Aspirin Rescues Wnt-Driven Stem-like Phenotype in Human Intestinal Organoids and Increases the Wnt Antagonist Dickkopf-1

Karen Dunbar,1,2,3,a Asta Valanciute,1,3,a Ana Cristina Silva Lima,1,3 Paz Freile Vinuela,1,2,3 Thomas Jamieson,4 Vidya Rajasekaran,1,2,3 James Blackmur,1,2,3 Anna-Maria Ochocka-Fox,1,2,3 Alice Guazzelli,1,3 Patrizia Cammareri,1,3 Mark J. Arends,1,3 Owen J. Sansom,4,5 Kevin B. Myant,1,3 Susan M. Farrington,1,2,3 Malcolm G. Dunlop,1,2,3 and Farhat V. N. Din1,2,3

1Cancer Research UK Edinburgh Centre, MRC Institute of Genetics & Molecular Medicine, University of Edinburgh, Western General Hospital, Edinburgh; 2MRC Human Genetics Unit, MRC Institute of Genetics & Molecular Medicine, University of Edinburgh, Western General Hospital, Edinburgh; 3Institute of Genetics and Molecular Medicine, University of Edinburgh, Western General Hospital, Edinburgh; 4Cancer Research UK Beatson Institute, Glasgow; and 5Institute of Cancer Sciences, University of Glasgow, Glasgow, United Kingdom

SUMMARY
Aspirin can reverse the Wnt-driven cystic phenotype in human organoids while reducing stem cell marker expression and increasing expression of Dickkopf-1, a Wnt antagonist often lost during CRC progression.

BACKGROUND &AIMS: Aspirin reduces colorectal cancer (CRC) incidence and mortality. Understanding the biology responsible for this protective effect is key to developing biomarker-led approaches for rational clinical use. Wnt signaling drives CRC development from initiation to progression through regulation of epithelial-mesenchymal transition (EMT) and cancer stem cell populations. Here, we investigated whether aspirin can rescue these invasive phenotypes associated with CRC progression in Wnt-driven human and mouse intestinal organoids.

METHODS: We evaluated aspirin-mediated effects on phenotype and stem cell markers in intestinal organoids derived from mouse (ApcMin/+ and Apcfl/fl) and human familial adenomatous polyposis patients. CRC cell lines (HCT116 and Colo205) were used to study effects on motility, invasion, Wnt signaling, and EMT.

RESULTS: Aspirin rescues the Wnt-driven cystic organoid phenotype by promoting budding in mouse and human Apc deficient organoids, which is paralleled by decreased stem cell marker expression. Aspirin-mediated Wnt inhibition in ApcMin/+ mice is associated with EMT inhibition and decreased cell migration, invasion, and motility in CRC cell lines. Chemical Wnt activation induces EMT and stem-like alterations in CRC cells, which are rescued by aspirin. Aspirin increases expression of the Wnt antagonist Dickkopf-1 in CRC cells and organoids derived from familial adenomatous polyposis patients, which contributes to EMT and cancer stem cell inhibition.
CONCLUSIONS: We provide evidence of phenotypic biomarkers of response to aspirin with an increased epithelial and reduced stem-like state mediated by an increase in Dickkopf-1. This highlights a novel mechanism of aspirin-mediated Wnt inhibition and potential phenotypic and molecular biomarkers for trials. (Cell Mol Gastroenterol Hepatol 2021;11:465–489; https://doi.org/10.1016/j.jcmgh.2020.09.010)

Keywords: Epithelial-Mesenchymal Transition; Stem Cells; Organoids; Migration; Invasion.

Colorectal cancer (CRC) is the third most common cancer and second cause of cancer death worldwide. The marginal improvement in survival for advanced disease highlights the need to pursue complementary approaches to target aberrant signaling underlying CRC initiation and progression. Compelling data show that aspirin decreases CRC incidence by 40%–50% and further suggest that post-diagnosis ingestion may delay progression and improve survival. Understanding the biology responsible for the protective effect is key to developing biomarker-led precision prevention strategies.

Canonical Wnt signaling is central to gastrointestinal homeostasis and maintenance of the intestinal stem cell niche. Dysregulated Wnt signaling, primarily driven by adenomatous polyposis coli (APC) mutations, is fundamental to cancer initiation in both sporadic CRC and familial adenomatous polyposis (FAP). Activation by Wnt ligands protects cytoplasmic β-catenin from destruction, leading to its nuclear translocation and T-cell factor (TCF)/lymphoid enhancer factor–dependent Wnt target gene induction. Wnt signaling also governs both normal and cancer stem cell (CSC) regulation. CSCs may arise from cancer cell dedifferentiation or from normal colonic stem cell transformation through increased intrinsic tumorigenesis or in response to microenvironmental cues. Promisingly, Apc restoration triggers differentiation and polyp regression and realigns crypt homeostasis, highlighting a dynamic process that may be exploited. Normally, secretion of endogenous Wnt antagonists serves to curtail excessive Wnt signaling. Whereas some secreted inhibitors that sequester Wnt ligands do not inhibit Wnt in APC mutant cells, Dickkopf-1 (DKK-1) does inhibit Wnt signaling either by disrupting the low-density lipoprotein receptor-related protein 6 (LRP6)-frizzled complex or by promoting inactive LRP6 receptor internalization. Ectopic expression studies of DKK-1 have established its role in intestinal crypt regulation. DKK-1 is down-regulated during adenoma to carcinoma development, with low expression correlating with increased tumorigenesis. Furthermore, DKK-1 is decreased in Western diet fed Mlh1−/− mice, a Lynch syndrome model, because of increased promoter methylation highlighting it is an early event.

In addition to direct intestinal stem cell regulation, increased Wnt remodels the tumor microenvironment to drive cancer progression through epithelial-mesenchymal transition (EMT). Poor CRC survival is associated with increased EMT-inducing gene expression and mesenchymal histology. Malignant cells co-opt EMT regulatory machinery to increase motility and metastatic properties through progressive loss of epithelial features, such as cell-cell junctions and polarity. The plasticity of this process leads to hybrid phenotypes and mesenchymal-epithelial transition, which establishes metastases. Furthermore, EMT induction by the transcription factors Snail, Slug, or Twist triggers a stem-like phenotype in several cancers. It is likely the response to oncogenic signaling in CRC, through intrinsic cell-specific plasticity and microenvironmental stimuli, determines the contribution of EMT to the acquisition of cancer stem properties.

Despite elevated Wnt being central to colorectal carcinogenesis, there are no inhibitors used clinically. Challenges in inhibitor development, including the Wnt requirement for homeostasis and cross-regulating networks, drive the search for “druggable vulnerabilities.” Segregation of CRCs by Wnt ligand dependency may identify a subgroup suitable for small molecule inhibition, but the majority of CRCs are ligand-independent through intracellular Wnt component mutation. Nonetheless, blocking Wnt-receptor activity in Apc null cells does inhibit Wnt signalling in a ligand-independent manner. Hence, a drug such as aspirin, which targets several nodes within Wnt signaling or multiple pathways to combat redundancy, may be more effective. Because of the limited clinical success of target-based drug discovery approaches, there is an accelerated drive toward disease-relevant phenotypic screening. Several pathways dysregulated in CRC, including Wnt, converge to regulate both CSCs and EMT, highlighting pro-neoplastic phenotypes that themselves may serve as response biomarkers. Here we investigate the inhibitory effect of aspirin on pro-neoplastic CSC and EMT phenotypes in CRC animal and human FAP organoid models and whether these effects can be attributed to inhibition of canonical Wnt signaling.

Results
Aspirin Reverses the Wnt-Driven Cystic Organoid Phenotype

Intestinal organoids are an invaluable tool for studying stem cell biology and Wnt signaling in a 3-dimensional ex vivo model. Increasingly organoid morphology can be

*Authors share co-first authorship.

