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ORIGINAL ARTICLE

Downregulation of ABCC3 activates MAPK signaling through accumulation of deoxycholic acid in colorectal cancer cells

Yukihiro Sato[1,2] | Minoru Kobayashi[2] | Masahiro Ohira[1] | Ryo Funayama[1] | Masamitsu Maekawa[3] | Hideaki Karasawa[2] | Ryosuke Kashiwagi[2] | Yayoi Aoyama[4] | Nariyasu Mano[3] | Shinobu Ohnuma[2] | Michiaki Unno[2] | Keiko Nakayama[1]

[1]Department of Cell Proliferation, ART, Graduate School of Medicine, Tohoku University, Sendai, Japan

[2]Department of Surgery, Graduate School of Medicine, Tohoku University, Sendai, Japan

[3]Department of Pharmaceutical Sciences, Tohoku University Hospital, Sendai, Japan

[4]Department of Investigative Pathology, Graduate School of Medicine, Tohoku University, Sendai, Japan

Correspondence
Keiko Nakayama, Department of Cell Proliferation, ART, Graduate School of Medicine, Tohoku University, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan.
Email: nakayak2@med.tohoku.ac.jp

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Abstract

ABCC3 (also known as MRP3) is an ATP binding cassette transporter for bile acids, whose expression is downregulated in colorectal cancer through the Wnt/β-catenin signaling pathway. However, it remained unclear how downregulation of ABCC3 expression contributes to colorectal carcinogenesis. We explored the role of ABCC3 in the progression of colorectal cancer—in particular, focusing on the regulation of bile acid export. Gene expression analysis of colorectal adenoma isolated from familial adenomatous polyposis patients revealed that genes related to bile acid secretion including ABCC3 were downregulated as early as at the stage of adenoma formation. Knockdown or overexpression of ABCC3 increased or decreased intracellular concentration of deoxycholic acid, a secondary bile acid, respectively, in colorectal cancer cells. Forced expression of ABCC3 suppressed deoxycholic acid-induced activation of MAPK signaling. Finally, we found that nonsteroidal anti-inflammatory drugs increased ABCC3 expression in colorectal cancer cells, suggesting that ABCC3 could be one of the targets for therapeutic intervention of familial adenomatous polyposis. Our data thus suggest that downregulation of ABCC3 expression contributes to colorectal carcinogenesis through the regulation of intracellular accumulation of bile acids and activity of MAPK signaling.

KEYWORDS
ABCC3, colorectal cancer, deoxycholic acid, MAPK signaling, MRP3

Abbreviations: ABC, ATP binding cassette; CDF, 5(6)-carboxy-2′,7′-dichlorofluorescein; CDFDA, 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate; COAD, colon adenocarcinoma; CRC, colorectal cancer; DCA, deoxycholic acid; DCA-NBD, 7-nitrobenz-2-oxa-1,3-diazole-labeled DCA; FAP, familial adenomatous polyposis; NSAID, nonsteroidal anti-inflammatory drug; OST, organic solute transporter; qPCR, quantitative PCR; READ, rectum adenocarcinoma; RNA-seq, RNA sequencing; SLC, solute carrier; TCGA, The Cancer Genome Atlas; UGT, UDP g lucuronosyltransferase.

Yukihiro Sato and Minoru Kobayashi contributed equally to this article.

1 | INTRODUCTION

Colorectal cancer is one of the leading causes of cancer-related mortality worldwide.[1,2] In most sporadic CRC cases, oncogenic transformation proceeds through a well-characterized adenoma–carcinoma sequence.[1–3] Normal epithelial cells thus progress to low grade adenoma, to high grade adenoma, and finally to cancer cells in a stepwise manner associated with acquired somatic mutations in oncogenes and tumor suppressor genes. In contrast, FAP, a hereditary CRC syndrome, is caused by inherited germline mutations in *APC*, which also initiates the adenoma–carcinoma sequence.[1] Familial adenomatous polyposis is characterized by hundreds to thousands of adenomas in the colon and rectum. Almost all FAP patients develop adenocarcinoma if not adequately treated.[2] In addition to the genetic contribution to CRC, environmental factors, especially dietary patterns, also affect the incidence of CRC.[2] High consumption of dietary fat is associated with an increased risk for CRC, which is attributed in part to altered metabolism of bile acids.[4]

Bile acids, which are synthesized in the liver from cholesterol, are crucial for food digestion and lipid absorption.[5,6] They function not only as natural detergents to emulsify lipids but also as signaling molecules to regulate their own synthesis and energy homeostasis. Hepatic primary bile acids are secreted into bile and then passed into the intestine, where a fraction of primary bile acids is converted by gut bacteria to secondary bile acids including DCA. Approximately 95% of bile acids is reabsorbed by ileal enterocytes and recycled back to the liver through a process called enterohepatic circulation. The remaining 5% of bile acids including DCA is further reabsorbed in the colon or excreted into feces.[5] Deoxycholic acid, the most abundant secondary bile acid in healthy individuals, has potent tumor-promoting activities.[4,5,7] Elevated concentrations of fecal and serum DCA are associated with higher incidence of CRC.[8–10] Deoxycholic acid also promotes proliferation of CRC cells in vitro and formation of adenocarcinoma in $Apc^{Min/+}$ mice, an FAP mouse model.[11] Deoxycholic acid is believed to exert the tumor-promoting activities through the regulation of multiple signaling pathways, including MAPK signaling.[6,12–14]

Nonsteroidal anti-inflammatory drugs have been extensively studied as a potential chemopreventive strategy against the development of CRC in FAP.[15,16] They have been shown to reduce the development of adenomas in $Apc^{Min/+}$ mice,[17–19] and to induce the regression of pre-existing adenomas in FAP patients.[16,20,21] Although it has been believed that the antitumorigenic activity of NSAIDs is primarily due to suppression of prostaglandin synthesis through inhibition of COX, NSAIDs can also exert antitumorigenic activity through suppression of Wnt/β-catenin signaling in a COX-dependent or independent manner.[22]

We previously investigated expression patterns of ABC transporters in chemotherapy-naive CRC patients and found that the expression of ABCC3 was downregulated in CRC tissue compared with surrounding normal tissue.[23] ABCC3 is a bile acid transporter expressed on the basolateral membrane of ileal enterocytes and colonocytes.[6,24–26] The Wnt/β-catenin signaling pathway, which is activated at an early stage of the adenoma–carcinoma sequence, represses ABCC3 expression.[23] These observations suggested that downregulation of ABCC3 expression can occur at an early stage of CRC progression, and that it might be beneficial for tumor growth in the context of the colorectal tumor microenvironment. However, it remained unclear whether downregulation of ABCC3 expression is evident in early CRC lesions such as adenomas, and how it contributes to the progression of CRC. We have now explored the potential role of ABCC3 in the regulation of CRC progression—in particular, focusing on the regulation of bile acid export in CRC cells.

2 | MATERIALS AND METHODS

2.1 | Surgical specimens

Colorectal specimens were obtained from seven FAP patients. Three patients developed advanced CRC and underwent total proctocolectomy at Tohoku University Hospital. This aspect of the study was approved by the Institutional Review Board of Tohoku University Graduate School of Medicine (2022-1-1056), and the patients provided written informed consent.

