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The effects of folic acid on global DNA methylation and colonosphere formation in colon cancer cell lines☆

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Abstract

Folate and its synthetic form, folic acid (FA), are essential vitamins for the regeneration of *S*-adenosyl methionine molecules, thereby maintaining adequate cellular methylation. The deregulation of DNA methylation is a contributing factor to carcinogenesis, as alterations in genetic methylation may contribute to stem cell reprogramming and dedifferentiation processes that lead to a cancer stem cell (CSC) phenotype. Here, we investigate the potential effects of FA exposure on DNA methylation and colonosphere formation in cultured human colorectal cancer (CRC) cell lines. We show for the first time that HCT116, LS174T, and SW480 cells grown without adequate FA demonstrate significantly impaired colonosphere forming ability with limited changes in CD133, CD166, and EpCAM surface expression. These differences were accompanied by concomitant changes to DNA methyltransferase (DNMT) enzyme expression and DNA methylation levels, which varied depending on cell line. Taken together, these results demonstrate an interaction between FA metabolism and CSC phenotype *in vitro* and help elucidate a connection between supplemental FA intake and CRC development.
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Keywords: Folate; Colorectal cancer; Colonospheres; DNMT; Cancer stem cell; DNA methylation

1. Introduction

Folate is the generic term for a group of essential B_9 vitamin compounds required for cellular biosynthesis and methylation [1]. Folic acid (FA) is the oxidized, more stable, synthetic form of folate primarily found in supplements and fortified foods [1]. Naturally occurring folate derivatives in their reduced forms are chemically unstable, which contributes to nutrient loss during harvesting, storage, and preparation [1,2]. Additionally, reduced folates require separation from polyglutamyl chains prior to absorption at the brush border, greatly reducing bioavailability [1–3]. In contrast, FA is conjugated to only one glutamate residue and has close to 100% bioavailability [1–3]. Once absorbed, FA must be converted to dihydrofolate via dihydrofolate reductase in the liver before being converted to tetrahydrofolate where it can enter the folate pool [1–3]. In 1998, sufficient evidence for folate's protective effects on preventing neural tube defects (NTD) subsequently led to the mandatory fortification of FA in grain products within Canada and the US [1,3]. Since then, a substantial beneficial effect on the original target, NTD, has been achieved but concerns have risen regarding the potential harmful effects of such chronically high FA levels [1,3,4]. The high bioavailability combined with its chemical stability has led to unnatural levels of unmodified FA directly entering the circulation [1,3]. One of the suggested risk factors associated with high FA intake is colorectal cancer (CRC) [1,3,4]. Further research is required to define the complex relationship between FA and CRC development to ensure safe and responsible fortification practices.

The role that B_9 vitamin folate and its synthetic form, FA, play in CRC development remains controversial [5–7]. Some epidemiological studies report that high dietary and blood folate levels inhibit CRC development [8,9]. However, more recent data from epidemiological and clinical trial studies suggest that high FA intake and subsequent high serum levels may actually increase cancer risk [10,11]. Rodent studies suggest that the effect of FA on CRC development is dependent on the underlying neoplastic status of the tissue [12]. FA supplementation before neoplastic transformation seems to be protective, while that following the formation of potentially undetectable preneoplastic colonic lesions may enhance CRC development [13]. Additionally, due to the stability and high bioavailability of FA postfortification, FA exposure may have been underestimated, significantly increasing FA levels in the population. Therefore, fortification of FA in Canada and

☆ Conflict of Interest. The authors declare that they have no conflict of interest.

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the US may be inadvertently increasing the risk for CRC in subpopulations vulnerable to colonic preneoplastic lesions.

The connection between folate and carcinogenesis is possibly a result of its role in both nucleotide biosynthesis and DNA methylation. Folate deficiency has been shown to result in purine and thymidine insufficiency, resulting in inadequate repair of DNA damage that impairs cellular proliferation; this is the basis for antifolate chemotherapeutics [14–16]. Additionally, folate is critical for the provision of methyl moieties that are used to synthesize *S*-adenosyl methionine (SAM), the universal methyl donor for DNA methylation [17]. Methylation of cytosine-guanine dinucleotides (CpG) is an epigenetic modification essential in maintaining chromosomal stability and regulating genetic expression in approximately half of all human genes [17]. DNA methyltransferases (DNMTs) are the enzymes responsible for establishing the original methylation pattern (*de novo* methylases DNMT3a and DNMT3b) and for maintaining it throughout subsequent cellular divisions (maintenance methylase DNMT1) [17]. Thus, FA plays a pivotal role in maintaining genomic integrity and gene expression profiles.

Aberrant DNA methylation is a hallmark of cancer, including CRC, and is characterized by both global hypomethylation [contributing to oncogenic gene activation, loss of heterozygosity, and chromosomal instability (CIN)] and simultaneous site-specific hypermethylation, contributing to the inactivation of tumor suppressors [17–20]. Disrupting DNA methylation patterns may allow cancer cells to manipulate gene expression in order to repress differentiation while simultaneously maintaining self-renewal, thus gaining a cancer stem cell (CSC) phenotype [21,22]. CSCs share properties with native stem cells and are vital in the development and perpetuation of tumor regrowth and metastasis [23]. Somatic stem cells utilize the same mechanism to maintain self-renewal as well as orchestrate cellular differentiation in a timely and accurate manner [21]. Global hypomethylation is a common and early event during CRC development [24], suggesting that it may be associated with disrupted CSC reprogramming in the colon. Random genomic hypomethylation alone may be sufficient to deregulate transcription factor activation, leading to ectopic gene expression and increased CSC characteristics such as poor differentiation, increased motility, and invasiveness [25,26]. Recent reports demonstrate that DNA methylation regulates expression of colon CSC surface proteins as well as targets downstream of Wnt signaling [27,28], a vital pathway involved in the maintenance of normal intestinal stem cells and the growth of CSC *in vitro* [29].

Targeting the CSC population independently from the bulk of the tumor may be an effective approach toward treating CRC [30]. The role of FA in the maintenance of colorectal CSCs has yet to be determined but may provide critical information on cancer development. In this investigation, we studied the effects of FA exposure on global DNA methylation and DNMT protein expression profiles in colon cancer cells *in vitro*. These changes correlated with altered cell proliferation under standard monolayer conditions and altered the ability of cells in nonadherent stem cell culture to generate colonospheres. Thus, varying levels of FA supplementation can alter CRC cell proliferation, DNA methylation, and stem cell phenotype *in vitro*.

