon cancer cell lines representing transition from highly differentiated to poorly differentiated, along with the levels of E-cadherin and vimentin, markers of epithelial-to-mesenchymal transition (EMT). NCI-H508 (duplicates), NCI-H716, NCI-H747, Colo320DM, DLD-1, HCT-15, and Colo201 cells were grown in RPMI-1640 medium; HT-29 and HCT-116 (duplicates) were grown in McCoy’s 5A medium; LoVo cells were grown F-12K medium; HT-29 and SW620 were used for azacytidine (AZA) treatment, methyl-DNA immunoprecipitation (MeDIP) analysis, and DNA methyltransferase (DNMT) siRNA experiments. Cells were cultured in media listed above, supplemented with both 10% FBS and 1% penicillin-streptomycin. These experiments were done before the recommendation for removal of antibiotics from complete media.

Table 1. Primers for MeDIP and RT-qPCR

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Forward 5’-3’</th>
<th>Reverse 5’-3’</th>
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</thead>
<tbody>
<tr>
<td>MeDIP primers</td>
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<td></td>
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<tr>
<td>FXR CpG</td>
<td>GTTTGAAGACAGCTGGGCAACAT</td>
<td>ATTTCGCGTCCAAAGCGGTCCTCTT</td>
</tr>
<tr>
<td>COL1A2 CpG</td>
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<td>RT-qPCR primers</td>
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<td></td>
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<tr>
<td>FXR</td>
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<td>OSTα</td>
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<td>OSTβ3</td>
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</tr>
<tr>
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<tr>
<td>DNMT 3b</td>
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</tr>
<tr>
<td>KRAS</td>
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<td>TGCCTGAGCGGTGTTTGTG</td>
</tr>
</tbody>
</table>

RT-qPCR, RT-quantitative PCR; meDIP, methyl-DNA immunoprecipitation; FXR, farnesoid X receptor; OSTα, organic solute transporter α; OSTβ, organic solute transporter β; DNMT, DNA methyltransferase; KRAS, V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog.
HT-29, SW620, SW1116, SK-CO-1, Colo201, and Colo320DM cells were used for small interfering KRAS (siKRAS) experiments with cells cultured as described above with 10% FBS and without antibiotics. Proteins and phosphoproteins from a panel of colon cancer cell lines were extracted and quantified by reverse-phase protein array (RPPA), as previously described (42). The RPPA cluster heat map of 15 cell lines based on the 172 proteins was generated with Partek Genomics Suite v 6.6 in which each column is a protein marker and each row a colon cancer cell line annotated with phosphatidylinositol-4,5-bisphosphate 3 kinase (PI3K) catalytic subunit α isoform (PI3KCA), and KRAS mutation status. Both rows and columns were clustered by Ward’s method based on rank (Spearman) dissimilarity (44a).

Azacytidine treatment. HT-29 and SW620 cells were plated at 10^6 cells in 10-cm plates and treated with or without 2 µg/ml AZA (Sigma, St. Louis, MO), a DNMT inhibitor, for 3 days, with fresh solutions prepared each day (n = 3). RNA was extracted as described below. COL1A2 and FXR mRNA levels were determined by RT-quantitative PCR (RT-qPCR). COL1A2 gene encodes for the collagen-Iα2 protein and has been shown to be methylated in colon cancer cell lines, SW620 (36). Therefore, the expression of this gene in response to AZA treatment was used as a positive control.

Fig. 2. Expression of FXR and FXR-target genes in human colon cancer samples. A: FXR immunohistochemistry (IHC) images of right (R) and left (L) real normal colon (no colon cancer history) from same patient; polyp (P) of descending colon with matched normal (N) tissue, and stage II (II) adenocarcinomas of the ascending colon and cecum and their matched normal (N) tissues. B: quantification of FXR labeling from IHC analysis. The % positively labeled nuclei plus intensity of nuclei staining, reported as the labeling index. Theoretical maximum labeling index would be 3 and minimum 0.0001. ● Outliers from 5–95% percentile. C: relative mRNA levels of FXR, organic solute transport (OSTβ), and OSTα in human colon cancer samples (n = 5–18). Data are expressed as means ± SE. ● Outliers from range defined by Tukey’s multiple comparison test. *P < 0.05.
MeDIP assay. MeDIP assay was performed as described (45). Briefly, ~4 μg of sheared DNA was incubated overnight at 4°C with either the 5-methylated-cytosine (5-mC) antibody (Diagenode, MAB-335MCE-100), or mouse IgG negative control. Then DNA samples with antibody were incubated with prewashed Dynabeads at 4°C for 5 h with rotation. The DNA-antibody-beads complex was washed and DNA were eluted. Immunoprecipitated DNA was phenol/chloroform extracted and dissolved in Tris-EDTA (TE) buffer.