2.2 | Cell culture and reagents

The CRC cell lines HT-29 (RRID: CVCL_0320) and SW620 (RRID: CVCL_0547) were obtained from ATCC, and authenticated by short tandem repeat profiling. HT-29 cells were cultured in McCoy's 5A medium (Thermo Fisher Scientific), and SW620 cells in DMEM (Nacalai Tesque), with each medium being supplemented with 10% heat-inactivated FBS (Thermo Fisher Scientific). All cell lines were maintained under a humidified atmosphere of 5% CO_2 at 37°C, and all experiments were undertaken with mycoplasma-free cells.

2.3 | Reverse transcription-qPCR analysis

Total RNA was isolated from tumor tissue or cultured cells with the use of an SV Total RNA Isolation System (Promega) and was subjected to RT with the use of a PrimeScript RT Reagent Kit (Takara Bio). Quantitative PCR analysis was carried out with Fast SYBR Green Master Mix (Thermo Fisher Scientific) and a StepOnePlus Real Time PCR System (Thermo Fisher Scientific). The data were normalized by the amount of glucuronidase beta (*GUSB*) or ribosomal protein large P0 (*RPLP0*) mRNA. Primer sequences are provided in Table S1.

For analysis of NSAIDs, HT-29 and SW620 cells were plated at a density of 1×10^6 per 100 mm dish (Corning) and were cultured for 48 h. The cells were treated with medium containing aspirin (Sigma-Aldrich), celecoxib (Sigma-Aldrich), or sulindac sulfide (Sigma-Aldrich) for 48 h. The treated cells were subjected to RT-qPCR analysis.

2.4 | Immunostaining

For immunohistochemistry of tumor tissue, paraffin-embedded sections were stained with primary Abs to ABCC3 (ab3375; Abcam) or to β-catenin (610153; BD Transduction Laboratories). Immune complexes were detected with peroxidase-labeled secondary Abs and 3,3′-diaminobenzidine (Histofine SAB-PO(M) Kit; Nichirei Bioscience), and the sections were counterstained with hematoxylin. For immunofluorescence analysis of HT-29 and SW620 cells, the cells were fixed with 3.7% formaldehyde and stained with primary Abs to ABCC3 (ab3375; Abcam). Immune complexes were detected with Alexa Fluor 488-labeled secondary Abs (Thermo Fisher Scientific), and the cells were counterstained with DAPI. Samples were observed with a BZ-9000 microscope (Keyence).

2.5 | Knockdown and forced expression of ABCC3 in CRC cells

The lentiviral vectors pRSI9-U6-(sh)-UbiC-TagRFP-2A-Puro and CSII-EF-MCS-IRES-Puro were used for knockdown or forced expression of ABCC3, respectively. HT-29 or SW620 cells were infected with recombinant lentiviruses encoding shRNA or ABCC3 protein, and the transfected cells were subjected to selection for 3 days in medium containing puromycin (Sigma-Aldrich) at 5 μg/mL. The sequences for the shRNAs are 5′-CGCTGATCTTACAACACTATT-3′ (ABCC3-A), 5′-GTGTACGTGGACCCAAACAAT-3′ (ABCC3-B), and 5′-CTTCGAAATGTCCGTTCGGTT-3′ (luciferase).

2.6 | Export assay for CDF and DCA-NBD

For export assay of CDF, HT-29 and SW620 cells were plated at a density of 2×10^4 per well in a 96-well black plate (PerkinElmer) and were cultured for 48 h. The cells were treated with HBSS (Nacalai Tesque) containing 10 μM CDFDA (Cayman Chemical) for 30 min, and then washed with HBSS. Samples were observed with a BZ-9000 microscope (Keyence). Fluorescent DCA-NBD was synthesized as described previously.[27] For export assay of DCA-NBD, HT-29 and SW620 cells were plated at a density of 1×10^4 per well in a 96-well black plate and were cultured for 48 h. The cells were treated with HBSS containing 20 μM DCA-NBD and Hoechst 33342 (1 μg/mL) for 30 min, and then washed with HBSS. Samples were observed with a BZ-X800 microscope (Keyence). Fluorescence intensities of DCA-NBD and Hoechst 33342 were measured for individual cells with the use of BZ-X Analyzer software (Keyence). Approximately 200 cells were analyzed for each sample in each of three independent experiments.

2.7 | Additional methods

Additional methods are described in Document S1.

2.8 | Statistical analysis

Data are presented as means ± SEM for the indicated number (n) of biological replicates, unless indicated otherwise, and they were subjected to statistical analysis with JMP Pro version 17.0.0 (JMP Statistical DIscovery LLC), R software version 4.0.3 (The R Foundation for Statistical Computing), or Excel for Microsoft 365 (Microsoft). A p value of <0.05 was considered statistically significant.

3 | RESULTS

3.1 | ABCC3 expression downregulated in early stage of CRC progression

As ABCC3 expression is regulated by Wnt/β-catenin signaling, which is activated in an early stage of the adenoma–carcinoma sequence,[1,28] we predicted that ABCC3 expression is downregulated in colorectal adenoma. To confirm this, we undertook RT-qPCR analysis of adenoma and adjacent normal tissue isolated from chemotherapy-naive FAP patients. As expected, we found that the abundance of *ABCC3* mRNA was reduced in adenoma relative to normal tissue (Figure 1A). Analysis of RNA-seq data (GSE81836) for organoid cultures prepared from adenomatous polyp or normal gut epithelium of $Apc^{Min/+}$ mice revealed that the abundance of *Abcc3* mRNA was also reduced in organoid cultures from adenoma relative to those from normal tissue (Figure 1B).[29] Furthermore, immunohistochemical analysis of adenoma and adjacent normal tissue isolated from three FAP patients showed that ABCC3 expression was decreased in adenoma, where β-catenin expression was increased, compared with normal epithelium (Figure 1C).

Not only adenoma but colorectal adenocarcinoma isolated from FAP patients also showed decrease in ABCC3 expression (Figure 1D). Furthermore, analysis of TCGA RNA-seq databases showed that expression of ABCC3 was reduced in COAD but not in READ relative to corresponding normal tissue (Figure 1E). These data thus indicated that ABCC3 expression was downregulated in the early stage as well as the late stage of CRC progression.

3.2 | Gene expression related to bile acid secretion is downregulated in colorectal adenoma and adenocarcinoma

ABCC3 mediates the export of bile acids in ileal enterocytes and colonocytes to contribute to enterohepatic circulation of bile acids.[6,25] To investigate expression of other genes related to bile acid secretion in adenoma, we again took advantage of RNA-seq data for organoid cultures from $Apc^{Min/+}$ mice (GSE81836). We selected 78 expressed genes that are associated with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway "bile secretion – Mus musculus (mmu04976)." Fifteen (19%) genes were expressed equally between

