
Publishers page: http://dx.doi.org/10.1158/0008-5472.CAN-18-2095

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Frizzled-7 is required for Wnt signaling in gastric tumors with and without Apc mutations.

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Running title: Targeting Wnt/Frizzled-7 signaling reduces gastric cancer.

Keywords: Frizzled-7, Wnt signaling, Gastric cancer, Targeted therapies

Funding: Funding is gratefully acknowledged from the following; National Health and Medical Research Council of Australia (NHMRC, 566679 and APP1099302 awarded to E. Vincan/T.J. Phesse/N. Barker), Melbourne Health project grants (605030 and PG-002 awarded to E. Vincan/T.J. Phesse/N. Barker/H. Clevers), Medical Research Council (MR/R026424/1 to T.J. Phesse), and Early career researcher grant (GIA-033 awarded to D.J. Flanagan), Cancer Council of Victoria project grants (CCV, APP1020716 awarded to E. Vincan/T.J. Phesse/N. Barker), CCV Fellowship awarded to D.J. Flanagan and Cardiff University/CMU Research Fellowship awarded to T.J. Phesse.
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Disclosures: Austin Gurney has ownership interests, including patents on the OMP-18R5 compound used in this study through OncoMed Pharmaceuticals. The remaining authors declare no conflict of interest.

Word count: 4199

Number of figures and tables: 6 main figures, 7 supplemental figures, 1 supplemental table

Abstract

A subset of gastric cancer (GC) patients have mutations in genes that participate in or regulate Wnt signaling at the level of ligand (Wnt) receptor (Fzd) binding. Moreover, increased Fzd expression is associated with poor clinical outcome. Despite these findings, there are no in vivo studies investigating the potential of targeting Wnt receptors for treating GC, and the specific Wnt receptor transmitting oncogenic Wnt signaling in GC is unknown. Here we use inhibitors of Wnt/Fzd (OMP-18R5/Vantictumab) and conditional gene deletion to test the therapeutic potential of targeting Wnt signaling in preclinical models of intestinal-type gastric cancer and ex vivo organoid cultures. Pharmacological targeting of Fzd inhibited the growth of gastric adenomas in vivo. We identified Fzd7 to be the predominant Wnt receptor responsible for transmitting Wnt signaling in human gastric cancer cells and mouse models of GC, whereby Fzd7-deficient cells were retained in gastric adenomas but were unable to respond to Wnt signals and consequently failed to proliferate. Genetic deletion of Fzd7 or treatment with Vantictumab was sufficient to inhibit the growth of gastric adenomas with or without mutations to Apc. Vantictumab is currently in phase Ib clinical trials for advanced pancreatic, lung, and breast cancer. Our data extend the scope of patients that may benefit from this therapeutic approach as we demonstrate that this drug will be effective in treating gastric cancer patients regardless of Apc mutation status.

Statement of significance

The Wnt receptor Fzd7 plays an essential role in gastric tumorigenesis irrespective of Apc mutation status therefore targeting Wnt/Fzd7 may be of therapeutic benefit to gastric cancer patients.
Introduction

Gastric cancer (GC) is a common malignancy, ranking in the top 4 of global cancer incidence [1]. Often due to advanced stage diagnosis, gastric cancer patients have a very poor 5-year survival rate [1]. This highlights a desperate need for novel clinical treatments as there are very few approved targeted therapies for GC [2, 3]. Gastric cancer is divided histologically into two groups; intestinal-type and diffuse-type, with intestinal-type being more prevalent. Members of the cell-surface Frizzled (Fzd) receptor family are deregulated or overexpressed in several cancer types, including GC [4]. Wnts are lipid-modified glycoproteins that initiate signal transduction by binding to Fzd via a palmitate group, which is appended by the palmitoyltransferase Porcupine (PORCN) [5, 6]. Wnts also bind cell surface co-receptors, such as Lrp5/6, forming a ternary complex [7]. Formation of the Wnt-receptor complex leads to inhibition of a multiprotein ‘destruction complex’ comprised of Axin, glycogen synthase kinase-3 (GSK3), calcium kinase-1 (CK1) and adenomatous polyposis coli (APC), which targets β-catenin for proteosomal degradation. Newly synthesised cytoplasmic β-catenin can now escape degradation, accumulate and translocate to the nucleus, where it forms a transcriptionally active complex with T-cell factor (TCF)/lymphoid enhancing factor (LEF) family of transcription factors to induce Wnt target gene transcription [8]. However, deregulated Wnt signaling can initiate cell transformation and subsequent carcinogenesis [8].

Furthermore, several Wnt/Fzd antagonists [9] are epigenetically silenced through promoter hypermethylation, including DKK3 (67.6% of gastric tumors [10]), sFRP1 (91%), sFRP2 (96%), sFRP5 (65%) [11], whilst others such as the E3 ligase RNF43, which regulates Fzd turnover on the cell surface [12], are mutated in 54% and 4.8% of microsatellite instable (MSI) and microsatellite stable (MSS) gastric tumors, respectively [13]. Exogenous re-introduction of sFRP or DKK can significantly reduce gastric tumor burden in APC or β-catenin-mutant gastric cancer cells by attenuating Wnt signaling [11, 14]. Critically, this provides proof-of-principle that modulation of ligand/receptor signaling components can further regulate Wnt signaling irrespective of downstream mutations that constitutively activate the pathway, which has been reported in colorectal cancer cells [15-18]. Together, these data strongly implicate a role for Wnt/Fzd in GC which could be exploited for targeted therapy.

