Farnesyl dimethyl chromanol targets colon cancer stem cells and prevents colorectal cancer metastasis

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The activation and growth of tumour-initiating cells with stem-like properties in distant organs characterize colorectal cancer (CRC) growth and metastasis. Thus, inhibition of colon cancer stem cell (CCSC) growth holds promise for CRC growth and metastasis prevention. We and others have shown that farnesyl dimethyl chromanol (FDMC) inhibits cancer cell growth and induces apoptosis in vitro and in vivo. We provide the first demonstration that FDMC inhibits CCSC viability, survival, self-renewal (spheroid formation), pluripotent transcription factors (Nanog, Oct4, and Sox2) expression, organoids formation, and Wnt/β-catenin signalling, as evidenced by comparisons with vehicle-treated controls. In addition, FDMC inhibits CCSC migration, invasion, inflammation (NF-kB), angiogenesis (vascular endothelial growth factor, VEGF), and metastasis (MMP9), which are critical tumour metastasis processes. Moreover, FDMC induced apoptosis (TUNEL, Annexin V, cleaved caspase 3, and cleaved PARP) in CCSCs and CCSC-derived spheroids and organoids. Finally, in an orthotopic (cecum-injected CCSCs) xenograft metastasis model, we show that FDMC significantly retards CCSC-derived tumour growth (Ki-67); inhibits inflammation (NF-kB), angiogenesis (VEGF and CD31), and β-catenin signalling; and induces apoptosis (cleaved PARP) in tumour tissues and inhibits liver metastasis. In summary, our results demonstrate that FDMC inhibits the CCSC metastatic phenotype and thereby supports investigating its ability to prevent CRC metastases.

Colorectal cancer (CRC) is a significant healthcare problem and is estimated to be the second leading cause of cancer-related deaths in the United States in 20191–3. Enhanced screening and improved therapies are being used; however, the survival rate at 5 years of CRC patients with late-stage disease is only 12.5%4,5. The process of colorectal cancer biology, starting from the normal colon epithelial cell to the metastatic colorectal cancer cell, is associated with the development and acquisition of genetic mutations that lead to the activation or suppression of specific genes6–10. One of the key signalling pathways that is disrupted in colon cancer formation is the Wnt/β-catenin signalling pathway. This disruption is caused by specific genetic alterations in genes in this pathway11–15. The most common mutations are related to the adenomatous polyposis coli (APC) gene, which is inactivated, and the β-catenin gene, which is activated16,17. The standard of care for treating patients with colorectal cancer includes surgical resection for early-stage disease and chemotheraphy with or without surgical resection for late-stage disease. Unfortunately, after undergoing potentially curative treatment, over half of all patients relapse in the form of distant metastasis16,19. In 85% of patients, relapse is diagnosed within the first 2.5 years after surgery and a third of patients are found to have new precursor legions of colorectal cancer at follow-up colonoscopy18,19. Metastasis of CRC is the most common reason for treatment failure because of CRC’s complicated mechanism of invasion, angiogenesis, and chemotaxis of the tumour microenvironment20–24. Our current understanding of the underlying process that leads to colorectal cancer relapse implicates specific cells that initiate cancer formation, referred to as cancer stem cells (CSCs). These cells are tumorigenic, metastatic, and resistant to the radiation and chemotherapies that are used to prevent CRC relapse25–28. Colorectal CSCs are histologically characterized by the expression of cell surface markers, such as CD24, CD44, CD133, LGR5, and ALDH1, which are key hallmarks of colorectal CSCs26,29–32. Given the critical role of the colorectal CSCs in CRC initiation, relapse, and metastasis, targeting these cells using novel agents may lead to both primary and secondary prevention of CRC.
Recently, farnesyl dimethyl chromanol (FDMC) was shown to interact with key signalling pathways that are important in cancer formation and metastasis33–36. FDMCs are a unique species that naturally occur in cereal grains and oils34,37–40. We and others have shown that FDMC inhibits proliferation and survival in different types of cancers, including CRCs40–48. Recently, we showed that FDMC prevented the development of colorectal cancer in Azoxymethane rat models of colon carcinogenesis and inhibited human colon cancer cell lines SW-620 and HCT-11649. However, the activity of FDMC on CRC-initiating cells has not been reported. In this study, we investigated the activity of FDMC on viability, survival, self-renewal, migration, invasion, inflammation, angiogenesis, apoptosis, and Wnt/β-catenin signalling in colon CSCs (CCSCs) and CCSC-derived spheroids or organoids in vitro. We also studied the in vivo effects of FDMC on metastatic tumour growth, inflammation, angiogenesis, liver metastasis, apoptosis, and Wnt/β-catenin signalling.

