

Perfluorooctanesulfonic acid exposure leads to downregulation of 3-hydroxy-3-methylglutaryl-CoA synthase 2 expression and upregulation of markers associated with intestinal carcinogenesis in mouse intestinal tissues

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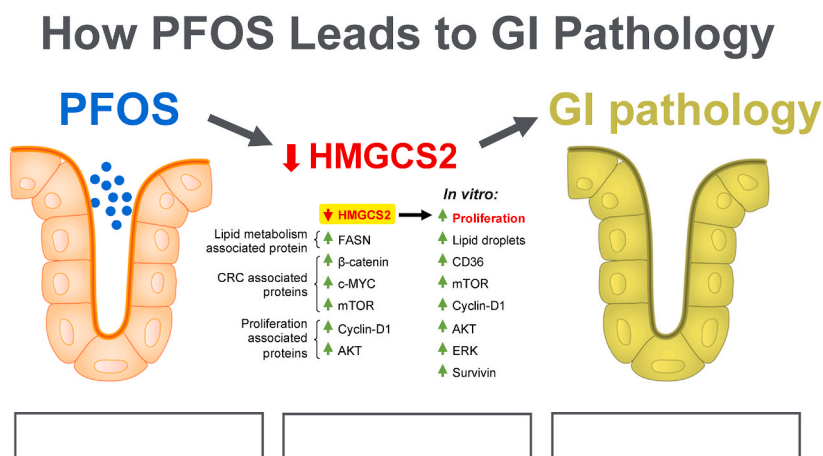
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HIGHLIGHTS

- PFOS exposure downregulates HMGCS2 protein expression in normal intestinal tissues.
- Inulin and pectin diets do not alleviate PFOS-induced HMGCS2 downregulation.
- PFOS exposure upregulates pathways and proteins associated with colon carcinogenesis.
- Loss of HMGCS2 increases proliferation and lipid accumulation in normal colon cells.
- Downregulation of HMGCS2 is observed in CRC and is associated with lower survival.

GRAPHICAL ABSTRACT



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ABSTRACT

Perfluorooctanesulfonic acid (PFOS) is a widely recognized environment pollutant known for its high bioaccumulation potential and a long elimination half-life. Several studies have shown that PFOS can alter multiple biological pathways and negatively affect human health. Considering the direct exposure to the gastrointestinal (GI) tract to environmental pollutants, PFOS can potentially disrupt intestinal homeostasis. However, there is limited knowledge about the effect of PFOS exposure on normal intestinal tissues, and its contribution to GI-associated diseases remains to be determined. In this study, we examined the effect of PFOS exposure on the gene expression profile of intestinal tissues of C57BL/6 mice using RNAseq analysis. We found that PFOS exposure in drinking water significantly downregulates mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCs2), a rate-limiting ketogenic enzyme, in intestinal tissues of mice. We found that diets containing the soluble fibers inulin and pectin, which are known to be protective against PFOS exposure, were ineffective in reversing the downregulation of HMGCs2 expression *in vivo*. Analysis of intestinal tissues also demonstrated that PFOS exposure leads to upregulation of proteins implicated in colorectal carcinogenesis, including β -catenin, c-MYC, mTOR and FASN. Consistent with the *in vivo* results, PFOS exposure leads to downregulation of HMGCs2 in mouse and human normal intestinal organoids *in vitro*. Furthermore, we show that shRNA-mediated knockdown of HMGCs2 in a human normal intestinal cell line resulted in increased cell proliferation and upregulation of key proliferation-associated proteins such as cyclin D, survivin, ERK1/2 and AKT, along with an increase in lipid accumulation. In summary, our results suggest that PFOS exposure may contribute to pathological changes in normal intestinal cells via downregulation of HMGCs2 expression and upregulation of pro-carcinogenic signaling pathways that may increase the risk of colorectal cancer development.

1. Introduction

Perfluoroalkyl and polyfluoroalkyl substances (PFAS) are a group of synthetic environmental pollutants widely used in industrial applications and consumer products (Gluge et al., 2020). These manufactured chemicals can contaminate air, soil and water during their production and use, and have frequently been detected in biological samples, such as breast milk and human blood (Centers for Disease Control and Prevention, 2022; Consumer Product Safety Commission, 2023). General population exposure mainly occurs through consuming contaminated water and food (Consumer Product Safety Commission, 2023). PFAS are pollutants that have been found globally in surface- and groundwater drinking-water resources (Evich et al., 2022; Sims et al., 2022). According to a study conducted by the U.S. Geological Survey, approximately 45% of drinking water in the US is contaminated by at least one type of PFAS (Smalling et al., 2023). These compounds are made of fluorinated carbon chains and the carbon-fluorine bond is one of the strongest bonds in organic chemistry, PFAS are extremely stable and resistant to environmental and metabolic degradation, resulting in their high bioaccumulation and toxicity potential (Cousins et al., 2020). Long-term exposure to PFAS has been increasingly associated with negative health outcomes, including human development, metabolic disorders, reduced immune responses and an increased risk of cancer (Zeng et al., 2019; Chen et al., 2020; Fenton et al., 2021; U.S. Environmental Protection Agency, 2023).

Perfluorooctanesulfonic acid (PFOS) is one of the most detected PFASs, with an estimated elimination half-life of 3.4–4.8 years in humans (Olsen et al., 2007; Li et al., 2018). Sampling of drinking water systems under the Unregulated Contaminant Monitoring Rule, conducted 2013–2015, showed PFOS concentrations ranged from 0.04 $\mu\text{g/L}$ (the minimum reporting level at this time) to 7 $\mu\text{g/L}$ (U.S. Environmental Protection Agency, 2022). In April 2024, EPA announced the final National Primary Drinking Water Regulation which enforced the maximum contaminant level of PFOS to be 4 ng per liter (ng/L) in drinking water by 2027 (U.S. Environmental Protection Agency, 2024). Because recent health effects studies show that PFOS can impact human health at exposure levels much lower, the U.S. Environmental Protection Agency currently has established that interim lifetime non cancer health advisory of 0.02 ng/L for PFOS can be applied to both short-term and chronic risk assessment scenarios (U.S. Environmental Protection Agency, 2022). The current studies also suggest that PFOS may be a carcinogen due to its pro-tumorigenic effects reported in multiple cancers, including breast, esophagus, glioblastoma, kidney, liver, lung and

prostate (Durham et al., 2023).

Importantly, the gastrointestinal (GI) tract is directly exposed to PFOS, where it can accumulate and impact intestinal homeostasis under both physiological and pathological conditions (Knutsen et al., 2018; Liang et al., 2021). A National Academies of Science, Engineering, and Medicine report provided evidence suggestive of an increased risk of ulcerative colitis associated with PFAS exposure (National Academies of Sciences, Engineering, and Medicine, 2022). Another study observed that PFOS induces inflammatory bowel disease (IBD)-like injury in rats, marked by inflammatory infiltration into the intestinal tissues (Liang et al., 2021). Consistent with these findings, several studies suggest that PFOS can influence the production of inflammatory cytokines, thereby contributing to chronic inflammation (Office of Environmental Health Hazard Assessment, 2010; Zhang et al., 2023). Notably, individuals with IBD have an elevated risk of colorectal cancer (CRC) development, a phenomenon attributed to chronic inflammation which can initiate and drive tumorigenesis (Lucafo et al., 2021). CRC represents a significant public health concern, especially given its global trend of increasing incidence among the younger population (Zaborowski et al., 2021). Possible causes of early-onset disease include dietary and lifestyle factors, as well as exposure to environmental contaminants (Adigun et al., 2023).