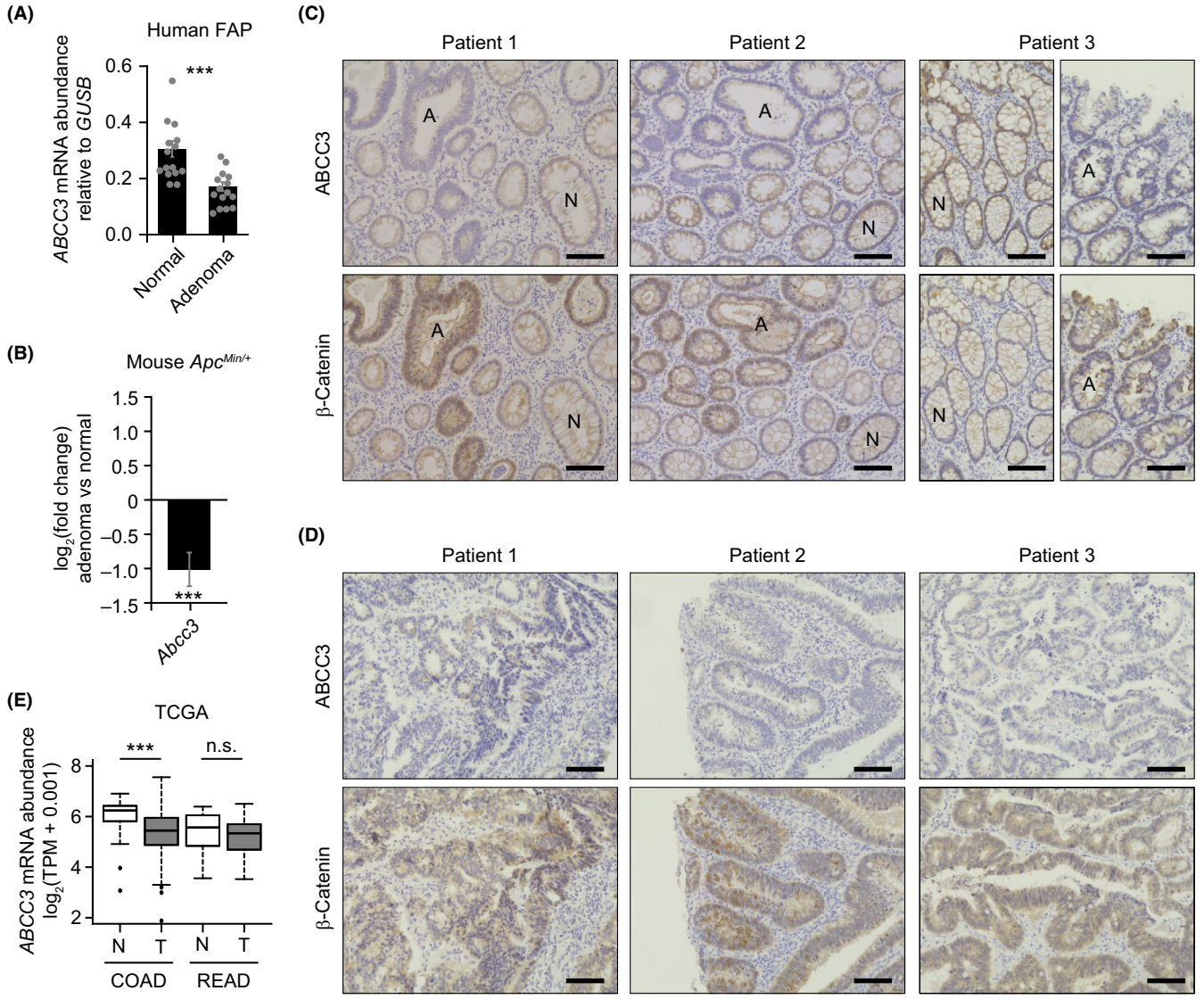


FIGURE 1 ABCC3 expression is downregulated in the early stage of colorectal cancer progression. (A) RT-quantitative PCR analysis of the expression of *ABCC3* in normal or colorectal adenoma tissue isolated from patients with familial adenomatous polyposis (FAP). Data are mean ± SEM ($n = 15$ tissue specimens from 5 patients). (B) Expression of *Abcc3* in organoid cultures prepared from adenomatous polyp of $Apc^{Min/+}$ mice. Data are mean ± SEM ($n = 3$) and expressed as $\log_2$ of the fold change relative to organoid cultures prepared from normal gut epithelium of $Apc^{Min/+}$ mice. (C, D) Representative immunohistochemical staining of ABCC3 (upper) or β-catenin (lower) in tissue sections (C) containing normal epithelium (N) and adenoma (A) or those (D) containing adenocarcinoma, respectively. Scale bar, 100 μm. (E) Box plots for *ABCC3* mRNA abundance based on transcripts per million (TPM) in normal (N) and primary tumor (T) tissue for colon adenocarcinoma (COAD) and rectum adenocarcinoma (READ). ***$p < 0.001$. n.s., not significant by paired *t*-test (A), by Wald test followed by Benjamini–Hochberg correction for multiple testing (B), or by Student's *t*-test (E); TCGA, The Cancer Genome Atlas.

organoid cultures from adenoma and those from normal gut epithelium. Notably, 46 (59%) genes were downregulated, whereas 17 (22%) genes were upregulated in organoid cultures from adenoma relative to those from normal gut epithelium (Figure 2A). Such downregulated genes included members of ABC transporters (Figure 2B), the SLC group of membrane transport proteins (Figure 2C), and UGTs (Figure 2D). Six downregulated genes were validated by RT-qPCR analysis in adenoma and normal tissue isolated from FAP patients, and we confirmed that expression of five of six genes was reduced in adenoma relative to normal tissue (Figure 2E). Furthermore, analysis of the TCGA RNA-seq databases showed that mRNA abundance of these five genes was lower in tumor tissue than in normal tissue in both COAD and READ (Figure 2F). Together, these results suggested that expression of relatively more genes related to bile acid secretion was downregulated in colorectal adenoma and adenocarcinoma.

3.3 | Downregulation of ABCC3 expression results in intracellular accumulation of DCA in CRC cells

We next sought to determine whether the downregulation of ABCC3 expression results in intracellular accumulation of bile acids

