Farnesoid X receptor represses matrix metalloproteinase 7 expression, revealing this regulatory axis as a promising therapeutic target in colon cancer

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The farnesoid X receptor (FXR) is a member of the nuclear receptor superfamily of bile acid–activated transcription factors and an important regulator of cell proliferation, apoptosis, and Wnt signaling. Down-regulated expression of FXR plays an important role in some malignancies such as colon cancer, and in rodent models of intestinal neoplasia, FXR knockout increases the size and number of colon tumors. These previous observations implicate FXR as a tumor suppressor, but the underlying molecular mechanisms are unclear. Employing complementary experimental approaches and using human colon cancer specimens, human and murine colon cancer cell lines, and FXR transgenic mice, here we identified an additional, potentially important role for FXR. We observed an inverse relationship between the expression of FXR and matrix metalloproteinase-7 (MMP7), a collagenase and signaling molecule consistently associated with colon cancer progression. We noted that *FXR* gene ablation increases MMP7 expression. Consistent with this finding, FXR overexpression and a dominant-negative FXR mutation reduced and augmented, respectively, MMP7 expression. Of note, *MMP7* was the only *MMP* gene family member whose expression was down-regulated after FXR activation. FXR-mediated regulation of *MMP7* transcription did not require heterodimerization with the retinoid X receptor (RXR), indicating that FXR represses *MMP7* expression independently of RXR. Last, we uncovered that FXR suppresses *MMP7* transcription by binding to a negative FXR-responsive element in the 5′ *MMP7* promoter, an event that inhibited colon cancer cell proliferation and invasion. These findings identify the *FXR-MMP7* axis as a potential therapeutic target for managing colon cancer.

The farnesoid X receptor (FXR;[2] encoded by *NR1H4*), a member of the nuclear receptor superfamily of ligand-activated transcription factors, is expressed at high levels in the mammalian liver and intestines. An important function of FXR is as a bile acid sensor that regulates feedback repression of hepatic bile acid synthesis (1–3). As a homodimer or heterodimer with the retinoid X receptor (RXR), FXR binds to the promoter regions of several genes involved in bile acid synthesis and transport, thereby regulating their expression and actions as well as modulating cholesterol and triglyceride metabolism. A key mechanism whereby FXR mediates feedback suppression of bile acid synthesis is by repressing the expression of cholesterol 7α-hydroxylase (CYP7A1), the rate-limiting enzyme in the metabolic pathway from cholesterol to bile acids.

In addition to naturally occurring bile acids (most potently chenodeoxycholic acid (CDCA; EC_{50} = 10–50 μM)) (4), more potent FXR agonists were developed (*e.g.* GW4064 (EC_{50} = 15 nM) (5), 6E-CDCA (EC_{50} = 99 nM) (6), and WAY-362450 (EC_{50} = 4 nM) (7)). The United States Food and Drug Administration approved a highly potent synthetic FXR agonist, obeticholic acid (EC_{50} = 99 nM), for the treatment of primary biliary cholangitis, a progressive disease associated with increased circulating and hepatic bile acid levels. FXR antagonists were also identified, including the plant-derived molecule guggulsterone (IC_{50} = 15 μM) (8) and a synthetic molecule, AGN34 (IC_{50} < 10 nM) (9). These agents provide useful experimental tools to parse the actions of FXR.

In addition to its essential role in regulating bile acid synthesis and lipid metabolism, emerging evidence supports an important role for FXR as an intestinal tumor suppressor (10). Compared with adjacent normal epithelium, colon adenomas express reduced levels of FXR; FXR levels are even further reduced in colon adenocarcinomas (11, 12). Diminished FXR expression in colon cancer is associated with advanced tumor stage and a worse prognosis (12, 13). In mouse models of colon

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This article contains Fig. S1.

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[2] The abbreviations used are: FXR, farnesoid X receptor; CDCA, chenodeoxycholic acid; 6E-CDCA, 6-ethylchenodeoxycholic acid; RXR, retinoid X receptor; MMP, matrix metalloproteinase; EGF, epidermal growth factor; EGFR, EGF receptor; QPCR, quantitative real-time PCR; IHC, immunohistochemistry; KO, knockout; IBABP, ileal bile acid–binding protein; FXRE, FXR-responsive element; EMSA, electrophoretic mobility shift assay; MNase, micrococcal nuclease; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ANOVA, analysis of variance; HSD, honestly significant difference.

neoplasia, FXR deficiency results in increased adenoma size and number (14, 15). Although these findings strongly implicate FXR as a tumor suppressor, the underlying molecular mechanisms are uncertain.

The 28-member matrix metalloproteinase (MMP) family is composed of zinc-dependent endopeptidases (collagenases, stromelysins, and gelatinases) that are secreted or membrane-bound proenzymes activated by proteolytic cleavage (16, 17). Besides degrading components of the extracellular matrix, including collagens and gelatins, MMPs, including MMP7, may regulate the release of cell surface molecules, including growth factors, growth factor receptors, cytokines, and cell adhesion molecules (18). Experimental data from several laboratories support a pivotal role for MMP7 in promoting cell proliferation and the development of intestinal tumors (19). Prenzel *et al.* (20) showed that MMP7 activity is critical for cross-talk between G protein–coupled and epidermal growth factor receptors (EGFRs).

In human colon cancer cells, we found that MMP7 mediates transactivation of EGFR by catalyzing the release of heparin-binding EGF-like growth factor, one of seven known EGFR ligands (21). In mouse models, Wilson *et al.* (22) showed that MMP7 deficiency reduces intestinal tumor formation. Others used animal models to show that MMP7 expression and activation is an important contributor to colon cancer metastasis (23–25). Notably, the stage and prognosis of human colon cancer correlate with increased MMP7 expression (26, 27), the inverse of what is reported for FXR expression (12, 13).

Previously, we reported that Src-mediated cross-talk between FXR and EGFR modulates ERK phosphorylation, providing one mechanism whereby FXR may regulate cell proliferation and tumorigenesis (28). Now, based on the wide range of transcriptional targets for FXR and the findings summarized above, primarily the inverse actions of FXR and MMP7 on colon neoplasia, we hypothesized that FXR directly regulates MMP7 expression. Herein, we report experimental findings supporting this hypothesis.

Results

Correlation between reduced levels of FXR and increased MMP7 expression in colon cancer

Lian *et al.* (29) used gene microarray to show that treating human AGS gastric cancer cells with CDCA substantially reduced levels of MMP7 mRNA. Nonetheless, the molecular basis of this FXR-mediated action and whether it extended to other types of gastrointestinal cancer were unexplored. To fill these gaps in knowledge and pursue this interesting observation, we examined the correlation between FXR and MMP7 expression in paired, fresh-frozen surgical specimens of colon cancer and adjacent normal colon tissues obtained from the same persons.

Using quantitative real-time PCR (QPCR) to measure mRNA and immunoblotting to measure protein levels, we detected an apparent inverse relationship between FXR and MMP7 gene and protein levels. Both mRNA (Fig. 1*A*) and protein (Fig. 1*B*) levels of FXR were substantially lower in tumors compared with normal mucosae obtained from the same patients. In contrast, compared with normal mucosae from the same patients, MMP7 mRNA and protein levels were substantially greater in the same tumors (Fig. 1, *A* and *B*). Moreover, when tumor levels of MMP7 protein were elevated, we detected overexpression of both pro-MMP7 (latent form) and cleaved MMP7 (active form) (Fig. 1*B*; this is most evident in cancer tissue samples from patients 2 and 3 that had the highest levels of MMP7 mRNA and protein expression). To measure the relationship between FXR and MMP7, we performed a correlation analysis. As shown in Fig. 1 (*C* and *D*), coefficients of determination for FXR *versus* MMP7 mRNA and protein were negatively correlated. Due to intratumor heterogeneity, we were not surprised to find small differences between mRNA and protein coefficients. Reassuringly, in control experiments, mRNA levels of *MMP1*, *MMP10*, and the retinoid x receptor (*RXR*) in tumor tissues compared with normal mucosa were not significantly different ($p > 0.05$; Fig. 1, *E–G*). These findings suggested a specific relationship between FXR and MMP7 expression levels.

To pursue these *in vivo* findings, we examined the relationship between levels of FXR and MMP7 expression in several human CRC cell lines, including HT-29 cells that were stably transfected with human FXR. In each tested cell line, we detected an inverse relationship between FXR and MMP7 expression (Fig. 1*H*).

To provide further evidence of an inverse relationship between FXR and MMP7 expression in colon cancer, we performed immunohistochemistry (IHC) using an additional 10 sets of paraffin-embedded formalin-fixed cancers, adjacent normal mucosae, and liver metastases from the same individuals with stage IV colon cancer. A senior gastrointestinal pathologist (C. B. D.) masked to experimental details assessed staining intensity (see "Experimental procedures"). As shown in Fig. 2 (*A* and *B*), IHC revealed significantly diminished FXR expression in primary and liver lesions. In contrast, MMP7 protein levels were increased significantly in primary and metastatic tumors (Fig. 2, *A* and *C*). These findings provide further evidence of an inverse relationship between FXR and MMP7 expression, consistent with a role for FXR as a potential regulator of *MMP7* gene transcription.