Results

FDMC suppresses cell growth of colon cancer stem cells (CCSCs), spheroids, and organoids. FDMC has been shown to exhibit antitumorigenic activity in a number of studies that include CRC cells41–43,47,48. However, our group is the first to have evaluated the effects of FDMC on CCSC and CCSC-derived spheroid and organoid growth. We treated CCSCs, spheroids, and organoids with FDMC in a concentration ranging from 10 to 100 μM for 72 h (for CCSCs), 5 days (for spheroids), and 6 days (for organoids). We measured cell viability using the CellTiter Glo assay and found that FDMC significantly (p < 0.001) decreased the viability of CCSCs, spheroids, and organoids in a concentration-dependent manner (Fig. 1A). The inhibitor concentrations required for 50% inhibition of cell proliferation (IC50) values were 50 ± 5 μM for CCSCs, spheroids, and organoids. We subsequently used IC50 (50 μM) of FDMC for other experiments. In addition, we evaluated the effects of FDMC (50 μM) on CCSC viability on soft agar by performing a colony formation assay; spheroid proliferation (Ki-67), using suspension 3D culture; and organoid growth (Ki-67) in organoids (*p < 0.02). Confocal microscopy data show that FDMC (50 μM) significantly inhibited anchorage-independent growth (luminescence units) (*p < 0.01). Results are mean ± standard error of the means (bars; n = 3).
as well as organoid formation ($p < 0.02$), as evidenced by comparisons with vehicle-treated controls (Fig. 1B–G), indicating the potential inhibition of CRC malignant transformation and growth.

**FDMC inhibits migration and invasion of CCSCs.** One of the hallmarks of cancer stem cells is high migratory and invasive properties leading to distant metastasis$^{26,50}$. Therefore, using a scratch assay, we examined whether FDMC would affect CCSCs’ ability to migrate or invade. We found that FDMC significantly ($p < 0.01$) suppressed cell mobility in CCSCs, which was evidenced by comparisons with vehicle-treated controls (Fig. 2A,B). Similarly, a transwell cell migration assay showed that FDMC significantly ($P < 0.001$) prevented CCSCs’ invasion into the matrix compared to that observed in vehicle-treated controls (Fig. 2C,D). In aggregate, our findings suggest that, in vitro, FDMC is able to suppress the movement of CRC stem cells from one location to another and the shift of CRC stem cells through the tissue microenvironment, leading to inhibition of distant metastasis.

**FDMC inhibits markers for inflammation, angiogenesis, and metastasis and induces apoptosis of CCSCs.** It is well-established that inflammation and angiogenesis are hallmarks of cancer and contribute to tumour progression$^{51}$. To investigate the effect of FDMC on inflammation and angiogenesis in vitro using CCSCs, we used Western blot analysis to examine the expression of inflammatory and angiogenic markers (NF-$\kappa$B, MMP9, and VEGF). Inflammatory marker NF-$\kappa$B expression was less in FDMC-treated CCSCs than in the vehicle-treated control, indicating that FDMC suppressed the inflammation (Fig. 3A). The expressions of metastatic marker MMP9 and angiogenic factor VEGF were depleted in CCSCs following FDMC treatment as compared with vehicle-treated control (Fig. 3A). Apoptosis (Annexin V/propidium iodide [PI], terminal deoxy-nucleotidyl transferase-mediated dUTP nick end labelling [TUNEL] assay, and Western blot for cleaved poly [ADP ribose] polymerase [C-PARP]) was induced in CCSCs after treatment with FDMC as compared with vehicle-treated controls (Fig. 3A–D).