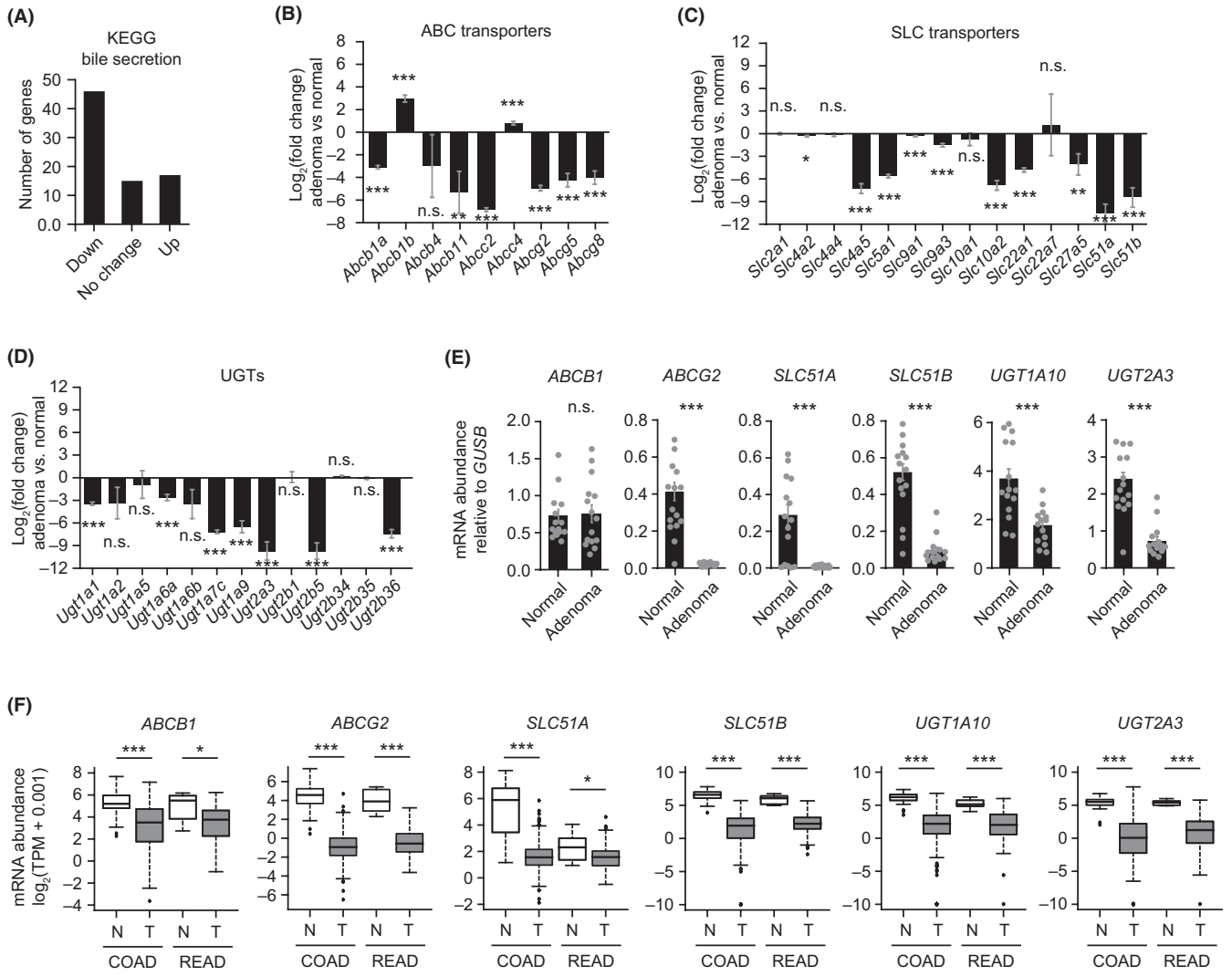


FIGURE 2 Gene expression related to bile acid secretion is downregulated in colorectal adenoma and adenocarcinoma. (A) Number of downregulated (down), unchanged (no change), or upregulated (up) genes associated with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway "bile secretion – Mus musculus (mmu04976)" in organoid cultures prepared from adenomatous polyp of $Apc^{Min/+}$ mice relative to those from normal gut epithelium. Expressed genes were defined as genes with base mean value >0. Downregulated and upregulated genes were defined as genes with $\log_2$ of the fold change <−0.5 or >0.5, respectively. (B–D) Expression of genes encoding (B) ATP binding cassette (ABC) transporters, (C) solute carrier (SLC) transporters, and (D) UDP glucuronosyltransferases (UGTs) in organoid cultures prepared from adenomatous polyp of $Apc^{Min/+}$ mice. (E) RT-quantitative PCR analysis of the expression for six selected genes in normal and colorectal adenoma tissue from patients with familial adenomatous polyposis. Data are mean ± SEM ($n = 15$ tissue specimens from 5 patients). (F) Box plots for mRNA abundance of six selected genes in normal (N) and primary tumor (T) tissue for colon adenocarcinoma (COAD) and rectum adenocarcinoma (READ). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. n.s., not significant by Wald test followed by Benjamini–Hochberg correction for multiple testing (B–D), by paired *t*-test (E), or by Student's or Welch's *t*-test (F).

in CRC cells. A human CRC cell line HT-29, which expresses endogenous ABCC3 (Figure 3A,B), was infected with recombinant lentiviruses encoding shRNAs against *ABCC3* mRNA (shABCC3 cells) to suppress ABCC3 expression. Cells harboring the empty vector or an shRNA against luciferase mRNA were examined as controls. The RT-qPCR (Figure 3A) and immunoblotting (Figure 3B) analyses revealed that ABCC3 expression was sufficiently reduced at both mRNA and protein levels in shABCC3 cells relative to control cells. To confirm the loss of transport activity in shABCC3 cells, we treated the cells with CDFDA, whose metabolite CDF is a known fluorescent substrate for ABCC3.[30] We found that CDF fluorescence intensity was markedly increased in shABCC3 cells compared with control cells (Figure 3C), indicating that ABCC3 transport activity was reduced in shABCC3 cells.

To examine whether the knockdown of ABCC3 expression results in intracellular accumulation of bile acids, we focused on DCA, a secondary bile acid produced by gut bacteria, as it has potent tumor-promoting activities.[4,5,7] We treated shABCC3 cells with DCA-NBD, a fluorescently-labeled DCA, together with a DNA staining dye Hoechst 33342, and fluorescence intensities of DCA-NBD and Hoechst 33342 were measured for individual cells by fluorescence microscopy (Figure 3D,E). The results showed that shABCC3 cells