Abbreviations used in this paper: APC, adenomatous polyposis coli; CRC, colorectal cancer; CSC, cancer stem cell; DKK-1, Dickkopf-1; EMT, epithelial-mesenchymal transition; F-actin, filamentous actin; FAP, familial adenomatous polyposis; GSK-3B, glycogen synthase kinase 3B; HRP, horseradish peroxidase; LGR5, leucine-rich repeat-containing G-protein coupled receptor 5; LRP6, low-density lipoprotein receptor-related protein 6; PBS, phosphate-buffered saline; RhoA, ras homolog gene family, member A; ROCK1, rho-associated protein kinase 1; ROCK2, rho-associated protein kinase 2; siRNA, small interfering RNA; TCF, T-cell factor; TROY, tumor necrosis factor receptor superfamily member 19; ZO-1, zona occludens 1.
exploited to uncover Wnt signaling mechanisms. Intestinal organoids are composed of the familiar budding projections representing crypt-like structures, crypts with the intervening cells mimicking villi. The budding projections are initiated by an Lgr5\textsuperscript{+} stem cell and maintained through microenvironment recapitulation by growth factors. This characteristic budding morphology is driven by Wnt signaling during intestinal homeostasis. Loss of the wild-type APC allele in the Apc\textsuperscript{Min/+} model drives increasingly cyst-like organoids reflecting the growth advantage. Excessive Wnt activation, via genetic silencing of APC or exogenous Wnt, promotes this cystic phenotype. Although the transcriptional response regulated by genetic inactivation of APC and exogenous Wnt3a differs, both result in cystic organoid morphology, highlighting the utility of this phenotype as Wnt activation readout irrespective of the underlying mechanism. Local Wnt signaling, critical for normal stem cell function and bud formation, is dysregulated in Apc\textsuperscript{Min/+.} Apc\textsuperscript{fl/fl}, and FAP adenoma organoids that grow in a characteristic cystic manner without budding structures (Fig. 1A). The selective advantage for cells that initiate cystic organoids over budding organoids provides a window to test preventive agents. Because trials of aspirin show benefit in sporadic and familial adenoma patients, we investigated whether aspirin alters the Wnt-driven cystic phenotype in organoids. Exposing small intestinal organoids grown from an Apc\textsuperscript{fl/fl} mouse to 2 mmol/L aspirin for 12 days increased the percentage of wild-type phenotype (non-cystic) organoids, identified as those budding or with ruffled edges, compared with the untreated population (Fig. 1B, Supplementary Videos 1a and 1b). We next translated these effects to human organoids from macroscopically normal (non-adenomatous) and adenomatous colon tissue from FAP patients. The presence of cystic organoids in the normal mucosa sample may relate to the germline mutation or the presence of microadenomas due to loss of both APC copies. Longer exposure to 0.5 mmol/L aspirin for 29 days increased the percentage of budding organoids in populations derived from both “normal” and adenomatous colonic mucosa from FAP patients (Fig. 2A). In addition, treatment of Apc\textsuperscript{Min/+} mice with aspirin for 4 weeks in vivo produced organoids that grew with a budding phenotype, in the absence of aspirin, compared with untreated Apc\textsuperscript{Min/+} intestinal organoids, which remained spherical (Fig. 2B). Hence, we present robust evidence that aspirin rescues the cystic phenotype characteristic of constitutively active Wnt signaling due to a mutant ApC background.

Aspirin Increases Wnt Antagonist in Colon

Aspirin Decreases Stem Cell Marker Expression and Reduces Wnt Signaling
The organoid models’ results suggest that aspirin mediates phenotype rescue by modulating Lgr5\textsuperscript{+} stem cell populations or Wnt signaling gradients required for efficient organoid budding. Lgr5, a Wnt target gene and receptor for the Wnt agonist R-spondin, is the principal stem cell marker in intestinal tissue, but others, such as nuclear necrosis factor receptor superfamily member 19 (TROY), are also validated intestinal stem cell markers. Basal Lgr5 and TROY transcript expression are increased in Apc\textsuperscript{Min/+} mice with aspirin for 4 weeks reducing the number of Lgr5 RNA transcripts per organoid (Fig. 3E). These findings were replicated in vivo, with the treatment of Apc\textsuperscript{Min/+} mice with aspirin significantly reduced Lgr5 protein expression compared with untreated mice (Fig. 3F). Wnt is the predominant pathway responsible for stem cell niche maintenance. Aspirin treatment caused a slight reduction in the total β-catenin protein expression in small intestinal adenomas from Apc\textsuperscript{Min/+} mice (Fig. 5A). Furthermore, aspirin treatment reduced transcript levels of Tcf7, the gene encoding Tcf1 protein that is a β-catenin co-transcriptional regulator, in Apc\textsuperscript{Min/+} crypts and Apc\textsuperscript{fl/fl} organoids (Fig. 5B). Nuclear β-catenin binds to the TCF transcription factor family to initiate Wnt target gene transcription, including several EMT genes. Interestingly, contrary to the expectation of EMT at advanced disease stages, increased vimentin and decreased E-cadherin are observed in adenomas from several Apc-driven mouse models. Aspirin increased E-cadherin and reduced vimentin expression in small intestinal adenomas from Apc\textsuperscript{Min/+} mice (Fig. 5C and D). These observations further support that aspirin promotes a more epithelial phenotype in vivo and contributes to EMT inhibition through aspirin-mediated Wnt inhibition.

Aspirin Inhibits Wnt and Epithelial-Mesenchymal Transition While Reducing Migration and Invasion of Colorectal Cancer Cells
These in vivo observations were confirmed in Colo205 cells, with aspirin reducing expression of both β-catenin and its targets c-myc and Lgr5, while increasing E-cadherin expression (Fig. 6A). Further experiments confirmed that aspirin increases both protein and transcript expression of the epithelial marker E-cadherin in HCT116 and Colo205 cells (Fig. 6B and C). Aspirin also increased expression of a
second epithelial marker, zona occludens 1 (ZO-1), but to a lesser extent than E-cadherin (Fig. 6D). Aspirin exposure reduced nuclear Snail protein expression, a key transcription factor that represses E-cadherin to drive EMT, while increasing cytoplasmic E-cadherin protein expression (Fig. 6E). These results indicate that aspirin is promoting an enhanced epithelial phenotype.

Increased migratory and invasive capabilities are characteristic of cells undergoing EMT and disease progression. Exposure to aspirin (0.5 and 3 mmol/L) reduced wound closure in HCT116 and Colo205 cells grown in both low (0.5%) and normal (10%) serum conditions (Fig. 7A and B). In low serum conditions cellular proliferation is inhibited; hence effects on wound closure more closely reflect alterations in migration rather than proliferation, thus increasing confidence that aspirin inhibits CRC cell migration. This is particularly important because aspirin has known antiproliferative effects especially at higher concentrations (Fig. 7C). Cellular invasion was modeled by using organotypic invasion assays using collagen-fibroblast matrices. Aspirin decreased both the distance invaded and the overall percentage of HCT116 and Colo205 cells invading, which was normalized to the noninvading population to remove any antiproliferative bias (Fig. 8A and B).

Consistent with inhibitory effects on invasion, aspirin exposure (0.5 and 3 mmol/L) reduced the distance single HCT116 and Colo205 cells travelled in 24 hours (Fig. 8C and D). Ras homolog gene family, member A (RhoA) signaling has been implicated in regulating CRC cell migration, invasion, and metastasis. Aspirin exposure reduced protein expression of the 2 kinases, ROCK1 and ROCK2 (Fig. 9A), and reduced levels of phosphorylated coflin in both HCT116 and Colo205 cells (Fig. 9B). Switching the balance between phosphorylated and unphosphorylated coflin alters F-actin stabilization and cycling, which drives cell movement. Aspirin treatment reduced the overall F-actin expression, although no changes in F-actin stress fiber formation were observed (Fig. 9C). These results demonstrate that aspirin can inhibit several invasive traits commonly associated with EMT and disease progression.
Aspirin Treatment Rescues Wnt-Driven Epithelial-Mesenchymal Transition and Stem Cell Changes in Colorectal Cancer Cells

We used the glycogen synthase kinase 3β (GSK-3β) inhibitor CHIR-99021 to hyperactivate Wnt signaling and investigate whether the aspirin-mediated inhibitory effects on EMT and stem cell markers are Wnt-regulated. Aspirin treatment abrogates CHIR-99021–mediated Wnt activation by increasing GSK-3β and β-catenin phosphorylation (Fig. 10A). The CHIR-99021–mediated increase in β-catenin and its targets, Axin2 and c-myc, are reversed on aspirin exposure (Fig. 10B). Wnt activation promotes a mesenchymal stem-like phenotype with decreased E-cadherin and increased Snail and Lgr5 expression, which was attenuated by aspirin in HCT116 cells (Fig. 10C). Increased E-cadherin has been shown to buffer excessive β-catenin, thus limiting hyperactivated Wnt and further promoting an epithelial phenotype.40 Because of the increase in E-cadherin on aspirin treatment, we investigated the E-cadherin–β-catenin interaction. CHIR-99021 treatment led to a reduction in E-cadherin bound β-catenin that was rescued with aspirin treatment as demonstrated by co-immunoprecipitation experiments (Fig. 10D). The novel observation that the aspirin-mediated E-cadherin increase is paralleled by greater E-cadherin–β-catenin binding further supports the hypothesis that aspirin promotes an epithelial phenotype through Wnt inhibition.
The addition of CHIR-99021 to organoids grown from FAP normal tissue further promoted the cystic phenotype identified in Wnt hyperactive tissues, with all organoids appearing cystic after 15 days of treatment. This alteration in cystic: budding organoid ratio was rescued by exposure to aspirin (Fig. 10E). Furthermore, aspirin as a single agent...
or after CHIR-99021 reduced the expression of β-catenin and the stem cell markers, SOX9 and Lgr5 (Fig. 10F). Taken together, we show that aspirin treatment reverses the Wnt-driven EMT and stem phenotype in both CRC cell lines and organoids derived from human colonic mucosa from FAP patients.