A CpG island located roughly 3 kb upstream of the FXR gene transcription start site (TSS) was identified by MethPrimer (Fig. 1; Ref. 18). This region was assessed for DNA methylation by MeDIP analysis. DNA precipitated by the 5-mC antibody was analyzed by qPCR using SYBR green chemistry (n = 2). Primers were designed to amplify a methylated CpG islands within the FXR promoter and the COL1A2 promoter (positive control; Ref. 36). Primer sequences are listed in Table 1. Primers designed to amplify a nonmethylated housekeeping gene (UBE2B) were used as a negative control and have been previously reported (40).

siRNA knockdown. DNMT1 and 3B are enzymes associated with aberrant DNA methylation (34). Therefore, siRNA knockdown of DNMT1 and/or 3B in SW620 cells was done. SMARTpool siRNAs to knock down expression of DNMT1 or DNMT3B, and nontargeting siRNA, were obtained from Dharmacon (LaFayette, CO). SW620 cells were plated at 30% confluence in a six-well plate (n = 2) and reverse transfected with siDNMT1, siDNMT3B, or nontargeting siRNA with use of Turbofect siRNA transfection reagent (Fermentas, Glen Burnie, MD). After 96 h, total RNA was extracted and mRNA of DMNT 1, DMNT 3B, FXR, and COL1A2 (positive control) were measured by RT-qPCR analysis.

Western blot. Thirty micrograms of whole cell lysates from a panel of colon cancer cell lines listed above were used for analysis. Primary anti-FXR mouse monoclonal antibody (R&D Biosystems), anti-E-cadherin mouse monoclonal antibody (Cell Signaling, Boston, MA), anti-vimentin rabbit monoclonal antibody (Cell Signaling, Boston, MA) was used for protein detection. The Western Lighting Plus ECL (PerkinElmer, Waltham, MA) was used for protein labeling. The Western blot images were scanned and band densities quantified by AlphaInnotech’s AlphaView image analysis software (ProteinSimple, Santa Clara, CA).

RNA extraction and RT-qPCR. Complimentary DNA prepared from human colon samples (OriGene) were used to measure mRNA levels of FXR, OSTx, and/or OSTβ. Total RNA from colon cancer cell lines was performed with TRIzol reagent (Invitrogen, Carlsbad, CA) and cDNA prepared by standard RT-PCR methods with random primers (Fermentas). cDNA prepared from AZA-treated cells, DNMT 1 and 3B siRNA experiments, and siKRAS experiments were used to measure mRNA levels of FXR, COL1A2, and/or KRAS. The primer sequences are listed in Table 1. The primer sequences for COL1A2 are as previously reported (36). All real-time qPCR reactions were done using standard SYBR green chemistry and an ABI Prism 7900 Detection system (Applied Biosystems, Foster City, CA).

The mRNA levels of these genes were normalized to hβ-actin or hGAPDH mRNA levels.

gDNA extraction. Genomic DNA (gDNA) was extracted from HT-29 and SW620 for MeDIP analysis. Briefly, trypsinized cancer cells lysed in DNA lysis buffer (10 mM Tris pH 7.5, 10 mM EDTA, 10 mM NaCl, 0.5% sodium dodecyl sulfate) plus 1 mg/mL protease K, and incubated at 55°C for 5 h to overnight, and ethanol precipitated. gDNA was further purified by standard phenol/chloroform extraction methods. Purified gDNA was bionically sonicated to 200–1,000 bp, and column purified by use of standard PCR purification kits (Fermentas).

Statistical analysis. Quantitative PCR data are expressed as means ± SE. One-way ANOVA was used with adjustment for multiple comparisons within model using Tukey’s method. Unpaired two-way Student’s t-test was used when comparing two groups.