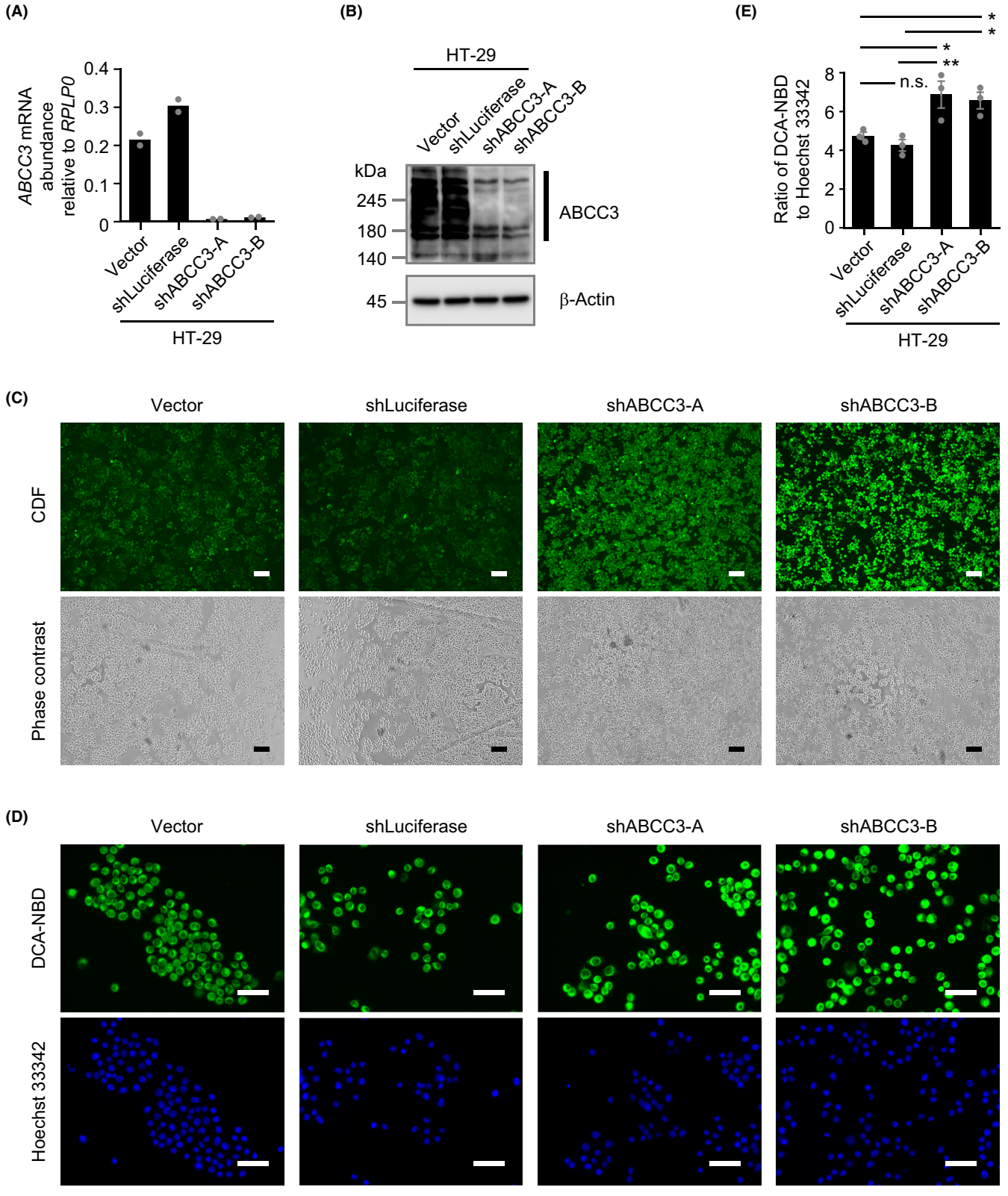


FIGURE 3 Downregulation of ABCC3 expression results in intracellular accumulation of deoxycholic acid (DCA) in colorectal cancer cells. (A, B) HT-29 cells infected with a control lentivirus (Vector), a lentivirus encoding shRNA against luciferase (shLuciferase), or lentiviruses encoding shRNAs against ABCC3 (shABCC3) were subjected to (A) RT-quantitative PCR analysis or (B) immunoblot analysis of ABCC3. Data are means ($n=2$) in (A). β-Actin expression was analyzed as loading control in (B). A vertical line indicates the position of ABCC3 signals in (B). (C, D) Representative fluorescence analysis of (C) 5(6)-carboxy-2′,7′-dichlorofluorescein (CDF) or (D) 7-nitrobenz-2-oxa-1,3-diazole-labeled DCA (DCA-NBD) in HT-29 cells manipulated as in (A). Scale bar, 100 μm in (C) or 50 μm in (D). (E) Quantification of fluorescence intensity of DCA-NBD as in (D). Data are mean ± SEM ($n=3$ independent experiments) and expressed as the ratio of DCA-NBD intensity to Hoechst 33342 intensity. $^{*}p<0.05$, $^{**}p<0.01$. n.s., not significant by one-way ANOVA followed by Dunnett's post hoc test.

exhibited higher DCA-NBD fluorescence compared with control cells. Our results thus suggested that downregulation of ABCC3 expression resulted in intracellular accumulation of DCA in CRC cells.

3.4 | Overexpression of ABCC3 reduces intracellular concentration of DCA in CRC cells

As a complementary approach to knockdown analysis, we also tested the effect of ABCC3 overexpression on intracellular DCA level in SW620 cells as well as HT-29 cells (Figure 4A). SW620 is a CRC cell line that expresses negligible levels of the endogenous ABCC3 protein. Although HT-29 cells express ABCC3 endogenously (Figure 3A,B), the exogenous ABCC3 was much more abundant than endogenous ABCC3 (Figure 4A). Immunofluorescence analysis revealed that the exogenous ABCC3 protein was predominantly localized at the cell surface (Figure 4B). Furthermore, treatment of cells with CDFDA showed that CDF fluorescence was reduced in ABCC3-expressing cells compared with vector control cells (Figure 4C), indicating that the exogenous ABCC3 has an export activity at the cell surface. Control SW620 cells showed stronger CDF fluorescence than did control HT-29 cells, probably due to lower endogenous ABCC3 expression in SW620 cells than in HT-29 cells (Figure 4C, data not shown). Of note, expression of ABCC3 significantly reduced fluorescence intensity of DCA-NBD in both HT-29 and SW620 cells (Figure 4D,E). These data thus indicated that the increased amount of ABCC3 expression reduced intracellular concentration of DCA in CRC cells.

3.5 | ABCC3 expression suppresses DCA-induced phosphorylation of ERK1/2 MAP kinases

We next determined the roles of ABCC3 expression in the progression of CRC. Given that ABCC3 expression is downregulated during the development of colorectal adenoma (Figure 1), it might contribute to the regulation of cell proliferation in CRC cells. To evaluate the potential role of ABCC3 expression in proliferation of CRC cells, we undertook growth assays of HT-29 and SW620 cells with knockdown or forced expression of ABCC3. ABCC3 overexpression did not affect the ability of CRC cells to proliferate in vitro (Figure 5A). In contrast, the two types of ABCC3-knockdown cells showed a decrease in cell proliferation. However, cell proliferation was also reduced in shLuciferase cells as well. Effects of shRNA introduction seems to have influenced cell proliferation ability (Figure 5B). We noticed that RNA-seq data from ABCC3-knockdown cells showed altered expression of many genes and pathways, suggesting that some substrates of ABCC3 have affected diverse signaling pathways, and have not induced any clear phenotype on cell proliferation (Figure S1, Table S2).

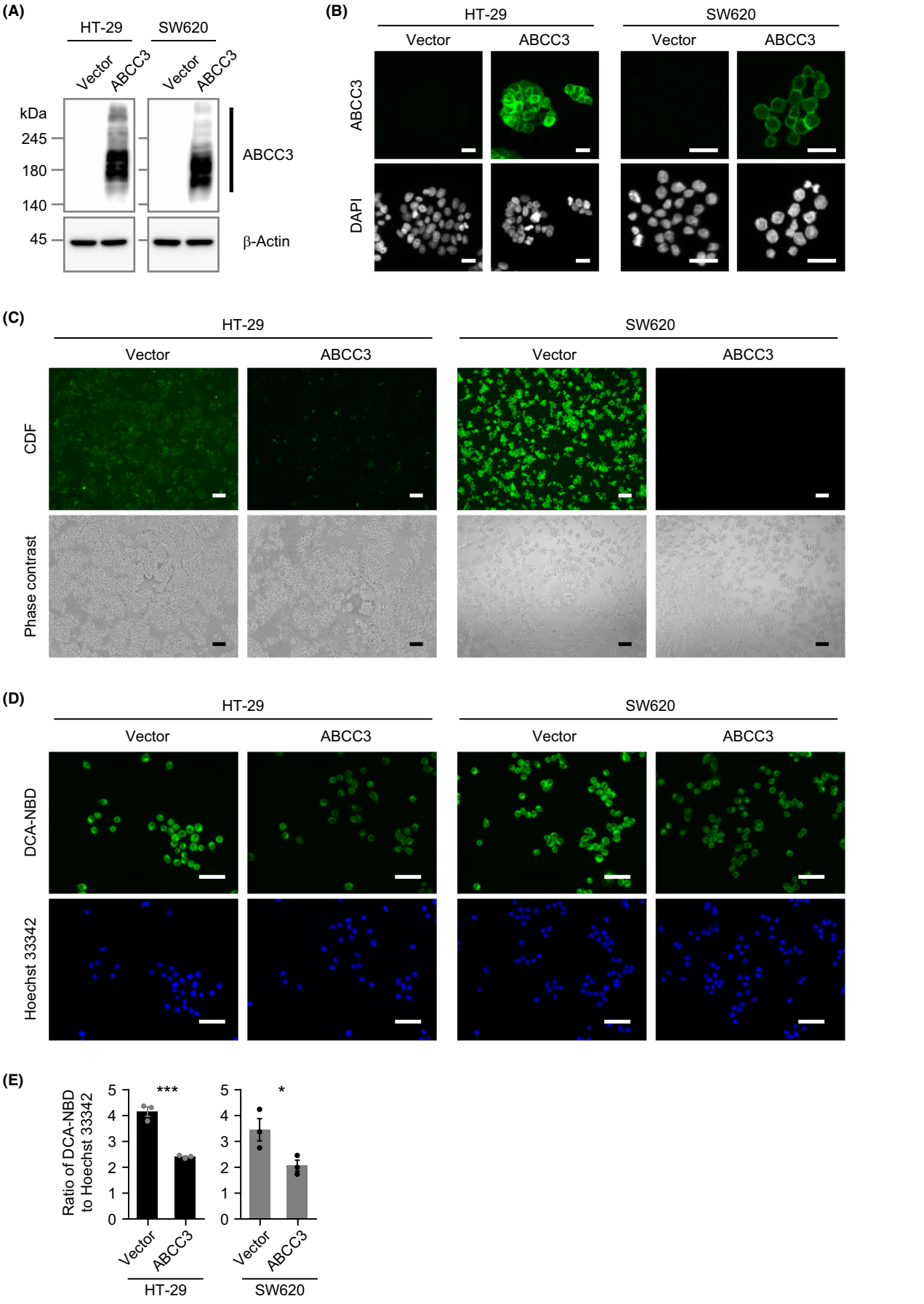
Bile acids including DCA have been shown to activate the MAPK signaling pathway in CRC cells.[6,14,31] As MAPK activity markedly increases during the development of colorectal adenomas,[1,28] we explored the possibility of the effect of ABCC3 expression on DCA-induced activation of MAPK signaling. To this end, we treated HT-29 and SW620 cells stably expressing the exogenous ABCC3 with 300 μM DCA for 90 or 45 min, respectively. The concentration of DCA and the duration of treatment were determined based on the conditions under which DCA most effectively induced the activation of the MAPK signal in each cell line. Then, we analyzed phosphorylation levels of ERK1/2 at Thr202 and Tyr204 by immunoblot analysis as a measure of MAPK activity. While DCA treatment induced phosphorylation of ERK1/2, their phosphorylation levels were modestly but significantly lower in ABCC3-expressing cells than in vector control cells in both HT-29 and SW620 cells (Figure 5C,D). Furthermore, a time course analysis of SW620 cells revealed that ABCC3-expressing cells showed lower maximum phosphorylation at 60 min after treatment than did control cells (Figure 5E). We also analyzed ERK1/2 phosphorylation levels in ABCC3-knockdown cells. We observed that ABCC3 knockdown induced relatively higher phosphorylation levels of ERK1/2 than control vector after DCA treatment, however, the difference was not significant (data not shown).