**FDMC alters morphology and suppresses self-renewal capacity, organoid formation, and pluripotency transcription factors in CCSCs.** We investigated whether FDMC could also affect morphology and stem cell self-renewal capacity (stemness), organoid formation, and pluripotency transcription factors. We found that FDMC-treated CCSCs became spherical in shape, unlike vehicle-treated CCSCs (Fig. 4A). FDMC inhibited in vitro self-renewal (eg, spheroid formation) and organoid formation of CCSCs, which were
less than in the vehicle-treated controls (Fig. 4B,C). As shown in the Western blot data in Fig. 4D, FDMC inhibits the expression of Nanog, Oct4, and Sox2 in CCSCs, as evidenced by comparisons with vehicle-treated CCSCs. Taken together, these results indicate that FDMC can target CRC stem cells to suppress their self-renewal, pluripotency, and tumouroid genesis.

FDMC inhibits Wnt/β-catenin activity in CCSCs, spheroids, and organoids and induces apoptosis. The role of Wnt/β-catenin signalling in the homeostasis of CSCs in human CRC patients and in the orthotopic mouse model of CRC is well documented. Induction of Wnt/β-catenin signalling is crucial for colorectal tumour growth and metastasis. Therefore, we first investigated the Wnt/β-catenin activity in 3T3 cells with β-catenin/TCF reporter activity (luciferase units) and CCSCs using Wnt receptor agonist Wnt3a and antagonist DKK1. β-catenin/TCF reporter activity (luciferase units) was induced by the agonist (Wnt3a) and decreased by the antagonist (DKK1) in 3T3 cells (Fig. 5A). FDMC (50 µM) treatment for 24 h significantly suppressed the agonist-induced receptor activity in 3T3 cells. WNT3a treatment induced β-catenin expression in 3T3 cells as well as in CCSCs, whereas DKK1 treatment abolished the Wnt-induced induction of β-catenin in both cells. FDMC almost completely suppressed the Wnt-induced β-catenin expression in both cells (Fig. 5B,C). FDMC (50 µM) treatment to CCSC-derived spheroids and organoids resulted in significantly less β-catenin expression and greater induction of apoptosis compared with vehicle-treated control (Fig. 5C–G).

FDMC inhibits colorectal tumour growth, metastasis, inflammation, angiogenesis, and β-catenin expression and induces apoptosis in mice. To investigate the effects of FDMC on colorectal tumour growth and metastasis in vivo, we inoculated the cecum of mice with human CCSCs that express luciferase and closely monitored for tumour growth and liver metastasis for 4 weeks. Our results demonstrate that FDMC significantly decreased human CCSC tumour growth and prevented CCSC metastasis to the liver (Fig. 6). In addition, FDMC significantly decreased the proliferation index in the tumours. Western blot and histology data in Fig. 6E–G further depict the suppression of inflammatory (NF-kB), angiogenic (VEGF and CD31), and metastatic (MMP9) markers as well as induction of apoptosis (C-PARP) in the CRC tumour tissue.
of mice treated with FDMC as compared with vehicle-treated mice. Taken together, these results indicate that FDMC suppresses CCSC-mediated tumorigenesis and liver metastasis and induces apoptosis in mice.