In a prior study, our group analyzed the effects of PFOS in C57BL/6J mice exposed to PFOS in drinking water and fed diets supplemented with cellulose as a control versus soluble fibers inulin and pectin as a potential mitigation strategy for PFOS exposure (Deng et al., 2022). This study demonstrated that PFOS exposure alters a gene transcription profile and lipid metabolism in the mouse livers, and markedly changes the composition of intestinal microbiota. Incorporation of soluble fibers such as inulin and pectin in diets partially normalize the PFOS-induced changes suggesting that a high fiber diet can be a potential mitigation strategy for PFOS exposure (Deng et al., 2022). In the present study, we performed RNAseq analysis on biobanked intestinal tissues to explore the effects of PFOS exposure in drinking water on transcriptional profile of intestinal epithelium. We tested the hypothesis that PFOS exposure disrupts intestinal homeostasis, potentially leading to pathological changes such as inflammation, altered signaling and metabolic pathways that may increase the risk of GI-associated diseases including CRC. Understanding the impact of PFOS exposure on GI pathology can provide valuable insights to guide the development of potential mitigation interventions to reduce the harmful effects of this environmental pollutant.

2. Materials and methods

2.1. Diets and study design

The diet specifications and the study design were described

previously by Deng (Deng et al., 2022) and are summarized in Fig. 1A. Briefly, wild-type male C57BL/6J mice were fed diets supplemented with cellulose as a control, inulin or pectin. PFOS (heptadecafluorooctanesulfonic acid potassium salt; 77,282; Sigma-Aldrich, St. Louis, MO, USA; purity >98%) was mixed with drinking water and given

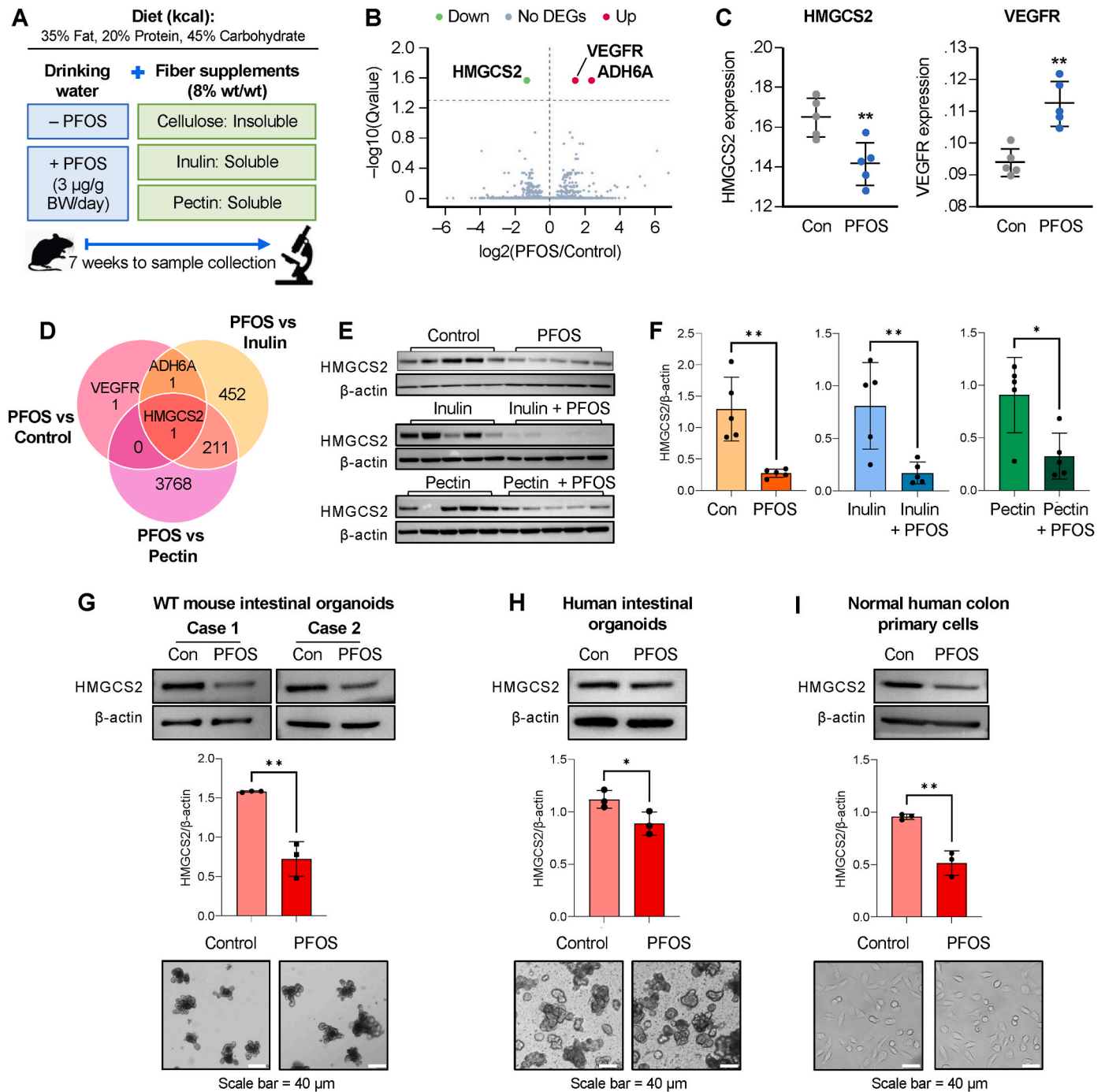


Fig. 1. PFOS exposure downregulates HMGCS2 levels. (A) Schematic of experimental design: seven-week-old male C57BL/6 mice were placed on an irradiated diet supplemented with cellulose (control), inulin or pectin. PFOS exposure occurred through daily oral ingestion of 3 µg/g body weight via drinking water. The mice were maintained on their respective diets for a 7-week period. (B) Volcano plot of differentially expressed genes (DEGs) between mice exposed to PFOS and the control (cellulose) group. Red and green dots represent upregulated and downregulated genes, respectively. (C) mRNA expression levels of HMGCS2 and VEGFR, normalized to GAPDH in intestinal tissues, were measured by qRT-PCR. Data are presented as mean ± SD (**p < 0.01). (D) Venn diagram displaying overlapping genes identified in the intestines of mice exposed to PFOS and fed with different diets. (E) Western blot and (F) densitometric quantification analysis of HMGCS2 protein levels on intestinal tissues of mice exposed to PFOS and treated with cellulose (control), inulin- or pectin-supplemented diets. (G) Western blot, densitometric quantification analysis of HMGCS2 protein levels, and representative images of wild-type mouse intestinal organoids treated with 1 µg/mL of PFOS for 15 days, (H) human intestinal organoids treated with 1 µg/mL of PFOS for 30 days, and (I) normal human colon primary cells treated with 1 µg/mL of PFOS for 6 months (n = 3). The levels of HMGCS2 expression were determined based on normalization of each band to β-actin (Scale bar: 40 µm).

to the animals *ad libitum* for 7 weeks. The exposure dose of PFOS corresponded to 3 µg/g body weight per day. At the end of the experiment, the samples were collected and stored at −80 °C for analysis.