We undertook growth assays of HT-29 and SW620 cells with knockdown or forced expression of ABCC3 at DCA concentrations of 25 and 100 μM (Figure S2), which are considered physiological concentrations.[32,33] In a previous study,[34] 25 μM DCA was found to promote cell proliferation, but no similar results were observed in the present experiments. Deoxycholic acid (100 μM) inhibited cell proliferation in both cell lines, and we did not observe the difference of inhibitory effect of DCA on cell proliferation. In growth assays, it was not possible to evaluate whether ABCC3 affects DCA-mediated cell proliferation, but the results of the immunoblot analysis showed that ABCC3 affects DCA-mediated activation of MAPK signaling.

3.6 | Nonsteroidal anti-inflammatory drugs increase ABCC3 expression in CRC cells

Having established that ABCC3 expression reduced the activity of MAPK signaling in response to DCA exposure, we sought to investigate whether ABCC3 expression could serve as a potential target for therapeutic intervention for FAP. We then focused on NSAIDs, including aspirin, celecoxib, and sulindac sulfide, that have been extensively studied as a potential chemopreventive strategy against the development of CRC in FAP.[15,16] As NSAIDs suppress Wnt/β-catenin signaling that downregulates ABCC3 expression,[22,23] we expected that NSAIDs could increase ABCC3 expression.

To examine whether NSAIDs increase ABCC3 expression in CRC cells, HT-29 and SW620 cells were treated with increasing concentrations of aspirin, celecoxib, and sulindac sulfide, and *ABCC3* mRNA expression was analyzed by RT-qPCR. The results showed that all except sulindac sulfide for SW620 cells increased *ABCC3* mRNA expression in a dose-dependent manner (Figure 6A). These results indicated that NSAIDs increased ABCC3 expression in CRC cells. Nonsteroidal anti-inflammatory drugs might therefore contribute to reduced risk of adenoma in FAP patients, at least in part, through


(A)
HT-29
SW620
Vector
ABCC3
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245
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4
5
Vector
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FIGURE 4 Overexpression of ABCC3 reduces intracellular concentration of deoxycholic acid (DCA) in colorectal cancer cells. (A) HT-29 or SW620 cells infected with a control lentivirus (Vector) or a lentivirus encoding ABCC3 were subjected to immunoblot analysis of ABCC3 and β-actin (loading control). A vertical line indicates the position of ABCC3 signals. (B) Representative immunofluorescence analysis of ABCC3 in HT-29 or SW620 cells manipulated as in (A). Scale bar, 20 μm. (C, D) Representative fluorescence analysis of (C) 5(6)-carboxy-2′,7′-dichlorofluorescein (CDF) or (D) 7-nitrobenz-2-oxa-1,3-diazole-labeled deoxycholic acid (DCA-NBD) in HT-29 or SW620 cells manipulated as in (A). Scale bar, 100 μm in (C) or 50 μm in (D). (E) Quantification of fluorescence intensity of DCA-NBD as in (D). Data are mean ± SEM ($n = 3$ independent experiments) and expressed as the ratio of DCA-NBD intensity to Hoechst 33342 intensity. $^{*}p < 0.05$, $^{***}p < 0.001$ (Student's t test).