**Discussion**

FDMC is a natural compound from the family of vitamin E molecules that has received significant interest for its potential as an agent to prevent and treat cancer. We and others have demonstrated that FDMC has significant anticancer activities in several models. Despite the reports of FDMC anticancer activity, the role of FDMC in CCSC-mediated metastatic CRC, has remained obscure. Therefore, the present study investigated the effects of FDMC on human CCSC growth, self-renewal, migration, invasion, inflammation, angiogenesis, and survival in vitro and metastatic CCSC tumour growth, inflammation, angiogenesis, and survival in vivo. CCSCs have been shown to be the key component within colon cancer tumours that lead to the development of metastasis and resistance to chemotherapy. This is the first report to show that FDMC inhibited the growth of human CCSCs in 3D suspension culture (spheroid formation or self-renewal capacity, one of the characteristics of CSCs) in soft agar (colonization or malignant transformation), and in Matrigel (organoid) formation. The maintenance of stemness, pluripotency, and self-renewal capacity in CCSCs is actively regulated by putative transcription factors such as Nanog, OCT4, SOX2, and KLF4. Our data clearly show that FDMC down-regulated the expression of Nanog, OCT4, and SOX2 in CCSCs, targeting the CSCs’ growth, tumorigenesis, and metastatic spread. Moreover, the expression of OCT4 and SOX2 has been shown to be elevated in colorectal tumours and expression was correlated with liver metastasis and predicted poor patient survival. Knock-down of OCT4 and SOX2 has been shown to inhibit colorectal tumour growth, deplete CSC populations, and induce apoptosis, supporting our proposal to target CCSC survival and self-renewal using FDMC.

Figure 4. Effect of FDMC on morphology, self-renewal capacity, organoid formation, and expression of pluripotency transcription factors in CCSCs, as evidenced by comparisons with vehicle-treated controls (V = vehicle). (a) Microscopic image shows that CCSCs became spherical after 24 h of FDMC (50 µM) treatment, in contrast to vehicle-treated cells. (b) Microscopic image shows that FDMC (50 µM) inhibited self-renewal (spheroid formation) of human CCSCs (CD24+CD44+LGR5+) grown in ultralow non-adherent plate containing stem cell-specific medium in 3D culture. (c) FDMC (50 µM) significantly inhibited the number of spheroids compared to vehicle (*p < 0.0005). (d) Microscopic image shows that FDMC (50 µM) inhibited CCSC-derived organoids formation when grown in Matrigel and stem cell-specific organoid medium in 3D culture. (e) FDMC (50 µM) significantly inhibited the number of organoids compared to vehicle (*p < 0.0002). (f,g) Western blot data show that FDMC (50 µM) significantly inhibited CCSC expression of the stem cell pluripotency transcription factors Nanog, Oct4, and Sox2 compared to vehicle (*p < 0.05 and **p < 0.02). Data are from 3 independent experiments.
Figure 5. Effects of FDMC on Wnt/β-catenin activity in CCSCs, spheroids and organoids, and induction of apoptosis, as evidenced by comparisons with vehicle-treated controls (V = vehicle). (a) Wnt receptor activity (luciferase units) significantly induced by agonist (Wnt3a) (\(p<0.01\)) and decreased by antagonist (DKK1) (\(p<0.01\)) in 3T3 cells. FDMC (50 µM) treatment for 24 h significantly suppressed agonist-induced receptor activity (\(p<0.01\)) in 3T3 cells. (b) Western blot data show that Wnt receptor agonist Wnt3a treatment induced β-catenin expression, whereas Wnt receptor antagonist DKK1 treatment abolished the Wnt3a-induced induction of β-catenin in 3T3 cells. FDMC inhibited Wnt3a-induced β-catenin expression in 3T3 cells. (c) Western blot data show that Wnt receptor agonist Wnt3a treatment induced β-catenin expression, whereas Wnt receptor antagonist DKK1 treatment abolished the Wnt3a-induced induction of β-catenin in CCSCs. FDMC inhibited Wnt3a-induced β-catenin expression in CCSCs. (d,e) Confocal microscopy data show that FDMC (50 µM) treatment to CCSC-derived spheroids significantly depleted β-catenin expression (green immunofluorescence staining) and induced apoptosis (cleaved caspase 3; red immunofluorescence staining; \(*p<0.01\) and \(**p<0.02\)). (f,g) Confocal microscopy data show that FDMC (50 µM) treatment to CCSC-derived organoids significantly depleted β-catenin expression (green immunofluorescence staining) and induced apoptosis (cleaved caspase 3; red immunofluorescence staining; \(*p<0.05\) and \(**p<0.01\)). Results are mean ± SEM (bars; n = 3).
One of our novel findings was that FDMC induced apoptosis in CCSCs, spheroids, organoids, and CRC tumours, which are important, as CCSCs are well known to be resistant to chemo and radiation therapies. Our data further demonstrate that FDMC significantly inhibited the migration and invasion of CCSCs, which are the hallmarks of the cancer stem cell behaviour that precedes distant metastasis. Therefore, it appears that FDMC prevents CCSC metastasis to the liver by mediating some of the necessary steps of tumour metastasis, such as migration, invasion, and angiogenesis. CCSCs are known to contribute to tumour angiogenesis, and they generate angiogenic factors such as VEGF to induce angiogenesis. Earlier studies showed that FDMC inhibited VEGF-induced angiogenesis in colorectal cancer cells in vitro. However, to our knowledge, this is the first report showing that both in vitro and in vivo, FDMC significantly inhibited colorectal tumour inflammation and angiogenesis in CCSCs and metastatic colon tumours by inhibiting NF-kb, VEGF, and MMP9 levels. These data further reflect the anti-inflammatory and antiangiogenic activity of FDMC.