2. Materials and Methods

2.1. Tissue culture

Human CRC cell lines HCT116, LS174T, and SW480 (purchased from the American Type Culture Collection, Manassas, VA, USA) were cultured in Roswell Park Memorial Institute 1640 media (RPMI-1640; Life Technologies, Burlington, ON, Canada), containing 10% fetal bovine serum (FBS; Life Technologies) and 1% gentamycin (Sigma-Aldrich, Oakville, ON, Canada) in a 37°C humidified incubator with 5% CO_2. After 24 h, individual cultures were treated with folate-free RPMI-1640 (RPMI-1640, no FA; Life Technologies) supplemented with 10% dialyzed FBS (DFBS; Life Technologies), 1% gentamicin, and FA (Sigma-Aldrich) dissolved in 1 M NaOH. Treatment media contained a final concentration of 0 mg/L (deficient), 4 mg/L (control), or 16 mg/L (excess) FA. Cells were resupplemented with media every 1–2 days, harvested after 7 days of treatment, and used for assays described below. The cell lines chosen for this study represent various molecular pathways associated with colorectal carcinogenesis. HCT116 and LS174T cells are both microsatellite instable (MSI), which refers to the genomic instability that occurs in the cells particularly within repetitive regions [31]. MSI can occur due to a mutation resulting in nonfunctional mismatch repair machinery or, more commonly, silencing of the machinery altogether as a result of promoter methylation [32]. This results in the accumulation of unaddressed errors during DNA replication. In addition, HCT116 cells show a CpG island methylator phenotype (CIMP) characterized by epigenetic instability [31]. SW480 cells are characterized as microsatellite stable (MSS) with a CIN phenotype [31]. Neoplasms with CIN phenotypes develop from the accumulation of structural and numerical chromosomal errors [32]. Genotypically, SW480 cells carry mutation in both p53 and adenomatous polyposis coli (APC) while HCT116 and LS174T maintain the wild-type genes [31,33].

2.2. Extraction and analysis of intracellular folates

The method of Kashani *et al.* was adopted for these analyses [34]. Treated cells were trypsinized and washed three times with cold PBS. Cell pellets were resuspended in 0.05 M potassium phosphate buffer, pH 6.5, containing 0.2 M mercaptoethanol, and stored at −80°C until analysis. For extraction of intracellular folates, cell suspensions were boiled for 5 min then cooled at 4°C for 5 min. Resulting cell homogenates were centrifuged at room temperature for 15 min at 1000*g* and supernatants were incubated with 10% (by volume) charcoal-treated rat serum at 37°C for 2 h to convert folate polyglutamate forms into monoglutamates. Protein was precipitated with 70% methanol saturated with sodium ascorbate and the precipitates were removed by centrifugation at 2000*g* at 4°C for 15 min. The samples were dried in flowing nitrogen gas at 37°C and kept on dry ice. Just before injection to high-performance liquid chromatography (HPLC), the samples were resuspended in 0.25 ml of 0.05 M potassium phosphate buffer, pH 6.5. The suspensions were filtered through a 0.45-μm membrane filter and 0.1 ml was injected into the reverse-phase column (SUPELCOSIL LC-18, 25 cm×4.6 mm, 5 μm). HPLC was carried out using a Waters HPLC system equipped with a HP Series 1050 UV detector set to 290 nm. The mobile phase was 10% acetonitrile in 20 mM phosphate buffer, pH 3.3, pumped at a flow rate of 0.5 ml/min. FA (MDL=0.03 mg/L), tetrahydrofolic acid (MDL=0.1 mg/L), and 5-methyltetrahydrofolic acid (MDL=0.05 mg/L) were measured and expressed as values per 10^6 cells.

2.3. Extraction and analysis of intracellular SAM and S-adenosyl homocysteine (SAH)

Analysis of SAM and SAH was performed based on a previously published method with some modifications [35]. Treated cells were trypsinized and washed twice with cold PBS and cell pellets were stored at −80°C until analysis. We added 1 ml cold 0.5 M $HClO_4$ containing 0.3% (w/v) $Na_2S_2O_5$ and 0.1% (w/v) EDTA to the cells on ice and sonified for 15 s. Samples were left on ice for 1 h to allow macromolecules to precipitate, and samples were centrifuged at 9000*g* for 15 min. The resultant supernatant was kept at −20°C until analysis. Before analysis, pH was adjusted to 4–5 by NaOH. The samples were filtered through a 0.45-μm membrane filter and 0.1 ml was injected into the reverse-phase column (SUPELCOSIL LC-18, 25 cm×4.6 mm, 5 μm). The HPLC measurements were carried out using a Waters HPLC system equipped with a HP Series 1050 UV detector set to 254 nm. The mobile phase was 18% acetonitrile in 20 mM phosphate buffer, pH 4.5, and was pumped at a flow rate of 0.5 ml/min. SAM (MDL=1.0 mg/L) and SAH (MDL=1.0 mg/L) were measured and expressed as values per 10^6 cells.

2.4. Proliferation assays

Cell number and viability were measured using Trypan blue exclusion and methyl thiazolyl tetrazolium (MTT) assay (both from Sigma-Aldrich). A total of 5×10^3 cells were transferred to individual wells on a 96-well plate and treated with different treatment media as described above. After 7 days, MTT assay was performed according to manufacturer's directions. The absorbance of each well was then measured using a 96-well colorimetric plate reader at a test wavelength of 570 nm and reference wavelength of 690 nm, and percent viability was calculated as (corrected optical density of deficient or excess FA-treated cells/corrected optical density of 4 mg/L FA-treated cells)×100.

2.5. Colonosphere limiting dilution analysis

Cells maintained under standard media conditions as described above were pelleted at 350*g* for 4 min and resuspended in serum-free stem cell media (SCM) consisting of Dulbecco's modified Eagle medium:nutrient mixture F12 (Life Technologies), 10% B27 supplement (Life Technologies), 10 ng/ml fibroblast growth factor, 20 ng/ml epidermal growth factor, and 1% gentamycin (all Sigma-Aldrich). Cell suspension was then plated in 96-well ultralow adhesion plates (Corning, Corning, NY, USA) at concentrations of 1, 10, and 100 cells per well and cultured for 15 days with media changes every 3–4 days. The number of colonosphere-positive wells was then counted to quantify the frequency of sphere formation.

2.6. FA exposure and colonosphere formation

After 7 days of incubation in FA-supplemented RPMI as described above, treated cells were plated in 96-well ultralow adhesion plates (Corning) at 10 cells per well in 200 μl. Wells were resupplemented with media as described above. After 15 days, the number of colonosphere-positive wells was then counted to quantify the frequency of sphere formation.