FXR, E-cadherin, vimentin, and Western blot bands were plotted as a ratio over actin loading control. E-cadherin and vimentin ratios are plotted against FXR ratios. Spearman correlation analysis was used to assess the correlation between continuous variables.

All TCGA data points are plotted with single lines representing group means. Statistical analysis comparing the means between two groups was done by Student’s t-test and Mann-Whitney U-test.

The expression levels of FXR and 172 other proteins in 15 colorectal cancer cell lines were profiled RPPA. The correlations between FXR densitometry ratio and 172 other proteins were assessed by using Spearman correlations on a protein-by-protein basis. To account for multiple testing, we estimated the false discovery rate (FDR) by the beta-uniform mixture method (32).

Linear mixed models with fixed effect of tissue groups were used to assess the differences in the expression of FXR between and among groups as determined by IHC analysis. A subject/individual-level random effect was included in the linear model where appropriate to account for the correlation between the matched pairs of normal and tumor or polyp for the same samples. We also examine for batch effects of three sets of data (MDACC colorectal cancer, Arizona colorectal cancer, MDACC polyp) and found no significant batch effect. Pairwise comparisons between groups were carried out by least squares estimate of the means with adjustment for within-model multiple comparisons by Tukey’s method. We used quantile-quantile plots to examine the normality assumption of the residuals of the linear models. IHC analysis was converted to log scale for statistical analysis but reported as arithmetic values in figures. All the analyses were performed with SAS 9.3 and GraphPad Prism 6.01.

RESULTS

FXR expression decreased in human colon cancers. FXR labeling is less in the left colon compared with the right, in polyps compared with matched normal colon, and in both stage II adenocarcinomas compared with matched normal samples (Fig. 2A). There was no statistical difference in FXR expres-

<table>
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<tr>
<th>Patient</th>
<th>Normal FXR LI</th>
<th>Polyp FXR LI</th>
<th>% of Normal</th>
<th>Polyp Nuclear β-Catenin</th>
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<td>1</td>
<td>0.586</td>
<td>0.069</td>
<td>12</td>
<td>+</td>
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<tr>
<td>2</td>
<td>0.59</td>
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<td>0</td>
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<tr>
<td>3</td>
<td>0.449</td>
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<td>4</td>
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<td>0.63</td>
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<tr>
<td>5</td>
<td>0.744</td>
<td>0.164</td>
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<td>6</td>
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<td>8</td>
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<tr>
<td>9</td>
<td>0.536</td>
<td>0.287</td>
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LI, labeling index.

Table 2. β-Catenin-positive polyp and tumor samples

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<thead>
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<th>Polyp</th>
<th>Tumor</th>
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<tr>
<td></td>
<td></td>
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<tr>
<td>Samples with positive nuclear β-catenin</td>
<td>2</td>
</tr>
<tr>
<td>Total samples</td>
<td>9</td>
</tr>
<tr>
<td>% Positive</td>
<td>22.2%</td>
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Fig. 3. FXR Western blot and reverse-phase protein array (RPPA) cluster analysis and in human colon cancer cell lines. A: Western blot analysis of FXR, E-cadherin, vimentin, and actin in a panel of colon cancer cell lines. B: densitometry ratios of E-cadherin or vimentin over actin were plotted on the y-axis against FXR ratios on the x-axis. Correlation coefficients and P values are from linear regression and Spearman correlation analysis. C: a subset of samples (≥0.25 vimentin/actin ratio) appeared to have a linear and negative correlation to FXR expression. Correlation analysis was redone by using samples with ≥0.25 vimentin/actin ratio. The cells included in this third analysis were NCI-H716, HT-29, Caco-2, SW480, SW620, SW1116, SK-CO-1, and LoVo. D: FXR/actin ratios were used for cluster analysis and heat map generation with RPPA data from colon cancer cell lines. Blue is relatively low (≤0.10) and yellow high (≥0.20) values. Cells cluster as high FXR (cluster 1) and low FXR (cluster 2). Mutational analysis of phosphatidylinositol-4, 5-bisphosphate 3 kinase (PI3KCA) and V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) for each cell line is also shown in this figure. Red, green, and gray boxes indicate mutant, wild-type, or unavailable genotypes.
sion between normal human colons (real normal) and matched normal tissues of polyps and adenocarcinomas (data not shown). Therefore, data from real normal and matched normal tissues were combined for analysis. The expression of FXR decreased down the colonic tract with the cecum (highest FXR expression), ascending colon, and transverse colon having statistically higher levels of FXR expression than their immediate distal segments (Fig. 2B; P < 0.05). The trend of FXR downregulation between normal tissue, polyps, and stage I, II, and IV adenocarcinomas was similar between the right and left colon (data not shown). Therefore, data from right and left colon were combined for analysis.