FXR gene ablation increases MMP7 expression

To determine whether ablation of *FXR* increased MMP7 expression *in vivo*, we measured MMP7 expression in FXR knockout mice. As shown in Fig. 3, we detected increased MMP7 protein and mRNA levels in intestinal tissues and liver homogenates from FXR knockout (KO) mice (B6.129X1(FVB)-Nr1h4^{tm1Goz}/J). Compared with tissues from WT mice, MMP7 protein levels were robustly increased in intestinal tissues (Fig. 3, *A* and *B*) and liver homogenates (Fig. 3, *C* and *D*) from FXR KO mice. Both pro-MMP7 (latent form) and cleaved MMP7 (active form) were increased in tissues from FXR KO mice (Fig. 3*A*). In addition, *MMP7* mRNA levels were increased in intestinal tissues from FXR KO mice (Fig. 3*E*). Collectively, these findings supported an inverse relationship between FXR and MMP7 expression.

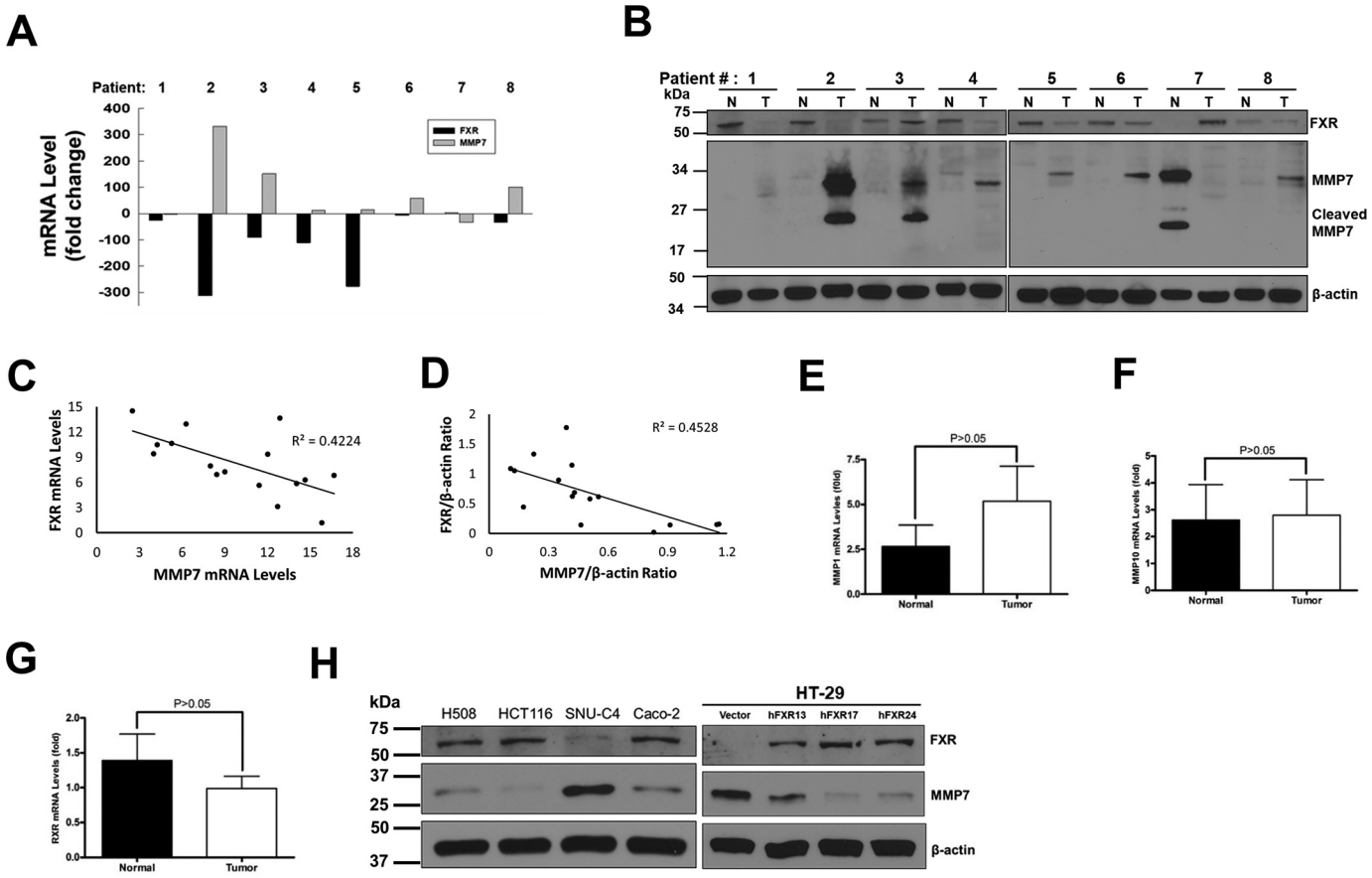

Figure 1. Inverse relationship between FXR and MMP7 mRNA and protein levels. *A*, mRNA levels were determined by QPCR in eight sets of paired human surgical specimens of colon tumors and adjacent normal mucosae. See "Experimental procedures." *B*, protein levels were determined by immunoblotting in the same eight sets of paired human surgical specimens of colon tumors and adjacent normal mucosae. *C* and *D*, the levels of FXR mRNA (*C*; $n = 16$) and protein (*D*; $n = 16$) were negatively correlated with the levels of MMP7 mRNA ($p = 0.0372$) and protein ($p = 0.0306$). *E–G*, mRNA levels of *MMP1* (*E*), *MMP10* (*F*), and *RXR* (*G*) in paired human CRC surgical specimens ($n = 10$) were not significantly different in colon cancer *versus* adjacent normal tissue. *H*, protein levels of MMP7 and FXR in human CRC cell lines and HT-29 cells stably transfected with human FXR. We measured mRNA levels by QPCR. Values represent mean ± S.E. (*error bars*) Quantification of relative protein levels was performed by ImageJ software.

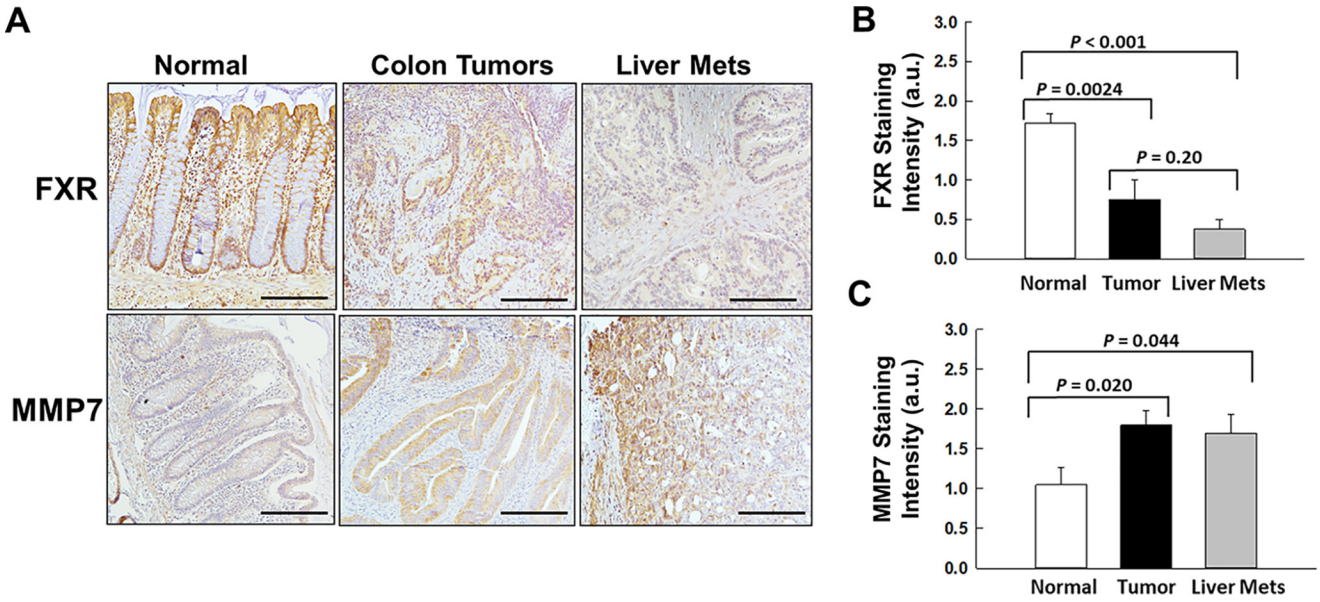

Figure 2. Negative correlation between FXR and MMP7 expression levels. *A*, immunohistochemical staining of paired human surgical specimens of adjacent normal mucosa, primary colon tumors, and metastatic liver lesions from the same person. *B* and *C*, graded staining intensities of FXR (*B*) and MMP7 (*C*) proteins in paired human surgical specimens of adjacent normal mucosa, primary colon tumors, and metastatic liver lesions from the same persons. Values represent means ± S.E. (*error bars*) from 10 samples. *a.u.*, arbitrary units. *Scale bars*, 50 μm.