2.7. Western blotting

Cells treated with FA for 7 days were pelleted and disrupted with lysis buffer (Cell Signaling Technology, Massachusetts, USA) containing protease inhibitors (Sigma-Aldrich). Cell lysate was incubated for 5 min on ice before centrifugation at 12,000g for 15 min at 4°C. Supernatant was collected and the protein concentration was quantified using the Bio-Rad DC Protein Assay Kit. We loaded 50 μg of total protein/sample into a 7.5% polyacrylamide gel and subject it to electrophoresis at 125 V for 80 min. Separated protein was then transferred onto a methanol-activated polyvinylidene difluoride membrane, blocked for 1 h at room temperature with either 5% (w/v) nonfat milk or 5% BSA in Tris-buffered saline/Tween 20 (TBS-T), and incubated overnight at 4°C with primary antibodies diluted in 5% (w/v) nonfat milk or BSA. After incubation, membranes were washed with TBS-T, incubated with secondary antibodies in 5% (w/v) nonfat milk in TBS-T for 1 h at room temperature, washed, and subjected to chemiluminescent HRP substrate Luminata Forte (Millipore, Darmstadt, Germany). Membranes were imaged using the Bio-Rad ChemiDoc XRS+ system and densitometric analysis was performed using the Image Lab Software (Bio-Rad). Molecular weight of proteins was determined by comparison with GeneDirex BLUeye Prestained Protein Ladder (FroggaBio Inc., Toronto, ON, Canada). Densitometric analysis was done using the Bio-Rad Image Lab Software. DNMT band density was normalized to α-tubulin band density for semiquantitative analysis of protein levels. Primary antibodies included mouse-anti-α-tubulin (1:600,000; Sigma-Aldrich), rabbit-anti-DNMT1 (1:2000; Cell Signaling), rabbit-anti-DNMT3a (1:500; Cell Signaling), and rabbit-anti-DNMT3b (1:500; Cell Signaling). Secondary antibodies included HRP-labeled goat-antimouse antibody (1:20,000; Sigma-Aldrich) and HRP-labeled goat-antirabbit antibody (1:20,000; Sigma-Aldrich).

2.8. DNA isolation

Genomic DNA was isolated from FA-treated cells using DNeasy Blood and Tissue Kit (Qiagen, Venlo, Limburg, Netherlands) according to the manufacturer's protocol. The concentration and purity were determined by measuring the absorbance at 230, 260, and 280 nm using a Nanodrop ND-1000 (Thermo Scientific, Waltham, MA, USA).

2.9. DNA methylation quantification

Global DNA methylation was quantified using EpiSeeker methylated DNA Colorimetric Quantification Kit (Abcam, Cambridge, UK) according to the manufacturer's directions, except that 300 ng of DNA per reaction was used. Absolute and relative methyl-cytosine content was then calculated using the supplied formula.

2.10. Flow cytometry analysis

CD133, CD166, and EpCAM staining was performed following FA treatment as described above. Briefly, cells were collected by trypsinization and fixed in 4% paraformaldehyde for 10 min at 37°C then chilled on ice for 1 min. Approximately 1×10^6 cells per treatment were blocked in 0.5% BSA/PBS for 10 min in room temperature then incubated for 10–30 min at room temperature in PE-conjugated human-CD133 (Miltenyi Biotec, San Diego, CA, USA), PerCP-conjugated human-EpCAM/TROP1 (R&D Systems, Inc., Minneapolis, MN, USA), and Alexa Fluor 488-conjugated human ALCAM/CD166 (R&D Systems, Inc.) antibodies diluted 1:22, 1:20, and 1:40, respectively, in 0.5% BSA/PBS. Cells were collected and resuspended in PBS for analysis using a BD Accuri C6 flow cytometer (BD Biosciences, Mississauga, ON, Canada). Unstained cells were analyzed to correct for background fluorescence and to establish gating parameters for each cell line. In total, 1×10^4 events were counted per sample.

2.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). Nonparametric Kruskal–Wallis and Dunn's multiple comparison tests were used to determine if differences existed between treatments. Linear regression was used to analyze changing intracellular FA levels in response to medium supplementation. At least three biological replicates were used for each statistical analysis, and treatments were considered significantly different if statistical tests produced a P value of ≤.05.

3. Results

3.1. Cellular growth

HCT116 and LS174T cells treated with 16 mg/L FA had significantly increased cellular proliferation compared to 0 mg/L FA conditions (P=.0127 and 0.0459, respectively; Fig. 1A). These results are in agreement with results from Trypan blue exclusion experiments showing that HCT116 and LS174T cells treated with 16 mg/L FA had a significantly higher number of viable cells compared to 0 mg/L FA conditions (P=.0407 and 0.0136, respectively; Fig. 1B). Although no significant differences in the proliferation SW480 cells were detected by the MTT assay (Fig. 1A), a significant increase in viable cells following 4 mg/L FA treatment compared to 0 mg/L FA was shown (P=.0225; Fig. 1B).

3.2. Intracellular folate

To measure whether exposure to higher FA supplementation in cell medium actively changed cellular folate levels, HPLC analysis was done to quantify intracellular folate levels. HCT116, LS174T, and SW480 cells all demonstrate a significant positive correlation in intracellular folate levels with increasing FA media supplementation (P=.0023, P=.0459, and P=.0002, respectively; Fig. 2A–C). Despite the lack of FA added to the 0 mg/L treatment group, these cells still

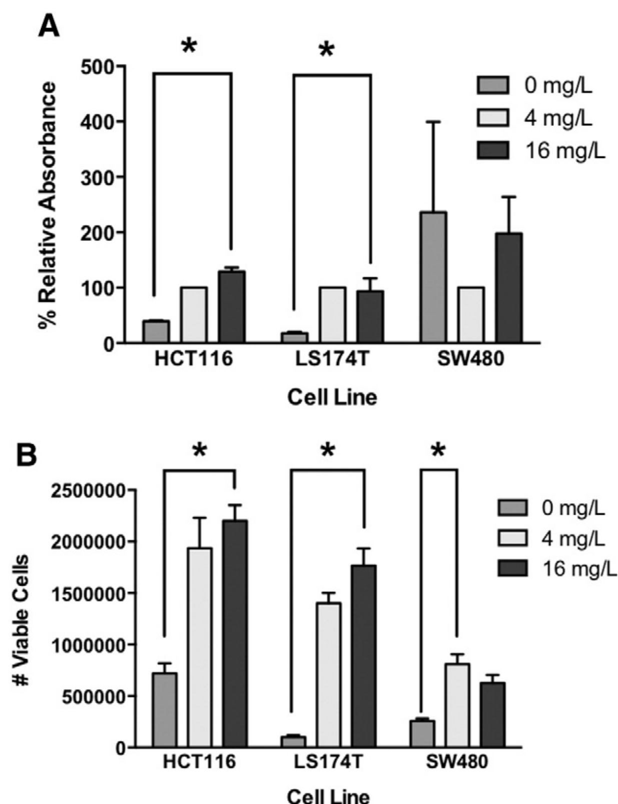

Fig. 1. FA levels influenced CRC cell proliferation in a dose-dependent and cell-line-dependent fashion. (A) Relative proliferation of FA-treated cells quantified via MTT assay, normalized to absorbance of 4 mg/L FA treatment. Percent viability was calculated as (corrected optical density of deficient or excess FA-treated cells/corrected optical density of 4 mg/L FA-treated cells)×100. A total of 16 mg/L FA significantly increased proliferation of HCT116 and LS174T cells compared to the 0 mg/L FA treatment (*P=.0127 and 0.0459, respectively). (B) Cellular proliferation quantified by measuring viable cells via Trypan blue exclusion assay. A total of 16 mg/L FA in HCT116 and LS174T cells and 4 mg/L FA in SW480 cells significantly increased proliferation compared to the 0 mg/L FA (*P=.0407, 0.0136, and 0.0225, respectively). The data represent the mean±S.E. of three independent experiments in triplicate.