2.2. RNA isolation and RNA-sequencing

Intestinal tissues were preserved at −80 °C and total RNA from mice exposed to PFOS and fed diets supplemented with cellulose, inulin or pectin were isolated using a RNeasy Plus kit (#74134, Qiagen, Germantown, MD, USA). mRNA samples from 4 mice were used for RNAseq analysis of control (cellulose), PFOS (cellulose), pectin and pectin/PFOS groups. mRNA samples from 3 mice were used for the inulin and inulin/PFOS groups due to unsatisfactory mRNA quality in one sample. Library preparation, sequencing and bioinformatics analysis were performed by Beijing Genomics Institute (Cambridge, MA, USA, <https://www.bgi.com/global/>). DNBSEQ platform was used for sequencing. FASTQ data first went through data quality control process to remove low-quality reads, reads with adaptor sequences, and reads with high levels of N base using SOAPnuke (Chen et al., 2018). HISAT (Kim et al., 2015) was used to align the clean reads to the reference genome, and Bowtie 2 (Langmead and Salzberg, 2012) was used to align the clean reads to the reference genes. The average mapping ratio with reference genome was 97.13%; with gene is 81.84%; 18,472 genes were identified. Reference Genome Version: GCF_000001635.27_GRCm39 (NCBI). The gene expression quantification was obtained based on mapping results with RSEM (Li and Dewey, 2011). The differential gene expression analysis was performed using DESeq2 software (Love et al., 2014). Bioinformatics analysis was also verified by the University of Kentucky Markey Cancer Center's Biostatistics and Bioinformatics Shared Resource Facility. The data presented in this publication have been deposited in NCBI's Gene Expression Omnibus and are assessable through GEO Series accession number GSE248402 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE248402>).

2.3. Real-time reverse transcription–polymerase chain reaction (RT-PCR) analysis

cDNA from total RNA isolation was synthesized using a high-capacity cDNA reverse transcription kit (#4368814, Applied Biosystems, Bedford, MA, USA). RT-PCR reactions were performed using a TaqMan Gene Expression Master Mix (#4369016, Applied Biosystems) TaqMan® MGB probes, FAM™ dye-labeled for mouse HMGCS2 (Mn00550050_m1), VEGFR (Mn0122242_m1) and GAPDH (Mn99999915_g1) were purchased from Thermo Fisher (Waltham, MA, USA), and used following the instructions provided by the manufacturer. mRNA expression levels were measured in a StepOnePlus Real Time PCR system (Applied Biosystems).

2.4. Organoid isolation and treatment

Mouse intestinal organoids were isolated as previously described (Li et al., 2023). Human organoids were isolated following the protocol of Sato et al. (2009) and cultivated in IntestiCult-SF Organoid Growth Medium (StemCell #100–0340). The organoid cultures were treated with 1 µg/mL of PFOS for 15 days (mouse) or 30 days (human) and then collected for protein analysis.

2.5. Cell culture and transfection

The normal human colon primary (NHCP) cell line was obtained from Celprogen, Torrance, CA, USA (#36037–08) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin–streptomycin. The cells were cultivated in a humidified atmosphere containing 5% CO₂ at 37 °C. The cells were exposed to a concentration of 1 µg/mL of PFOS over a 6-month period, with PFOS

being replenished during every medium change. Upon reaching 80% confluence, the cells were trypsinized and split at a 1:10 ratio. Stable HMGCS2 knockdown NHCP cells were established using Mission lentiviral transduction particles purchased from Sigma-Aldrich (TRCN0000045859 [shRNA#1] and TRCN0000429288 [shRNA#2]). pLKO.1-puro non-mammalian shRNA was used as a non-target control (NTC). Cells were transfected with 8 µg/mL of polybrene and selected with 10 µg/mL of puromycin. HMGCS2 knockdown was confirmed by western blotting.

2.6. Tissue collection

Normal and CRC tissues were collected from consented patients with stage II–IV CRC who had undergone surgery at the University of Kentucky Medical Center (IRB # 52,094, PI: Zaytseva) and biobanked in Dr. Zaytseva's laboratory. Frozen tissues were homogenized using cold stainless-steel beads in Cell Lysis Buffer (#9803, Cell Signaling, Danvers, MA, USA) supplemented with protease inhibitor cocktail (#11836170001, Roche, Indianapolis, IN, USA) and 1 mM PMSF (#93482, Sigma, St. Louis, MO, USA) and stored at −80C for analysis.

2.7. Protein extraction and Western blot analysis

Human and mouse tissues, organoids and NHCP cells were lysed using Cell Lysis Buffer (#9803, Cell Signaling, Danvers, MA, USA) supplemented with protease inhibitor cocktail (#11836170001, Roche, Indianapolis, IN, USA) and 1 mM PMSF (#93482, Sigma, St. Louis, MO, USA). Equal amounts of protein were separated on SDS-PAGE and transferred onto PVDF membranes, followed by blocking with 5% non-fat dry milk. The membranes were probed with the following primary antibodies: HMGCS2 (#ab137043), FASN (#3180), p-mTOR (#2971), c-Myc (#5605), total β-catenin (#610154), active β-catenin (#7270), survivin (#2808), cyclin D1 (#ab134175), total Erk1/2 (#4695), phospho-Erk1/2 (#4376), total AKT (#2920), phospho-AKT (#4056), CD36 (#ab133625) and β-actin (#A5441). Then, membranes were incubated with the appropriate secondary antibodies (rabbit #7074 or mouse #7076) and protein bands were detected with Immobilon Western Chemiluminescent HRP substrate (#WBKLS0500, Sigma). The quantification of band intensities was performed using ImageJ software.

2.8. Cell viability assay

NHCP cells were seeded onto 96-well plates at a concentration of 1 × 10³ cells per well, followed by incubation at 37 °C for 6 days. Then, 100 µL of medium containing 10 µL of PrestoBlue cell viability reagent (#A13262, Thermo Fisher) was added to the wells and incubated for 2 h at 37 °C and protected from light. The fluorescence was measured at 560 nm excitation and 590 nm emission using a microplate reader (Varioskan LUX, Thermo Fisher).

2.9. Confocal microscopy

NHCP cells were seeded on an 8-well coverslip µ-slide (#80807, Ibidi, Fitchburg, WI, USA) for 24 h. Subsequently, the cells were washed with PBS and incubated with the fluorescent fatty acid analog BODIPY FL (#D3822, Thermo Fisher) and Hoechst 33,342 (#62249, Thermo Fisher) for 30 min at 37 °C. Afterwards, cells were washed three times with PBS and fixed with 4% paraformaldehyde for 20 min. Images were captured using a Nikon A1 Confocal Microscope.

2.10. CRC tissue microarray

The immunoreactivity score of HMGCS2 expression was assessed in CRC tumors and adjacent normal tissue collected from patients diagnosed with stage I–IV who had surgery at UK Chandler Medical Center (Tumor Microarray ID BH 15991A, n = 55 normal, n = 16 stage I, n = 9

stage II, $n = 10$ stage III and $n = 5$ stage IV). The score was conducted blindly by a GI pathologist. Immunoreactivity score was calculated by multiplying values for staining intensity (0 = no staining; 1 = weak; 2 = moderate and 3 = strong staining) with values for the percentage of positive cells (0 = no positive cells; 1 = 0–10%; 2 = 11–50%; 3 = 51–100% positive cells).