Figure 4. Aspirin reduces stem cell marker expression in vivo. (A1) Images of RNAscope staining for Lgr5 RNA expression in control and aspirin-treated (400 mg/kg aspirin by oral gavage for 4 weeks) ApcMin/+ mouse tissue. (A2) Quantification of number of Lgr5 dots per μm² of adenoma tissue (24 control and 30 aspirin-treated adenomas) from cohort of 5 ApcMin/+ control and 4 ApcMin/+ aspirin-treated mice. (B1) Immunohistochemistry images of lysozyme expression in control and aspirin-treated (2.6 mg/mL aspirin in drinking water for 7 days) ApcMin/+ mouse tissue. (B2) Quantification of lysozyme staining in adenoma tissue (50 control and 34 aspirin-treated adenomas) from cohort of 5 ApcMin/+ control mice and 4 ApcMin/+ aspirin-treated mice. Microscope objective magnification noted in bottom left corner of image. Graphs represent individual data plots with overlay of mean and standard deviation. Statistical significance determined by Student t test. Asterisks denote P value (*<.05, **<.01, ***<.001, ****<.0001). Scale bar = 50 μm.

Figure 3. (See previous page). Aspirin reduces stem cell marker expression in organoids. (A) Transcript expression of Lgr5 and TROY in untreated wild-type (C57BL/6J) mouse organoids and Apclox/lox mouse organoids treated with 2 mmol/L aspirin for 12 days. Lgr5 and TROY transcript levels are normalized to GAPDH transcripts and expressed as fold-change compared with untreated control sample. Data represent 1 mouse per experimental condition. (B1) Images of organoids derived from Apclox/lox mouse small intestine treated with 2 mmol/L aspirin for 12 days and stained with RNAscope probes for either Lgr5 or TROY. (B2) Quantification of organoids from 2 independent experiments (Lgr5-1, 54 control and 39 aspirin-treated organoids; Lgr5-2, 67 control and 25 aspirin-treated organoids; TROY-1, 92 control and 25 aspirin-treated organoids; TROY-2, 70 control and 31 aspirin-treated organoids). Quantification of RNAscope staining presented as a positivity score normalized to area. (C) Transcript expression of Lgr5 and TROY in isolated ApcMin/+ mouse crypts treated with 400 mg/kg aspirin by oral gavage for 4 weeks. Lgr5 and TROY transcript levels are normalized to GAPDH transcripts and expressed as fold-change compared with untreated control sample. Data represent 1 control and 1 aspirin-treated ApcMin/+ mouse. (D) Lgr5 transcript expression in organoids derived from human colonic tissue treated with 2 mmol/L aspirin for 24 hours. Lgr5 transcript levels are normalized to 18S transcripts, and data are expressed as fold-change compared with untreated control sample. Data represent organoids derived from 3 individual patients: a non-neoplastic lesion, sporadic neoplastic lesion, and adenoma tissue from a FAP patient. (E) Immunoblotting of Lgr5 protein abundance in organoids derived from human colonic tissue and treated with 2 mmol/L aspirin for 4 hours. Data from 3 individual human patient samples (2 normal colonic tissue samples and 1 colonic adenoma tissue sample). Microscope objective magnification noted in bottom left corner of image. Graphs represent individual data plots with overlay of mean and standard deviation. Statistical significance determined by Student t test. Asterisks denote P value (*<.05, **<.01, ***<.001, ****<.0001). Scale bar = 50 μm. NM, normal mucosa.
Aspirin Increases Expression of the Wnt Inhibitor, Dickkopf-1

Studies reporting aspirin-induced Wnt inhibition have generally focused on destruction complex machinery and downstream gene effects. The loss of endogenous Wnt antagonists during colorectal carcinogenesis is well-recognized and may impact on survival. We focused on the Wnt inhibitor DKK-1 because it is a specific canonical Wnt antagonist. DKK-1 acts as a potent Wnt inhibitor by binding to the transmembrane receptor LRP6, thus preventing the LRP6-frizzled interaction and blocking canonical Wnt activation.41 DKK-1 negatively correlates with EMT, and overexpression promotes an epithelial phenotype paralleled by decreased invasive capabilities of CRC cells.42 Aspirin treatment markedly increases DKK-1 expression while decreasing LRP6 expression in CRC cells, and this is paralleled by a decrease in secreted DKK-1 in the media (Fig. 11A and B). The aspirin-mediated increase in DKK-1 is modestly attenuated, in relative terms, by CHIR-99021 because it decreases DKK-1 in untreated samples (Fig. 11A and B). Aspirin increases nuclear DKK-1 expression in HCT116 cells, which is associated with reduced nuclear c-myc and Snail protein expression (Fig. 11C). Aspirin treatment increased DKK-1 transcript expression in organoids from FAP patients’ normal mucosa and adenoma tissue (Fig. 11D). Because DKK-1 expression can be silenced because of methylation, we used human fetal colonic organoids to minimize potential epigenetic effects in human adult colon. Aspirin treatment of fetal organoids significantly increased DKK-1 transcript expression (Fig. 11E), and although no alteration of the cystic phenotype was expected because of the shorter duration, there was a significant reduction in organoid size compared with controls (Fig. 11F). Using small interfering RNA (siRNA) to DKK1, we observe that siDKK1 shows an increase in β-catenin and c-myc, a decrease in E-cadherin expression, and no change in Snail in untreated cells (Fig. 11G). Because the siRNA DKK-1 knockdown is incomplete, we observe partial abrogation of aspirin-mediated effects on EMT and stem markers. We observe that aspirin decreases stem function using clonogenicity assays with a reduction in Apc<sup>flox/flox</sup> organoid size and number at 7 and 14 days, respectively (Fig. 12A and C). Furthermore, we confirm that human recombinant DKK-1 similarly decreases Apc<sup>flox/flox</sup> organoid size and number (Fig. 12D–F). Overall, our data robustly show that aspirin increases DKK-1 expression in CRC cells and in FAP organoids, which is associated with decreased stem cell function.

Discussion

In addition to its CRC prevention role, aspirin may confer a survival benefit after cancer diagnosis. The challenge remains in delineating the dominant signaling pathways responsible and identifying biomarkers with clinical utility. Here, we show that aspirin rescues the Wnt-driven cystic organoid phenotype and reduces stem cell marker activity in colonic epithelium and adenomas from FAP patients. We also find that aspirin decreases the migratory and invasive capabilities of CRC cells while promoting an epithelial phenotype both in vitro and in mouse models. In exploring underlying mechanisms, we demonstrate for the first time that strikingly aspirin increases expression of the endogenous Wnt inhibitor, DKK-1, which contributes to the aspirin-mediated Wnt inhibition, decreases stem cell function, and enhances the epithelial phenotype.

The budding intestinal organoid phenotype recapitulates normal Wnt gradients during intestinal homeostasis.27,43 We report the novel observation that aspirin rescues the characteristic aberrant Wnt-driven cystic phenotype by promoting budding in human normal mucosa and adenoma colonic organoids from FAP patients. This phenotype transition has been attributed to a concomitant decrease in proliferation and an increase in the elastic module that occurs during differentiation.44 Cystic growth of intestinal organoids is further attributed to stem cell dysregulation due to hyperactivated Wnt. Both increased stem cell and mesenchymal marker expression are associated with poor CRC survival.5,46 Reports suggest aspirin decreases stem cell markers in CRC cell lines.47 Here, we establish in vivo relevance by using several Apc-driven mouse and human FAP organoid models to show that aspirin reduces both stem cell marker expression and function. Ectopic Paneth-like cells are observed in murine Apc-deficient colonic epithelium.48 That aspirin decreases lysozyme expression, a Paneth cell marker, strongly suggests it also may be remodeling the stem cell niche generated by Paneth cell ligands and growth factors.29 Interestingly, Paneth cell metaplasia, proposed as an early CRC marker, is observed.