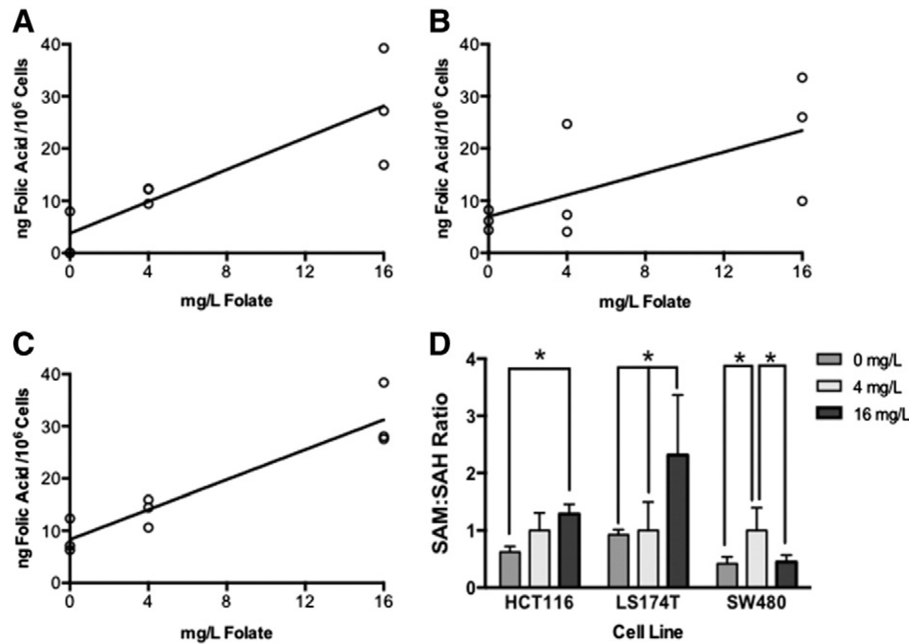

Fig. 2. Intracellular folate and methionine compounds SAM and SAH were measured using HPLC. Linear regression of intracellular folate levels in response to FA media supplementation in (A) HCT116 cells (*P*=.0023), (B) LS174T cells (*P*=.0459), and (C) SW480 cells (*P*=.0002). (D) Graph representing relative intracellular SAM:SAH ratios in response to FA treatment. HCT116, LS174T, and SW480 cells all showed a significant increase in SAM:SAH ratio in response to increasing FA (*P*=.0036, *P*=.00196, and *P*=.015, respectively). However, this effect was mitigated following 16 mg/L FA in SW480 cells.

exhibited detectable levels of folate, which ranged between 90% and 72% less than the highest treatment group (16 mg/L FA). This may be explained by the minute quantities of folate found in the DFBS supplement added to the medium.

3.3. Intracellular SAM and SAH

To measure whether FA media supplementation had a physiological consequence on methionine cycle intermediates, HPLC analysis was done to quantify relative SAM and SAH levels. HCT116 and LS174T cells both showed increasing SAM:SAH ratios with increasing FA media levels, with the highest supplementation level (16 mg/L) being 60–50% greater than the corresponding lowest supplementation level (0 mg/L) (*P*=.0036 and *P*=.00196, respectively; Fig. 2D). SW480 cells showed a significantly higher SAM:SAH ratio at the standard FA supplementation level (4 mg/L FA) compared to the deficient group (0 mg/L) (*P*=.015); however, this effect was mitigated at the highest supplementation level (16 mg/L) (Fig. 2D).

3.4. DNMT1, DNMT3a, and DNMT3b protein expression

Western blot analysis showed that increasing FA supplementation in general resulted in increased DNMT1, decreased DNMT3a, and no significant effect on DNMT3b protein expression, with the exception of SW480 DNMT1 protein expression. In HCT116 and LS174T cells, 16 mg/L FA supplementation increased DNMT1 protein expression by 58–57% (*P*=.0027 and *P*=.0048, respectively) and reduced SW480 cell DNMT1 protein expression by 60% (*P*=.0108) compared to 0 mg/L FA-treated cells (Fig. 3A). HCT116 cells showed the only significant change in DNMT3a protein expression, with 85% reduction in the 16 mg/L FA-supplemented group compared to 0 mg/L (*P*=.001; Fig. 3B). SW480 cells also showed a suggestive, albeit nonsignificant, 50% reduction in DNMT3a expression when supplemented with 16 mg/L FA compared to 0 mg/L FA (Fig. 3B). DNMT3a protein was undetectable in LS174T cells under all FA treatment levels (Fig. 3B). No significant differences were detected regarding changing DNMT3b protein levels; however, SW480 cells showed a nonsignificant inverse dose response to FA level with a 64% reduction in DNMT3b protein level following 16 mg/L FA supplementation compared to 0 mg/L FA (Fig. 3C). HCT116 cells also showed a similar nonsignificant decrease in DNMT3a protein level in the 4 mg/L FA group compared to 0 mg/L (Fig. 3C).

3.5. DNA methylation

We observed an inverse dose response between genomic DNA methyl-cytosine content and FA supplementation (Fig. 4). The methyl-cytosine content of DNA from HCT116 and LS174T cells was 46% and 57% higher, respectively, under 0 mg/L FA conditions compared to cells treated with 16 mg/L FA (*P*=.0341 and *P*=.0341, respectively) (Fig. 4). No significant effects of FA treatment on global DNA methyl cytosine were detected in SW480 cells.

3.6. Colonosphere formation

Monolayer cultured cells were passaged at concentrations of 1, 10, and 100 cells per well in a 96-well ultralow adhesion plate (Corning) supplemented with SCM, to assess their ability to grow as colonospheres (Fig. 5A). All cell lines showed a high ability to proliferate under these conditions, and the efficiency of colonosphere formation significantly increased with number of cells originally plated (*P*≤.05; Fig. 5B). From these data, it was determined that 10 cells per well was the optimal number to assess differences in colonosphere formation in response to FA treatment.