2.11. Statistical analysis

The Biostatistics and Bioinformatics Shared Resource Facility assisted with TCGA and tumor microarray data analysis and validation of RNAseq analysis. Statistical significance between the control and PFOS treated groups was analyzed using *t*-test. Comparisons between the NTC and HMGCS2sh groups were performed using one-way ANOVA and Dunnett's post-test. Differences in densitometric analysis between normal and CRC tissues were assessed using a paired *t*-test. Comparisons of immunoreactive scores were conducted using a linear mixed model. Statistical analyses were performed using GraphPad Prism (version 9.4.1).

3. Results

3.1. PFOS exposure downregulates HMGCS2 expression in intestinal tissues of mice, mouse and human organoid models, and normal human intestinal cells

Mice were administered diets incorporating different fibers: cellulose (control, insoluble), inulin (soluble) or pectin (soluble) and subjected to PFOS exposure in drinking water for a period of 7 weeks (Deng et al., 2022). (Fig. 1A). The RNAseq analysis of intestinal epithelium revealed that expression of HMGCS2, a rate-limiting ketogenic enzyme in the synthesis of ketone bodies, is significantly downregulated in mice exposed to PFOS as compared to a control group (Fig. 1B). In contrast, expression of vascular endothelial growth factor receptor (VEGFR) and alcohol dehydrogenase 6A (ADH6A) are significantly upregulated (Fig. 1B). qRT-PCR analysis further confirmed that HMGCS2 is downregulated and VEGFR is upregulated in mouse intestinal tissues due to PFOS exposure (Fig. 1C). We excluded ADH6A from confirmational analysis since this gene is mouse specific. The further analysis, as demonstrated by Venn diagram showing numbers of overlapping and non-overlapping genes differentially expressed among three different diet groups, revealed that HMGCS2 is the only gene displaying differential expression due to PFOS exposure across all three groups (Fig. 1D). Western blot analysis on intestinal tissues confirmed that HMGCS2 is downregulated at the protein level in PFOS exposed samples across all diet groups (Fig. 1E and F). To further support that reduction of HMGCS2 expression is due to PFOS exposure, wild type mouse normal intestinal organoids were exposed to 1 $\mu\text{g/mL}$ of PFOS in complete culture medium, and Western blot analysis was performed after a 15-day exposure period. The results on organoids established from two different mice demonstrate that PFOS exposure significantly reduces expression of HMGCS2 (Fig. 1G). Moreover, PFOS exposure also decreases the level of HMGCS2 in human normal intestinal organoids treated with PFOS for 30 days (Fig. 1H) and in normal human colon primary (NHCP) cell line treated with PFOS for 6 months (Fig. 1). The morphology of the cells shows no apparent differences between the control and PFOS groups in mouse organoids (Fig. 1G), in human organoids (Fig. 1H) and in NHCP cells (Fig. 1I), suggesting the absence of toxicity associated with PFOS treatment. Together, these results demonstrated that PFOS exposure decreases expression of HMGCS2 in intestinal cells.

3.2. PFOS exposure induces changes in gene expression and upregulates the levels of proteins associated with carcinogenesis in intestinal tissues of mice

The analysis of RNAseq data resulted in identification of three genes

which are significantly changed in the control (cellulose) group due to PFOS exposure; however, PFOS exposure led to significant changes in multiple genes in the inulin and pectin diet groups (Fig. 1D). Therefore, to identify which pathways are dysregulated by PFOS, we performed KEGG analysis on RNAseq data from the inulin and pectin groups. We have identified signal transduction, cancer, lipid metabolism and immune pathways as the top pathways significantly altered by PFOS exposure in both the pectin and inulin groups (Fig. 2A). To assess the impact of PFOS exposure independent of dietary influences, we examined key proteins within the signaling pathways identified in the KEGG analysis in the control and PFOS-treated group on cellulose diet. Interestingly, we found that PFOS upregulates fatty acid synthase (FASN), a key enzyme in *de novo* lipid synthesis and a well-established oncogenic protein associated with CRC (Zaytseva et al., 2012; Zaytseva, 2021; Drury et al., 2022) (Fig. 2B and C). Moreover, we found that PFOS exposure induces the expression of other pro-oncogenic proteins linked to CRC, including β -catenin, c-Myc, p-mTOR, as well as proteins involved in cellular proliferation, such as cyclin D1 and AKT (Nguyen et al., 2020; Huang and Yang, 2022). In contrast, the levels of pERK seem to be lower in the PFOS group (Fig. 2B and C).

3.3. HMGCS2 knockdown in a normal primary colon cell line results in enhanced proliferation and lipid accumulation

To investigate the functional role of HMGCS2 in NHCP cells we used shRNA-mediated knockdown of HMGCS2. As shown in Fig. 3A, knockdown of HMGCS2 leads to cells with significantly increased cell viability. Consistent with our finding in intestinal tissues of mice demonstrating that PFOS-mediated decrease in HMGCS2 expression is associated with an increase in Cyclin D, survivin, AKT and mTOR upregulation and activation, the proteins well known to drive cellular proliferation (Fig. 2B) and knockdown of HMGCS2 also upregulate Cyclin D and survivin expression and activity of AKT and mTOR (Fig. 3B and C). In addition, we observed activation of pERK 1/2 in HMGCS2 knockdown cells. While FASN expression remained unchanged in these cells, we observed upregulation of CD36 expression, a fatty acid transporter (Fig. 3B and C), and an increase in lipid accumulation, as shown by BODIPY staining (Fig. 3D).

3.4. Colorectal cancer is associated with downregulation of HMGCS2 expression

To assess HMGCS2 expression in human specimens and the role of this protein in disease, we utilized public databases. Analysis of The Cancer Genome Atlas data shows that HMGCS2 mRNA expression is lower in CRC tissues compared to normal mucosa (Fig. 4A). Moreover, according to the Protein Atlas data, the decrease of expression of HMGCS2 is notably associated with significantly lower five-year survival rates in patients with colon cancer (Fig. 4B). To validate these results, we assessed the expression of HMGCS2 in matched normal mucosa and CRC tissues in 15 randomly selected cases and showed that HMGCS2 is downregulated in CRC compared to adjacent normal tissues (Fig. 4C and D). Additionally, the expression of HMGCS2 protein was analyzed in a larger number of CRC patients and adjacent normal mucosa through tumor microarray staining. We observed a decrease in HMGCS2 levels corresponding to the tumor stage, with the stages III and IV exhibiting lower levels of HMGCS2 expression as compared to normal mucosa (Fig. 4E and F). Interestingly, in some cases we observed a partial loss of HMGCS2 expression in different parts of the tumor as pointed by the arrows in tumor tissues from case #1582 (stage IV) (Fig. 4F).