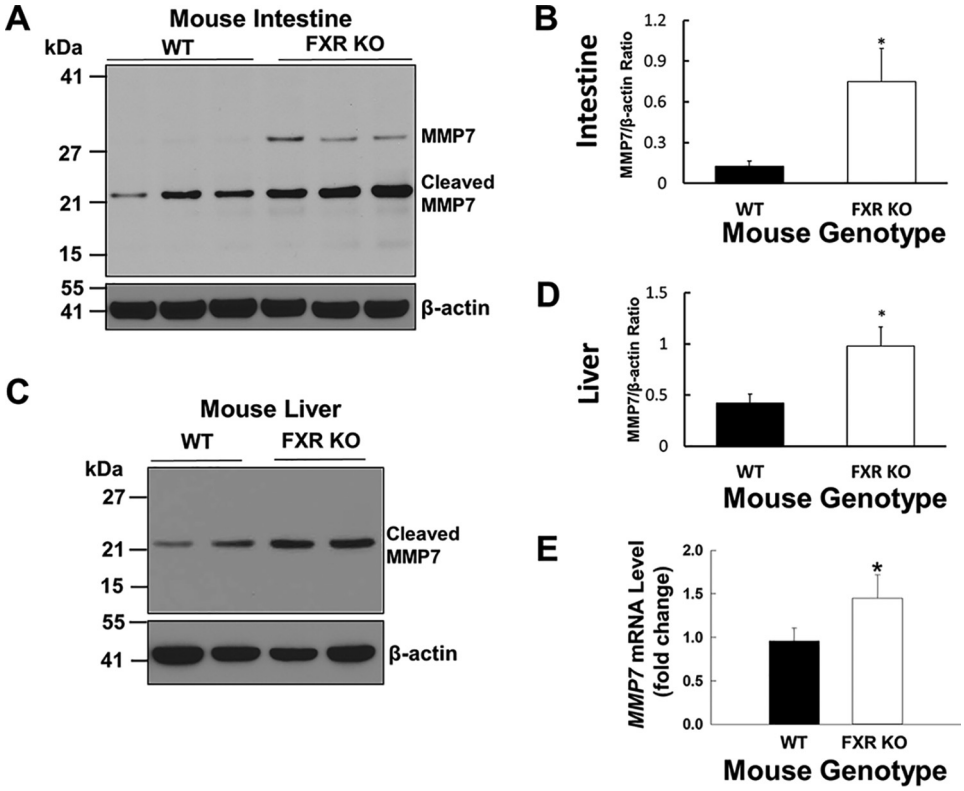

Figure 3. Increased MMP7 expression in FXR knockout mice. *A*, MMP7 immunoblots from intestines of WT and FXR KO mice ($n = 3$ each). *B*, MMP7/β-actin ratios in the mouse intestines ($n = 3$). *C*, MMP7 immunoblots from livers of WT and FXR KO mice ($n = 2$ each). *D*, MMP7/β-actin ratios in the mouse livers ($n = 3$). *E*, *MMP7* mRNA levels in the mouse intestines. mRNA and protein levels were determined by QPCR and immunoblotting, respectively. Quantification of relative protein levels was performed by ImageJ software. Band density was quantified and normalized to β-actin levels. Values represent means ± S.E. (*error bars*). *, $p < 0.05$ *versus* control.

FXR overexpression and dominant-negative FXR mutation alters MMP7 expression

When overexpressed, the W469A mutation in the FXR DNA-binding domain results in dominant-negative effects similar to those observed with *FXR* gene ablation (31). As described previously (28), after transfecting HT-29 human colon cancer cells with these plasmids, we successfully generated several stably transfected clones overexpressing either FXR or W469A. Overexpressing FXR in HT-29 cells (Fig. 4*A*) robustly reduced MMP7 protein (Fig. 4*A*) and mRNA levels (Fig. 4*B*) and diminished cell proliferation (Fig. 4*C*). In contrast, overexpressing dominant-negative FXR in HT-29 cells (Fig. 4*D*) increased MMP7 protein (Fig. 4*D*) and mRNA (Fig. 4*E*) levels and augmented cell proliferation (Fig. 4*F*). Moreover, as shown in Fig. 4*G*, using ELISA to measure MMP7 levels revealed that overexpressing FXR reduced the levels of secreted MMP7 in cell supernatants, whereas overexpressing dominant-negative FXR W469A increased levels of active MMP7 in supernatants. Moreover, siRNA knockdown of FXR in HT-29 cells increased MMP7 protein expression (Fig. 4*H*). As a negative control, siRNA knockdown of *MMP7* in HT-29 cells did not alter FXR expression (Fig. 4*I*).

To determine whether FXR activation affects enzymatic activity, we performed casein zymography (32) (see "Experimental procedures") using HT-29 whole-cell lysates and supernatants. As a negative control, we used *MMP7* siRNA-transfected HT-29 cells with drastically reduced MMP7 expression (Fig. 4*I*). As shown in Fig. 4*J*, activation of FXR with CDCA dose-dependently reduced hydrolysis of casein, providing direct evidence of reduced MMP7 enzymatic activity in both HT-20 whole-cell lysates (Fig. 4*J*, *left*) and HT-29 cell media (Fig. 4*J*, *right*). As an additional demonstration that activating FXR reduces active MMP7, we used ELISA to measure active secreted MMP7 in cell media. As shown in Fig. 4*K*, treating HT-29 cells with the FXR agonist CDCA significantly reduced levels of active MMP7 in cell supernatants.

Last, we used xenograft models in nude mice to explore whether FXR overexpression *in vivo* increased MMP7 expression (28). We found that MMP7 protein levels were strikingly reduced in xenografts derived from HT-29 cells stably transfected with human FXR (Fig. 4*L*). Collectively, the experiments shown in Fig. 4 provide compelling evidence that FXR expression governs MMP7 expression in colon cancer.

FXR activation down-regulates MMP7 gene expression at the transcriptional level

To explore the mechanism underlying this regulation of MMP7 by FXR, we examined the effects of FXR activation on MMP7 in human colon cancer cell lines. In HT-29 cells, the FXR agonist CDCA dose-dependently decreased MMP7 mRNA (Fig. 5*A*) and protein (Fig. 5*B*) levels. Likewise, FXR activation with another agonist, GW4064, also dose-dependently reduced MMP7 mRNA (Fig. 5*C*) and protein levels (Fig. 5*D*). In contrast to the findings in HT-29 cells, in SW620 human