HCT116 cells treated with 0 mg/L FA significantly produced 82.8% and 84.4% fewer colonosphere-positive wells compared to cells treated with 4 mg/L (*P*=.0411) and 16 mg/L (*P*=.0315) FA, respectively (Fig. 6). LS174T cells treated with 0 mg/L FA produced 54.7% and 53.1% fewer colonosphere-positive wells compared to cells treated with 4 mg/L (*P*=.0087) and 16 mg/L (*P*=.0832) FA, respectively (Fig. 6). For SW480 cells, however, 0 mg/L FA reduced colonosphere-positive wells by only 18% and 14.8% compared to cells treated with 4 mg/L (*P*=.0448) and 16 mg/L (*P*=.1646) FA, respectively (Fig. 6).

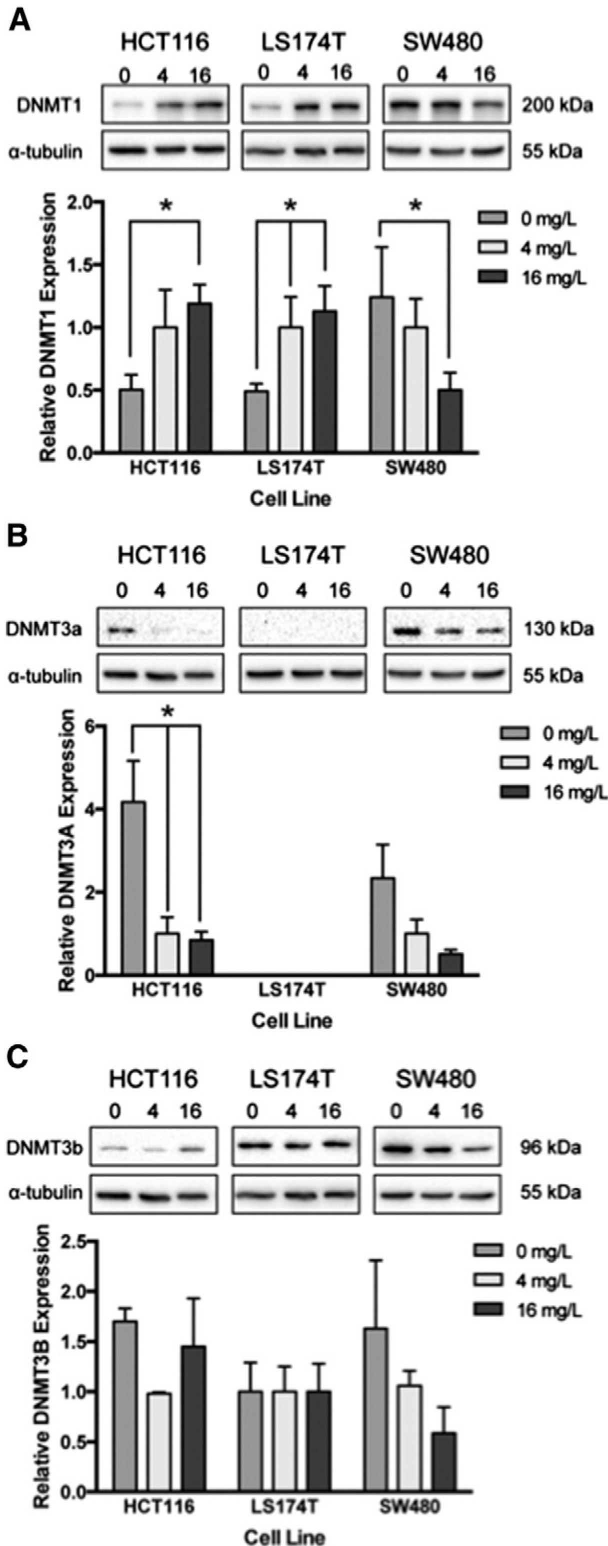

Fig. 3. Western blot analysis of DNMT protein expression in response to FA supplementation. Representative western blot and densitometric analysis of (A) DNMT1, (B) DNMT3a, and (C) DNMT3b protein expression normalized to α-tubulin then to respective protein expression in the 4 mg/L FA group. (A) A total of 16 mg/L FA significantly increased DNMT1 in HCT116 and LS174T cells and reduced it in SW480 cells (P=.0027, P=.0048, and P=.108, respectively). (B) At total of 16 mg/L FA significantly reduced DNMT3a protein expression in HCT116 cells (P=.001). LS174T cell exhibited no detectable levels of DNMT3a protein in all FA treatments. (C) No significant differences were detected in DNMT3b protein expression in response to FA treatment.

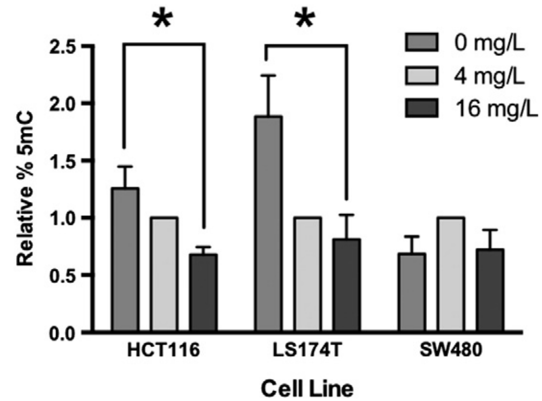

Fig. 4. FA supplementation reduced while FA deficiency increased global genomic methyl-cytosine content. The percentage of 5-methyl cytosine (% 5mC) of each sample was normalized to the 4 mg/L FA group for each cell line. A total of 16 mg/L of FA significantly reduced global methyl-cytosine content in HCT116 and LS174T cells compared to the 0 mg/L FA conditions (*P=.0104 and P=.0434, respectively). The data represent the mean±S.E. of three independent experiments in triplicate.