4. Discussion

PFOS is one of the most commonly detected chemicals among the PFAS group of contaminants known as “forever chemicals” (Wee and

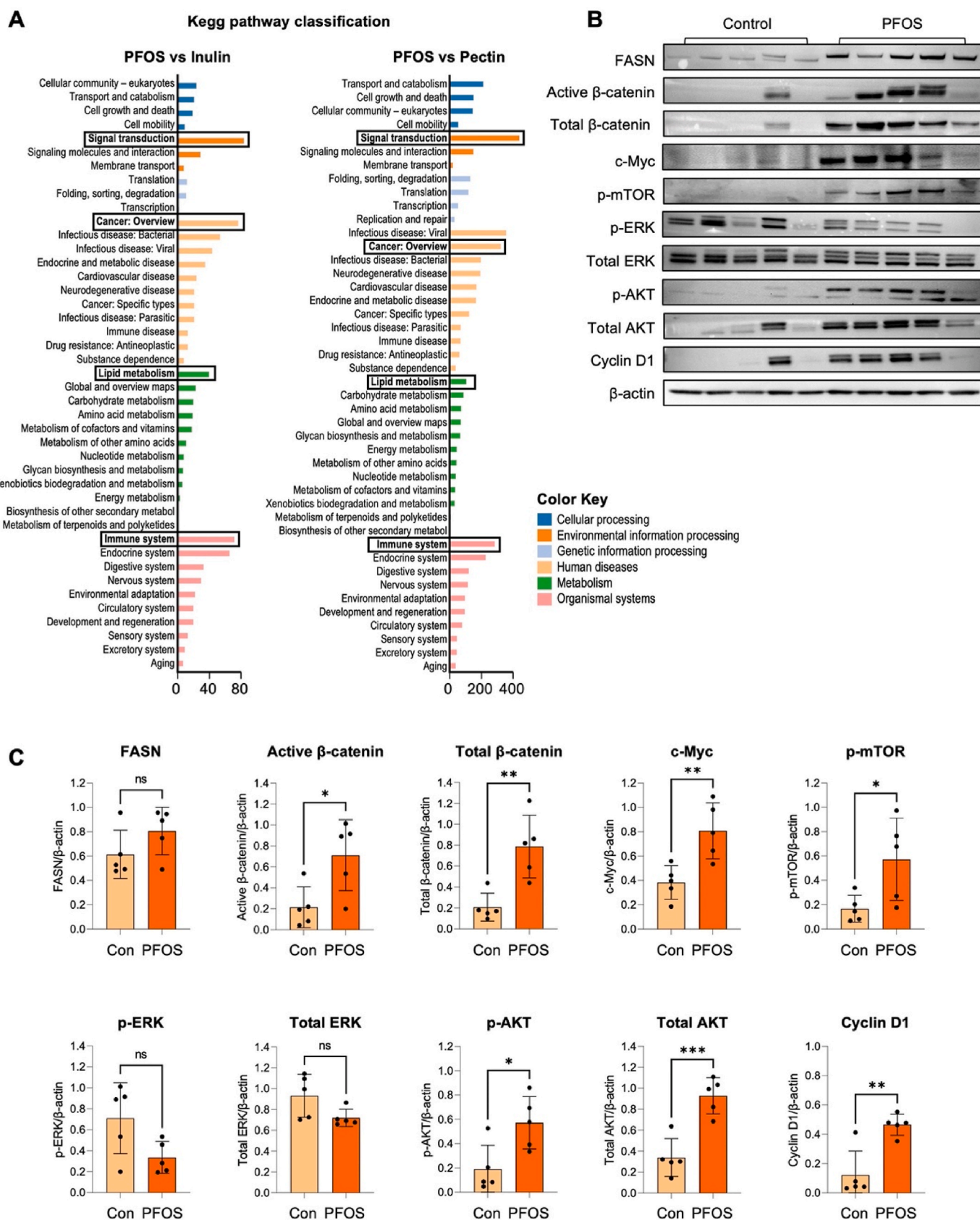


Fig. 2. PFOS exposure upregulates expression of proteins associated with intestinal carcinogenesis. (A) KEGG pathway classification in mice exposed to PFOS and kept on inulin or pectin diets. (B–C) Western blot and densitometric quantification analysis of mouse intestinal tissues. Control (cellulose) versus PFOS (cellulose) groups.