To associate FXR expression with development of polyps, a low sample number of polyps and two colon tumors (positive control) IHC samples were labeled for β-catenin nuclear localization. Two of nine (22.2%) polyp samples stained positively for nuclear β-catenin, compared with two of two colon tumors (100%; Table 2A). Only one of nine polyps showed both a decrease in FXR expression (defined by decreased expression) and higher levels of FXR expression (defined by increased expression > 50% from normal) and positive nuclear localization of β-catenin (Table 3).

FXR mRNA levels were reduced 6- to 10-fold stages I, II, and III colon adenocarcinoma (Fig. 2C), indicating that FXR silencing occurs at the transcriptional level. FXR mRNA in stage IV samples was not reduced compared with normal, likely because of small sample size. Reduction of mRNA levels of FXR target genes NR1H4 and NR1H5 was only significant for NR1H4 mRNA for stage I and III. There was an overall decreased trend in these target genes all four stages, but most were found not to be statistically significant, likely because of the low sample number.

**FXR expression is negatively correlated to vimentin and PI3K signaling.** There was no statistically significant positive or negative correlation between FXR, E-cadherin, or vimentin expression in a panel of colon cancer cell lines (Fig. 3, A and B). However, there is a subset of vimentin (≥0.25 ratio of vimentin/actin) samples that negatively correlated to FXR expression. It is clear that many of the colon cancer cell lines labeled negative for vimentin expression (Fig. 3A; designated < 0.25 vimentin/actin ratio). Therefore, using samples that label positive for vimentin (≥0.25 ratio of vimentin/actin; Fig. 3C), FXR was significantly and negatively correlated with vimentin expression (r = −0.864, P = 0.001).

RPPA data from colon cancer cells were used for cluster analysis with FXR expression measured by Western blot. The colon cancer cells clustered into a high-FXR group (cluster red), including LoVo, SW1417, SK-CO-1, and LDL-1 cells, and low FXR group (cluster blue), including Colo320, Caco-2, HCT-116, HCT-15, SW948, SW403, SW1116, LS174T, SW48, and SW620 cells (Fig. 3D). Furthermore, RPPA genes separated into two clusters: cluster 1 (red) and cluster 2 (blue). The FXR column lies within cluster 2 (indicated by ↓ and labeled FXR). Mutational status (green = WT and red = mutant genotypes) of PI3KCA and KRAS, two commonly mutated genes in colon cancer (1), showed that four of eight cells (with PI3KCA mutation status) in cluster 2 (low FXR expression) carried a mutation in their PI3KCA gene compared with only one of four cell lines in cluster 1. These appeared to be no difference in KRAS mutation status between these two clusters. Spearman correlation analysis comparing FXR protein levels measured by RPPA analysis identified 23 and 5 proteins that significantly correlated, either positively (positive r value) or negatively (negative r value), respectively, with FXR expression when using a FDR cutoff values of 20 and 10% (Table 4). The most statistically significant positively and negatively correlated genes were BCL-x and eEF2. It should be noted that many of the mediators of PI3K signaling, including PI3KCA (PI3Kp110), mammalian target of rapamycin, negatively and significantly correlated to FXR expression.

**DNA methylation regulates FXR expression.** Mutation of the NR1H4 gene was first investigated as a potential cause of FXR silencing in three different cell lines: HT-29, Caco-2, and SW620. These cells show different baseline expression of FXR and no mutations within the entire gene and 5-kb promoter to account for FXR silencing in colon cancer (data not shown). TCGA data shows only two colon cancer samples have a NR1H4 gene variant and no samples have loss of NR1H4 copy number indicating the conservation of the NR1H4 gene locus in colon tumors (4). Sequence analysis of the FXR promoter revealed a putative CpG island located in the promoter of NR1H4 gene (Fig. 1). This island is located ~3 kb upstream of the NR1H4 gene TSS and has 11 predicted CpG islands. Complete methylation of this site was observed in ~12% TCGA colon cancer samples (4).