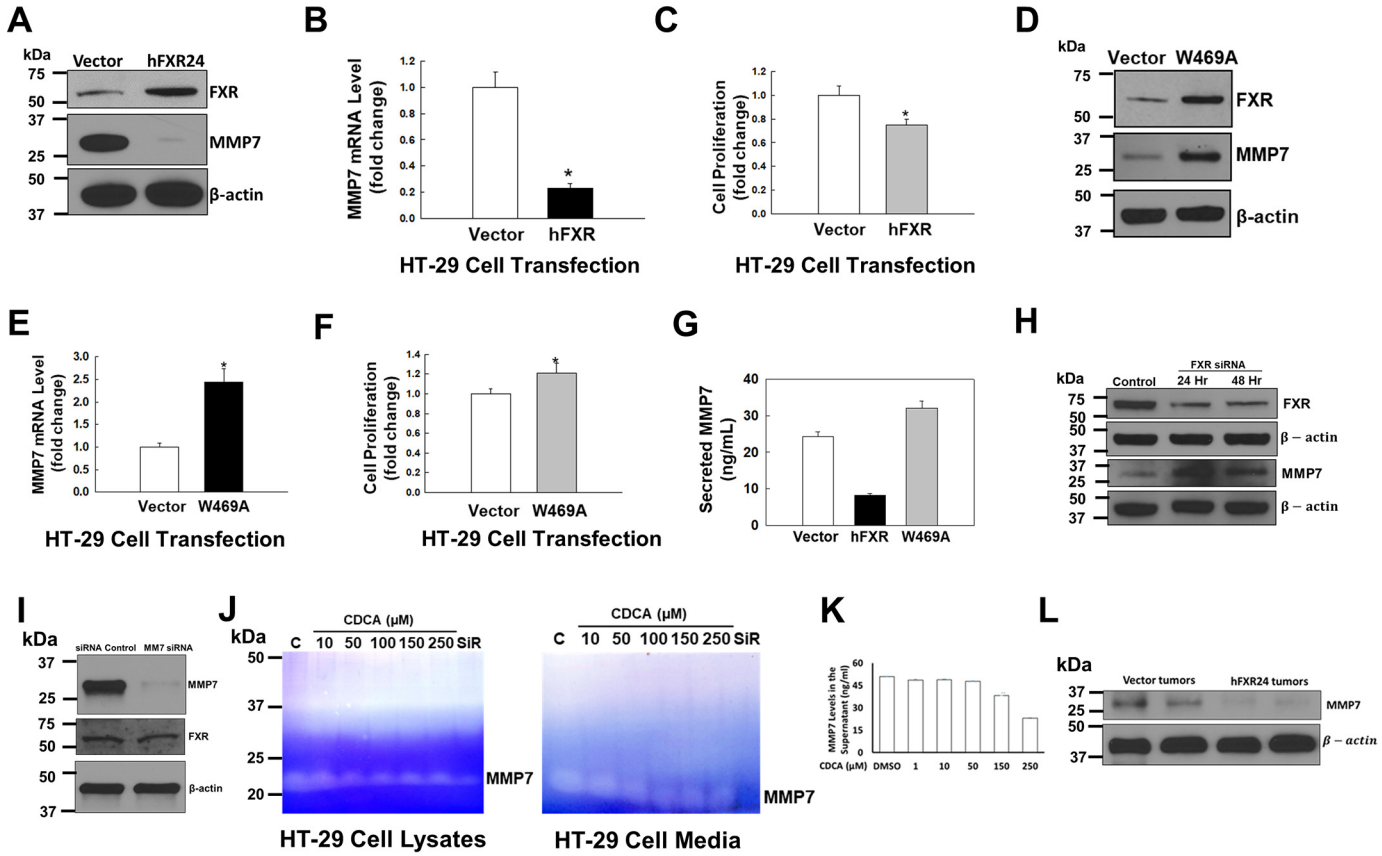

Figure 4. Effects of FXR overexpression or inhibition on *MMP7* gene expression. *A–C*, FXR and MMP7 protein (*A*), *MMP7* mRNA (*B*), and cell proliferation (*C*) in HT-29 cells stably transfected with human full-length FXR. *D–F*, FXR and MMP7 protein (*D*), *MMP7* mRNA (*E*), and cell proliferation (*F*) in HT-29 cells stably transfected with human dominant-negative mutant FXR W469A. *G*, effect of CDCA on secreted MMP7 levels in vector-transfected HT-29 cells or FXR-overexpressed or W469A-overexpressed HT-29 cells. *H*, FXR and MMP7 protein levels from FXR siRNA-transfected HT-29 cells for 24 or 48 h. *I*, MMP7 and FXR protein levels from *MMP7* siRNA-transfected HT-29 cells for 24 h. *J*, dose responses in HT-29 cells for CDCA-induced reduction in MMP7 activity measured in whole-cell lysates (*left*) or supernatants (*right*) using casein zymography. *MMP7* siRNA (*SiR*)-transfected HT-29 cells were used a negative control. *K*, active (secreted) MMP7 levels in the supernatants of HT-29 cells treated with CDCA measured by ELISA. *L*, MMP7 protein levels in xenografts generated from either vector alone or human full-length FXR. Values represent means ± S.E. (*error bars*) from three experiments. For *B*, *C*, *E*, and *F*, *, $p < 0.05$ *versus* vector alone or DMSO control in unpaired *t* tests. One-way ANOVA identified significant variance in *G* ($p < 0.001$) and *K* ($p < 0.001$). Tukey's HSD post hoc tests showed significant differences ($p < 0.05$) between any two groups except 1 μM *versus* 10 μM, 1 μM *versus* 50 μM, and 10 μM *versus* 50 μM.

colon cancer cells that lack FXR expression (Fig. S1*B*), treating cells with CDCA and GW4064 did not alter MMP7 mRNA or protein levels (Fig. 5, *E–H*). As a positive control, in HT-29 cells, the expression of an FXR target gene, ileal bile acid–binding protein (IBABP), was dose-dependently up-regulated by the FXR agonists CDCA (Fig. 5*I*) and GW4064 (Fig. 5*J*). In contrast, in SW620 cells, CDCA (Fig. 5*K*) and GW4064 (Fig. 5*L*) did not alter levels of IBABP mRNA levels. Moreover, CDCA and GW4064 did not alter *MMP1* or *MMP10* mRNA levels in HT-29 cells (Fig. S1, *C–F*), suggesting that this action of FXR is specific to *MMP7*.

As the active form of MMP7 is largely secreted into the culture media, we detected almost exclusively pro-MMP7 (latent form) after immunoblotting HT-29 cell lysates. Adding guggulsterone, a specific FXR antagonist, abolished FXR agonist-induced repression of MMP7 expression (Fig. S1*A*). Taken together, these results are compatible with the hypothesis that FXR represses MMP7 expression at the transcriptional level.

To investigate whether FXR alters mRNA stability, we treated HT-29 cells with actinomycin D (1 μg/ml) and used QPCR to measure whether CDCA altered MMP7 levels. As shown in Fig. 5*M*, CDCA treatment did not alter *MMP7* mRNA levels in human colon cancer cells (half-life of ~16 h) (33). This result indicates that activating FXR does not alter the stability of *MMP7* mRNA and supports the conclusion that FXR acts as a transcriptional repressor for MMP7.

FXR regulation of MMP7 transcription does not require RXR

FXR can bind to its target promoters as either a homo- or heterodimer with another nuclear factor, RXR. To determine whether RXR is required for the actions of FXR as a repressor of MMP7 expression, we treated cells with three RXR antagonists, K00083, HX531, and UVI3003. None of these RXR antagonists altered the effects of CDCA on MMP7 expression (Fig. 5, *N* and *O*). Based on these findings, we conclude that FXR most likely binds to the MMP7 promoter as a homodimer and does not require RXR. Our finding that RXR agonists (retinoid acid and SR11237) did not reduce MMP7 expression (Fig. S1*G*) supports this conclusion.

MMP7 is the only MMP gene down-regulated by FXR activation

To determine whether FXR down-regulates other *MMP* genes, we examined the effects of activating FXR in HT-29 cells

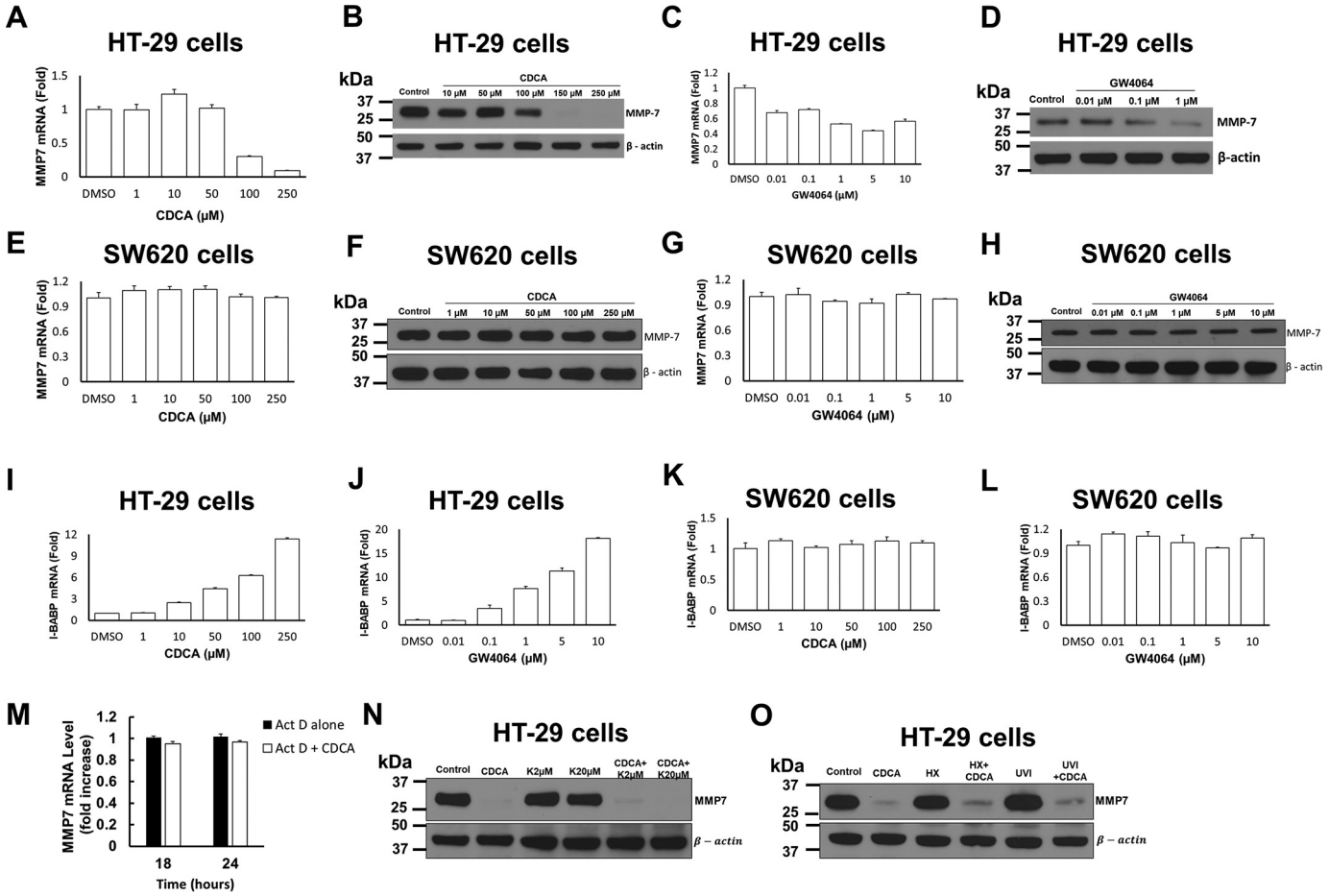

Figure 5. Effects of FXR activation on MMP7 expression. *A* and *B*, dose responses in HT-29 cells for CDCA-induced reduction in *MMP7* mRNA (*A*) and protein (*B*). *C* and *D*, dose responses in HT-29 cells for GW4064-induced decrease in *MMP7* mRNA (*C*) and protein (*D*). *E* and *F*, absence of dose response for CDCA-induced changes in *MMP7* mRNA (*E*) and protein (*F*) levels. *G* and *H*, absence of dose response for GW4064-induced changes in *MMP7* mRNA (*E*) and protein (*F*) levels. *I* and *J*, dose responses in HT-29 cells for CDCA-induced (*I*) or GW4064-induced (*J*) increases in *IBABP* mRNA. *K* and *L*, absence of dose response in SW620 cells for CDCA-induced (*K*) and GW4064-induced (*L*) changes in *IBABP* mRNA levels. *M*, *MMP7* mRNA levels with actinomycin D (1 μg/ml) treatment in the presence or absence of 200 μM CDCA. *N* and *O*, effects of RXR inhibitor K00083 (*N*), HX531 (*O*), and UVI3003 (*O*) on CDCA (200 μM)-induced decrease of MMP7 protein level in HT-29 cells. We measured mRNA and protein levels by QPCR and immunoblotting, respectively. We harvested cells at 6 h (for *IBABP* mRNA) and 24 h (for *MMP7* mRNA and immunoblotting) after the addition of test agents. Values represent mean ± S.E. (*error bars*) from three experiments. One-way ANOVA analysis: $p < 0.0001$ (*A*), $p < 0.0001$ (*C*), $p < 0.0001$ (*D*), $p < 0.0001$ (*F*), $p = 0.2263$ (*G*), $p = 0.1380$ (*H*), $p = 0.2661$ (*J*), and $p = 0.0899$ (*L*). Tukey's HSD post hoc tests showed significance ($p < 0.05$) between any two groups in *A*, *C*, *I*, and *J*, except 1 μM *versus* DMSO, 10 μM *versus* DMSO, 50 μM *versus* DMSO, 10 μM *versus* 1 μM, 50 μM *versus* 1 μM, and 50 μM *versus* 10 μM (in *A*); 0.1 μM *versus* 0.01 μM, 10 μM *versus* 0.01 μM, and 10 μM *versus* 1 μM (in *C*); 1 μM *versus* DMSO (in *I*); and 0.01 μM *versus* DMSO (in *J*).

on mRNA levels for all of the known *MMP* genes. Of 28 known *MMP* genes, HT-29 cells only expressed *MMP1*, *-7*, *-8*, *-9*, *-10*, *-14*, *-19*, *-21*, *-26*, and *-28* at levels that were reliably detected by QPCR. Activating FXR did not alter the expression levels of any of these genes (Fig. S1*H*). In addition, activating FXR did not alter the expression of *Myc*, *COX2*, or cyclin D1 (*CCND1*) (Fig. S1*H*). These data provide strong evidence that FXR is a specific transcriptional repressor of *MMP7*.