We recently demonstrated that Frizzled-7 (Fzd7) regulates stem cell function in the gastric and intestinal epithelium [19, 20]. In addition, FZD7 is abundantly expressed in human gastric cancer tissue [21-23], which is also associated with poor patient outcome [24]. Despite compelling
evidence implicating Fzd receptors in GC, there has been no formal investigation of the therapeutic benefit of targeting Fzd receptors in GC in vivo. These types of in vivo studies are crucial to fully understand the potential of novel therapeutic strategies due to the complex cellular and molecular interactions of a tumor, which can directly inform clinical trials and cannot be replicated in vitro. Our results demonstrate that Fzd receptors, specifically Fzd7, are rate-limiting for the growth of gastric adenomas with or without Apc mutations in vivo. These findings have significant clinical utility as targeted Fzd therapeutics (OMP-18R5/Vantictumab), currently being tested in other solid cancer types (http://www.oncomed.com/Pipeline), can now be extended to GC patients with and without APC mutations.

Materials and Methods

Mice
The Tff1CreERT2[25], Fzd7fl/fl[20], Apc580 (Apcfl/fl)[26], c-Mycfl/fl[27], Rosa26LacZ[28] and gp130F/F[29] are previously described. Mice were interbred to generate compound mice with appropriate alleles on an inbred C57Bl/6 genetic background. Mice were co-housed using appropriate littermates as controls. All animal experiments were approved by the Animal Ethics Committee, Office for Research Ethics and Integrity, University of Melbourne.

Treatments
In vivo Cre induction was performed in 8-10 week old mice with a single daily intraperitoneal (ip) injection of 2mg of tamoxifen/mouse/day over four consecutive days. gp130F/F mice aged 8-9 weeks were injected ip with 20mg/kg of OMP-18R5 (OncoMed) or vehicle control (2.5%DMSO+IgG) twice weekly over the course of 30 days at which point animals were sacrificed and tissues harvested.

Tumor xenografts
A total of 4x10^6 cells in 100µl of PBS were injected subcutaneously into the hind flank of 6-8 week old nude mice (nu(ncr)-foxn1 nu/nu). 7 mice were used for each cohort which were treated with 20mg/kg OMP-18R5 or vehicle control (2.5%DMSO+IgG) once tumors were palpable, five days following injection of cells. Xenografts were measured with calipers twice a week to monitor tumor growth.
Tissue collection and histological analysis
Mouse stomachs were isolated, flushed with PBS, fixed overnight at 4°C in 10% neutral buffered formalin (NBF) and processed for immunohistochemistry and immunofluorescence as we previously described [20, 30, 31], with antibodies used on Table S2.

Isolation and culture of normal and tumor organoids
Organoids were cultured from mouse stomachs as previously described [31]. Adenomas from gp130F/F mice were isolated from the stomach, washed in PBS, roughly minced and incubated in digestion buffer (Dispase I (125µg/ml), Collagenase IV (75U/ml) and DMEM+2.5% FCS) at 37°C until epithelial fragments dissociate from tumor bulk. Dissociated cells were passed through a 70µM cell strainer, counted, centrifuged and resuspended in Matrigel. In vitro Cre recombinase was activated by treating gastric organoid cultures with 100nM 4-hydroxytamoxifen (4-OHT) as previously described [31]. R-Spondin and Wnt conditioned medium were withdrawn from Tff1Cre;Apcfl/fl organoid cultures following 4-OHT treatment. Differential interference contrast (DIC) images were captured as Z-sections and final image generated as previously described [20, 32].

RNA extraction and analysis
Gastric glands were homogenized in TRizol and total RNA purified, DNase treated, quantified and subjected to quantitative reverse transcriptase PCR (qRT-PCR). qRT-PCR and calculating gene expression levels relative to the house-keeping gene 18S (2-∆∆CT) were performed as previously described [16].

MTT assay
Following treatment, gastric organoids were mechanically dissociated, washed with ADF, resuspended in fresh Matrigel and seeded in a flat bottom 96 well tissue culture plate for enumeration using the MTT assay performed exactly as we previously described [19, 20].

Cell culture and transfection
Human gastric cancer cell lines (MKN28, MKN74, MKN7, MKN1, AGS and MKN45) were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum (FCS) (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) and L-Glutamine (Invitrogen) and were not taken past passage 15 for experimental use. All cells were tested for Mycoplasma, authenticated and cultured at 37°C in 5% CO₂. Gastric cancer cells were transfected with Short-
hairpin RNA (shRNA) and expression constructs designed to knockdown and stably express FZD7 respectively, as previously described [16, 33] or MSCV-MYC from Addgene (18119).