A panel of cDNAs prepared from 11 different colon cancer cell lines treated with a DNMT inhibitor, AZA, was used to measure mRNA levels of FXR. This preliminary screen revealed 6 of 11 of the colon cancer cell lines showed a 1.5-fold increase (relative threshold for significance) in FXR expression, ranging from 1.7- to 2.33-fold (1.2- and 50-fold for HT-29 and SW620 cells), after AZA treatment. To confirm this, the colon cancer cell lines HT-29 and SW620 were treated with AZA and mRNA levels of FXR and COL1A2 were measured (n = 3, Fig. 4A). AZA treatment significantly increased mRNA levels of both FXR and COL1A2 in SW620
cells six- and eightfold, respectively (*P < 0.05), but not in HT-29 cells.

Methylation of the FXR promoter CpG island was assessed by MeDIP analysis on gDNA isolated from HT-29 and SW620 cells. Identification of a methylated CpG island near the TSS of COL1A2 gene was first confirmed (36). A housekeeping gene region that is not methylated (UBE2B) was used as a negative control for this assay (40). The results confirmed methylation COL1A2 promoter and the FXR gene promoter in HT-29 and SW620 cells, with relative enrichment levels of 400- and 600-fold over IgG, but not negative control region (Fig. 4, *P < 0.05).

SW620 cells have lower basal levels of FXR expression compared with HT-29 cells and responded to AZA treatment (Fig. 4A); therefore, these cells were used for DNMT siRNA knockdown to determine the molecular machinery responsible for FXR downregulation. Knockdown of DNMT 1 and 3B by >80% (Fig. 4C; *P < 0.05) was sufficient to increase mRNA levels of FXR 6.7- and 7.3-fold, respectively, and positive control gene COL1A2 7- and 6.6-fold, respectively (Fig. 4C; *P < 0.05).

Methylation of FXR promoter in clinical colon tumors. As noted previously, TCGA data revealed that roughly 12% of colon tumors have a fully methylated FXR promoter (determined by a methylation β value > 0.6). Clinical methylation and expression of NR1H4, KRAS mutational status, MSI, hypermutated status, and CIMP were obtained from previous report (4). Data analysis showed the methylation of the FXR promoter does not correlate with FXR expression (Fig. 5A), and there was no statistically significant difference in FXR expression in tumors with fully methylated promoters (Fig. 5B). FXR expression also did not significantly change in tumors with KRAS mutations, MSI-H status, hypermutated status, or CIMP (Fig. 6, A–D).

TCGA data showed no statistically significant difference in the frequency of full FXR promoter methylation (β-value > 0.6) and KRAS mutation status, MSI-H status, hypermutated status, or CIMP status (Table 5). Partial methylation of CpG
islands has been shown to have transcription inhibition effects (5, 10). Taking this into consideration, we assessed differences in the degree of FXR promoter methylation. Levels of methylation of FXR promoter was statistically higher in MSI-H and hypermutated but not KRAS mutant or CIMP tumors (Fig. 6, A–D). Interestingly, although not statistically significant, there was a decreased trend in FXR promoter methylation in KRAS mutant vs. nonmutant tumors (Fig. 6A).

**Effects of KRAS signaling of FXR expression.** Because FXR expression in colon cancer cell lines negatively correlated with PI3K signaling in RPPA data, FXR expression was measured in PI3K isogenic cell lines (37). There was no difference in FXR expression in cells with no vs. high PI3K signaling (indicated by phospho-AKT; data not shown).

However, knockdown of KRAS signaling in PI3K isogenic cells resulted in dramatic increase in FXR levels in KRAS WT and mutant cells, measured by Western blot analysis (data not shown). Expanding this in more cells showed KRAS siRNA treatment significantly increased FXR mRNA levels in five of six colon cancer cell lines (Fig. 7A). Interestingly, this increase was greater in KRAS mutant cells (SW620, SK-Co-1, and SW1116) than KRAS WT cells (HT-29). KRAS mutational status of these cells can be found in Fig. 3D. HT-29 cells are WT for KRAS (31).