Sixty percent of CRC patients experience cancer recurrence following remission. Relapse from colorectal cancer remission is often associated with the development of metastasis. The process of metastasis involves the reactivation of dormant cancer cells from the primary tumour that have to undergo multiple steps to accomplish a clinically relevant metastatic tumour. These steps include proliferation, invasion, motility, and modulation of the tumour microenvironment, such as the induction of angiogenesis and suppression of immune clearance. Our in vitro and in vivo experiments demonstrated that FDMC inhibited the metastasis marker MMP9. In our mouse model of metastasis (involving cecum-injected CCSCs), FDMC significantly inhibited tumour volume, tumour weight, and liver metastases compared with vehicle-treated controls. The analysis of the CCSC-derived tumours revealed that FDMC treatment not only restricted tumour growth by inhibiting pluripotency transcription factors (OCT4 and SOX2), inflammation (NF-kB), and angiogenesis (VEGF) but also induced apoptosis (C-PARP). The role of Wnt signalling in the homeostasis of CSCs in human CRC and tumorigenesis in mice has been demonstrated. Our data also show that FDMC significantly inhibited Wnt/β-catenin receptor activity and β-catenin expression in vitro and in vivo.

In summary, we found that FDMC inhibits CCSC growth, self-renewal, pluripotency, migration, invasion, inflammation, angiogenesis, and Wnt/β-catenin signalling and induction of apoptosis in vitro. It also inhibits metastatic tumour growth, inflammation, angiogenesis, Wnt/β-catenin signalling, and liver metastasis spread and induces apoptosis in vivo. These data indicate a novel antimetastatic potential of FDMC in targeting CCSCs, and hence, possible clinical use for the prevention and treatment of metastatic CRC.

Materials and methods

Materials. As previously described in Husain et al., all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. FDMC was obtained from Davos Life (Helios, Singapore and American River, Hadley, MA). L-Glutamine, penicillin, streptomycin, and HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) buffer were purchased from Mediatech (Herndon, VA). Foetal bovine serum (FBS) was purchased from HyClone (Logan, UT) and ethanol (100%) from Aaper Alcohol and Chemical (Shelbyville, KY). Human colon cancer stem cells (CCSCs CD24+, CD44+, LGR5+, and ALDH1+) and growth media (10% FBS) was purchased from HyClone (Logan, UT) and ethanol (100%) from Aaper Alcohol and Chemical (Shelbyville, KY). Human colon cancer stem cells (CCSCs CD24+, CD44+, LGR5+, and ALDH1+) were purchased from Celprogen (Torrance, CA). Phosphate-buffered saline (PBS), GlutaMAX, N2 supplement, B27 supplement, and advanced Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) were purchased from Corning (Belford, KY). Human colon cancer stem cells (CCSCs CD24+, CD44+, LGR5+, and ALDH1+) were purchased from Celprogen (Torrance, CA). Human epidermal growth factor (EGF), fibroblast growth factor 4, Wnt3a, R-Spondin, and Noggin were purchased from R&D Systems (Minneapolis, MN). Bovine serum albumin (BSA), N-acetyl cysteine, nicotinamide, gastrin, A83-01, SB202190, prostaglandin E2, and Y-27632 were purchased from Sigma (St. Louis, MO). Growth factor-reduced Matrigel was purchased from Corning (Belford, MA), pimocin from InvivoGen (San Diego, CA), basic fibroblast growth factor, and EGF from Peprotech (Rocky Hill, NJ), and basement membrane extract (BME) from Trevigen (Gaithersburg, MD).