**Soft agar colony assay**
Cells were cultured in 60mm tissue culture dishes until 50% confluency and transfected with 5µg of plasmid DNA using Lipofectamine LTX (Invitrogen) following manufacturer’s instructions. After 48hrs incubation, cells were washed in PBS, detached using trypsin, resuspended RPMI+10%FCS, counted and mixed with pre-warmed 1% agar/RPMI culture medium to a final concentration of 500 cells/well of a 6-well plate. Once agar/cell suspensions solidified, cultures were overlaid with RPMI+10% FCS culture medium and incubated at 37°C in 5% CO₂ for 14 days. For Wnt inhibition experiments, cells were treated with OMP-18R5 (10µg/ml), IWP-2 (10µM) [34] or vehicle control (2.5%DMSO+IgG) 3 days after plating. Treatments were removed and replaced every 4 days over the 2 weeks. Cells were fixed in 4%PFA and stained with crystal violet and colonies consisting of ≥50 cells scored and imaged.

**Genomic recombination PCR**
Conventional PCR to detect the Fzd7 and Apc mutant alleles following recombination in genomic DNA extracted from compound transgenic mice was performed as previously described [20, 35]. See also supplementary experimental procedures.

**Luciferase assay**
Cells were cultured in 24-well tissue culture plates until 50% confluency and transfected with a total of 1µg plasmid DNA/well (500ng of SuperTOPflash or SuperFOPflash TCF reporter plasmids expressing firefly luciferase [36], plus 500ng of either “control” or “treatment” DNA, plus 2ng of renilla luciferase plasmid to normalize transfection efficiency). Cells were transfected using Lipofectamine LTX with Plus reagent (Invitrogen) according to manufacturer’s instructions. Cells were harvested 48hr later and analysed using the dual luciferase reporter assay system (Promega). Ratio of luciferase/renilla reporter activity was calculated and results expressed relative to control cultures.

**Analysis of gastric adenocarcinoma genomic dataset**
Analysis of somatic mutations and copy number alterations (CNA) for a panel of 21 Wnt pathway genes was performed on the TCGA stomach adenocarcinoma dataset [37] using the cBioPortal.
platform [38]. Only samples with sequencing and CNA data were assessed across all molecular subtypes, n = 287.

**Statistical analysis**

Data are expressed as mean ± SEM, where mean represents number of mice (≥ 3 per genotype) or number of independent experiments (≥3). Statistical tests used are Mann-Whitney with Prism7 (GraphPad software) where P values of ≤ 0.05 were considered significant. Heatmap generated in R version 3.0.2 using the heatmap function in the stats base package. Raw Ct values were transformed to delta Ct values using β2M as housekeeping gene.

**Results**

**Gastric cancer cells require cell intrinsic Wnt signaling for growth**

Gastric cancer, like many malignancies, is genetically heterogeneous, which complicates identifying non-redundant signaling pathways suitable for targeted therapy. To investigate the expression of Fzd receptors, which transmit oncogenic Wnt signals, we performed qRT-PCR for all 10 mammalian Fzd genes on a panel of human GC cell lines. Several Fzd receptors were abundantly expressed, including FZD7 (Figs. 1A, B and Supplementary Fig. S1A-D), suggesting these might be attractive therapeutic targets. Although the pan-Fzd antibody, OMP-18R5 (Vanticumab) has shown efficacy in several solid cancer types [39], its therapeutic potential for GC has not been explored. MKN28 (APC mutant), MKN74 (APC mutant) and MKN45 (APC wild-type) GC cells treated with OMP-18R5 formed significantly fewer anchorage-independent colonies compared to vehicle control treated cells (Figs. 1C, D and Supplementary Fig. S1E). Of note, MKN28 and MKN45 cells grown as conventional 2D monolayers do not show growth inhibition following OMP-18R5 treatment (Supplementary Fig.1F and G), which highlights the importance of testing drug efficacy in conditions that better mimic tumor biology. This suggests that cell intrinsic Wnt ligands are required for the 3D-growth of GC cells, which we confirmed by treatment with IWP-2, which prevents Wnt secretion [40] (Figs. 1C and D). TOPflash assays and qRT-PCR demonstrate that either IWP-2 or OMP-18R5 treatment inhibit Wnt signaling in GC cells (Figs. 1E-H). These data demonstrate cell intrinsic secretion of Wnt ligands and Fzd receptor availability are required for the sustained growth of GC cells. To determine whether Fzd regulates the growth of established gastric tumors, MKN28 and MKN45 cells were subcutaneously injected into the hind flanks of nude mice and allowed to develop into palpable gastric tumors. Compared to vehicle control treated gastric tumor xenografts, OMP-18R5 treated mice had significantly smaller gastric tumors (Supplementary Figs. 2A-D), which demonstrates Fzd inhibition is sufficient.
to block the initiation (Figs 1C and D) and progression (Supplementary Figs. 2A-D) of human gastric cancer cells.

**Inhibiting Fzd receptors limits gastric tumorigenesis in vivo**

We next utilised the well-characterised *gp130<sup>F/F</sup>* mouse-model of intestinal-type gastric tumorigenesis [29, 41], which develop prominent antral lesions with adenomatous hyperplasia to explore the relative expression of Fzd receptors. Compared to normal gastric epithelium, upregulation of several Fzds was observed in *gp130<sup>F/F</sup>* gastric adenomas (Figs. 2A-C), supporting expression levels observed in human GC cells (Figs. 1A, B, and Supplementary Figs. S1A-D).