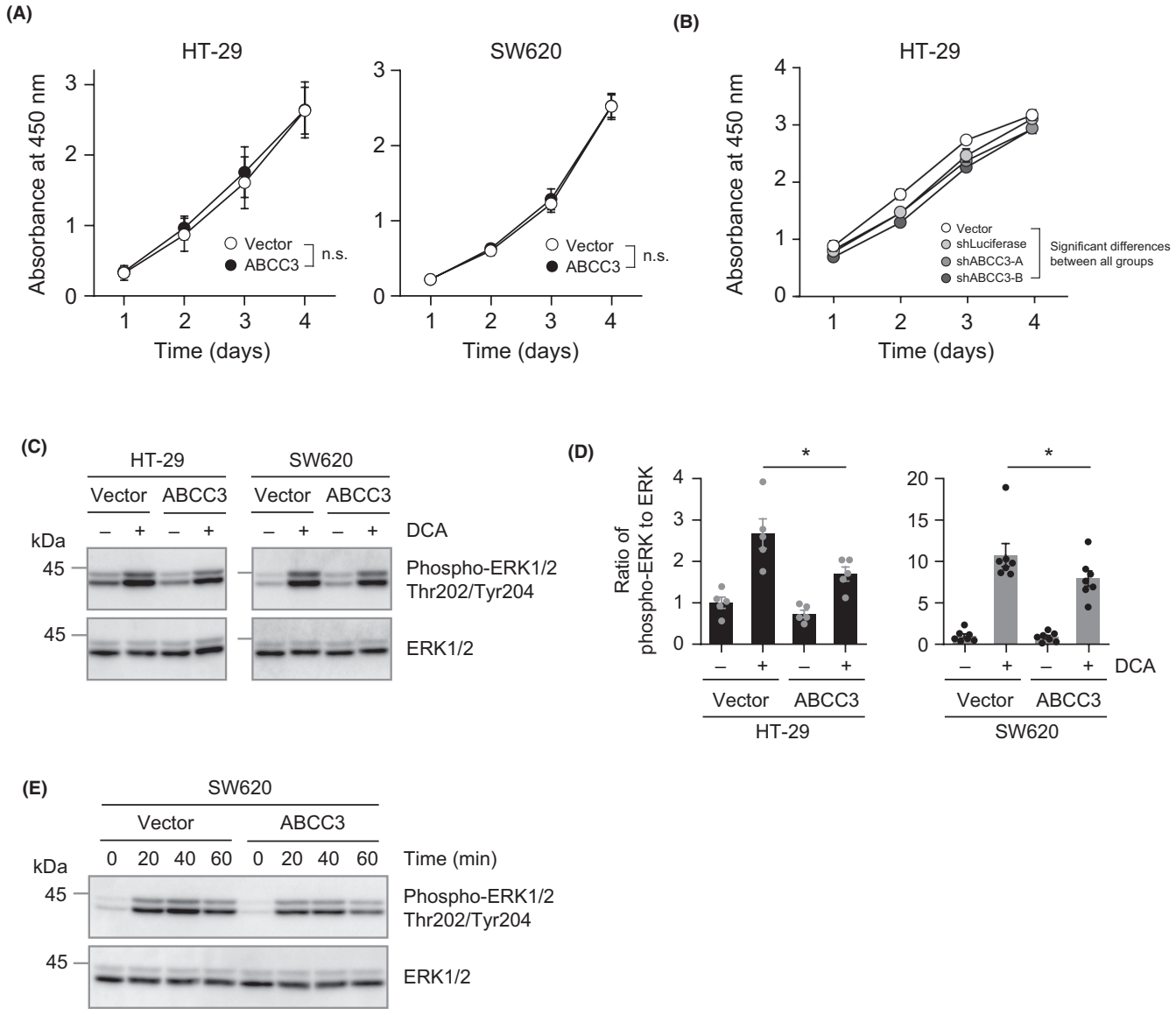
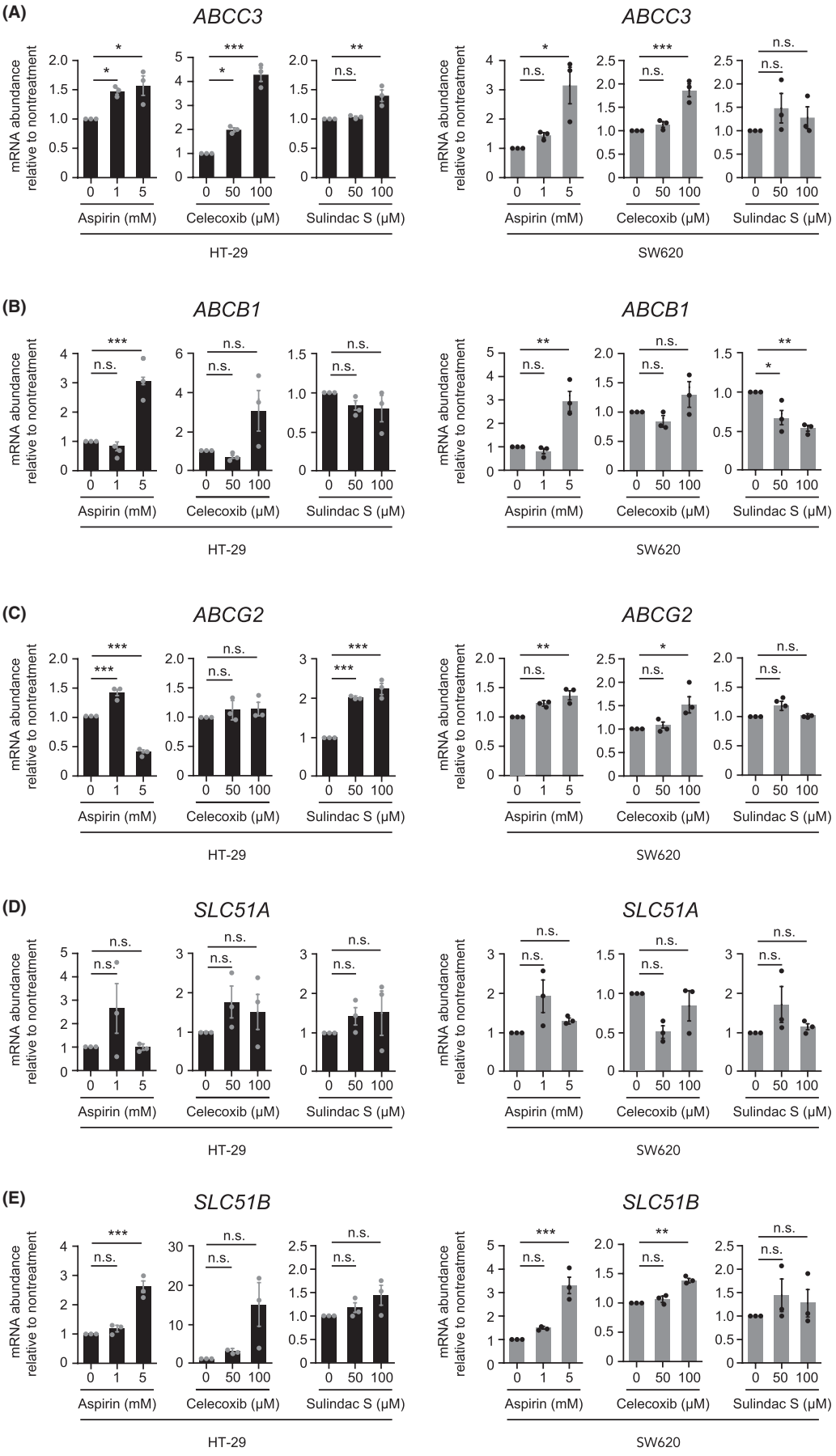


FIGURE 5 ABCC3 expression suppresses deoxycholic acid (DCA)-induced phosphorylation of ERK1/2 MAP kinases. (A) Growth curves for HT-29 or SW620 cells infected with a control lentivirus (Vector) or a lentivirus encoding ABCC3. (B) Growth curves for HT-29 cells infected with a control lentivirus (Vector), a lentivirus encoding shRNA against luciferase (shLuciferase), or lentiviruses encoding shRNAs against ABCC3 (shABCC3). (C) HT-29 or SW620 cells manipulated as in (A) were treated with (+) or without (−) 300 μM DCA and were subjected to immunoblot analysis of phospho-ERK1//2 (Thr202/Tyr204) and ERK1/2. (D) Quantification of phosphorylation level of ERK1/2 as in (B). Data are means ± SEM and are expressed as the ratio of phospho-ERK1/2 intensity to ERK1/2 intensity; $n = 5$ for HT-29 cells or $n = 7$ for SW620 cells. (E) SW620 cells manipulated as in (A) were treated without (0 min) or with 300 μM DCA, and lysed at 20, 40, or 60 min after addition of DCA. Cell lysates were then subjected to immunoblot analysis of phospho-ERK1//2 (Thr202/Tyr204) and ERK1/2. $^{*}p < 0.05$. n.s., not significant by two-way ANOVA (A, B) or by Wilcoxon rank sum test (D).


(A)
ABCC3
ABCC3
(B)
ABCB1
ABCB1
(C)
ABCG2
ABCG2
(D)
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SLC51B
SLC51B
mRNA abundance relative to nontreatment
Aspirin (mM)
Celecoxib (μM)
Sulindac S (μM)
HT-29
SW620
n.s.