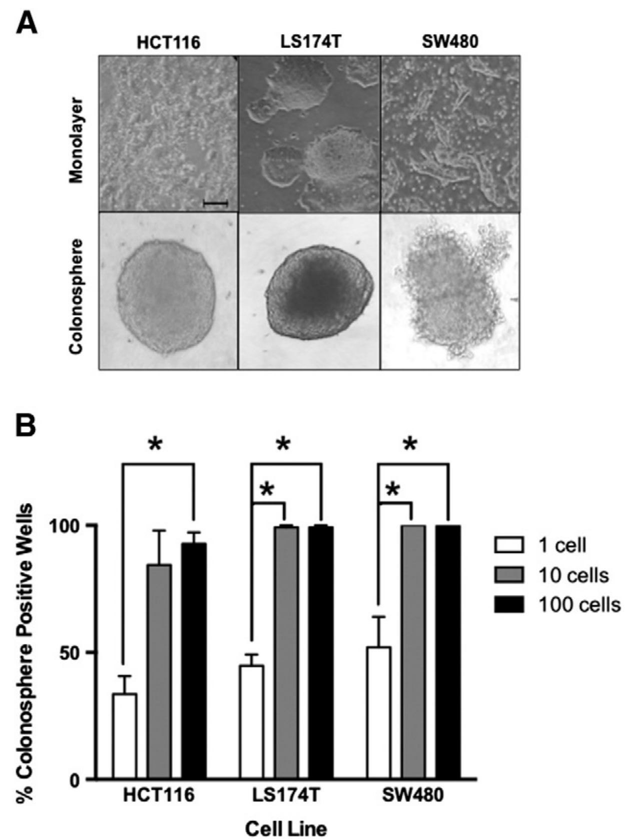

Fig. 5. Colonosphere formation of HCT116, LS174T, and SW480 cells when grown in SCM under low-adhesion conditions. (A) Representative photomicrographs of HCT116, SW480, and LS174T cells grown in monolayer (top) and in colonosphere culture for 10 days (bottom). Scale bar represents 100 μm. (B) Limiting dilution analysis of 1, 10, and 100 cells per well. HCT116 cells plated at a density of 100 cells per well had significantly higher colonosphere yields than at 1 cell per well (*P=.0217), while LS174T and SW480 cells plated at densities of both 10 and 100 cells per well had significantly higher colonosphere yields than at 1 cell per well (*P=.0233 and P=.0102, respectively). The data represent the mean±S.E. of four independent experiments.

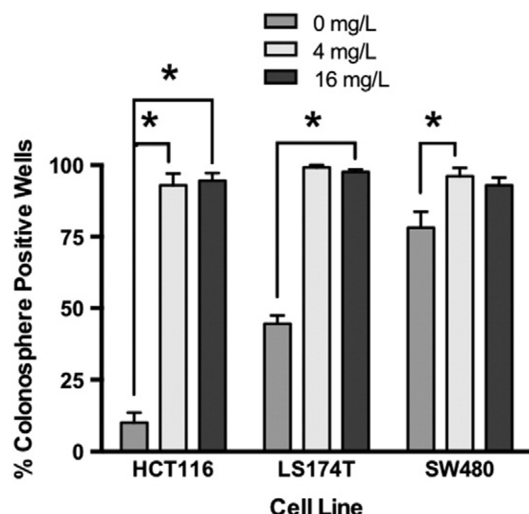

Fig. 6. FA deficiency impairs colonosphere formation in HCT116, LS174T, and SW480 cells. HCT116 cells treated with either 4 mg/L FA or 16 mg/L FA generated significantly higher colonosphere yields than cells treated with 0 mg/L FA (*P=.0411 and P=.0315, respectively). LS174T and SW480 cells treated with 4 mg/L FA generated significantly higher colonosphere yields than 0 mg/L FA-treated cells (*P=.0087 and P=.0448, respectively). The data represent the mean±S.E. of four independent experiments.

3.7. EpCAM, CD166, and CD133 expression

EpCAM, CD166, and CD133 surface proteins were expressed in all CRC cell lines to varying degrees. HCT116 cells expressed high levels of all surface markers (Fig. 7) on nearly 100% of cells despite changes in FA exposure. CD133 and CD166 expression by SW480 and LS174T cells was lower than that seen in HCT116 cells (Fig. 7A). LS174T cells show trends toward reduced surface expression of all the markers with increasing FA treatment; however, no significant changes were detected (P>.01; Fig. 7B and C). The proportion of SW480 cells with positive CD133 expression decreased with increasing FA at 4 mg/L FA and the median CD133 fluorescence in these cells was highest at 0 mg/L. No other significant changes were detected (Fig. 7B and C).

4. Discussion

We employed an *in vitro* model to investigate the association between FA-induced changes in DNA methylation and CSC reprogramming as revealed by CRC colonosphere formation and growth. Cells were grown in media containing 0, 4, or 16 mg/L of FA for 7 days, after which point they were passaged to suspension culture in serum-free media for 15 days. After 7 days, a significant correlation between FA supplemented in the media and intracellular FA levels was observed in all cell lines. This was accompanied by subsequent physiological changes in downstream methionine cycle intermediates SAM and SAH. These findings along with the previous characterization of these *in vitro* functional folate levels with transformed CRC cells support the suitability of these levels to model isolated FA-dependent modulation [36–38].

FA deficiency significantly reduced cellular proliferation in HCT116 and LS174T cells and survival in HCT116, LS174T, and SW480 cells. This response is most likely attributed to compromised folate-dependent genomic integrity, leading to cell cycle arrest and apoptosis. Folate depletion has been shown to increase dUMP:dTMP ratios, which increases uracil misincorporation during DNA synthesis [39]. Uracil residues in close proximity on opposing strands can trigger double-stranded breaks during DNA repair [40,41]. In response, DNA damage signals the cell to inhibit cell cycle progression. Increased expression of cell cycle modulating proteins p16, p21, and p15 has been reported in FA-depleted colon epithelial cell lines [42]. Additionally, human lymphocytes grown in folate-deficient conditions show cell cycle arrest in S-phase when uracil misincorporation occurs [43]. The difference in proliferative abilities between HCT116 and LS174T, both p53 wild-type cell lines, and SW480, a p53 mutant cell line, suggests that cell cycle arrest following intracellular folate depletion may be p53 dependent. However, consistent changes in viability between these cell lines following folate depletion suggest that folate-deficiency-triggered cell death occurs in a p53-independent manner.

To date, FA-dependent modulation of DNA methylation patterns appears to be highly specific to the tissue, cell type, stage of transformation, and even genetic locus [17,37,44,45]. Colon tissue seems to be particularly resistant to folate-induced changes in genomic methylation, a response that may be a result of robust SAM:SAH ratios despite changes to methyl donor availability [46]. Sustained SAM:SAH ratios during folate deficiency may be a result of up-regulated compensatory mechanisms such as the selenium or choline/betain pathways [36]. Alternatively, in response to FA deficiency, HCT116 has been shown to up-regulate key mediators of the folate cycle, particularly methylenetetrahydrofolate reductase (MTHFR), the enzyme responsible for converting 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate [38]. As a result, HCT116 cells preferentially shuttle folate pools to the methionine cycle over nucleotide biosynthesis in an attempted to maintain SAM-dependent methylation reactions [38]. However, our data show that, while FA has a significant dose-dependent effect on SAM:SAH ratios, it also has an inverse dose effect on global genomic methylation levels in HCT116 and LS174T CRC cells. This suggests that FA-induced epigenetic changes in these cells are associated with SAM- and SAH-independent pathways and that the SAM:SAH ratio may not be a reliable biomarker for genomic methylation. This hypothesis is supported by recent studies examining more severe forms of folate deficiency in rats, which show that, while such conditions reduce SAM:SAH ratios, paradoxically, an increase in global methylation is observed in the colon [45]. Our results are also consistent with other studies showing that *in vitro* FA deficiency can result in both site-specific hypermethylation and global hypermethylation [37,44]. A possible explanation for FA-, SAM-, and SAH-independent effects on DNA methylation may be via the modulation of the methylation machinery [47].