Expression of Wnt ligands and target genes are also increased in *gp130<sup>F/F</sup>* gastric adenomas compared to non-adenoma gastric epithelium (Figs. 2A-C, Table S1). To determine if Fzd inhibition could also reduce the growth of antral gastric adenomas *in vivo*, we treated 8-week-old *gp130<sup>F/F</sup>* mice, which at this age have small antral gastric adenomas (Supplementary Fig. S2E), with OMP-18R5 twice a week for 30 days, following published protocols (Supplementary Fig. S3A) [39]. Gastric adenomas were significantly smaller and fewer in OMP-18R5-treated *gp130<sup>F/F</sup>* mice compared to vehicle control treated mice (Figs. 2D-F), which was associated with a significant reduction in the expression of Wnt target genes and cell proliferation (PCNA IHC) (Figs. 2G-J). As previously reported [39], no toxicity was observed in OMP-18R5-treated mice, which displayed consistent bodyweight, no signs of morbidity and no reduction in proliferation of normal non-adenoma gastric epithelial cells for the duration of treatment (Supplementary Figs. S3B-D). These data strongly suggest Fzd receptors are rate-limiting for the growth of gastric adenomas *in vivo*, and in human GC cells *in vitro*. Given that Wnts and Fzds can be expressed by non-epithelial cells, we established gastric organoids from *gp130<sup>F/F</sup>* antral adenomas using defined culture conditions to determine if the anti-growth effects observed in *gp130<sup>F/F</sup>* mice following OMP-18R5 treatment was systemic or cell intrinsic. *gp130<sup>F/F</sup>* gastric adenoma organoids treated with OMP-18R5 or IWP-2 displayed reduced viability (MTT assay) and growth compared to vehicle control treated organoids (Figs. 2K-M). This data confirms that Wnt ligands and Fzd receptors are required cell intrinsically for the growth of gastric adenoma cells *ex vivo*.

**Targeted FZD7 knockdown reduces gastric cancer colony formation**

Inhibition of cell growth following OMP-18R5 treatment suggest that one of several Fzds targeted by OMP-18R5 (FZD1, 2, 5, 7 and 8) is responsible for transmitting Wnt signals to GC cells. Gene expression analysis narrows this down to *FZD2* and/or *FZD7*, as *FZD1, FZD5* and *FZD8* are undetectable in these cell lines (Figs. 1A and B). We have previously shown that Fzd2 is unable...
to compensate for the loss of Fzd7 in the intestinal epithelium [20], which may indicate Fzd7 plays a predominant role in Wnt signal transmission in gastric tissue. Indeed, FZD7 is commonly upregulated in a variety of different cancer types, including gastric cancer, which is associated with poor clinical outcome [24, 42]. To determine the specific requirement of FZD7 for the growth of human GC cells we performed colony formation assays. Cells transfected with FZD7-targeted shRNA (shFZD7) [16] had a marked decrease in colony growth, compared to scrambled shRNA (shSCRAM) or empty vector (EV) controls (Figs. 3A and B), associated with decreased Wnt signaling (Figs. 3C and D). These data suggest that Fzd7 is the predominant Wnt receptor transmitting oncogenic Wnt signaling in GC cells. Importantly, growth inhibition following FZD7-knockdown was rescued by co-transfection with a full-length FZD7 expression construct [33], demonstrating the specificity of the shRNA and FZD7-regulated growth in human GC cells (Supplementary Figs. S4A and B).

Conditional deletion of Fzd7 from gp130^{F/F} gastric tumors reduces cell proliferation
To determine the functional requirement of Fzd7 for gastric adenoma growth in vivo, we conditionally deleted Fzd7 in the gastric adenomas of 8-week old Tff1Cre^{ERT2/+};gp130^{F/F};Fzd7^{fl/fl} mice (Cre^{+};gp130^{F/F};Fzd7^{fl/fl}) (Supplementary Fig. S4C), which allows robust recombination in these adenomas [25]. Tamoxifen injected Cre^{+};gp130^{F/F};Fzd7^{fl/fl} mice developed significantly smaller and fewer antral gastric adenomas than their Cre-negative (Cre^{-};gp130^{F/F};Fzd7^{fl/fl}) tamoxifen-treated littermates (Figs. 3E-G and Supplementary Fig. S4D), supporting our previous in vitro experiments demonstrating FZD7 inhibition is sufficient to block gastric adenoma growth (Figs. 3A-D).