Cell culture and growth. Human (parental) colon cancer stem cells were grown in human colon cancer stem cell complete growth medium (M36112-39PS). As previously described in Husain et al., cells were cultured at 37 °C in a humidified atmosphere of 5% CO2 and 95% O2.

Cell viability (CellTiter-Glo) assay. Human (parental) colon CSCs (CD24+, CD44+, LGR5+, and ALDH1+; Celprogen) were grown in human colon CSC complete growth medium (M36112-49PS). Two thousand cells (100 µL) were seeded in 96-well plates (Costar, Corning, NY) for 24 h to attach overnight. As previously described in Husain et al., cells were then incubated for 72 h with various concentrations of FDMC (10−5 to 10−4 M) or ethanol (<5%) vehicle as control. To test spheroid viability, 1000 spheroids (100 µL) were added to each well of the nonadherent plates (Corning, NY). The cells were then incubated for 5 days with various concentrations of FDMC (10−5 to 10−4 M) or ethanol (<5%) vehicle as control medium. To test organoid viability, organoids were diluted to 50 organoids/µL in growth medium containing 5% BME. First, a 96-well plate was coated with 30 µL BME then 20 µL of organoid cell suspension were added to each well of the plate. The following day, different concentrations of FDMC (10−5 to 10−4 M) or ethanol (<5%) vehicle as control (50 µL) were added to each well and then incubated at 37 °C in a humidified atmosphere of 5% CO2 and 95% O2 for 6 days, as previously described in Husain et al. CellTiter-Glo reagent (100 µL) was added to each well, mixed on an orbital shaker, and incubated at room temperature for 30 min, and luminescence was recorded in a Multimode Plate Reader (BioTek, Winooski, VT).

Cell migration and invasion assay. The cell migration assay was performed as previously described in Husain et al. Cell migration was performed by scratch test or wound-healing assay. CCSCCs were seeded in 6-well plates and cultured to 100% confluence. Wounds were generated in the cell monolayer using a small plas-
at 24 h there are minimal effects on the death of these specific cells. As previously described by Yuan et al.68, the 24 h. Cells were harvested, and 10⁵ cells were transferred to 5 mL tubes containing PBS (100 μL), and then 2 μL (parental) colon CSCs were plated and treated concurrently with vehicle (5% ethanol) or FDMC (50 μM) for described in Husain et al.46 In brief, 100 000 human (parental) colon cancer stem cells (CD24+, CD44+, LGR5+, by flow cytometry.

Apoptosis assay. The apoptosis assay was performed as previously described by Husain et al.49. Human (parental) colon CSCs were plated and treated concurrently with vehicle (5% ethanol) or FDMC (50 μM) for 24 h. Cells were harvested, and 10⁵ cells were transferred to 5 mL tubes containing PBS (100 μL), and then 2 μL of PI and 5 μL of annexin V-FITC (BD Bioscience) were added and mixed. The tubes were incubated for 15 min at 37 °C for 5 days. A number of spheroids were formed, counted under microscope, and photographed.