![Figure 5. (See previous page). Aspirin reduces β-catenin and EMT markers in vivo.](image)

(A) Immunohistochemistry images and quantification of control and aspirin-treated (400 mg/kg aspirin by oral gavage for 4 weeks) Apc<sup>Min/+</sup> mouse tissue stained for β-catenin expression. (B) Transcript expression of Tcf7 in Apc<sup>Min/+</sup> mouse crypts treated with 400 mg/kg aspirin for 4 weeks and Apc<sup>flox/flox</sup> mouse organoids treated with 2 mmol/L aspirin for 12 days. Tcf7 transcript levels are normalized to GAPDH transcripts and expressed as fold-change compared with untreated control sample. Data represent 1 mouse per experimental condition. (C) Immunohistochemistry images and quantification of control and aspirin-treated (400 mg/kg aspirin by oral gavage for 4 weeks) Apc<sup>Min/+</sup> mouse tissue stained for E-cadherin expression. (D) Immunohistochemistry images and quantification of control and aspirin-treated (400 mg/kg aspirin by oral gavage for 4 weeks) Apc<sup>Min/+</sup> mouse tissue stained for vimentin expression. Quantification of β-catenin (21 control and 30 aspirin-treated adenomas), E-cadherin (37 control and 23 aspirin-treated adenomas), and vimentin (29 control and 16 aspirin-treated adenomas) staining in adenoma tissue from cohort of 5 Apc<sup>Min/+</sup> control and 4 Apc<sup>Min/+</sup> aspirin-treated mice. Microscope objective magnification noted in bottom left corner of image. Scale bar = 50 μm. Graphs represent individual data plots with overlay of mean and standard deviation. Statistical significance determined by unpaired Student t test. Asterisks denote P value (*<.05, **<.01, ***<.001, ****<.0001).
**A**

| Aspirin 24h (mM) | Colo205 | HCT116 |
|------------------|---------|--------|
| 0                | ![Image](image1.png) | ![Image](image2.png) |
| 0.5              | ![Image](image3.png) | ![Image](image4.png) |
| 3                | ![Image](image5.png) | ![Image](image6.png) |

**B**

| Aspirin 24h (mM) | HCT116 |
|------------------|--------|
| 0                | ![Image](image7.png) |
| 0.5              | ![Image](image8.png) |
| 3                | ![Image](image9.png) |

**C**

**CDH1 transcript abundance**

| 3mM Aspirin | 24h | 48h |
|-------------|-----|-----|
| -           | ![Image](image10.png) | ![Image](image11.png) |
| +           | ![Image](image12.png) | ![Image](image13.png) |

**D1**

- **E-cadherin**
- **DAPI**
- **Merge**

**D2**

Quantification of IF expression

- Control
- 0.5mM Aspirin

**E**

| HCT116 | Cytoplasmic/Nuclear |
|--------|---------------------|
| Aspirin 16h (mM) | C | N | C | N | C | N |
| 0       | ![Image](image14.png) | ![Image](image15.png) | ![Image](image16.png) | ![Image](image17.png) | ![Image](image18.png) | ![Image](image19.png) |
| 0.5     | ![Image](image20.png) | ![Image](image21.png) | ![Image](image22.png) | ![Image](image23.png) | ![Image](image24.png) | ![Image](image25.png) |
| 3       | ![Image](image26.png) | ![Image](image27.png) | ![Image](image28.png) | ![Image](image29.png) | ![Image](image30.png) | ![Image](image31.png) |
| **Snail** | ![Image](image32.png) | ![Image](image33.png) | ![Image](image34.png) | ![Image](image35.png) | ![Image](image36.png) | ![Image](image37.png) |
| **E-cadherin** | ![Image](image38.png) | ![Image](image39.png) | ![Image](image40.png) | ![Image](image41.png) | ![Image](image42.png) | ![Image](image43.png) |
| **GAPDH** | ![Image](image44.png) | ![Image](image45.png) | ![Image](image46.png) | ![Image](image47.png) | ![Image](image48.png) | ![Image](image49.png) |
| **Lamin-B** | ![Image](image50.png) | ![Image](image51.png) | ![Image](image52.png) | ![Image](image53.png) | ![Image](image54.png) | ![Image](image55.png) |
| **β-actin** | ![Image](image56.png) | ![Image](image57.png) | ![Image](image58.png) | ![Image](image59.png) | ![Image](image60.png) | ![Image](image61.png) |
Aspirin increases Wnt antagonist in colon

Aspirin reduces Wnt signaling and promotes an epithelial phenotype in CRC cells.

Materials and Methods

Animal Studies

All animal experiments were approved by either the University of Edinburgh or University of Glasgow ethics committee and performed under a UK Home Office project license. Tissues from 2 separate ApcMin/+ mouse cohorts were analyzed in the production of this article. A 4-week treatment cohort consisted of 12 C57BL/6J mice and 12 ApcMin/+ mice. Each group was separated into 6 aspirin-treated and 6 control mice. All treatment groups were composed of 3 male and 3 female mice except the C57BL/6J aspirin treatment group, which contained 1 male and 5 female mice. Treatment commenced at 6 weeks and lasted 4 weeks. Mice were administered distilled water or 400 mg/

Figure 6. (See previous page). Aspirin reduces Wnt signaling and promotes an epithelial phenotype in CRC cells. (A) Immunoblotting of β-catenin, c-myc, Lgr5, and E-cadherin protein abundance in Colo205 cells treated with 0.5 or 3 mmol/L aspirin for 24 hours. (B) Immunoblotting of E-cadherin protein expression in HCT116 cells treated with 0.5 or 3 mmol/L aspirin for 24 hours. (C) E-cadherin (CDH1) transcript expression in HCT116 and Colo205 cells treated with 3 mmol/L aspirin for either 24 or 48 hours. CDH1 transcript levels are normalized to GAPDH transcripts and expressed as fold-change compared with untreated control sample. Data represent 3 independent experiments. (D1) Immunofluorescence images of E-cadherin and zona occludens 1 staining in HCT116 cells treated with 0.5 mmol/L aspirin for 24 hours. (D2) Quantification of E-cadherin and zona occludens 1 staining in HCT116 cells treated with 0.5 mmol/L aspirin for 24 hours. Staining intensity quantified as mean grey intensity per cell. Data represent 2 independent experiments. (E) Immunoblotting of E-cadherin and Snail protein abundance in cytoplasmic/nuclear extracts from HCT116 cells treated with 0.5 or 3 mmol/L aspirin for 16 hours. Immunoblotting data representative of 3 independent experiments. Microscope objective magnification noted in bottom left corner of image. Scale bar = 50 μm. Graphs represent individual data plots with overlay of mean and standard deviation. Statistical significance determined by unpaired Student t test. Asterisks denote P value (*<.05, **<.01, ***<.001, ****<.0001).
kg aspirin by oral gavage daily. A 7-day treatment cohort consisted of 9 Apc<sup>Min/+</sup> mice, 4 aspirin-treated (1 male and 3 females) and 5 controls (5 females). Treatment commenced once symptoms of tumor burden, specifically pale feet, became apparent and continued for 7 days. Aspirin was administered via the drinking water at 2.6 mg/mL. Mice
were culled after treatment by schedule 1 methods and tissue formalin-fixed and paraffin-embedded for histologic analysis.