Absence of traditional MMP7 FXR-responsive elements (FXREs)

Traditional nuclear hormone receptor–binding sequences consist of 6-bp half-sites, arranged as direct, inverted, or everted repeats. Several computational tools, including NHR-Scan, can be used to predict *in silico* the presence of nuclear hormone receptor–binding sequences in the human genome (34). To identify possible FXR-binding elements in the *MMP7* promoter, we performed NHR-Scan (http://www.cisreg.ca/cgi-bin/NHR-scan/nhr_scan.cgi)[3] using the 5.0-kb *MMP7* promoter sequence. The scan revealed seven potential traditional FXREs in the *MMP7* promoter upstream of the transcription start site. However, ChIP analyses of these sites in HT-29 cells did not reveal enhanced binding by FXR, suggesting that these were not active sites (Fig. S1*I*)

Identification of a nontraditional FXR-binding element in the MMP7 promoter

In addition to traditional FXREs, nontraditional negative FXREs are increasingly recognized, the majority located in the 5′ promoter region (35). To seek a potential nontraditional FXR-binding element on the *MMP7* promoter, we performed analyses using *MMP7* promoter-luciferase constructs con-

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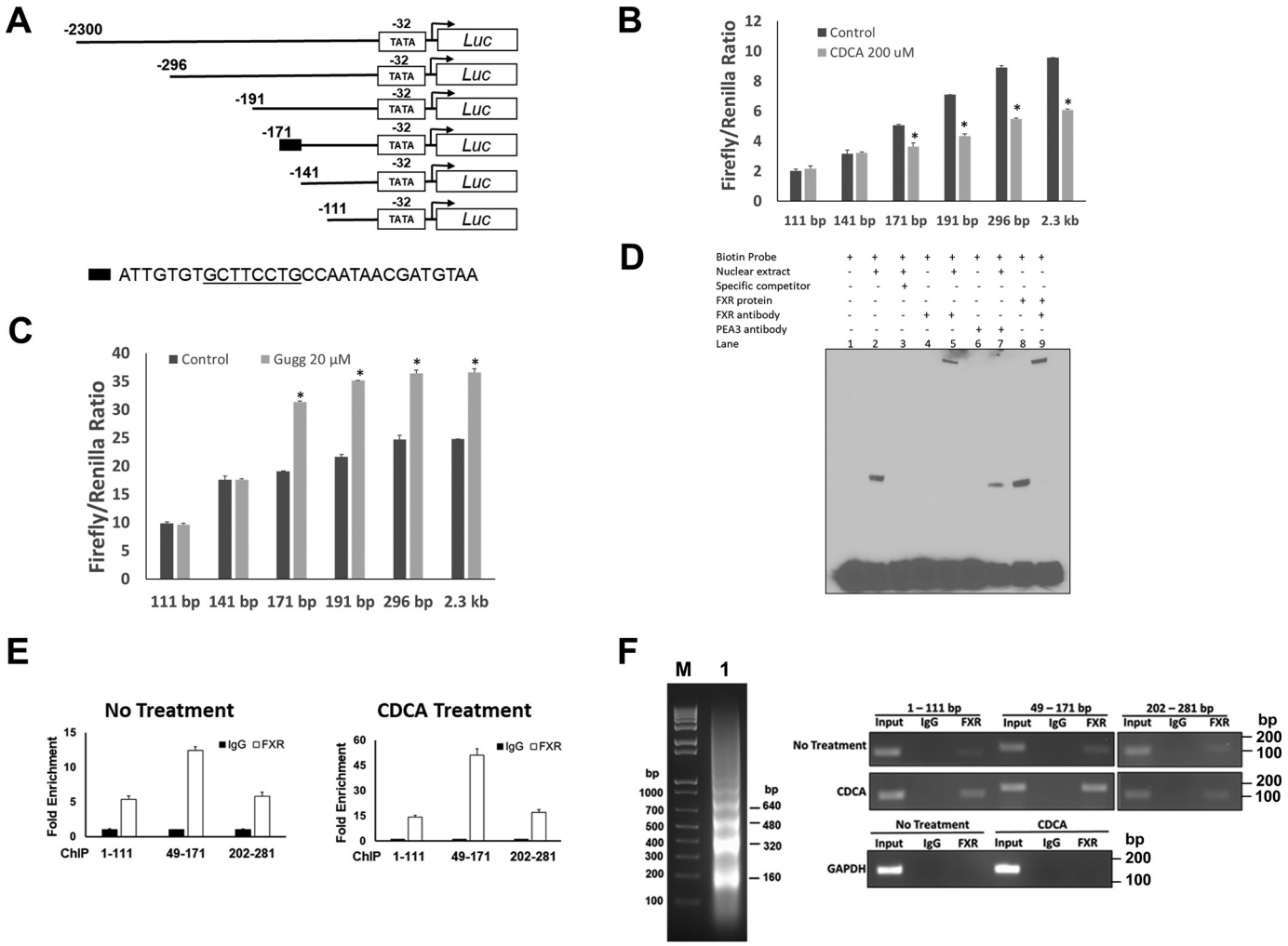

Figure 6. Negative FXRE in MMP7 promoter. *A*, *schematic* of the MMP7 promoter constructs and the negative response element marked by the *small black rectangle*. The PEA-3–binding sequence is *underlined*. *B*, luciferase assay of MMP7 promoter with CDCA (200 μM) treatment in HT-29 cells. *C*, luciferase assay of MMP7 promoter with guggulsterone treatment in HT-29 cells. CDCA was added to cells (for an additional 24 h) transiently transfected for 24–48 h with MMP7-luc plasmids with control pGL4.14-Renilla luciferase vectors. *D*, we performed EMSA using 3′-biotin-labeled probes and nuclear extracts from HT-29 cells. *E*, ChIP assay using random DNA fragments generated by MNase digestion in HT-29 cells without CDCA treatment or after treatment with 200 μM CDCA for 24 h. *F* (*left*), fragmentated genomic DNA (*M*, molecular marker; *lane 1*, DNA after MNase digestion (see "Experimental procedures") from HT-29 cells. *Right*, agarose gel images of QPCR products from ChIP assays using MNase-digested DNA. GAPDH was used as a control. *MMP7* promoter positions and DNA sequences of QPCR primers are listed in Table 1. *, $p < 0.05$ *versus* control. *Error bars*, S.E.

taining either 2.3 or 296 bp of *MMP7* promoter sequences upstream of the transcription start site (see "Experimental procedures"). We found this negative FXRE in HT-29 cells is located between 141 and 171 bp of the *MMP7* 5′ promoter (Fig. 6*A*). CDCA-induced MMP7 inhibition was absent when we reduced the size of the promoter sequence from 171 to 141 bp (Fig. 6*B*). The sequence between 141 and 171 bp is 5′-ATTGTGTGCTTCCTGCCAATAACGATGTAA. The increase in luciferase activity induced by the FXR antagonist, guggulsterone, requires the same 30-bp promoter element (Fig. 6*C*). Although the presence of antagonists alone should not increase gene expression, endogenous FXR agonists appear to maintain basal levels of FXR activation (36).

To determine whether human FXR can bind to this 30-bp negative *MMP7* FXRE, we performed gel shift assays. Using double-stranded 3′-end biotin-labeled probes in electrophoretic mobility shift assays (EMSAs) that contained this 30-bp fragment, we detected strong binding of the probe by both HT-29 nuclear extracts and recombinant human FXR protein, an effect abolished by specific competitors (Fig. 6*D*). Anti-FXR antibody caused a supershift of this complex, also abolished with the addition of a specific competitor. Although this 30-bp element also contains a PEA3-binding site (*i.e.* 5′-GCTTCCTG-3′) (Fig. 6*A*), anti-PEA3 antibody did not induce a supershift, suggesting that FXR binds to this 30-bp element and displaces PEA3.

Next, we used EMSAs to identify the minimal sequence required for FXR binding. Reduced-length sequences from this 30-bp element (16-bp oligonucleotide (5′-ATTGTGTGCTTCCTGC), 20-bp oligonucleotide (5′-ATTGTGTGCTTCCTGCCAAT, or 21-bp oligonucleotide 5′-TTCCTGCCAATAACGATGTAA)) failed to cause gel shifts with either recombinant human FXR or HT-29 nuclear extracts (data not shown). These results indicate that the entire 30-bp *MMP7* FXRE is required for FXR binding.