CSCCs organoid culture. The cells were grown in RPMI medium supplemented with GlutaMAX, penicillin/streptomycin (100 μg/mL), and varying concentrations of defined FBS (0% for the first 24 h, followed by 0.2% [second day] and 2% [third day]). On the fourth day, the cells were grown in DMEM/F12 medium containing 2% FBS, fibroblast growth factor 4 (500 ng/mL), and Wnt3a (500 ng/mL).90. The spheroids formed in 3D culture were then suspended in growth factor-reduced Matrigel (Corning) and pipetted (25 μL) on the bottom of the 24-well plate and incubated for 10 min at 37 °C. Five hundred microliters of DMEM/F12 organoid culture medium containing penicillin/streptomycin, 10 mM HEPES, GlutaMAX, 50% Wnt, 10% Noggin, 1 × B27, 1.25 mM n-Acetyl Cysteine, 10 mM Nicotinamide, 50 ng/ml human EGF, 10 mM Gastrin, 500 nM A83-01, 3 μM SB202190, 10 mM Prostaglandin E2, 100 μg/mL Primocin, and 10 μM of Y-27632 added to the wells of the plate without touching the Matrigel dome and plates were incubated at 37 °C for 10 days36. The media were refreshed every 2 to 3 days. Organoids treated with vehicle and FDMC were photographed under microscope.

Wnt/β-catenin receptor activity in 3T3 cells and CCSCs. The 3T3 cell line expressing luciferase reporter gene under the control of Wnt-responsive promoters (T-cell factor/lymphoid enhancer factor) were grown and maintained in DMEM medium (Enzo Life Science, Farmingdale, NY). The cells (10 000) were added to each well of the 96-well plate (Costar, Corning, NY) in growth medium and grown to 60% confluency. One hundred μL of PBS, 100 ng/mL Wnt receptor agonist (WNT3a) alone, agonist plus antagonist (DKK1) at a concentration of 100 ng/mL, and agonist plus FDMC (50 μM) prepared in assay medium were added to the control.
Orthotropic CCSC microinjection to cecum wall in a mouse model of metastasis. We tested whether direct orthotopic cell microinjection, between the mucosa and the muscularis externa layers of the wall of cecum in nude mice, induces tumour foci in sites corresponding to those most often observed to be the location of metastases in humans. This technique required the use of needle under binocular guidance. Female Athymic nude mice (6 weeks old, 20–23 g), obtained from Charles River (Wilmington, MA), were kept in our animal facility for 1 week of quarantine. After 1 week, mice were anesthetized with isoflurane, and their ceca were exteriorized via laparotomy. Luciferase-expressing CCSCs (1 × 10^5) were suspended in 50 µL of PBS and placed into a sterile syringe for each animal. We slowly injected the cell suspension at a depth of 5 mm into the wall of each cecum under a binocular lens, with the syringe at an approximately 30° angle. Afterward, we applied slight pressure with a cotton stick at 2 mm from the injection point in the direction of the needle. We pulled the needle out and cleansed the area around the injection with 3% iodine to avoid the unlikely seeding of refluxed tumour cells into the abdominal cavity. After injection, the gut was returned to the abdominal cavity and closed with surgical sutures and staples.

Animals and treatments. As described in Husain et al., 1 week after CCSC microinjection, mice were randomly divided into 2 treatment groups as follows: (1) control group, which received oral gavage of 100 µL of vehicle (ethanol-extracted olive oil) twice daily for 4 weeks and (2) FDMC group, which received 200 mg/kg FDMC oral gavage twice daily for 4 weeks. Mouse tumour volume was measured once per week (luciferase bioluminescence) using an IVIS 200 (Xenogen) in vivo imaging system machine. After 4 weeks of treatment, animals were sacrificed by CO2 inhalation, and tumour weights and liver metastases were recorded. One half of the tumour was immediately frozen in liquid nitrogen and stored at −80 °C for biochemical analyses; the other half was fixed in buffered formalin for histologic analyses. The care and use of the animals included in this study were approved by the Moffitt Cancer Centre Institutional Laboratory Animal Care and Use Committee and were conducted following National Institutes of Health guidelines.

Cell and tissue protein extraction and protein determination. As previously described in Husain et al., cells and tumour tissues were washed 3 times in cold PBS (pH 7) and then lysed in protein extraction reagent radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific, Rockford, IL) that contained phosphatase and protease inhibitor cocktail. Protein concentration was determined using bicinchoninic acid assay reagents (Pierce, Rockford, IL), in accordance with the manufacturer’s instructions.