Fzd7 deficient cells are retained in gastric tumors and fail to proliferate
The growth of gp130^{F/F} gastric adenomas requires Stat3 [43]. Therefore we performed p-Stat3 IHC and Socs3 qRT-PCR which identified no alterations in Stat3 activity, and did not cause the reduced growth of gastric adenomas in Cre^{+};gp130^{F/F};Fzd7^{fl/fl} mice (Figs. 4A and B). This identifies that Fzd7-mediated Wnt signaling is rate-limiting for Stat3-driven gastric adenomas, which have no Wnt-activating mutations. Deletion of Fzd7 in normal, non-transformed gastric epithelium causes repopulation with Fzd7-proficient cells [19]. To monitor if repopulation occurs in Cre^{+};gp130^{F/F};Fzd7^{fl/fl} adenomas, we performed PCR for the recombined Fzd7 floxed allele (Fzd7^{Δ}), which we have previously shown is lost during repopulation in the normal gastric epithelium following Fzd7 deletion [19]. However, in gastric adenomas of Cre^{+};gp130^{F/F};Fzd7^{fl/fl} mice 30 days post tamoxifen, we detect robust recombination of the Fzd7^{Δ} allele, demonstrating
that Fzd7 deleted cells are retained in these adenomas (Fig. 4C). In support, the expression of Fzd7 and many Wnt pathway components and target genes remain low in these adenomas (Fig. 4D, Supplementary Fig. S4E and Table S1). This suggests that the mechanism underlying smaller gastric adenomas following Fzd7 deletion is due to retention of Fzd7-deficient cells in the adenoma that are unable to respond to proliferative Wnt signals, and thus fail to proliferate (Fig. 4E). To investigate this further, we performed IHC on serial sections to detect recombined (β-gal+, Fzd7 deleted) cells and proliferating cells (PCNA+) in Cre⁺;gp130⁺Fr/Fz,f,Fzd7fl/fl;LacZ mice and observed a marked co-localisation of non-proliferative (PCNA⁻) cells with recombined cells (β-gal⁺) (Fig. 4F).

To monitor cellular changes following Fzd7 deletion in Cre⁺;gp130⁺Fr/Fz,f,Fzd7fl/fl mice, IHC for apoptosis (Caspase-3) and differentiation (Muc5a and Gastrin) was performed (Supplementary Fig. S4F). Muc5a⁺ and Gastrin⁺ cells were increased following Fzd7 deletion in Cre⁺;gp130⁺Fr/Fz,f,Fzd7fl/fl mice compared to Fzd7-proficient gastric adenomas (Cre⁺;gp130⁺Fr/Fz,f,Fzd7fl/fl). This also suggests that gastric adenomas do not repopulate following Fzd7 deletion, as repopulation in the normal gastric epithelium following Fzd7 deletion is associated with reduced cell differentiation [19]. No change in the frequency of Caspase-3⁺ cells was observed (Supplementary Fig. S4D), indicating that deletion of Fzd7 from adenoma cells does not trigger apoptosis.

**Cell intrinsic Wnt signaling via Fzd7 is required for Wnt-driven gastric adenomas**

The gp130⁺Fr mice and MKN45 GC cells are wild-type for APC, and have no known Wnt-activating mutations, suggesting that targeting Fzd7 may be effective in gastric adenomas and GC cells without mutations to the Wnt pathway. However, some of the GC cell lines that responded to Fzd therapy (MKN28 and MKN74) have mutant APC (https://portals.broadinstitute.org/ccle), suggesting that Fzd therapies can be effective in gastric adenomas with and without mutant APC.

In silico analysis of GC patient datasets identify mutations in several genes that regulate Wnt signaling, demonstrating that this pathway is aberrantly activated in GC (Supplementary Fig. S5A). To functionally investigate this, gastric organoids established from Tff1 CreERT2/⁺;Apcfl/fl (Cre⁺;Apcfl/fl) mice were treated with tamoxifen, to truncate Apc, and showed significant increase in growth and proliferation (Fig. 5A), which was confirmed by Ki-67 staining and increased cell viability (MTT assay) (Figs. 5A-C). A concordant increase in Wnt target gene expression was observed in hyperproliferative Apc mutant organoids (Fig. 5D). Treatment of Apc mutant organoids with IWP-2 or OMP-18R5 prevented upregulation of the Wnt pathway and blocked
organoid proliferation (Figs. 5A-D), demonstrating that cell intrinsic Wnt secretion and Fzd receptors are required for gastric cells to activate Wnt signaling and regulate growth, even after mutation of Apc (Figs. 5A-D).

Fzd7 expression was increased in Apc mutant gastric organoids and subsequently downregulated in IWP-2 or OMP-18R5 treated organoids (Fig. 5E), therefore we examined whether Fzd7 is responsible for transmitting Wnt signaling in Apc mutant gastric adenoma cells in vivo. 30 days following tamoxifen, Cre⁺;Apc⁺/+;Fzd7⁺/+ mice developed multiple, large intestinal-type gastric adenomas with extensive hyperplasia in the antral stomach (Figs. 6A and B), which were not observed in tamoxifen-treated Cre⁺;Apc⁺/-;Fzd7⁺/+ mice (Fig. 6A). Remarkably, co-recombination of Apc and Fzd7 alleles in Cre⁺;Apc⁺/-;Fzd7⁺/+ mice inhibited the ability of Apc mutant cells to develop antral adenomas (Figs. 6A and B). Gastric adenomas of Cre⁺;Apc⁺/-;Fzd7⁺/+ mice had significantly less PCNA⁺ cells compared to Cre⁺;Apc⁺/+; mouse (Figs. 6A and C). In common with gp130⁺/+ tumours, deletion of Fzd7 in Apc deficient gastric adenomas also results in retention of Fzd7-deficient cells as monitored by expression of the Fzd7Δ allele (Fig. 6D).