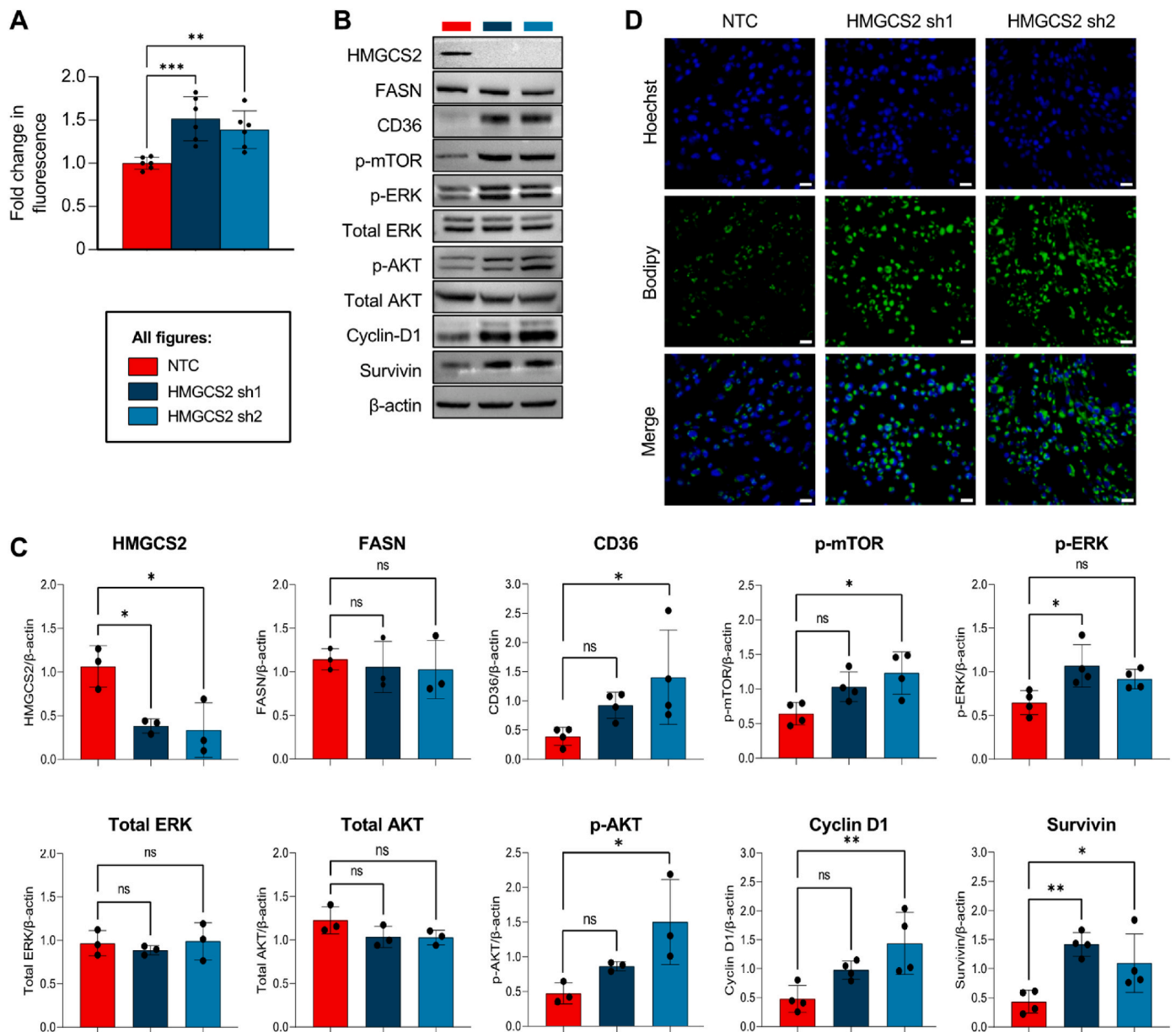


Fig. 3. HMGCS2 knockdown increases cell proliferation and lipid accumulation. (A) Cell viability of NTC and HMGCS2 knockdown normal human colon primary cells measured by Presto Blue assay. Data are presented as mean $\pm$ SD. The one-way ANOVA followed by Dunnett's post-test was used to analyze statistical significance (** $p \leq 0.01$, *** $p \leq 0.001$). (B–C) Western blot and densitometric quantification analysis for HMGCS2 and proliferation/carcinogenic markers. At least 3 replicates were performed for each protein (D) Cells were stained with BODIPY FL (green) and Hoescht (blue) before being processed for confocal analysis (Scale bar: 25 μ m).

Aris, 2023). Although increasing evidence has demonstrated that PFOS exposure may be harmful to human health, there is currently limited available information regarding its impact on the GI tract. In this current study, for the first time, we report that exposure to PFOS in drinking water leads to downregulation of HMGCS2 expression in the intestinal tissues of wild-type mice. Notably, the incorporation of soluble fibers, inulin and pectin, into the diet has no effect on the PFOS-induced downregulation of HMGCS2 protein levels. Additionally, we show that PFOS exposure in drinking water leads to upregulation of proteins associated with colorectal carcinogenesis in the intestinal tissues of these mice. These findings provide a novel insight to how PFOS exposure is linked to adverse health outcomes in the context of GI pathology. Furthermore, this study demonstrates that HMGCS2 plays an important role in regulating cellular proliferation and lipid homeostasis in human normal colon cells *in vitro*. Finally, we show that HMGCS2 protein levels

are downregulated in human CRC compared to normal adjacent tissues. These data collectively suggest an important role of PFOS-mediated downregulation of HMGCS2 expression in pathological alterations of the intestinal epithelium. A summary of our findings is shown in Fig. 5.

The mitochondrial enzyme HMGCS2 is the rate-limiting step in the production of ketone bodies (KBs) (Hwang et al., 2022). Among KBs, β -hydroxybutyrate (BHB) predominates as the most abundant within the circulating KB pool (Dabek et al., 2020). HMGCS2 is primarily expressed in the liver but it is also found in extrahepatic tissues, including colon (Camarero et al., 2006; Nishitani et al., 2022), where it exerts a significant role in maintaining intestinal homeostasis. For instance, HMGCS2 sustains intestinal stem cell numbers and lineage-balanced differentiation. Loss of HMGCS2 compromises intestinal stemness and regeneration after injury, a condition partially restored by the exogenous administration of BHB (Cheng et al., 2019). Another study showed that TNF α

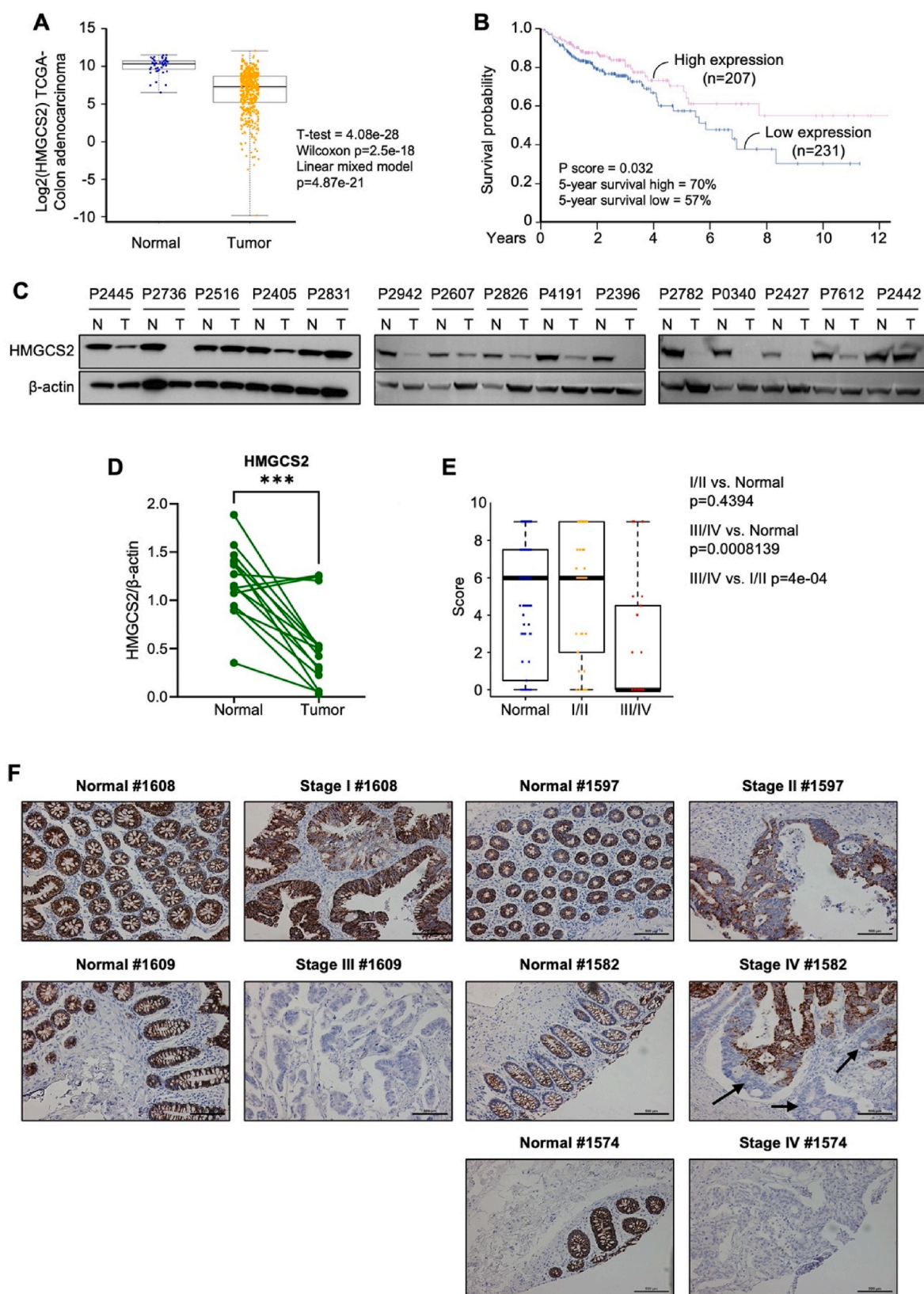


Fig. 4. HMGCS2 mRNA and protein levels are downregulated in CRC. (A) HMGCS2 mRNA expression in colorectal cancer patient samples from the TCGA dataset, comprising 22 normal tissues and 215 tumor samples. (B) 5-year survival analysis of patients with colorectal cancer based on HMGCS2 protein levels (source proteintatlas.org). (C) Western blot analysis and (D) densitometric quantification of HMGCS2 protein levels in 15 randomly selected CRC cases. Matched normal mucosa (N) and tumor tissues (T). β-actin was used as an endogenous loading control. (E) Distribution of HMGCS2 immunoreactivity score was analyzed in tumor tissues from patients diagnosed with CRC stage I-IV. The linear mixed model was used to analyze statistical significance. (F) Representative images of HMGCS2 expression in matched normal and tumor tissues.