**Organoid Culture**

The culture of colonic organoids from human tissue has been described previously. Human colonic mucosa and adenomas were removed during surgery. After washing in phosphate-buffered saline (PBS), mucosa was incubated in crypt chelating solution (1 × PBS, 45 mmol/L sucrose, 55 mmol/L D-sorbitol, 500 μmol/L DL-dithiothreitol, 5 mmol/L EDTA) for 1 hour at 4°C to dissociate the crypts. Crypts were dissociated from the mucosa by vigorous washing in PBS. Crypts were pelleted, washed, and embedded in BD Matrigel basement membrane matrix (BD Biosciences, San Jose, CA; 356234). Organoids were maintained in human colon mucosa medium (Advanced DMEM/F12 [Gibco, Wal-tham, MA; 12634028], 1 × GlutaMax [Gibco, 35050038], 1 mol/L Hepes [Gibco, 15630106], 100 IU/mL penicillin and 100 μg/mL streptomycin [Lonza, Basel, Switzerland; 09-757F], 1 × B27 [Gibco, 17504044], 1 mmol/L N-acetyl-L-cysteine [Sigma-Aldrich, St Louis, MO; A7250], 10 mmol/L nicotinamide [Sigma-Aldrich, 72340], 10 nmol/L gastrin [Sigma-Aldrich, G9020], 50 ng/mL epidermal growth factor [Sigma-Aldrich, E9644], 100 ng/mL mouse Noggin [Peprotech, 250-38], and 1 μg/mL mouse R-spondin 1 [R&D, 5036-WN]), which was replaced every 2–3 days with passage 1:4 every 5–7 days. Adenomas were incubated in digestion media (Advanced DMEM/F12 [Gibco, 12634028], 1 × GlutaMax [Gibco, 35050038], 1 mol/L Hepes [Gibco, 15630106], 100 IU/mL penicillin and 100 μg/mL streptomycin [Lonza, 09-757F], 1 mg/mL collagenase [Sigma-Aldrich, SCR103], 1 × Y27632 [Tocris, Bristol, UK; 1254], 20 ng/mL hyaluronidase [Sigma-Aldrich, H3506]) at 37°C for 1 hour. The solution was placed through 70-μm cell strainer to remove fibrous material, and cells were washed and embedded in BD Matrigel basement membrane matrix (BD Biosciences, 356234). Organoids were maintained in human colon adenoma medium without human Wnt-3a and with the addition of 1 × Y27632 (Tocris, 1254), which was replaced every 2–3 days with passage 1:4 every 5–7 days. Mouse small intestine was removed after culling by schedule 1 methods and 356234). Organoids were mechanically dissociated by vigorous washing in PBS, pelleted, and embedded in BD Matrigel basement membrane matrix (BD Biosciences, 356234). Fetal organoids were maintained in human colon mucosa medium, which was replaced every 2–3 days with passage 1:4 every 5–7 days.

**Live Cell Imaging**

Images were acquired using 10 × objective on a Zeiss Axio-Observer Z1 inverted microscope with ASI MS-2000 XY stage (Applied Scientific Instrumentation, Eugene, OR). Samples were illuminated using brightfield and acquired on a Retiga6000 CCD camera (Qimaging, Surrey, BC, Canada). Organoids were maintained at 37°C using a custom-made environmental enclosure (MRC Human Genetics Unit imaging facility). A 5% CO2 atmosphere was maintained by an Okolabs chamber (Okolabs, Ottaviano, Italy). Images were captured every 1 hour for 48 hours, and videos were created using Image J software (https://imagej.nih.gov/ij/).

**RNAscope**

Organoids were mechanically removed from BD Matrigel basement membrane matrix using a p200 pipette to disrupt the Matrigel. Organoids were washed in PBS, pelleted, and fixed with 4% paraformaldehyde (Thermo Scientific, 28906) for 30 minutes at room temperature. After fixation, organoids were washed in PBS twice and then dehydrated by sequential incubations in 25%, 50%, and 70% ethanol for 15 minutes each. The organoid pellet was embedded in 4% agarose and then paraffin embedded. For RNAscope, 5-μm sections were cut from formalin-fixed paraffin-embedded blocks of organoids or mouse tissue. RNAscope is commercially available from Advanced Cell Diagnostics (ACD, Newark, CA). Here, the probes used were mouse TROY (420241) and mouse Lgr5 (312171). Before

**Figure 7.** (See previous page). **Aspirin reduces cell migration in CRC cells.** (A) Brightfield images of wound closure assays and quantification of percentage of wound closure over 48 hours in Colo205 and HCT116 cells maintained in 0.5% serum. (B) Brightfield images of wound closure assays and quantification of percentage of wound closure over 48 hours in Colo205 and HCT116 cells maintained in 10% serum. Wound closure assay data representative of 3 independent experiments, each containing 3 technical replicates. Scale bar = 100 μm. (C) Growth curves of HCT116 and Colo205 cells treated with various concentrations of aspirin (0.1, 0.5, 1, 3, or 5 mmol/L) over 72 hours. Quantification presented as number of cells per mL. Data represent 3 independent experiments. *Error bars* represent the standard error. Graphs represent individual data plots with overlay of mean and standard deviation. Statistical significance determined by Student’s t test. Asterisks denote P value (∗<.05, **<.01, ***<.001, ****<.0001).
Figure 8. Aspirin reduces cell invasion and motility in CRC cells. (A) Brightfield images of hematoxylin-eosin stained organotypic invasion assays and quantification of percentage of cells invading and maximum distance invaded over 7 days with Colo205 cells. (B) Brightfield images of hematoxylin-eosin stained organotypic invasion assays and quantification of percentage of cells invading and maximum distance invaded over 7 days with HCT116 cells. Scale bar = 200 μm. Organotypic invasion assay data representative of 3 independent experiments, each containing 2 technical replicates. (C) Chemotaxis plots of cell movement and quantification of accumulated distance over 24 hours in Colo205 cells treated with 0.5 or 3 mmol/L aspirin and maintained in 0.5% or 10% serum. (D) Chemotaxis plots of cell movement and quantification of accumulated distance over 24 hours in HCT116 cells treated with 0.5 or 3 mmol/L aspirin and maintained in 0.5% or 10% serum. Single cell motility data represent 3 independent experiments, each with 2 technical replicates. Microscope objective magnification noted in bottom left corner of image. Graphs represent individual data plots with overlay of mean and standard deviation. Statistical significance determined by Student t test. Asterisks denote P value (*<.05, **<.01, ***<.001, ****<.0001).
treatment, target retrieval and peroxidase block of formalin-fixed paraffin-embedded sections were performed using RNAscope 2.5 HD Reagent kit-RED (ACD, 322350) according to manufacturer’s instructions. After detection of the probes using 6 different amplification reagents, mRNA transcripts were visualized using diaminobenzidine substrate kit (Vector Laboratories, Burlingame, CA; SK-4100). Slides were counterstained with Gills hematoxylin (Sigma-Aldrich, GHS132). Slides were mounted using DPX mounting medium (Sigma-Aldrich, ACD, 322350).
1005790500, and slides were imaged using a Nano-Zoomer S60 digital slide scanner (Hamamatsu, Hamamatsu City, Japan). Quantification of RNAscope from adenoma tissue was completed manually using Image J software (https://imagej.nih.gov/ij/) to count all dots within an adenoma and normalized to the area. Quantification of RNAscope from organoids was completed using QuPath software. All well-formed organoids present on the slide were analyzed by setting threshold values (for different intensities of diaminobenzidine staining to account for the number of amplification agents attach on the probes) and then calculating the number of positive cells.

**A**

| Condition          | pβ-catenin (S33/S37/T41) | β-catenin | pGSK3β (S9) | GSK3β | β-actin |
|--------------------|--------------------------|-----------|-------------|-------|---------|
| 3mM Aspirin (16h)  | -                        | +         | -           | -     | +       |
| 3μM CHIR-99021 (16h) | -                        | +         | -           | -     | +       |

**B**

| Condition          | β-catenin | Axin2 | β-actin | c-myc | β-actin |
|--------------------|-----------|-------|---------|-------|---------|
| 3mM Aspirin (24h)  | +         | +     | +       | -     |         |
| 3μM CHIR-99021 (24h)| +         | +     | -       | -     |         |

**C**

| Condition          | E-cadherin | β-actin | Snail | GAPDH | Lgr5 | β-actin |
|--------------------|------------|---------|-------|-------|------|---------|
| 3mM Aspirin (24h)  | 35         | 100     | 35    | 100   | 55   | 100     |
| 3μM CHIR-99021 (24h)| 35         | 100     | 35    | 100   | 55   | 100     |

**D1**

| Condition          | INPUT | IP: E-cadherin |
|--------------------|-------|----------------|
| m-sprrn (24h)      | -     | -              |
| 3μM CHIR-99021 (24h) | -     | +              |

| Condition          | E-cadherin | β-catenin | GAPDH | IgG |
|--------------------|------------|-----------|-------|-----|
| INPUT              | 35         | 100       | 35    | 25  |
| IP: E-cadherin     | 35         | 100       | 35    | 25  |

**D2**

| Condition          | INPUT | IP: β-catenin |
|--------------------|-------|---------------|
| 3mM Aspirin (24h)  | -     | -             |
| 3μM CHIR-99021 (24h)| -     | +             |

| Condition          | E-cadherin | β-catenin | GAPDH | IgG |
|--------------------|------------|-----------|-------|-----|
| INPUT              | 35         | 100       | 35    | 25  |
| IP: β-catenin      | 35         | 100       | 35    | 25  |