As expected, Wnt signaling is increased in gastric adenomas of Cre⁺;Apc⁺/-; mice, however, Wnt signaling is not elevated in the non-adenoma antral epithelium of Cre⁺;Apc⁺/-;Fzd7⁺/+ mice (Fig. 6E). This is supported by IHC for the surrogate markers of active Wnt signaling, β-catenin and Myc (Supplementary Fig. S6A). IHC revealed a decrease in Muc5a⁺ and Gastrin⁺ cells following Apc mutation (Supplementary Fig. S6B), while tamoxifen-treated Cre⁺;Apc⁺/-;Fzd7⁺/+ mice display a modest restoration of mucus-secreting and gastrin-producing cells, similar to that observed in Cre⁺;gp130⁺/+;Fzd7⁺/+ mice (Supplementary Fig. S4D). Collectively, these data demonstrate that Apc-mutant gastric phenotypes require functional Fzd7.

Fzd7-dependant Myc expression is required for the growth of gastric adenomas.

The transcription factor c-Myc is a well-characterised β-catenin/TCF target gene in the gastrointestinal tract as c-Myc is required for all intestinal tumor phenotypes following Apc-mediated activation of Wnt signaling [35]. Myc is upregulated in our gastric adenoma mouse models and human GC cell lines, and inhibition of Fzd7 prevents this upregulation (Figs. 2H, 3C, 4D, 5D and 6E). Conditional deletion of c-Myc in Tff1CreERT2/+;Apc⁺/-;cre⁺/+;Myc⁺/+ (Cre⁺;Apc⁺/-;Myc⁺/+ mice showed complete absence of antral adenoma formation and Wnt activation compared to Cre⁺;Apc⁺/+ mice (Supplementary Fig. S7), indicating Fzd7-dependant expression of Myc is required for the growth of Apc mutant gastric adenomas.
To determine whether elevated levels of MYC can rescue GC cell growth suppression following FZD7 knockdown, GC cells were co-transfected with FZD7shRNA and MSCV-MYC expression plasmids and grown as colonies in soft agar for 2 weeks. Compared to control (EV) transfected cells, co-transfected cells (FZD7shRNA and MSCV-MYC) showed no difference in the number of colonies formed (Supplementary Fig. S7G-I), which suggests that overexpression of MYC is able to rescue the growth suppressive effects of FZD7 knockdown in GC cells.

**Discussion**

Expression of Fzd receptors is deregulated in several cancers, including gastric cancer [4, 21, 42]. Here we show for the first time that Fzd receptors are rate-limiting for the growth of gastric adenomas *in vivo*. We further elucidate that Fzd7 is the predominant Wnt receptor transmitting cell-intrinsic Wnt signals in human GC cells.

*In vitro* studies have shown that targeted inhibition of Fzd is sufficient to block growth of GC cells [24, 44]. However, it is well documented that *in vitro* studies do not fully recapitulate the complex cellular and molecular interactions present in tumors [45]. Here, we demonstrate that gastric adenomas require Fzd7 for optimal growth using genetic and pharmacological strategies in two independent mouse models. Our findings support our previous work [39] demonstrating that targeting multiple Fzd receptors blocks the growth of several different cancers, which we now extend to GC. Using *ex-vivo* adenoma-derived organoids we demonstrate these anti-growth effects are cell intrinsic as OMP-18R5 blocks the growth of gastric adenoma-derived organoids in the absence of immune or stromal cells.

As previously observed in the normal gastric epithelium [19], genetic inhibition of Fzd7 in gastric adenomas induces upregulation of other Fzd genes (Table S1), however, these are insufficient to compensate and promote gastric adenoma growth. This suggests that specific targeting of Fzd7 is an attractive therapeutic strategy for the treatment of gastric cancer.

Deletion of Fzd7 in the normal gastric epithelium triggers repopulation [19] which could be a possible explanation for why Fzd7-deficient gastric adenomas are smaller. Epithelial repopulation is an effective tissue mechanism that helps the gastric epithelium to survive the harsh conditions of the stomach. Here we show that repopulation is not preserved in gastric adenomas, which contain aberrant cell signaling and tissue architecture, and therefore Fzd7-deficient cells remain in the adenoma but are unable to respond to Wnt signals and thus do not proliferate.
One feature of inflammation-associated tumors in the gastrointestinal tract is phosphorylated Stat3 (p-Stat3), which regulates many cancer hallmarks [43]. Gastric adenomas in gp130FF mice do not harbor any Wnt-activating mutations [41], however, they display high levels of Wnt signaling. Stat-3 has been shown to activate Wnt signaling, which would allow pathway activation in the absence of Wnt mutations in gp130FF adenomas [46, 47]. Indeed, Wnt and gp130/Stat3 signaling operate in parallel during gastric tumorigenesis as active p-Stat3 levels remain high in Fzd7 deleted adenomas, demonstrating that Wnt/Fzd7 signaling is rate-limiting for Stat3-driven gastric adenomas. Similarly, mTORC1 signaling is also rate-limiting for gp130FF adenoma growth independent of Stat3 [41].