Western blot analyses. Extracted proteins from cells and tumour tissues (40 µg) were resolved on 12.5% sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE, Bio-Rad, Hercules, CA) and a 5% stacking gel. As described in Pimiento et al., proteins were then electrotransferred onto nitrocellulose membranes. After blocking in 5% nonfat powdered milk for 1 h, the membranes were washed and treated overnight at 4 °C with antibodies to VEGE, MMP9, Nanog, Oct4, Sox-2, NF-kβ, C-PARP, β-catenin, ubiquitin and β-actin (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA; Cell Signaling, Danvers, MA). As described in Husain et al., after blots were washed, they were incubated with horseradish peroxidase-conjugated secondary antibody IgG (1:5000) for 1 h at room temperature. Washed blots were then treated with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) for positive antibody reaction. Membranes were exposed to radiographic film (KODAK, Midwest Scientific, St. Louis, MO) for visualization and densitometric quantization of protein bands using AlphaEaseFC software (Alpha Innotech, San Jose, CA).

Immunofluorescence staining of CCSCs expressing signalling proteins. Treated cells and spheroids/organoids were cytospin on glass slides then fixed in 4% paraformaldehyde in PME buffer overnight at 4 °C. Cells/spheroids were made permeable the next day via incubation in 0.5% Triton X-100 solution at 4 °C for 2 min and blocked with 2% BSA for 30 min. Slides were immunostained with primary antibodies (F-Actin, Ki-67, β-catenin, and cleaved caspase 3 (Cell Signalling Technology and Santa Cruz Biotechnology) for 2 h followed by secondary fluorescence antibodies (Alexafluor 594 and 488) and 4,6-diamidino-2-phenylindole (DAPI). The images were recorded using confocal microscopy (Leica Microsystems, Bannockburn, IL), and representative pictures of the slide field were taken with a digital camera (Diagnostic Instruments, Sterling Heights, MI).

Histologic evaluation. Histologic evaluation was performed as previously described in Husain et al. Formalin-fixed, paraffin-embedded tumour tissues were sectioned (4 µm) and stained with haematoxylin and eosin. Immunohistochemistry was performed using the Ventana Discovery XT automated system (Ventana Medical Systems, Tucson, AZ) with proprietary reagents as per manufacturer’s protocol. Briefly, slides were deparaffinised on the automated system with EZ Prep solution. Sections were heated for antigen retrieval. For immunohistochemistry, tissue sections were incubated with Ki-67 and CD31 at 1:4000 dilutions for 60 min. Detection was performed using the Ventana OmniMap kit. The expression percentage was recorded for each area and then averaged for each mouse. For CD31, sections were examined at low power to identify cancers and associated hot spots. The number of vessels per ×400 field was counted manually. Single cells and groups...
expressing CD31 were counted as vessels in addition to groups with lumens. Sections not showing a cancer were assessed for hot spots at low power.

**Statistical analyses.** The data were expressed as means ± standard errors of the means. As described in Husain et al. \(^1\), the data were analyzed statistically using unpaired t-tests or 1-way ANOVA (analysis of variance) where appropriate. ANOVA was followed by Duncan’s multiple range tests using SAS statistical software for comparisons between different treatment groups. Statistical significance was set at \( p < 0.05 \).

**Data availability**

The datasets generated during and/or analysed during the current study are available from the corresponding author upon any reasonable request.

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Author contributions
M.P.M. and K.H. conceived, planned, and designed the experiments. K.H. performed the experiments. K.H. and D.C. analysed the data. M.P.M., D.C., K.H. and C.S.Y. contributed reagents/materials/analysis tools. M.P.M., K.H. and C.S.Y. wrote the paper.

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Competing interests
Dr. Malafa is named as an inventor on US Patent “Delta-Tocotrienol Treatment and Prevention of Pancreatic Cancer” (June 26, 2007; OTML docket number 06A069) but does not have financial interest in the companies that have licensed this patent. Further patents are in development. The other authors declare no competing interests.

Additional information
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