The observed inconsistency between DNMT1 protein levels and global DNA methylation suggests that reductions in DNMT1 alone may not be sufficient to induce significant cellular hypomethylation in CRC cells that already harbor global genomic hypomethylation. Although DNMT1 is recognized as the primary maintenance methyltransferase, the other DNMT isoforms may be imperative for inducing *de novo* changes to the DNA methylation code. One study has shown that, while complete knockdown of DNMT1 in HCT116 cells resulted in a modest 20% reduction in global genomic methylation, the complete knockdown of both DNMT1 and DNMT3b in conjunction produced a >95% reduction in global genomic methylation [48]. Indeed, our results show that, in HCT116 cells, although FA deficiency reduced DNMT1 protein expression by more than 50%, it also increased DNMT3a protein expression by 80%, which may have mitigated any DNMT-dependent hypomethylation effects, resulting in net global hypermethylation. However, the method of methylation quantification in this study was limited to global analysis, which is not sensitive enough to detect gene-specific differences. FA levels modulate DNMT protein expression, an effect that is highly specific to both supplementation level and cell type. Previous work has shown that both p53 and APC status can act as regulatory factors in determining DNMT1 expression [49,50]. This may explain why p53 and APC mutant SW480 cells showed no significant DNMT response to FA exposure while HCT116 and LS174T do. However, in addition to altering DNMT protein expression, previous studies have shown that FA levels also affect DNMT enzyme activity and methyl CpG binding protein expression [47,51]. These results suggest that folate-dependent modulation of DNA methylation may not solely be a result of altered SAM levels in the cell

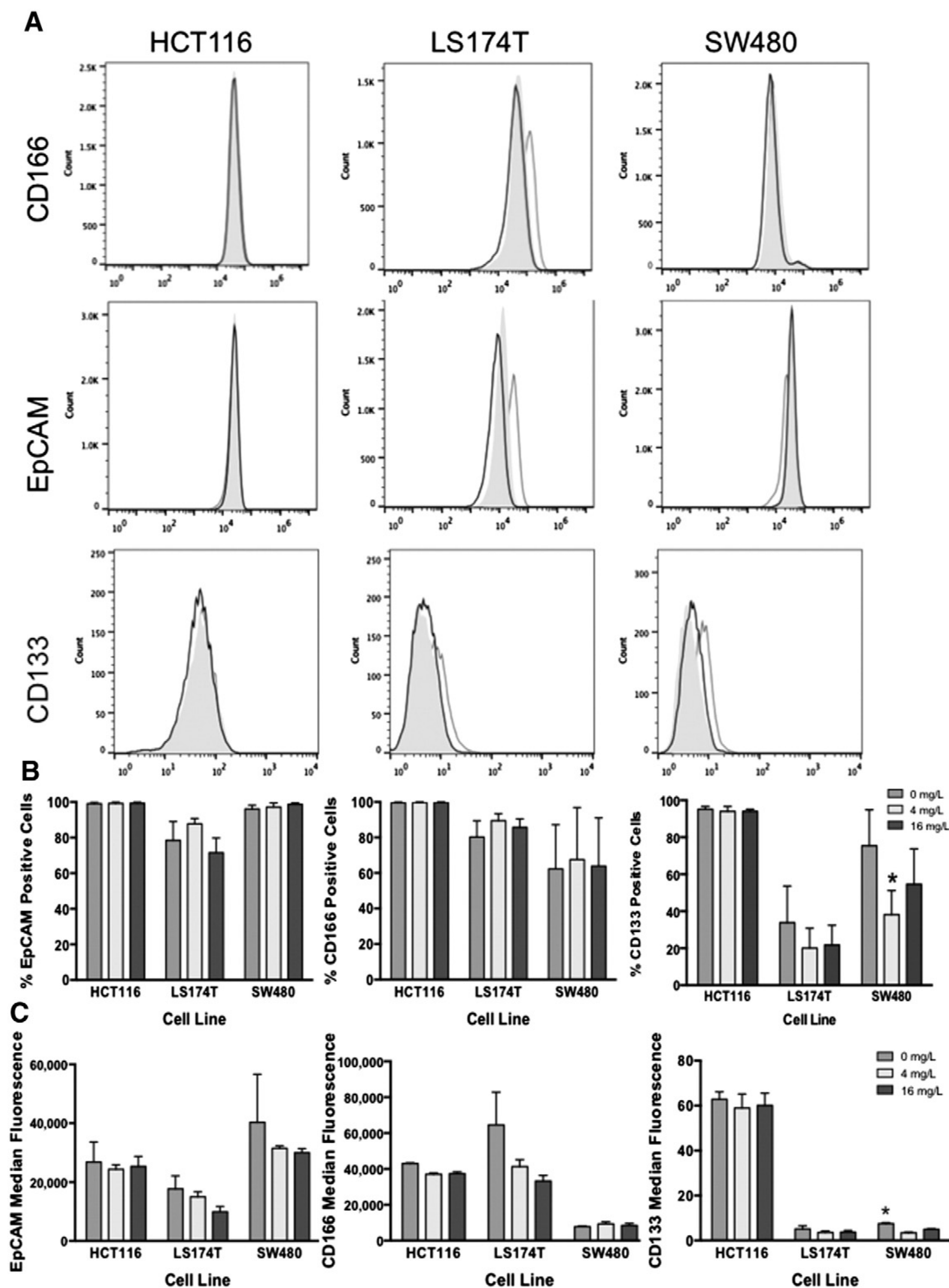

Fig. 7. Fluorescence-activated cell sorting showing CD133, CD166, and EpCAM expression in different CRC cells lines after 7 days FA treatment. (A) Representative flow diagrams for each cell line. Shaded plot indicates signal for cells cultured with 4 mg/L FA and gray and black lines represent signal from cells cultured with 0 mg/L and 16 mg/L FA, respectively. (B and C) Quantification of CD133, CD166 and EpCAM staining frequency and intensity. HCT116 cells showed the highest level of expression and the greatest proportion of CD133 positive cells; there were no significant effects on these parameters with FA exposure. LS174T and SW480 cells showed lower levels of CD133, and SW480 cells showed a significant reduction in CD133 positive cells in response to 4 mg/L FA treatment compared to 0 mg/L FA (*P<.05). No other significant effects were detected.

but also due to modulation of methylation machinery. The resultant downstream changes in DNA methylation may lead to aberrant genetic activation, having consequences for cellular function, differentiation, and survival [21,22].