**E**

Quantification of organoid phenotype derived from FAP normal mucosa

**F**

Organoids derived from FAP normal mucosa

| Condition          | β-catenin | SOX-9 | Lgr5 | β-actin |
|--------------------|-----------|-------|------|---------|
| 0.5mM Aspirin (36 days) | -         | +     | -    | +       |
| 3μM CHIR-99021 (36 days) | -         | +     | -    | +       |

**G**

| Condition          | β-catenin | SOX-9 | Lgr5 | β-actin |
|--------------------|-----------|-------|------|---------|
| 0.5mM Aspirin (15 days) | -         | +     | -    | +       |
| 3μM CHIR-99021 (15 days) | -         | +     | -    | +       |
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Immunohistochemistry
Formalin-fixed paraffin-embedded tissue blocks were cut into 5-μm-thick sections. Sections were deparaffinized by 3 × 5-minute washes in 100% xylene and sequentially washed in 3 × 100%, 1 × 90%, and 1 × 70% ethanol for 2 minutes each. Sections were incubated in water for 5 minutes to rehydrate. Antigen retrieval was completed by incubation in boiling 10% citrate buffer (Thermo Scientific, AP-9003). Endogenous blocking was achieved with incubation in 3% hydrogen peroxide (Sigma-Aldrich, H1009) for 10 minutes, followed by 5% goat serum (Thermo Scientific, 16210064) for 1 hour at room temperature. Primary antibody was diluted 1:200 in 5% goat serum (Thermo Scientific, 16210064) 0.01% Tween20 in PBS and incubated overnight at 4°C. After washing in 0.01% Tween20 in PBS, sections were incubated in EnVision horseradish peroxidase (HRP)-conjugated rabbit secondary antibody (Dako, Glostrup, Denmark; K400311-2) for 1 hour at room temperature. Sections were washed 3 × 10 minutes in 0.01% Tween20 in PBS. Signal was detected using diaminobenzidine substrate kit (Vector Laboratories, SK-4100). Sections were counterstained with Gills hematoxylin (Sigma-Aldrich, GHS132). Slides were mounted using DPX mounting medium (Sigma-Aldrich, 1005790500), and slides were imaged using a NanoZoomer S60 digital slide scanner (Hamamatsu). For quantification, an objective quantification method was used to avoid bias, and so all adenoma images were randomized, and quantification was conducted blinded. The objective staining intensity number (0, negative; 1, mild; 2, moderate; 3, intense) was multiplied by the percentage of adenoma tissue expressing that intensity to give a grading score for each adenoma. For example, an adenoma with 50% mild staining and 50% moderate staining would be attributed a grading score of 150.

Cell Culture
CRC cell lines HCT116 (CCL-247) and Colo205 (CCL-222) are available from the American type culture collection (ATCC). HCT116 and Colo205 cells were grown as a monolayer in either McCoy’s 5A Medium ( Gibco, 16600082) or Dulbecco modified Eagle medium ( Gibco, 41965039), respectively. Medium was supplemented with 10% fetal bovine serum (HyClone, Logan, UT; 12379802), 100 IU/mL penicillin and 100 μg/mL streptomycin (Lonza, 09-7577F). Cells were regularly tested for mycoplasma, with only mycoplasma free cell lines used for experimentation.

Antibodies
Antibodies recognizing E-cadherin (24E10) (3195), Snail (C15D3) (3879), ROCK1 (C8F7) (4035), ROCK2 (D1B1) (9029), coflin (D3F9) (5175), phospho-cofilin (Ser3) (7762) (3313), phospho-β-catenin (S33/37/41) (9561), phospho-GSK-3β (Ser9) (D85E12) (5558), GSK-3β (D552Z) (12456), Axin2 (76G6) (2151), Sox-9 (D8G8H) (82630) ZO-1 (D6L1E) (13663), and DKK-1 (D5V6L) (48367) were obtained from Cell Signaling Technology (Danvers, MA). Additional antibodies used were E-cadherin (BD Transduction Laboratories, 610181), β-catenin (BD Transduction Laboratories, 610153), c-myc (Abcam, Cambridge, United Kingdom; ab32072), GPCR GPR49 (Lgr5) (Abcam, ab75850), GAPDH (GeneTex, Irvine, CA; GTX627408), Lamin B (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA; sc-6216), and β-actin (C4) (Santa Cruz Biotechnology, sc-47778). Secondary HRP-conjugated antibodies used were rabbit immunoglobulin G HRP-linked (GE Healthcare, Chicago, IL; GENA934) and mouse immunoglobulin G HRP-linked (GE Healthcare, GENA931).

Compound Treatments
Aspirin (Sigma-Aldrich, A2093) was added to cell culture medium at indicated concentrations (between 0.1 and 3 mmol/L) for the indicated duration. CHIR99021 (Tocris, 4423) is a GSK-3 inhibitor that was added to cells at a concentration of 3 μmol/L for 24 hours.

Wound Healing Assays
Cells were grown as a monolayer until 90% confluent, at which point a “wound” was created using a p200 pipette tip. Cells were washed in PBS twice to remove cellular debris before the addition of medium containing either 0.5% or 10% fetal bovine serum. Compound treatments were added as indicated, and brightfield images of the wound were taken at 0, 24, and 48 hours using a Zeiss Axiovert 100

Figure 10. (See previous page). Aspirin reduces Wnt-driven mesenchymal and stem marker expression in CRC cell lines and FAP organoids. (A) Immunoblotting of phospho-β-catenin (S33/S37/T41), β-catenin, phospho-GSK3β (S9), and GSK3β protein abundance in HCT116 cells treated with 3 mmol/L aspirin, 3 μmol/L CHIR-99021, or combination for 16 hours. (B) Immunoblotting of β-catenin, Axin2, and c-myc protein abundance in HCT116 cells treated with 3 mmol/L aspirin, 3 μmol/L CHIR-99021, or combination for 24 hours. (C) Immunoblotting of E-cadherin, Snail, and Lgr5 protein abundance in HCT116 cells treated with 3 mmol/L aspirin, 3 μmol/L CHIR-99021, or combination for 24 hours. Immunoblotting data representative of 3 independent experiments. (D1) Immunoprecipitation using E-cadherin antibody in HCT116 cells treated with 3 mmol/L aspirin, 3 μmol/L CHIR-99021, or combination for 24 hours. Immunoprecipitation data representative of 3 independent experiments. (D2) Immunoprecipitation using β-catenin antibody in HCT116 cells treated with 3 mmol/L aspirin, 3 μmol/L CHIR-99021, or combination for 24 hours and immunoblotting of β-catenin and E-cadherin from input lysates and immunoprecipitation elutes. Immunoprecipitation data representative of 3 independent experiments. (E) Quantification of percentage of budding organoids derived from human FAP patient normal colonic mucosa tissue treated with 0.5 mmol/L aspirin, 3 μmol/L CHIR-99021, or combination for 15 days in vitro. Organoid data represent 1 individual human patient sample. (F) Immunoblotting of β-catenin, SOX-9, and Lgr5 protein abundance in organoids derived from human FAP patient normal colonic mucosa tissue treated with 0.5 mmol/L aspirin, 3 μmol/L CHIR-99021, or combination for 36 days in vitro. Organoid data represent 1 individual human patient sample. Graphs represent individual data plots with overlay of mean and standard deviation. Statistical significance determined by Student t test. Asterisks denote P value (*<.05, **<.01, ***<.001, ****<.0001).
Organotypic collagen-based invasion assays were performed as previously described. To prepare 12 matrices, approximately $1 \times 10^6$ fibroblast cells were suspended in 3 mL of 10× minimum essential medium (Gibco, 11430030), which was then added to 25 mL of 2 ng/mL rat tail collagen that had been adjusted to pH 7.2 using 0.22 mol/L NaOH. The fibroblast-collagen mixture was plated into 12 × 35 mm plastic cell culture plates and allowed to set. Dulbecco modified Eagle medium containing 10% fetal bovine serum was added to each fibroblast-collagen matrix, and matrices were left to contract for 8 days, with medium replaced every second day. Fibroblast-collagen matrices were transferred to 24-well cell culture plates, and CRC cells were seeded on top of matrices in complete cell culture medium. Once cells were confluent, the fibroblast-collagen matrices were transferred to sterile stainless steel grids, which allowed the bottom of the matrices to be in contact with medium but not the CRC cells on top of the matrices. Compound treatments were added to the medium. Cells were left to invade matrices for 7 days before the fibroblast-collagen matrices were fixed in 4% paraformaldehyde (Thermo Scientific, 28908), paraffin-embedded, and sections stained with hematoxylin-eosin. Sections were imaged using a Zeiss Axioplan 2 microscope with a $10 \times$ objective. Quantification of the number of invaded cells and the distance of invasion was performed manually using the Image J software (https://image.j.nih.gov/ij/). The percentage of invaded cells was normalized to the total cell number. Telomerase immortalized fibroblasts were used to create the fibroblast-collagen matrices and were provided by John Dawson (IGMM, Edinburgh).