**DISCUSSION**

FXR is an adopted nuclear receptor responsible for regulating free BA levels in both liver and intestine and has been suggested to be a potential tumor suppressor for colon cancer development (7, 21, 25). Studies have shown that mice deficient in FXR have increased colonic tumorigenesis and that the FXR antitumorigenic effects are at least partially due to BA-independent mechanisms, namely by regulating intestinal integrity and inflammation and protection from genotoxic compounds (13, 21, 25, 44). FXR has also been suggested to suppress colon tumorigenesis by increasing apoptosis and suppressing epidermal growth factor receptor-mediated cell proliferation (21, 25, 30).

This study revealed that FXR is downregulated at the transcriptional level in colon adenomas and virtually silenced in all stages of adenocarcinoma. Previous reports have suggested FXR was downregulated in a stage-dependent manner (6, 16) compared with our findings that indicate that FXR was decreased in adenomas and almost completely silenced in stage I adenocarcinomas. Furthermore, FXR expression was positively and negatively correlated to expression of collagen IV and vimentin, markers for mesenchymal cell phenotype (14),...
and negatively correlated to PI3K, cyclin E1, and other oncogenic signaling mediators (46). This implicates that FXR is either a strong marker for cell differentiation or that FXR regulates these signaling pathways.

Mutations within WNT signaling cascade have been widely accepted as a major initiating event in colon cancer development. Our analysis showed that increased β-catenin nuclear localization was observed in only 22.2% of the polyps and adenomas we analyzed, compared with 66% (6 of 9) polyps having decreased FXR expression. The direct association between β-catenin activation and FXR silencing remains unclear and larger sample size and detailed analysis will be needed in the future to determine the degree of association.

Tumor suppressor genes can be silenced in cancer cells through acquired silencing mutations, epigenetic mechanisms, or transcriptional silencing through oncogenic signaling. We detected no genetic mutations within the FXR gene in colon cancer cell lines or clinical colon tumors (data not shown) that could account for the silencing and TCGA data support that genomic alterations of the FXR gene are a rare event in colon cancer. However, inhibition of DNMT 1/3B activity increased FXR mRNA levels in malignant colon cancer cell lines suggesting DNA methylation is a contributing mechanism for FXR silencing in colon cancer. We confirmed functional methylation of the FXR promoter within colon cancer cell lines and

Table 5. Frequency of NR1H4 promoter methylation with different colon tumor subtypes

<table>
<thead>
<tr>
<th>Tumor Subtype</th>
<th>NR1H4 meth β &gt; 0.6</th>
<th>NR1H4 meth β &lt; 0.6</th>
<th>Frequency</th>
<th>Fisher Exact Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>26</td>
<td>210</td>
<td>0.124</td>
<td></td>
</tr>
<tr>
<td>KRAS wt</td>
<td>15</td>
<td>113</td>
<td>0.682</td>
<td>0.37</td>
</tr>
<tr>
<td>KRAS mut</td>
<td>7</td>
<td>84</td>
<td>0.574</td>
<td></td>
</tr>
<tr>
<td>N/A</td>
<td>4</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSH-H</td>
<td>6</td>
<td>25</td>
<td>0.231</td>
<td>0.23</td>
</tr>
<tr>
<td>MSH-L</td>
<td>18</td>
<td>148</td>
<td>0.120</td>
<td></td>
</tr>
<tr>
<td>MSS</td>
<td>2</td>
<td>36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N/A</td>
<td>0</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIMP-H</td>
<td>6</td>
<td>30</td>
<td>0.231</td>
<td>0.76</td>
</tr>
<tr>
<td>CIMP-L</td>
<td>7</td>
<td>46</td>
<td>0.143</td>
<td></td>
</tr>
<tr>
<td>Cluster3</td>
<td>8</td>
<td>69</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cluster4</td>
<td>5</td>
<td>65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N/A</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypermut</td>
<td>5</td>
<td>29</td>
<td>0.227</td>
<td>0.35</td>
</tr>
<tr>
<td>Nonhypermut</td>
<td>17</td>
<td>168</td>
<td>0.147</td>
<td></td>
</tr>
<tr>
<td>N/A</td>
<td>4</td>
<td>13</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

meth, Methylation; wt, wild-type, mut, mutated; N/A, not applicable; MSH-H, microsatellite instability-high; MSH-L, microsatellite instability-low; MSS, microsatellite stability; CIMP-H, CpG island methylator phenotype-high; CIMP-L, CpG island methylator phenotype-low; hypermut, hypermethylated; Nonhypermut, nonhypermethylated.