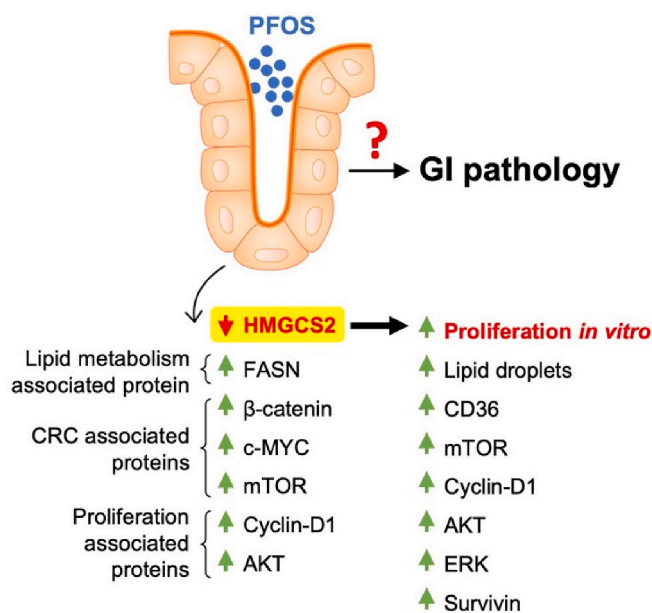


Fig. 5. The potential mechanisms of how PFOS exposure by drinking water alters intestinal homeostasis. PFOS exposure in drinking water leads to downregulation of HMGCS2 and upregulation of FASN, β -catenin, c-MYC, mTOR, cyclin-D1 and AKT protein levels. Furthermore, loss of HMGCS2 results in increased cell proliferation and upregulation of CD36, mTOR, cyclin-D1, AKT, ERK and survivin, along with an increase in lipid accumulation. Further studies are warranted to determine if PFOS exposure can increase the risk of disease-associated GI pathology.

inhibits BHB production by reducing HMGCS2 expression in intestinal cells. Overexpression of HMGCS2 attenuates TNF α -induced apoptosis and inflammatory responses (Kim et al., 2021). Consistent with these findings, HMGCS2 downregulation is observed in intestinal pathological conditions such as IBD (Kim et al., 2021; Martin-Adrados et al., 2023) and CRC (Zou et al., 2019). Moreover, KBs exhibit anti-inflammatory, antioxidant and anti-cancer properties in CRC (Rojas-Morales et al., 2020; Dmitrieva-Posocco et al., 2022; Hwang et al., 2022). On the other hand, PFOS has been linked to the induction of chronic inflammation, immunosuppression, oxidative stress and cellular proliferation (Sarah Elmore et al., 2021; Zhang et al., 2023). Previous reports suggest PFOS involvement in ulcerative colitis and IBD conditions (Liang et al., 2021; National Academies of SciencesE.and Medicine, 2022), which are recognized risk factors for CRC development (Lucafo et al., 2021). These findings suggest a possible mechanistic link between PFOS-induced downregulation of HMGCS2 and GI pathology.

The biological effects induced by PFOS have been suggested to be mediated through activation of nuclear receptors, including peroxisome proliferator activated receptor alpha (PPAR α), constitutive androstane receptor (CAR) and pregnane X receptor (PXR) (Sarah Elmore et al., 2021). Notably, PPAR α positively regulates HMGCS2 gene expression (Grabacka et al., 2016). Interestingly, activation of PXR or CAR has been observed to suppress HMGCS2 expression (Nakamura et al., 2007; Shizu et al., 2020, 2021). The proposed mechanism suggests that both PXR and CAR inhibit PPAR α -dependent gene expression by competing for recruitment of the transcription coactivator PGC1 α (Shizu et al., 2020, 2021). In addition, several studies have provided evidence indicating that PFOS can increase the activity of estrogen receptors (Sarah Elmore et al., 2021), which potentiate the induction of c-MYC (Mukherjee and Conrad, 2005; Rehman et al., 2022). Also, a study found that HMGCS2 is a direct target of c-MYC, leading to the repression of HMGCS2 transcriptional activity in intestinal cells (Camarero et al., 2006). In line with these observations, our study shows upregulation of c-MYC protein levels in the intestinal tissues of PFOS-exposed mice. Collectively, these

findings contribute to the understanding of potential mechanisms through which PFOS may mediate the downregulation of HMGCS2.

Our results demonstrate that the dietary supplement containing the soluble fibers inulin and pectin has no effect on the PFOS-induced downregulation of HMGCS2. Short-chain fatty acids constitute the primary end products of dietary fiber fermentation (den Besten et al., 2013). The major short-chain fatty acids formed by the gut bacteria are acetate, propionate and butyrate (Nogal et al., 2021). In contrast to pectin, inulin increases the relative production of propionate and butyrate over the production of acetate (den Besten et al., 2013). Butyrate, a key energy source for colonocytes, possesses anti-inflammatory properties and enhances the epithelial barrier in intestinal cells, thereby maintaining the epithelial homeostasis (Parada Venegas et al., 2019; Salvi and Cowles, 2021). A depletion of butyrate-producing bacteria and lower intestinal concentrations of butyrate have been observed in IBD patients (Marchesi et al., 2007; Huda-Faujan et al., 2010; Machiels et al., 2014). Butyrate undergoes β -oxidation to form Acetyl-CoA, subsequently used in the citric acid cycle or serving as the substrate for HMGCS2 in the production of KBs (Helenius et al., 2015). As demonstrated by Xu et al. butyrate can regulate the activity of HMGCS2 and the KB levels. Administration of sodium butyrate effectively increases blood BHB levels in mice. Further analysis reveals that sodium butyrate activates HMGCS2, without altering protein levels, through SIRT-5-mediated desuccinylation for KB production (Xu et al., 2023). Additionally, a recent study showed that BHB reduces the proliferation of colonic crypt cells and potently suppresses intestinal tumor growth (Dmitrieva-Posocco et al., 2022). In light of these findings, the downregulation of HMGCS2 induced by PFOS may decrease the potential beneficial effects of diets supplemented with inulin and pectin.