To confirm the EMSA results, we performed ChIP assays in HT-29 cells using endogenous FXR. As shown in Fig. 6

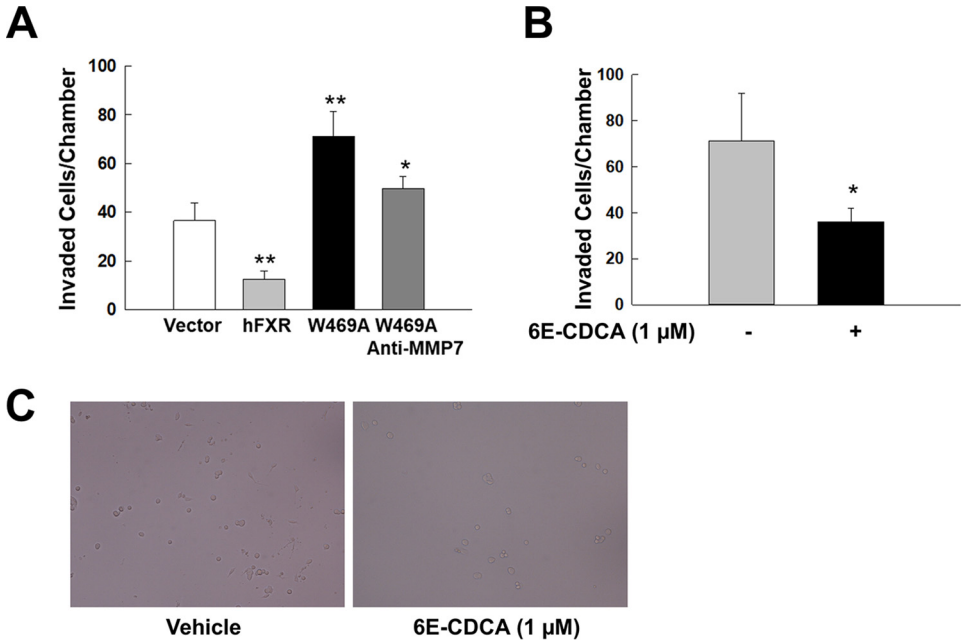

Figure 7. FXR overexpression inhibits colon cancer cell invasion. *A*, we performed Boyden chamber invasion assays using either HT-29 cells stably transfected with vector alone or FXR, FXR W469A, or FXR W469A treated with neutralizing anti-MMP7 antibody. *B*, Boyden chamber invasion assay using mouse colon cancer cell line MC38Luc1 with or without FXR activation by 6E-CDCA. *C*, representative microscopic images of invaded MC38Luc1 cells with and without FXR activation by 6E-CDCA (magnification, ×100). Values represent mean ± S.E. (*error bars*). *, $p < 0.05$ compared with W469A. **, $p < 0.05$ compared with control.

(*E* and *F*), in ChIP assays using chromatin generated by micrococcal nuclease (MNase) digestion that yielded genomic DNA fragments primarily between 160 and 480 bp in size, we detected enriched binding of endogenous FXR to the 49-171-bp region of the *MMP7* promoter; this was augmented by treatment with CDCA. We also detected enrichment, with and without CDCA treatment, in the regions from 1 to 111 bp and 202 to 281 bp, presumably due to sequences overlapping with the adjacent 141–171-bp region. These results are consistent with our previous conclusions from the luciferase assays and EMSAs that the 30-bp sequence from 141 to 171 bp represents the negative FXRE in the *MMP7* promoter.

Overexpressing FXR inhibits colon cancer cell invasion in vitro

To determine whether FXR activation or overexpression affects tumor cell invasion, a functional outcome involving MMP7, we performed collagen-based Boyden chamber cell invasion assays. Overexpressing FXR strongly inhibited HT-29 cell invasion (Fig. 7*A*). In contrast, overexpressing dominant-negative FXR W469A increased cell invasion (Fig. 7*A*). Likewise, as shown in Fig. 7 (*B* and *C*), FXR activation with an agonist, 6E-CDCA, inhibited invasion of the murine colon cancer cell line MC38. In addition, as shown in Fig. 7*A*, treating cells with neutralizing anti-MMP7 antibody significantly reduced the invasion of FXR W469A-transfected HT-29 cells, a finding that confirmed that MMP7 plays a role in FXR-dependent cell invasion.

Discussion

A strong body of epidemiological and animal data supports the concept that bile acids are risk factors for colon cancer. Colon cancer risk is also associated with the consumption of a so-called "Western diet," rich in carbohydrates and saturated fatty acids (37–39). Studies show that individuals consuming a Western diet and those with colon cancer both have elevated fecal levels of secondary bile acids, primarily lithocholic and deoxycholic acids (40–43). Provocative but controversial epidemiological studies identified a relationship between the increase in intestinal bile acid levels following cholecystectomy and an elevated risk of colon cancer; other findings dispute such an association (44, 45). Nonetheless, in rodent models of colon cancer, either instilling exogenous bile acids into the intestines or increasing the spillage of endogenous bile acids into the colon consistently promotes advanced neoplasia (46–48). Despite this incriminating body of literature, a clear understanding of the molecular mechanisms underlying the association between increased intestinal bile acid levels and colon neoplasia has remained elusive.

It seemed reasonable to assume studies would find that FXR, a master regulator of bile acid metabolism and transport, plays an important in bile acid-induced promotion of colon neoplasia. Instead, the preponderance of evidence suggests that FXR inhibits intestinal tumorigenesis by *bile acid–independent* mechanisms. For example, work by Degirolamo *et al.* (49) suggested that FXR deficiency, not elevated bile acid levels, mediates susceptibility to intestinal tumorigenesis; the tumor-promoting activity of bile acids was independent of their ability to activate intestinal FXR (15, 49). We and others showed that several bile acids, including native and conjugated deoxycholic acid and lithocholic acid, promote colon neoplasia by functional interaction with M3 muscarinic receptors, which are overexpressed in the majority of colon cancers; this mechanism is independent of FXR (48, 51–54). Overall, the prevailing evi-

dence favors the conclusion that bile acids have no direct bearing on the effects of FXR as an intestinal tumor suppressor, a role that is also independent of FXR acting as a regulator of bile acid metabolism.

Bile acid–independent action of FXR as a suppressor of intestinal tumorigenesis and progression appears to involve multiple signaling pathways (28, 55). In animal models, Modica *et al.* showed FXR activation promotes intestinal epithelial cell apoptosis and Wnt signaling (15). In previous *in vitro* and *in vivo* work, we showed that FXR activation reduced human colon cancer cell proliferation by inhibiting Src-mediated cross-talk between FXR and EGFR, which reduced ERK activation (28). In the present study, we employed multiple complementary experimental approaches and used colon cancer tissue, human and murine colon cancer cell lines, human xenografts, and an FXR transgenic mouse to identify a potentially important novel role for FXR. We found that FXR directly regulates MMP7 expression by acting as a transcriptional repressor.

Starting by consistently demonstrating the relationship between reduced FXR levels and increased MMP7 expression in colon cancer, we showed that *FXR* gene ablation increases MMP7 expression. FXR overexpression and dominant-negative FXR mutation reduced and augmented MMP7 expression, respectively. We also provided evidence that regulation of *MMP7* gene transcription by FXR does not require it to partner with RXR. Finally, MMP7 is the only member of the large *MMP* gene family down-regulated by activating FXR. Notably, in the course of these studies, we identified a novel nontraditional negative FXR-responsive element in the *MMP7* 5′ promoter.

Although transcriptional co-regulator complexes are often composed of both activators and co-repressors (56), others have shown that FXR acts as a transcriptional repressor via a distinct mechanism; FXR binds to negative FXREs as a monomer and competes with transcriptional activators to suppress transcription of multiple genes, including *ApoAI*, *ApoCII*, *OAT2*, and *UGT2B4* (57–60). Similarly, by binding to a PEA3-binding site, FXR transcriptionally represses *MMP7* expression by displacing the transcriptional activator PEA3. Collectively, these findings fill key gaps in knowledge regarding the mechanisms whereby FXR modulates colon cancer progression and affirm FXR as a promising therapeutic target in colon cancer.

Classical nuclear hormone receptor–binding sequences consist of repeats with the core sequence AGGTCA separated by one or more nucleotides. The traditional FXREs include direct repeats separated by *n* number of nucleotides (DR*n*), inverted repeats (IR*n*), and everted repeats (ER*n*). However, nontraditional FXRE promoter elements were also identified in several human genes (35); these include FXR-binding sites in the 5′ promoters of *ApoAI* and *UGT2B7* (35). In this study, we demonstrated a novel negative FXRE in the *MMP7* gene promoter. Although the majority of traditional FXREs are located in the 5′ promoter regions upstream from the transcription start sites (35), some FXREs are located in distant 5′ promoter region, in introns, or in the 3′ region (35). Although we did not identify traditional FXREs within the 5.0-kb *MMP7* 5′ promoter sequence, it is possible that additional FXREs exist in other regions of the *MMP7* gene.

It is noteworthy that FXR appears to regulate *MMP* gene expression differentially. In the liver, which predominantly expresses MMP2 and MMP9, but not MMP7, activating FXR in hepatic stellate cells increased MMP2 expression 2-fold, possibly by reducing levels of TIMP1 and TIMP2 (61). However, in gastric tissue, activating FXR down-regulated MMP7 expression (29). In our study, activating FXR in human colon cancer cells that express high levels of MMP7, but very low levels of MMP9 and MMP2, resulted in suppression of MMP7 expression and activity. Although the molecular mechanisms underlying this divergence in FXR-mediated *MMP* gene regulation are poorly understood, we speculate that it may be related to MMP7 being the only member of the MMP family that functions as both a signaling molecule/growth factor and as an enzyme.