FIGURE 6 Nonsteroidal anti-inflammatory drugs increase expression of bile acid transporters in colorectal cancer cells. (A–E) RT-quantitative PCR analysis of the expression of bile acid transporters in HT-29 or SW620 cells treated without (0) or with increasing concentrations of aspirin, celecoxib, or sulindac sulfide (Sulindac S). Data are mean ± SEM ($n = 3$) and expressed as mRNA abundance of bile acid transporters relative to nontreatment. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. n.s., not significant by one-way ANOVA followed by Dunnett's post hoc test.

upregulation of ABCC3 expression and suppression of bile acid-induced MAPK signaling. Additionally, we investigated whether NSAIDs affect the expression of other transporters (ABCB1, ABCG2, SLC51A, and SLC51B), which are thought to function as bile acid transporters in colonocytes. The results showed that the expression of several transporters was increased by treatment with NSAIDs as well as ABCC3 (Figure 6B–E).

4 | DISCUSSION

We found that expression of ABCC3 was downregulated as early as at the stage of adenoma formation, which resulted in intracellular accumulation of DCA in CRC cells. Forced expression of ABCC3 reduced intracellular DCA and DCA-induced activation of MAPK signaling. These results suggest that downregulation of ABCC3 expression contributes to colorectal carcinogenesis through the regulation of intracellular accumulation of bile acids and activity of MAPK signaling. We also found that ABCC3 could be one of the targets by NSAIDs for a therapeutic intervention for FAP.

Previous gene expression analysis of ABCC3 was somewhat controversial in that the abundance of *ABCC3* mRNA was reduced[23,35] or unchanged[36] in colorectal adenoma and adenocarcinoma compared with normal tissue.[37] We here determined that ABCC3 expression was downregulated in colorectal adenoma and adenocarcinoma relative to normal tissue isolated from FAP patients in our hospital. This result was consistent with our reanalysis of RNA-seq data for GSE81836 and TCGA. The abundance of *Abcc3/ABCC3* mRNA was lower in organoid cultures prepared from adenomatous polyp of $Apc^{Min/+}$ mice or in COAD tissues than in corresponding normal controls. The discrepancy observed between previous studies might be due in part to differences in carcinogenic pathways between CRC patients, as ABCC3 expression is downregulated through the Wnt/β-catenin signaling pathway.[23] Colorectal cancer tumors without mutations in Wnt/β-catenin signaling might not show downregulation in ABCC3 expression. In particular, the details of the serrated pathway that develops from serrated adenoma have been clarified in recent years. The mechanism we have elucidated might not be applicable in CRCs that develop in the serrated pathway, and therefore, we need to continue to examine the mechanism in detail.

ABCC3 is a bile acid transporter expressed on the basolateral membrane of ileal enterocytes and colonocytes, where it cooperates with OSTα/β heterodimers to export bile acids toward the portal circulation.[6,24–26] Although ABCC3 has been shown to transport several conjugated forms of bile acids, including glycocholate and taurocholate,[38–40] it remained unclear whether ABCC3 has an ability to transport DCA. We showed that knockdown or overexpression of ABCC3 increased or decreased intracellular DCA-NBD fluorescence, respectively, strongly suggesting that ABCC3 had an ability to transport DCA in CRC cells. However, DCA-NBD is a fluorescent NBD-labeled DCA, and therefore we cannot exclude the possibility that NBD moiety might affect substrate recognition by ABCC3. Future studies are thus warranted to determine whether ABCC3 has an ability to transport unlabeled DCA. This issue could be addressed by in vitro transport assay with plasma membrane vesicles that overexpress ABCC3, or by real-time monitoring of intracellular bile acids with a Förster resonance energy transfer (FRET)-based sensor for bile acids.[41,42]

We found that expression of genes related to bile acid secretion—including genes encoding ABC transporters, SLC transporters, and UGTs—was downregulated in colorectal adenoma and adenocarcinoma compared with normal tissue. The ABC and SLC transporters mediate the transport of bile acids across plasma membrane, whereas UGTs enhance water solubility and excretion of bile acids through conjugation with glucuronic acid.[43] Notably, two SLC transporters, OSTα and OSTβ (encoded by *SLC51A* and *SLC51B*, respectively), are major bile acid exporters in ileal enterocytes and colonocytes.[25,26] Simultaneous downregulation of ABCC3, OSTα, and OSTβ thus could lead to excessive intracellular accumulation of bile acids in colorectal adenoma and adenocarcinoma. Although overexpression of ABCC3 alone only modestly affected activity of MAPK signaling in HT-29 and SW620 cells, it will be of interest to analyze the potential effects of ABCC3, OSTα, and OSTβ in combination. In addition, we plan to conduct further detailed examinations of other ABC transporters as they might also be involved in the oncogenic mechanism mediated by DCA and MAPK signaling. Furthermore, it would be also noteworthy to analyze the effects of ABCC3 downregulation on other signaling pathways besides MAPK signaling, as DCA has been shown to activate PI3K-AKT, nuclear factor-κB, and Wnt/β-catenin signaling pathways.[44–47]

Our study has several limitations. First, although we found that ABCC3 suppressed DCA-induced activation of MAPK signaling in CRC cells in vitro, its functional significance in vivo remains unclear. Second, HT-29 and SW620 cells harbor the *BRAF* V600E and *KRAS* G12D mutations, respectively, which lead to constitutive activation of MAPK signaling. These mutations might affect the responsiveness of DCA-induced MAPK signaling in those cells. Finally, in the growth assay of HT-29 cells with knockdown of ABCC3, the effect of ABCC3 expression changes on cell proliferation could not be properly evaluated, and it was unable to demonstrate reproducibility in experiments using ABCC3-knockdown cells to induce phosphorylated ERK by DCA. Detailed verification is needed after the optimization of the experimental conditions.

AUTHOR CONTRIBUTIONS

Yukihiro Sato: Conceptualization; funding acquisition; investigation; writing – original draft; writing – review and editing. **Minoru Kobayashi:**

Conceptualization; funding acquisition; investigation; writing – original draft; writing – review and editing. **Masahiro Ohira:** Investigation. **Ryo Funayama:** Conceptualization; funding acquisition; investigation; writing – original draft; writing – review and editing. **Masamitsu Maekawa:** Resources; supervision; writing – review and editing. **Hideaki Karasawa:** Resources; supervision; writing – review and editing. **Ryosuke Kashiwagi:** Supervision. **Yayoi Aoyama:** Investigation. **Nariyasu Mano:** Resources; supervision; writing – review and editing. **Shinobu Ohnuma:** Resources; supervision; writing – review and editing. **Michiaki Unno:** Resources; supervision; writing – review and editing. **Keiko Nakayama:** Conceptualization; funding acquisition; investigation; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

M.U. is an Editorial Board Member of *Cancer Science*. The other authors declare no conflict of interest.

ETHICS STATEMENT

Approval of the research protocol by an institutional review board: This study was approved by the institutional review board of Tohoku University Graduate School of Medicine (2022-1-1056).

Informed consent: Written informed consent was obtained from all participants.

Registry and the registration no. of the study/trial: N/A.

Animal studies: N/A.

ORCID

Minoru Kobayashi https://orcid.org/0000-0003-4340-8535
Hideaki Karasawa https://orcid.org/0000-0003-0358-3398
Shinobu Ohnuma https://orcid.org/0000-0002-0205-8957
Michiaki Unno https://orcid.org/0000-0002-2145-6416
Keiko Nakayama https://orcid.org/0000-0003-0134-6401

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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