Organotypic Invasion Assays

Organotypic collagen-based invasion assays were performed as previously described. To prepare 12 matrices, approximately $1 \times 10^6$ fibroblast cells were suspended in 3 mL of 10× minimum essential medium (Gibco, 11430030), which was then added to 25 mL of 2 ng/mL rat tail collagen that had been adjusted to pH 7.2 using 0.22 mol/L NaOH. The fibroblast-collagen mixture was plated into 12 × 35 mm plastic cell culture plates and allowed to set. Dulbecco modified Eagle medium containing 10% fetal bovine serum was added to each fibroblast-collagen matrix, and matrices were left to contract for 8 days, with medium replaced every second day. Fibroblast-collagen matrices were transferred to 24-well cell culture plates, and CRC cells were seeded on top of matrices in complete cell culture medium. Once cells were confluent, the fibroblast-collagen matrices were transferred to sterile stainless steel grids, which allowed the bottom of the matrices to be in contact with medium but not the CRC cells on top of the matrices. Compound treatments were added to the medium. Cells were left to invade matrices for 7 days before the fibroblast-collagen matrices were fixed in 4% paraformaldehyde (Thermo Scientific, 28908), paraffin-embedded, and sections stained with hematoxylin-eosin. Sections were imaged using a Zeiss Axioscipt 2 microscope with a $10 \times$ objective. Quantification of the number of invaded cells and the distance of invasion was performed manually using the Image J software (https://image.j.nih.gov/ij/). The percentage of invaded cells was normalized to the total cell number. Telomerase immortalized fibroblasts were used to create the fibroblast-collagen matrices and were provided by John Dawson (IGMM, Edinburgh).

Single Cell Motility Assays

Cells were grown as a monolayer until approximately 40% confluence. Cell culture medium was replaced with medium containing either 0.5% or 10% fetal bovine serum and indicated compound treatments. Brightfield images were taken on a live cell imaging microscope, Zeiss Axioscipt 200 microscope. A $10 \times$ objective was used with images captured every 30 minutes for 24 hours. Cell movement was analyzed, and distance was calculated using manual tracking and chemotaxis plugins for Image J software (https://image.j.nih.gov/ij/). Ten randomly selected cells were tracked for each well. Each experiment contained 2 technical replicates for each compound treatment, with 3 biological replicate experiments completed.

Protein Extractions

Cells were washed and scraped in ice-cold PBS and then pelleted at 1800 rpm for 5 minutes. For whole cell protein extractions, cell pellets were resuspended in whole cell lysis buffer (20 mmol/L Tris-HCl [pH 7.5], 150 mmol/L NaCl, 1 mmol/L Na2 EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 50 mmol/L sodium fluoride, 5 mmol/L beta-glycerophosphate, 50 mmol/L calyculin A, 1 mmol/L Na3VO4, 1× Roche complete EDTA-free protease inhibitor cocktail [Sigma-Aldrich, 1183617001], 1× Roche PhosSTOP [Sigma-Aldrich, 4906845001]) and incubated on ice for 30 minutes. Lysates were clarified at 13,000 rpm for 20 minutes. For cytoplasmic/nuclear protein extractions, cell pellets were resuspended in cytoplasmic lysis buffer (20 mmol/L Tris-HCl [pH 7.5], 0.1 mmol/L Na2 EDTA, 2 mmol/L MgCl2, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 0.3 μg/mL benzamidinchloride, 10 μg/mL trypsin inhibitor, 1% NP40, 50 mmol/L beta-2-mercaptoethanol, 1 mmol/L phenylmethylsulfonyl fluoride, 1× Roche complete EDTA-free protease inhibitor cocktail [Sigma-Aldrich, 1183617001], 1× Roche PhosSTOP [Sigma-Aldrich, 4906845001]) and incubated for 2 minutes at room temperature and then 10 minutes on ice. Cytoplasmic lysate was centrifuged at 3000 rpm for 5 minutes. The nuclear pellet was washed in detergent free cytoplasmic buffer 3 times. The residual pellet was resuspended in nuclear lysis buffer (20 mmol/L Hepes [pH 8], 0.4 mol/L NaCl, 25% glycerol, 1 mmol/L
A Clonogenicity assay

Control

2 mM Aspirin

B Clonogenicity assays: Organoid number

1st generation (Day 7)  2nd generation (Day 14)

- Control  ○ 2 mM Aspirin

Number of organoids

C Clonogenicity assays: Organoid size

1st generation (Day 7)  2nd generation (Day 14)

- Control  ○ 2 mM Aspirin

Organoid size (pixels)

D Clonogenicity Assay

Control

250 ng/ml hrDKK1 (Treat day 0)

250 ng/ml hrDKK1 (Treat day 0 and 7)

E Apofox/fox organoids: Number

- Control  ○ 250 ng/ml hrDKK1 (Treat day 0)

△ 250 ng/ml hrDKK1 (Treat day 0 and 7)

Number of organoids

F Apofox/fox organoids: Size

- Control  ○ 250 ng/ml hrDKK1 (Treat day 0)

△ 250 ng/ml hrDKK1 (Treat day 0 and 7)

Organoid size (pixels)
Nuclear lysate was clarified for membrane integrity, and then incubated on ice for 30 minutes.

Dry ice, thawed, vortexed 3 times to disrupt nuclear membranes. The sample was frozen on dry ice, 4°C for 10 minutes before clarifying at 13,000 rpm for 20 minutes. Protein concentrations were determined as previously described. For each reaction 5 μL of anti-E-cadherin (BD Transduction Laboratories, 610181) or anti-β-catenin (BD Transduction Laboratories, 610153) antibody was added to 800 μL sample buffer (Thermo Scientific, 10003D) containing 5% beta-2-mercaptoethanol was added to samples. Samples were incubated at 95°C for 10 minutes before immunoblotting.

**Immunoblotting**

Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel and transferred to polyvinylidene difluoride 0.2 µm transfer membrane (Thermo Scientific, 88520) using a Trans-blot SD semidry electrophoretic transfer cell (Bio-Rad, Hercules, CA; 1703940). Membranes were washed in PBS and blocked with 5% non-fat milk solution for 1 hour at room temperature. Membranes were washed in PBS containing 0.01% Tween20 and then incubated in primary antibody diluted in 5% bovine serum albumin solution containing 0.02% sodium azide for 16 hours at 4°C. Membranes were washed in PBS containing 0.01% Tween20 and then incubated with HRP-conjugated secondary antibody diluted in 5% non-fat milk solution containing 0.01% Tween20 for 1 hour at room temperature. After 3 × 10-minute washes in PBS containing 0.01% Tween20, antigen-antibody complexes were visualized by chemiluminescence using the Western blotting luminol reagent (Santa Cruz, sc-2048).

**Immunoprecipitation**

For immunoprecipitation, cells were lysed in an immunoprecipitation specific lysis buffer (40 mmol/L Hepes [pH 7.5], 150 mmol/L NaCl, 1 mmol/L Na2EDTA, 10 mmol/L sodium pyrophosphate, 50 mmol/L glycerophosphate, 50 mmol/L sodium fluoride, 1 mL supernatant and incubated on a rotating wheel for 1183617001 and 1 × Roche PhosSTOP [Sigma-Aldrich, 4906845001]) for 45 minutes on ice before clarifying at 13,000 rpm for 20 minutes. Protein concentrations were measured using Pierce Coomassie Bradford protein assay kit (Thermo Scientific, 23200). Protein concentrations were adjusted to 1–3 μg/mL, and NuPAGE 4–12% SDS polyacrylamide gel was run at 150 V for 1 hour. After 3 × 10-minute washes in PBS containing 0.01% Tween20, antigen-antibody complexes were visualized by chemiluminescence using the Western blotting luminol reagent (Santa Cruz, sc-2048).

**Pull-down of Dickkopf-1 Protein From the Medium**

Cells were treated with compounds as indicated. After treatment, the medium was collected from cells and incubated with StrataClean Resin (Agilent Technologies, Santa Clara, CA; 400714) in accordance with manufacturer’s instructions. Briefly, 10 μL of StrataClean Resin was added to 1 mL supernatant and incubated on a rotating wheel for 1 hour at 4°C. The resin was pelleted, and supernatant was removed. The remaining resin was incubated in 50 μL NuPAGE 2 × LDS sample buffer (Thermo Scientific, NP0007) containing 5% beta-2-mercaptoethanol at 7°C for 10 minutes. Samples were separated by sodium dodecyl sulfate–polyacrylamide gel, and DKK-1 protein was detected by immunoblotting using anti-DKK-1 antibody.