Currently, there are limited therapeutic options for advanced colon cancer; although approaches targeting EGFR and vascular endothelial growth factors may be effective initially, their efficacy commonly wanes within months. These agents do not meaningfully alter 5-year survival rates (~10%) (62–64). Moreover, use of these agents is frequently limited by toxicity resulting from off-target side effects; EGFR is expressed widely in nonintestinal epithelial cells (*e.g.* dermal epithelial cells) (65). These comments highlight the urgent need for novel therapeutic approaches.

In addition to colon cancer, matrix metalloproteinases play important roles in the progression of a variety of solid tumors and have been considered potential therapeutic targets for at least 20 years (19, 66). Interest in targeting MMPs was diminished when several synthetic MMP inhibitors failed to improve colon cancer survival in late-stage clinical trials. Reappraisal of these trials attributed failure to nonselective targeting that also activated MMPs that promoted neoplasia; given the large MMP family and the myriad cell functions they impact, it is perhaps not surprising that some MMPs are tumor suppressors (67). Although recent headway was achieved in developing selective MMP inhibitors (*e.g.* MMP9) (68, 69), MMP7 is not presently a selectively druggable target.

Our finding of a novel molecular mechanism whereby FXR acts as a transcriptional repressor of MMP7 provides the rationale for an entirely new therapeutic approach to targeting MMP7 via the regulatory role of FXR. Selective FXR agonists with favorable safety profiles show great promise in treating chronic liver diseases. These include obeticholic acid (Ocaliva®), approved by the United States Food and Drug Administration in 2016 to treat primary biliary cholangitis (70), and EDP-305, successful in late-stage clinical trials to treat nonalcoholic steatohepatitis with liver fibrosis. Moreover, to minimize potential systemic toxicity, an intestine-specific FXR agonist, fexeramine, may prove to be a useful therapeutic agent specifically in colon cancer (71).

In conclusion, we report our finding that FXR transcriptionally represses MMP7 expression by interacting with a novel nontraditional negative FXR-responsive element in the *MMP7* 5′ promoter. Given the current limited options for treating advanced colon cancer, our findings identify the *FXR-MMP7* axis as a new promising therapeutic target. Because combination therapy is commonly required to prevent or treat cancer

effectively, identifying additional genes that interact with the *FXR-MMP7* axis is also warranted.

Experimental procedures

Materials

Chemicals—We purchased GW4064 from Calbiochem and all other chemicals from Sigma-Aldrich or Thermo Fisher Scientific.

Human samples—De-identified paraffin and frozen sections from surgically resected human colon cancers and adjacent normal colon tissue and metastatic liver lesions from the same persons were obtained from the Department of Pathology at the University of Maryland Medical Center. This study was approved by the institutional review board at the University of Maryland Baltimore and abides by the Declaration of Helsinki principles.

Cell lines and cell culture

Human colon cancer cell lines HT-29, SW620, HCT116, SNU-C4, Caco-2, and mouse colon cancer cell line MC38 were purchased from the American Type Culture Collection (ATCC) and maintained in growth media supplemented with 10% fetal bovine serum. HT-29, HCT116, and SNU-C4 were grown in McCoy's 5A medium. SW620, Caco-2, and MC38 were grown in Leibovitz's L-15, Eagle's minimum essential, and Dulbecco's modified Eagle's growth media, respectively. We passaged adherent cells weekly at subconfluence after trypsinization and maintained cultures in incubators at 37 °C in an atmosphere of 5% CO_2 and 95% air.

Experimental animals

WT C57BL/6J mice and FXR knockout mice (B6.129X1 (FVB)-Nr1h4^{tm1Goz}/J) were purchased from the Jackson Laboratory. For all experiments, we used 6-week-old male mice. We housed mice under identical conditions in a pathogen-free room with free access to commercial rodent chow and water; we allowed mice to acclimatize in the vivarium for 2 weeks prior to experiments. This study was approved by the Office of Animal Welfare Assurance from the University of Maryland School of Medicine and the Research Development Committee at the Veterans Affairs Maryland Health Care System.

Plasmids and stable transfections

Generation of stably transfected human HT-29 colon cancer cell lines using the plasmid pCDNA3.1hFXR was described previously (28). To generate cell lines stably overexpressing either full-length human FXR or a dominant-negative FXR mutant, we first constructed plasmids pcDNA3.1hFXR and pcDNA3.1FXRW469A using pCMX-hFXR and pCMX-hFXR W469A (a gift from Dr. David Mangelsdorf, University of Texas Southwestern School of Medicine), which contain the full-length human FXR cDNA and the dominant-negative FXR mutant protein W469A, respectively.

QPCR

We performed QPCR and quantification of mRNA levels as described previously (33). We confirmed the specificity of amplifications by melting curve analysis and calculated relative levels of mRNA according to the standard $\Delta\Delta Ct$ method. We normalized expression values by comparison with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). We listed QPCR primer sequences used in this study in Table 1.

FXR and MMP7 siRNA transfections

We purchased FXR (FXR SMARTpool: ON-TARGETplus L-003414-00 and Nontargeting siRNA 1 D-001810-01) and MMP7 siRNA (MMP7 Silencer Select assay ID S8856 and Silencer Select negative control 1) from Dharmacon and Invitrogen, respectively, and performed siRNA transfections using Lipofectamine as per the manufacturer's instructions (Thermo Fisher Scientific). HT-29 cells were transfected for 24–48 h before preparation of RNA and protein extracts for QPCR and immunoblotting, respectively.

Subcloning and luciferase assays

MMP7 5′ promoter plasmids were constructed using PCR-generated fragments using the pGL4.14 plasmid (Promega) and pGL2.hmat-2.3 and pGL2.hmat-296 that contain 2.3 and 296 bp of MMP7 promoter (gifts from Dr. Lynn Matrisian, Vanderbilt University). For all constructs, we used reverse PCR primer GTGAGAAGATCTTGCTAGTGACTGCAGAAATT; for the 191-bp construct, we used the forward PCR primer GTGAGACTCGAGATCCTTTGAAAGACAAATAC;forthe171-bpconstruct, we used GTGAGACTCGAGATTGTGTGCTTCCTGCCA; for the 141-bp construct, we used GTGAGACTCGAGTACTTCCTCGTTTTAGTT; and for the 111-bp construct, we used PCR primer GTGAGACTCGAGCACATACTTTCAAAGTTCTG. We confirmed constructs by DNA sequencing. Vectors were transfected into HT-29 cells for 24 h, followed by the addition of either CDCA (200 μM) or guggulsterone (20 μM) for an additional 24 h. Luciferase activity was measured using a SpectraMax spectrophotometer with a 96-well microplate reader.

Antibodies and immunoblotting

Immunoblotting was performed as described previously (72). To ensure equal loading of protein samples, protein concentrations of cell lysates were determined using the BCA Protein Assay kit (catalogue no. 23227, Thermo Scientific, Inc.). 10–20 μg of protein was loaded into each lane. We purchased rabbit anti-human FXR (Santa Cruz Biotechnology, Inc., catalogue no. SC-1205, lot K3009, H1904 for immunoblotting and SC-1204, lot C2404 for immunoprecipitation), goat anti-human MMP7 polyclonal antibody (R&D Systems, catalogue no. AF907, lot DPS0910111, DPS091308, DPS0710081), rabbit anti-mouse MMP7 monoclonal antibodies (Cell Signaling, catalogue no. 3801), and mouse monoclonal anti-β-actin antibodies (Sigma-Aldrich, catalogue no. A1978, lot 087M4850V). After probing with the primary antibodies, immunoblots were incubated with horseradish peroxidase–conjugated secondary antibodies and visualized by chemiluminescence (Pierce) with the Chemi-Doc^{TM} Touch Imaging System (Bio-Rad). To avoid oversaturation of bands and to work within the linear ranges only, we performed semiquantifications using Quantity One software (Bio-Rad).