Our study demonstrates that PFOS exposure alters cancer, lipid metabolism and immune system signaling pathways in wild-type mouse intestinal tissues. Moreover, PFOS upregulates protein levels of β -catenin, c-MYC, mTOR and FASN, suggesting the involvement of PFOS in the activation of proteins associated with GI carcinogenesis. In CRC, the frequent hyperactivation of the Wnt signaling pathway is a pivotal initiating and driving event (Schatoff et al., 2017). The central effector in the Wnt canonical pathway is β -catenin. Upon activation, β -catenin is translocated to the nucleus, where it activates Wnt target genes, including c-MYC and CCND1 (gene encoding cyclin D1) (Schatoff et al., 2017). Activation of these genes contributes to the dysregulation of cell proliferation and cell cycle control, two crucial aspects implicated in cancer development and progression (Wang et al., 2008; Montalto and De Amicis, 2020). In addition, activation of Wnt/ β -catenin signaling decreased protein and mRNA levels of HMGCS2 and BHB production in CRC cells and wild-type intestinal organoids (Kim et al., 2019).

mTOR is also a well-known pathway contributing to the initiation and progression of CRC (Alqurashi et al., 2013). Multiple signals activate the mTOR pathway, such as the classical upstream activator AKT (Lagasse, 2013; Wang and Zhang, 2014). The oncogenic activation of PI3K/AKT/mTOR signaling has been suggested to stimulate *de novo* lipogenesis via upregulation of SREBP1, a master transcriptional regulator of lipogenic enzymes such as FASN (Porstmann et al., 2008; Caron et al., 2015; Yi et al., 2018). Furthermore, Wang et al. reported that PI3K/AKT/mTOR signaling cooperates with HMGCS2 in the regulation of intestinal cell differentiation. The authors demonstrated that inhibition of mTOR increases HMGCS2 protein levels and BHB production, contributing to intestinal cell differentiation. Conversely, an increase in ketogenesis inhibits mTOR signaling and induces differentiation (Wang et al., 2017).

Previous studies have reported that PFOS can induce metabolic perturbation and disrupt lipid metabolism in liver of mice (Deng et al., 2022). Lipid metabolism has a profound impact on intestinal inflammation as well as CRC initiation and progression (Zaytseva, 2021; Andersen, 2022). FASN, a key enzyme involved in the *de novo* biogenesis of fatty acids, has been linked to the development of IBD (Matsuo et al.,

2014) and is a well-established hallmark of cancer (Vanauberg et al., 2023). Moreover, upregulation of FASN is associated with CRC cell proliferation, metastatic potential and poor survival of CRC patients (Zaytseva et al., 2012; Lu et al., 2019). In a previous study, it was reported that loss of HMGCS2 enhances lipogenesis and FASN expression in hepatocellular carcinoma (Wang et al., 2020). In contrast, our data show that knockdown of HMGCS2 in normal intestinal cells does not alter FASN protein levels. Instead, HMGCS2 knockdown promotes the overexpression of CD36 and accumulation of lipid droplets. Fatty acids are obtained either through endogenous synthesis or by exogenous uptake via cell surface receptors, such as CD36 (Zaytseva, 2021; Guerrero-Rodriguez et al., 2022). Fatty acids are incorporated into triglycerides, and excessive triglycerides, along with other lipid types like cholesterol, are stored in lipid droplets (Zaytseva et al., 2015; Zaytseva, 2021). Notably, accumulation of lipid droplets has been reported in CRC compared to normal colonic tissues (Zaytseva et al., 2015; Salita et al., 2022). Taken together, our results suggest that PFOS-induced upregulation of FASN is not mediated by HMGCS2. However, HMGCS2 seems to play a significant role in lipid metabolism, partially through the upregulation of CD36. Further studies are needed to better understand the mechanism of PFOS-induced lipid alterations in normal intestinal and cancer cells.

In the present study, we demonstrated that PFOS exposure and subsequent HMGCS2 downregulation induce upregulation of proteins associated with cellular proliferation, such as cyclin D and AKT in intestinal tissues of mice. Interestingly, we observed a similar upregulation of these proteins in human normal colon cells with HMGCS2 knockdown, and a decrease in HMGCS2 expression was accompanied by an increase in cell viability. These findings are consistent with other studies demonstrating that loss of HMGCS2 protein promotes proliferation and triggers upregulation of cyclin D expression (Wan et al., 2019; Wang et al., 2019; Mao et al., 2023). Together, these data suggest that PFOS-induced downregulation of HMGCS2 may contribute to the regulation of cellular proliferation.

Finally, our results are in accordance with previous studies that demonstrated lower HMGCS2 protein levels in CRC compared to normal mucosa and lower expression of HMGCS2 is associated with lower survival rate of CRC patients (Zou et al., 2019; Wei et al., 2022). We also demonstrated a trend of downregulation in HMGCS2 with the progression of tumor stages, particularly evident in stages III and IV where significantly lower levels of HMGCS2 expression were observed. In published studies, downregulation of HMGCS2 has been linked to advanced stages of hepatocellular carcinoma and poorly differentiated CRC, suggesting that HMGCS2 expression decreases with progression of cancer to more advanced stages and is associated with aggressiveness of disease (Camarero et al., 2006; Wang et al., 2019). Our data suggest that PFOS-mediated downregulation of HMGCS2 and upregulation of oncogenic proteins may be a crucial step in disrupting intestinal homeostasis and contributing to disease initiation.

In summary, this study is the first to demonstrate that PFOS exposure downregulates HMGCS2 levels in intestinal cells. Although incorporating inulin or pectin supplemented diets had no impact on the PFOS-induced downregulation of HMGCS2, diet-derived soluble fibers may still be protective against pathologies of carcinogenesis either directly or indirectly through mechanisms other than down-regulation of HMGCS2 (Song et al., 2015; Sivaprakasam et al., 2016; Vernia et al., 2021). Furthermore, PFOS induces the upregulation of oncogenic proteins associated with CRC and increases the expression of proliferation-associated proteins, suggesting the potential role of PFOS in promoting GI pathological changes and increasing risk of CRC. Overall, these findings provide novel insights into the mechanisms by which PFOS can contribute to the pathogenesis of the GI tract.

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CRediT authorship contribution statement

Josiane Weber Tessmann: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Conceptualization. **Pan Deng:** Writing – review & editing, Project administration, Methodology, Conceptualization. **Jerika Durham:** Writing – review & editing, Project administration. **Chang Li:** Project administration. **Moumita Banerjee:** Project administration. **Qingding Wang:** Writing – review & editing, Conceptualization. **Ryan A. Goettl:** Project administration. **Daheng He:** Project administration. **Chi Wang:** Writing – review & editing, Project administration. **Eun Y. Lee:** Methodology. **B. Mark Evers:** Writing – review & editing. **Bernhard Hennig:** Writing – review & editing, Methodology, Conceptualization. **Yekaterina Y. Zaytseva:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data have been deposited in NCBI's Gene Expression Omnibus and are assessable through GEO Series accession number GSE248402 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE248402>).

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Abbreviations

ADH6A	alcohol dehydrogenase 6A
BHB	β -hydroxybutyrate
CAR	constitutive androstane receptor
CRC	colorectal cancer
FASN	fatty acid synthase
GI	gastrointestinal
HMGCS2	3-hydroxy-3-methylglutaryl-CoA synthase 2
IBD	inflammatory bowel disease
KBs	ketone bodies
NHCP	normal human colon primary
PFAS	perfluoroalkyl and polyfluoroalkyl substances
PFOS	perfluorooctanesulfonic acid
PPAR α	peroxisome proliferator activated receptor alpha
PXR	pregnane X receptor
RT-PCR	real-time reverse transcription-polymerase chain reaction
VEGFR	vascular endothelial growth factor receptor

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