Recent large-scale sequencing of human gastric tumors has identified environmental and genetic factors associated with increased pathology, which include aberrant Wnt signaling [48-50]. Importantly, these genomic studies are yet to be validated with functional interrogation in vivo, which are essential to understand the therapeutic potential of targeting Wnt signaling in gastric cancer [21]. We and others have demonstrated that Fzd7 inhibition is sufficient to block Wnt signaling in cells with mutant APC [17, 51]. Interestingly, ~37% of APC mutant gastric tumors are mutant for RNF43 (regulates Fzd on the cell surface [12]), demonstrating that Fzd is deregulated in a subset of APC mutant gastric tumors (http://www.cbioportal.org/). Interestingly RNF43 and APC mutations are mutually exclusive in colon tumors suggesting that CRC and GC cells preferentially select different Wnt mutations that confer optimal or ‘just-right’ levels of Wnt signaling required for tumor growth [52, 53].

Furthermore, we have shown that Myc is required for the gastric adenoma phenotypes associated with Apc mutation. These findings are reminiscent of the role played by Myc in the intestinal epithelium following Apc mutation [35], and thus place the Wnt/Fzd7/Myc signaling axis as an attractive therapeutic target for gastric cancer. Encouragingly, next generation bromodomain (BET) inhibitors are effective in killing patient-derived GC cells [54]. Importantly, this provides justification for testing a combination of BET and Wnt inhibitors in GC, which we have previously shown is effective at blocking the growth of human colon cancer cells [55].

New generation PORCN inhibitors are in clinical trials for solid tumors, which our results show may be effective in gastric cancer, however these target the secretion of all Wnt ligands. Collectively, we demonstrate that targeted inhibition of Wnt receptors, specifically Fzd7, is rate-limiting for the growth of gastric adenomas with and without Apc mutations. This provides a broad
scope for the application of this therapeutic strategy for the treatment of GC, with potentially less side effects than targeting all Wnt secretion with PORCN inhibitors, and will directly inform clinical trials to treat GC patients with OMP-18R5 (Vantictumab), which only targets 5 out of the 10 Fzd family.

Acknowledgments: We thank Damian Neate and Jean Moselen for technical assistance. We also thank John Mariadason, Cameron Nowell, Christine Wells, Trevelyan Menheniott, Matthias Ernst, Stefen Glaser and Andy Giraud for gifting cell lines, plasmids, mutant mice, microscopy assistance and critical discussion.

References


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Figure Legends

Figure 1. Inhibition of Wnt or Fzd blocks gastric cancer cell growth.
A. qRT-PCR for FZD gene expression in MKN28 gastric cancer cells. Expression shown relative to housekeeper (β2M), n=4 biological replicates.
B. qRT-PCR for FZD gene expression in MKN74 gastric cancer cells. Expression shown relative to housekeeper (β2M), n=4 biological replicates.
C. Quantification of cell colonies (>50 cells) from MKN28 gastric cancer cells grown in agar for 2 weeks following treatment with vehicle control (2.5%DMSO+IgG), IWP-2 (10µM) or OMP-18R5 (10µg/ml). Treatments were replaced every 4 days for the duration of 2 weeks. Individual experiments were repeated three times. Colonies were counted with ImageJ (*= p<0.05, mean ±SEM, Mann-Whitney).
D. Quantification of cell colonies (>50 cells) from MKN74 gastric cancer cells grown in agar for 2 weeks following treatment with vehicle control (2.5%DMSO+IgG), IWP-2 (10µM) or OMP-18R5 (10µg/ml). Treatments were replaced every 4 days for the duration of 2 weeks. Individual experiments were repeated three times. Colonies were counted with ImageJ (*= p<0.05, mean ±SEM, Mann-Whitney).
E. TOPflash assay on MKN28 cells treated 24hrs with DMSO, IWP-2 (10µM) or OMP-18R5 (10µg/ml) (**= p<0.005, mean ±SEM, n=9 biological replicates, Mann-Whitney). Individual experiments were repeated three times.
F. TOPflash assay on MKN74 cells treated 24hrs with DMSO, IWP-2 (10µM) or OMP-18R5 (10µg/ml) (**= p<0.005, mean ±SEM, n=9 biological replicates, Mann-Whitney). Individual experiments were repeated three times.
G. qRT-PCR for CD44 in MKN28 and MKN74 cells described in E and F (mean ±SEM, n=6 biological replicates, Mann-Whitney). Individual experiments were repeated twice.
H. qRT-PCR for AXIN2 in MKN28 and MKN74 cells described in E and F (mean ±SEM, n=6 biological replicates, Mann-Whitney). Individual experiments were repeated twice.

Figure 2. Inhibition of Fzd receptors reduces cell intrinsic Wnt signaling and gastric adenoma burden.
A. qRT-PCR for Wnt ligands in gp130F/F adenomas compared to normal gastric epithelium (*= p<0.05, mean ±SEM, n=4 mice, Mann-Whitney).
B. qRT-PCR for Fzd receptors in gp130F/F adenomas compared to normal gastric epithelium (*= p<0.05, mean ±SEM, n=4 mice, Mann-Whitney).
C. qRT-PCR for Wnt target genes in gp130F/F adenomas compared to normal gastric epithelium (*= p<0.05, mean ±SEM, n=4 mice, Mann-Whitney).
D. Whole mount images of 8-9 week old gp130F/F mice treated with control IgG or OMP-18R5 over the course of 30 days and harvested. Black and white arrows show gastric tumors.
E. Weights of gastric adenomas from mice described in D (**= p<0.005, mean ±SEM, n=9 mice, Mann-Whitney).
F. Quantification of gastric adenomas in mice described in D (**= p<0.005, mean ±SEM, n=9 mice, Mann-Whitney).
G. qRT-PCR for Fzd receptors in mice described in D (**= p<0.005, mean ±SEM, n=9 mice, Mann-Whitney).
H. qRT-PCR for Wnt target genes in mice described in D (**= p<0.005, mean ±SEM, n=9 mice, Mann-Whitney).
I. Immunohistochemistry for PCNA on adenomas sections from mice described in D. Scale bars = 100µm.