Unlike HCT116 and LS174T, SW480 cells showed no significant methylation effect in response to FA exposure. The molecular pathway characteristics of each cell type may indicate the type of colorectal neoplasms that are vulnerable to FA-dependent epigenetic

modifications. As well as having a response to FA, HCT116 and LS174T cells are both MSI positive cancers while SW480 cells are MSS [31]. In addition, HCT116 cells also carry a CIMP phenotype and a known mutation in the gene coding for MTHFR, reducing its effectiveness [31,38]. Evidence suggests that folate status and MTHFR polymorphisms may be precursors associated with an increased risk of developing MSI and CIMP-type neoplasms [52–54]. Therefore, mutations to MTHFR or other folate cycle enzymes may leave cells vulnerable to folate status-dependent aberrant methylation leading to MSI and CIMP phenotypes. Hence, it would be expected that CIMP and MSI positive cancer cells such as HCT116 and LS174T have underlying features predisposing them to aberrant methylation and therefore may be more susceptible to FA-induced changes to genomic methylation than MSI negative cancers such as SW480.

The hierarchical theory of tumor development stipulates that a subpopulation of cells, termed CSCs or cancer progenitor/initiating cells, are responsible for driving tumorigenesis [30]. A characteristic of colorectal CSCs is their ability to grow into a floating multicellular sphere from a single cell (termed a colonosphere) under anchorage-independent conditions [29]. Here we show, for the first time, that deficient FA exposure during monolayer culture significantly reduced the ability of CRC cells to form such colonospheres. To the best of our knowledge, no other studies have shown that FA is required for efficient colonosphere formation.

Aside from altered intracellular FA levels, we found no consistent physiological modification in all cell lines that clearly alludes to mechanisms governing FA's effect on colonosphere formation. Both HCT116 and LS174T cells with elevated DNMT1 also had higher colonosphere forming potential under increased FA conditions. This has significant implications for the role of FA in cancer therapy, as manipulation of DNMT activity has shown promise as a therapeutic strategy against CSC populations. Zebularine, a DNMT inhibitor, selectively targets HCT116 cells with a CSC phenotype by reducing the ALDH and CD44/CD166 positive cells in the culture and also has a higher toxicity toward HCT116 colonospheres over monolayer cells [55]. The complete knockdown of DNMT1 in SW480 and HCT116 cells reduced expression of the CSC markers Sox and CD133, reduced the cancer initiating frequency, and produced xenograft tumors with reduced volume compared to DNMT1 wild-type cells [56]. However, here, we show that the DNMT1 expression profile in FA-treated SW480 cells was opposite to that of HCT116 and LS174T cells despite maintaining consistent colonosphere forming potential. This suggests that, while DNMT1 may contribute to the physiological changes necessary for colonosphere formation, it is not absolutely necessary.

The influence that the DNMT proteins have on CSC phenotype may be a result of their modifications to DNA methylation and the subsequent changes in genetic expression. Our data show that global methylation changes and colonosphere forming ability were concordant in two out of the three cell lines, SW480 cells being the exception. However, this does not rule out the possibility that potentially undetectable, site-specific differences took place in all FA-supplemented cells that may have enhanced colonosphere forming potential. Currently, the only other studies that have investigated FA-dependent stem cell proliferation involve neural stem cells. Yu *et al.* showed that FA promotes methylation changes in genes for key PI3K/Akt/CREB pathway proteins, which subsequently led to stimulated neural stem cell proliferation [57]. HCT116 and LS174T cells both harbor hyperactivating mutations in PI3K while SW480 cells maintain the wild-type gene [31]. Differences in PI3K activity may facilitate or exaggerate the effects of FA-dependent stem cell proliferation as seen by the greater response of HCT116 and LS174T cells compared to SW480 cells [31]. In another study, FA was shown to increase Notch signaling leading to increased neural stem cell proliferation [58]. The Notch signaling pathway along with Wnt, Hedgehog, and TGF-β signaling pathways are involved in maintaining normal colon stem cell renewal and have been implicated in CRC CSC development [59]. This is important when considering that SW480 cells carry a mutation in the APC gene, which functions as a Wnt protooncogene [33]. This results in a loss of β-catenin regulation and a constitutively active Wnt pathway [33]. Unregulated Wnt signaling may be a contributing factor to the relative high levels of stem cell proliferation despite differential FA exposure in SW480 cells [33]. Nevertheless, many of the key proteins involved in stem cell renewal pathways are epigenetically regulated [27,60–62]. Thus, deregulated protein transcription leading to the activation of stem cell pathways as a result of FA-induced methylation changes may facilitate stem cell reprogramming and colonosphere forming ability in these CRC cell lines.

Colonospheres from CRC cell lines have increased expression of putative CRC stem cell markers such as CD133, CD166, CD144, CD24, CD29, LGR5, and nuclear β-catenin as well as up-regulation of CSC associated pathways compared to their monolayer derived equivalents, which include the Wnt, Notch, and Hedgehog pathways [29,63]. Although we saw changes in CD133 expression with FA, the results were not concordant with colonosphere forming ability. For instance, CD133 was highly expressed on virtually all HCT116 cells and did not alter with FA exposure, yet there was a profound FA-dependent difference in the ability of HCT116 cells to generate colonospheres. Conversely, SW480 and LS174T cells showed overall low levels of CD133 expression despite highly efficient colonosphere forming ability. LS174T cells also exhibited a slight reduction in CD166 and EpCAM surface expression with increasing FA levels; however, these results did not reach significance. Taken together, these findings suggest that although EpCAM, CD166, and CD133 are used as "biomarkers" for CSCs in many types of cancer, including CRC [64], their utility as indicators of colonosphere forming ability (arguably a more relevant bioassay for CSCs) in cultured cell lines is questionable [65].

In summary, the association among DNMT expression, FA exposure, and CRC colonosphere growth outlined in this report provides a possible mechanism by which FA can modulate CRC development. Our data suggest that excessive FA intake from supplements and fortified foods over a prolonged period of time may contribute to aberrant methylation patterns. This could provide a survival advantage to preexisting colon neoplasms, perhaps as a consequence of both DNMT protein modulation and altered methyl donor availability in the cell. This association between FA intake, methylation, and DNMT levels in cancer cells is an important step in characterizing a potentially problematic relationship between nutrient oversupplementation and cancer progression. We believe that these effects pose significant implications for *in vitro* and *in vivo* CSC models, and future investigations are warranted.

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