**Immunofluorescence**

Cells were seeded on glass coverslips and treated as indicated. Cells were fixed in 4% paraformaldehyde (Thermo Scientific, 28906) for 15 minutes, incubated in permeabilization buffer (0.2% Triton X) for 10 minutes, and then blocking buffer (3% goat serum, 3% bovine serum albumin) added for 1 hour, all at room temperature. E-cadherin (24E10) (Cell Signaling Technology, 3195), or phenylmethylsulfonyl fluoride, 0.5% NP40, 1 × Roche complete EDTA-free protease inhibitor cocktail [Sigma-Aldrich, 118367001], and 1 × Roche PhosSTOP [Sigma-Aldrich, 4906845001]) for 45 minutes on ice before clarifying at 13,000 rpm for 20 minutes. Protein concentrations were determined as previously described. For each reaction 5 μL of anti-E-cadherin (BD Transduction Laboratories, 610181) or anti-β-catenin (BD Transduction Laboratories, 610153) antibody was added to 800 μL protein and incubated at 4°C for 24 hours. Dynabeads protein G (Thermo Scientific, 10003D) were equilibrated in immunoprecipitation lysis buffer, and then 50 μL was added to each reaction and incubated for 16 hours at 4°C. Beads were washed in PBS containing 0.02% Tween20 three times before elution by incubating beads in 100 μL NuPAGE 2 × LDS sample buffer (Thermo Scientific, NP0007) containing 5% beta-2-mercaptoethanol at 7°C for 10 minutes. Samples were separated by sodium dodecyl sulfate–polyacrylamide gel, and DKK-1 protein was detected by immunoblotting using anti-DKK-1 antibody.

**Figure 12.** (See previous page.) Aspirin and DKK-1 treatment reduce number and size of Apcflx/flx organoids in clonogenicity assay. (A) Brightfield images of organoids derived from Apcflx/flx mouse small intestine at day 14 of clonogenicity assay after treatment with 2 mmol/L aspirin. (B) Average number of organoids per well from clonogenicity assay at day 7 (first generation) and day 14 (second generation) after addition of 2 mmol/L aspirin on day 0. (C) Average size of organoids per well from clonogenicity assay at day 7 (first generation) and day 14 (second generation) after addition of 2 mmol/L aspirin on day 0. Aspirin clonogenicity assay data are representative of 6 technical replicates from organoids derived from 1 Apcflx/flx mouse tissue sample. (D) Brightfield images of Apcflx/flx organoids at day 14 of clonogenicity assay after addition of 250 ng/mL hrDKK1 as sole treatment on day 0 or double treatment on days 0 and 7. (E) Average number of organoids per well at day 14 of clonogenicity assay after addition of 250 ng/mL hrDKK1 as sole treatment on day 0 or double treatment on days 0 and 7. DKK-1 clonogenicity assay data represent 4 technical replicates from organoids derived from 1 Apcflx/flx mouse small intestine sample. Microscope objective magnification noted in bottom left corner of image. Scale bar = 300 μm. Graphs represent individual data plots with overlay of mean and standard deviation. Statistical significance determined by Student t test. Asterisks denote P value (*<.05, **<.01, ***<.001, ****<.0001).
ZO-1 (D6L1E) (Cell Signaling Technology, 13663), diluted 1:200, was incubated overnight at 4°C before incubation with goat anti-rabbit immunoglobulin G Alexa Fluor 488 antibody (Life Technologies, Carlsbad, CA; A32731) at 1:1000 for 1 hour at room temperature. For F-actin staining, after blocking cells were incubated with Alexa Fluor 488 phalloidin (Life Technologies, A12379) for 1 hour at room temperature. Coverslips were washed in PBS containing 0.01% Tween20 3 x 10 minutes before mounting. Cells were mounted with Vectashield mounting medium with DAPI (Vector Laboratories, H-1200-10). Cells were imaged on Zeiss Axioplan 2 using 10×, 40×, and 100× objectives maintaining consistent exposure settings for all treatment conditions. Quantification of immunofluorescence images was completed using Image J software (https://imagej.nih.gov/ij/). The mean gray area of each image was calculated and then divided by the number of nuclei per image to determine a value for staining intensity.

**Quantitative Real-Time Polymerase Chain Reaction**

After treatments, RNA was extracted using the Ribopure RNA isolation kit (Invitrogen, AM1924). RNA concentration was measured using a nanodrop and treated with RNase-free DNase (Promega, Madison, WI; M6101). cDNA synthesis was completed using M-MLV reverse transcriptase (Promega, M5313) following manufacturer’s instructions. Quantitative real-time polymerase chain reaction was completed in triplicate in a 10 μL reaction with 2 μmol/L forward primer, 2 μmol/L reverse primer, 50% SYBR green master mix (Applied Biosystems, 4309155), and 2 μL cDNA (diluted 1:5) using a Lightcycler 480 (Roche). Primers used were as follows: GAPDH (F) [5′-GCACCGTCAAGGGTGAAGAC-3′], GAPDH (R) [5′-TGGTGAAGACGGCAATGGGA-3′]; CDH1 (F) [5′-ATTCTGATTCTGCTGGCTTG-3′], CDH1 (R) [5′-AGTAGTCATAGTCTCCTGGTCTT-3′]; mouse Lgr5 (F) [5′-GAGCTACCC-CAACGCTTATGATC-3′], mouse Lgr5 (R) [5′-CATGGGACAAATGCAACTGAAG-3′]; human Lgr5 (F) [5′-AATGTTGCCTACAATAAAC-3′], human Lgr5 (R) [5′-GAGTATGGTAACCTGATGGAG-3′]; 18S (F) [5′-GAATCCGGTTGACCCCCATT-3′], 18S (R) [5′-CCCTCTGATGGAGGCGCGC-3′]; TROY (F) [5′-CTGGATTGTTCTCATCTGTCG-3′], TROY (R) [5′-GCTGTTCATTTCTCTGCTCCTATC-3′]; DKK1 (F) [5′-GCACCGTAGGAAAATTGGAAATG-3′], DKK1 (R) [5′-ATGGTGGCAAGGAGGGAATGGCA-3′]. The data were analyzed by using the comparative Ct method (ΔΔCt), with GAPDH or 18S as the endogenous control gene.

**Clonogenicity Assays**

Apflx/flox organoids were removed from Matrigel and incubated in trypsin for 20 minutes at 37°C to dissociate into single cells. Single cells in solution were passed through a 0.44-μm filter and manually counted using a Neubauer chamber. In each well of a 24-well plate, 1000 cells were plated in 10 μL BD Matrigel basement membrane matrix. After solidification of Matrigel, 500 μL mouse small intestine medium was added to each well, and treatment, 250 μg/mL human recombinant DKK-1 or 2 mmol/L aspirin, was added as indicated. Organoids were grown for 7 days and then imaged and manually counted. For a second generation, organoids were again removed from Matrigel, dissociated, counted, and replated as before. An additional treatment with 250 μg/mL human recombinant DKK1 was performed. Organoids were grown for another 7 days, imaged, and manually counted.

**Transfection of Small Interfering RNAs**

Cells were seeded into 6-well plates and grown to 70% confluence. Transfections were completed by using siRNAs that target human DKK-1 (Dharmacon, Lafayette,CO; E-003843-00-0005) or nonspecific siRNA (Dharmacon, E-001910-01-5) as a negative control. For each well transfected, 25 nmol/L siRNA was diluted in 150 μL OptiMem (Gibco, 31985062). Separately, 10 μL Lipofectamine2000 (Invitrogen, 11668030) was diluted in 150 μL OptiMem (Gibco, 31985062). After incubating both reaction mixtures for 5 minutes at room temperature, they were combined and incubated for an additional 20 minutes at room temperature. The transfection mixture was added dropwise to the cells, and cells were cultured for an additional 24 hours before addition of compound treatments with aspirin and/or CHIR-99021 for 24 hours.

**Statistics**

All statistical tests and graphs were completed using Prism 8 (https://www.graphpad.com). Scatter plots show individual data points with an overlay of the mean and standard deviation. Statistical significance was determined by Student t test, with P value <.05 deemed significant. Asterisks denote the P value (*<.05, **<.01, ***<.001, ****<.0001).

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