Table 1
QPCR primers and MMP7 promoter positions for ChIP experiments

Gene	Forward primer sequence (5′ to 3′)	Reverse primer sequence (5′ to 3′)
MMP1	AACTGCCAAATGGGCTTG	CCGTGTAGCATTCTGTCC
MMP2	CTGCAGGGCGCTACTTCT	TCACGCTCTTCAGACTTTGG
MMP3	CCAAGCAAATAGCTGAAGACTTT	TTTCTTTGCATTTGGGTCAA
MMP7	GGAGATGCTCACTTCGATGA	ATACCCAAAGAATGGCCAAG
MMP8	AGCAATTGACGCAGCTGTT	GGAAAGGCACCTGATATGCT
MMP9	GAGGTGGACCGGATGTTC	CCTGGCAGAAATAGGCTTTC
MMP10	TGAGCCTAAGGTTGATGCTG	GTCACCATCCTGGCATTG
MMP12	TTGGAGGTATGATGAAAGGAGA	TTTAGGCCCGATTCCTTG
MMP13	GAAGACTTCCCAGGAATTGG	AAATGGAATTTGCTGGCAT
MMP14	ACATCAAAGTCTGGGAAGGG	CCGGTTCTACCTTCAGCTTC
MMP19	TTAAGCTGCACCCAGATGAT	AGTGGGCAGCTCTGTCTCTT
MMP21	CTGGAATCCCAACACACAAC	ATCTTCTGTGAGGGCCTGAT
MMP26	GGTCAGCTTCAGACACTGGA	AGCTCTGATTCCCAGAGTGC
MMP28	CACTGCAGGAAAGATGGGT	GGAACCTCCAGCATCGAC
Mmp7 (mouse)	CTGTTCCCGGTACTGTGATG	TCACAGCGTGTTCCTCTTTC
I-BABP	CCACCCATTCTCCTCATCCCTCTGCTC	ACCAAGTGAAGTCCTGCCCATCCTG
FXR	CAGCAATTGTTATCCTGTCTCC	CAGGCTGGTGAATCTTACACA
RXRα	TTTAACCCTGACTCCAAGGG	CTCCAAGGAGGCATAGACCT
Fxr (mouse)	AGGAGTACGCTCTGCTCACA	CGCCTCTCTGTCCTTGATG
GAPDH	CCCCATGGTGTCTGAGCG	CGACAGTCAGCCGCATCTT
Gapdh (mouse)	GGTGAAGGTCGGTGTGAAC	TGATGGCAACAATCTCCACT
ChIP 1 (bp 1–111)	CACATACTTTCAAAGTTCTG	TGCTAGTGACTGCAGAAATT
ChIP 2 (bp 49–171)	CATTGTGTGCTTCCTGCCAAT	TCTCAGCCTCGAATGTGGAAA
ChIP 3 (bp 202–281)	CCTGAATGATACCTATGAGAGCA	TCTGCTGCATAAATTGACTCCA
ChIP 4 (bp 965–1072)	CACTGCACTGCCTATTCAT	TAAGAATGGAGTCATTGGGC
ChIP 5 (bp 1523–1678)	GAAACAGGGTTTTGCCATGT	GCCACAAATTGGGTGAAAAT
ChIP 6 (bp 1708–1810)	TGCAGTGGAGTGATCTCAGC	GGTGATGCGTGCCTGTAGT
ChIP 7 (bp 1912–2038)	GTATCTTTTAAAAACTCAAC	GTGAAGAATATTTTCATATG
ChIP 8 (bp 2805–2955)	TACACGCACCTGCCACAAC	GTGGCTCACGCCTGTAATC
ChIP 9 (bp 3765–3935)	CAAGTTGTTGAGAGCGAGCC	CTCTAGAGGGCATCAATCTC
ChIP 10 (bp 4278–4429)	TTTCCTAATATCTTTTCCCAGAGC	AGGGGTACGACTGGGACATT

Immunohistochemical analyses

We performed immunohistochemistry using paraffin-embedded blocks from paired human colon cancer surgical specimens. Quantification of staining intensities was performed as described previously (50) by an expert pathologist (C. B. D.) masked to experimental groups. IgG and secondary antibodies were used as negative controls. Both anti-FXR and anti-MMP7 antibodies used in IHC detected predominantly one band in HT-29 and human colon tumors (Fig. S1, *J* and *K*). In addition, nonspecific IgG and secondary antibodies alone did not show any specific staining (Fig. S1*L*).

Casein zymography

MMP7 enzymatic activity was analyzed by casein gel zymography as described (32). Briefly, whole-cell lysates or supernatants from HT-29 cells grown in a 10-cm culture dishes were prepared after CDCA treatment for 24 h and subjected to electrophoresis using 12% SDS-PAGE containing casein. 50 μg of protein was loaded into each lane. The gels were washed in Zymogram Renaturing Buffer (Novex, catalogue no. LC2670) for 30 min to remove SDS and incubated in Zymogram Developing Buffer (Novex, catalogue no. LC2671) for 24 h. The gels were then stained with 0.25% Coomassie Brilliant Blue and photographed.

MMP7 ELISA

HT-29 cells were seeded at 10^5 cells/2 ml of medium/well in 6-well plates. 24 h after the addition of different concentrations of CDCA, supernatants were collected and centrifuged at 500 rpm for 5 min. As described previously, MMP7 enzymatic activity was measured using the Human Total MMP7 Quantikine Elisa Kit (R & D Systems, catalogue no. RND-DMP700) following the manufacturer's instructions (33).

ChIP assays

We performed ChIP assays in human HT-29 colon cancer cells using the Pierce Magnetic ChIP Kit (catalogue no. 26157; Thermo Scientific Inc.) as per the manufacturer's instructions. Briefly, *in vivo* cross-linking was performed using 4 million cultured HT-29 cells using 1% formaldehyde. Cells lysis was performed using buffers containing proteinase inhibitor mixtures. Lysates were then digested with MNase (Pierce kit) to generate random DNA fragments from 160 to 640 bp with an intense ladder of bands at ~160, 320, and 480 bp, which correspond to 1, 2, and 3 nucleosome units (Fig. 6*F*, *left*). Chromatin was obtained after brief sonication to rupture nuclei. A 5-μl aliquot was removed for agarose gel analysis. Immunoprecipitation of cross-linked protein/DNA was performed overnight at 4 °C using antibodies against human FXR, RNA polymerase II (positive control), and rabbit or goat IgG (negative controls). Elution and reverse cross-links of protein/DNA complexes to free DNA were performed using the ChIP Elution Buffer without protein kinase K using a magnet. Eluted DNA was purified using DAN spin columns. Real-time quantitative PCR was performed using QPCR primers listed in Table 1 using eluted DNA and 1% input. For FXR activation, monolayer HT-29 cells were treated with 200 μM CDCA for 2 h prior to cross-linking. We used goat anti-human FXR antibody (Santa Cruz Biotechnology, catalogue no. SC-1204, lot C2404), rabbit anti-RNA Polymerase II (Thermo Scientific, kit component, catalogue number not specified), rabbit IgG (Thermo Scientific, kit component, catalogue number not specified), and goat IgG (Calbi-

ochem, catalogue no. N102, lot D00067506). GAPDH was used as a negative control.

EMSA

We performed EMSAs using the LightShift Chemilumnescent EMSA kit (Thermo Fisher Scientific) according to the manufacturer's instructions. We purchased recombinant human full-length FXR protein form Sigma. Binding reactions contained 10 mM Tris, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM $MgCl_2$, 50 ng/μl poly(dI·dC), 0.05% Nonidet P-40, 5 μM zinc sulfate, 1× proteinase inhibitor mixture (Sigma), and 5 pmol of 3′-end biotin-labeled DNA. Protein/DNA complexes were resolved on a 6% DNA retardation gel (Invitrogen). We labeled double-stranded oligonucleotides (forward sequence, 5′-ATTGTGTGCTTCCTGCCAATAACGATGTAA-3′; reverse sequence, 5′-TTACATCGTTATTGGCAGGAAGCACACAAT-3′) with biotin using the Biotin 3′-end DNA Labeling Kit (Thermo Scientific) using 20 fmol of each probe. Briefly, we performed incubations in a total volume of 20 μl of binding buffer (1× binding buffer: 2.5% glycerol, 5 mM $MgCl_2$, 50 ng/μl poly(dI·dC), 0.05% Nonidet P-40, 5 μM zinc sulfate, 1 μg of nuclear extract, and 5 μl of labeled probe) for 20 min at room temperature. For competitive inhibition, we used a >100-fold excess of specific oligonucleotides before adding labeled probes. For the supershift experiments, 1 μg of nuclear extract or 0.5 μg of FXR recombinant protein (Sigma, catalogue no. SRP3029, lot 037298123) was preincubated for 15 min at room temperature with 1 μg of anti-FXR antibody (Santa Cruz Biotechnology, catalogue no. SC-1204, lot C2404) or anti-PEA3 antibody (Santa Cruz Biotechnology, catalogue no. SC-166629, lot L0516 for Western blotting and catalogue no. SC-166629X, lot L0302 for immunoprecipitation) before adding biotin-labeled probes. The reactions were terminated by adding 5× loading buffer. 20 μl of mixed solution was loaded on a 6% DNA retardation gel (Invitrogen) and electrophoresed in 0.5× Tris borate–EDTA buffer at 100 V for 30 min. We transferred the protein/DNA complexes to positively charged nylon membranes (Invitrogen), and we detected gel shifts using the chemiluminescent nucleic acid detection module (Thermo Scientific).

Matrigel chamber invasion assay

We performed invasion assays using the Biocoat Matrigel Invasion Chamber Kit (BD Biosciences) as described previously (72). Briefly, we trypsinized and resuspended HT-29 human colon cancer cells in serum-free McCoy's 5A medium, placed cells in the upper chamber (5×10^4 cells/chamber), and then added the indicated agents or vehicle (control). McCoy's 5A medium containing 10% fetal bovine serum was placed in the lower chamber. We incubated cells for 48 h in a humidified atmosphere with 95% air and 5% CO_2 at 37 °C. We fixed and stained invasive cells using Diff-Quick™ stain. We removed noninvasive cells in the upper chamber by wiping with a cotton swab and counted all cells on the lower surface of the insert that had penetrated Matrigel using a light microscope. We performed each experiment in triplicate.

Statistical analysis

Data are expressed as mean ± S.E. of at least three independent experiments. We performed one-way ANOVA, Tukey's HSD post hoc analyses, and Student's unpaired *t* tests (SigmaPlot, Systat Software, Inc., San Jose, CA) and considered a p value < 0.05 to be statistically significant.

Author contributions—Z. P., J. C., C. B. D., and G. X. data curation; Z. P., C. B. D., and G. X. formal analysis; Z. P. and G. X. validation; Z. P. and G. X. investigation; Z. P. and G. X. visualization; Z. P., J. C., C. B. D., and G. X. methodology; Z. P. and G. X. writing-original draft; J.-P. R. and G. X. resources; J.-P. R. and G. X. funding acquisition; J.-P. R. and G. X. writing-review and editing; G. X. conceptualization; G. X. supervision.

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