Fig. 7. FXR and small interfering KRAS (siKRAS) expression in human colon cancer cell lines and the predicted role of FXR in colon cancer development. A: relative mRNA levels of FXR in colon cancer cell lines treated with siKRAS. B: relative mRNA levels of KRAS in colon cancer cell lines confirming knockdown of KRAS. Data are expressed as means ± SE. *P < 0.05 compared with nontargeting (NT) siRNA controls. Hyper-Me, hypermethylation. C: colon cancer is initiated by an acquired mutation within genes involved in WNT signaling (APC or β-catenin) or DNA repair signaling. Promotion to an adenoma often occurs through CpG island hypermethylation and acquired KRAS mutations (22). Our results indicate that FXR is also silenced during this early period of adenoma formation and correlates with EMT and oncogenic signaling of PI3K, suggesting that FXR silencing contributes to colon cancer progression and/or metastasis. If FXR expression is restored by inhibition of DNA methylation or KRAS signaling and activated by synthetic FXR ligands, this may help restore normal cancer cell phenotype, slow cancer progression, and/or sensitize cancer cells to chemotherapy.
detected full methylation of FXR promoter in 12% of clinical colon tumors.

DNA methylation is clearly not the only mechanism of FXR silencing in colon tumors since 80–90% of tumors still have low levels of FXR expression in the absence of FXR promoter methylation. RPPA analysis revealed that PI3K signaling, a common molecular feature of colon cancer (46), was negatively correlated with FXR expression and could also play a role in FXR silencing. However, PI3K isogenic cell lines had no difference in basal expression of FXR. Conversely, silencing of KRAS signaling, another well-known oncogenic event in colon cancer (1), in multiple colon cancer cell lines increased FXR expression. Sequence analysis of the FXR promoter by use of MATCH 1.0 [which utilized TRANFAC Public 6.0 (24)] revealed a predicted binding site for activator protein 1 (AP-1; composed of c-Jun and c-Fos heterodimer), a transcriptional mediator of the c-Jun NH2-terminal kinase signaling pathway. This pathway can be involved in RAS-initiated tumor formation (3). Thus it is possible that AP-1 mediates the effects of KRAS and potentially other signaling pathways for FXR expression in colon cancer.

TCGA data showed no molecular subtype, including FXR promoter methylation and KRAS mutation correlated with FXR expression. However, our results show that nearly all colon tumors, regardless of stage, had low to no FXR expression, illustrating the difficulty of detecting a molecular causal relationship. FXR promoter methylation was higher in MSI-H and hypermutated tumors and lower in KRAS mutant tumors. Hypermutated tumors often segregate with MSI-H tumors whereas KRAS tumors segregate with MSI-low and nonhypermutated (4, 35). Thus an inverse methylation pattern of FXR, higher methylation in MSI-H tumors and lower methylation in KRAS mutant tumors, suggests two distinct mechanisms of FXR silencing: 1) DNA methylation in MSI-H and 2) KRAS signaling in MSI-low tumors. No difference in FXR promoter methylation was detected between CIMP and non-CIMP tumors.

In conclusion, we have shown that FXR was downregulated very early in human colon cancer development, which was partly due to DNA methylation of the FXR promoter and increased KRAS signaling (Fig. 5B). Silencing of FXR alone is not sufficient to initiate colon cancer development, but activation of remnant FXR in healthy tissues may play an important role in preventing and inhibiting the promotion of colon cancer (21, 25, 44). Restoration of basal FXR expression through inhibition of DNA methylation or KRAS signaling, or through activation of residual FXR, might slow or prevent the progress of colon cancer either through direct antiproliferative or chemopreventative mechanisms. There are known FXR agonists, such as GW4064 and 6α-ethylchenodeoxycholic acid (6E-CDCA), that are currently in preclinical and clinical development for metabolic disorders (20, 29). It is conceivable that in situations where FXR is not completely absent these agents might be able to restore lost FXR activity in colon cancer resulting in inhibited tumor growth.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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