J. Quantification of PCNA+ cells from adenomas sections described in I (*= p<0.05, mean ±SEM, n=4 mice, Mann-Whitney).

K. Representative DIC images of gp130F/F adenoma-derived organoids treated with vehicle control (2.5%DMSO+IgG), IWP-2 (10µM) or OMP-18R5 (10µg/ml) and cultured for 5 days. Green arrows indicate viable organoids. Red arrows indicate dying/atrophic organoids. Scale bar = 200 µm

L. MTT viability assay performed on organoids described in K (*= p<0.05, mean ±SEM, n=3 biological replicates, Mann-Whitney).

M. Measurement (diameter) of organoids described in K. Measurements were quantified in ImageJ (*= p<0.05, mean ±SEM, n=3 biological replicates, Mann-Whitney).

Figure 3. Targeted inhibition of Fzd7 reduces gastric cancer clonogenicity and adenoma burden.

A. Representative DIC images of MKN28 and MKN74 cells transfected with empty vector (EV), scrambled (shSCRAM) or FZD7-specific shRNA (FZD7shRNA) and grown in agar. Scale bars = 200µm

B. Quantification of cell colonies from experiment described in A (*= p<0.05, mean ±SEM, n=3 biological replicates, Mann-Whitney). Individual experiments were repeated twice.

C. qRT-PCR for Wnt target genes on MKN28 and MKN74 cells transfected with empty vector (EV), scrambled (shSCRAM) or FZD7-specific shRNA (Fzd7shRNA) (*= p<0.05, mean ±SEM, n=3 biological replicates, Mann-Whitney).

D. TOPflash assay on MKN28 and MKN74 cells described in C (***= p<0.001, mean ±SEM, n=9 biological replicates, Mann-Whitney). Individual experiments were repeated three times.

E. Representative images of tamoxifen-treated Tff1CreERT2- (Cre−) or Tff1CreERT2+ (Cre+) stomachs following Fzd7 deletion. Black arrows indicate gastric tumors.

F. Weights of gastric adenomas per mouse described in E (***= p<0.005, mean ±SEM, n=7 mice, Mann-Whitney).

G. Quantification of gastric adenomas per mouse described in E (***= p<0.005, mean ±SEM, n=7 mice, Mann-Whitney).

Figure 4. Deletion of Fzd7 from gastric tumors decreases cell proliferation.

A. Immunohistochemistry (IHC) for p-Stat3 on adenoma sections from Fzd7fl/fl;gp130F/F mice (Cre or Cre+) 30 days after tamoxifen treatment. Scale bars = 100µm.

B. qRT-PCR for Socs3 on gastric adenomas from mice described in A (*= p<0.05, mean ±SEM, n=4 mice, Mann-Whitney).

C. Conventional PCR to detect recombination of Fzd7fl/fl allele (Fzd7Δ) in gastric adenomas from mice described in A.

D. qRT-PCR for Wnt target genes in gastric adenomas from mice described in A (***= p<0.005, mean ±SEM, n=7 mice, Mann-Whitney).

E. Quantification of PCNA+ cells from adenoma sections described in A (*= p<0.05, mean ±SEM, n=3 mice, Mann-Whitney).

F. Representative IHC images for β-galactosidase (detecting allelic recombination) and PCNA (proliferation) on serial sections from Tff1Cre−;Fzd7fl/fl;gp130F/F;LacZ or Tff1Cre+;Fzd7fl/fl;gp130F/F;LacZ mice 30 days following tamoxifen. Note, yellow dashed
lines demarcate areas of allelic recombination, which correspond to reduced proliferation and black dashed lines represent areas of non-recombined cells. Scale bars = 100µm.

Figure 5. Wnt/Fzd inhibition reduces Apc mutant gastric organoid proliferation.
A. Representative DIC and immunofluorescence images of Tff1Cre;Apc^{fl/fl} organoids treated for 24hrs with tamoxifen (tmx, 100nM), IWP-2 (10µM) or OMP-18R5 (10µg/ml). Green arrows indicate hyperproliferative organoids. Red arrows indicate growth-constrained organoids. Scale bars = 200µm.
B. MTT viability assay performed on organoid cultures described in A (**= p<0.001, mean ±SEM, n=3 biological replicates, Mann-Whitney). Individual experiments were repeated twice.
C. Measurement of organoid size (µm) from cultures described in A (**= p<0.001, mean ±SEM, n=3 biological replicates, Mann-Whitney).
D. qRT-PCR for Wnt target genes on organoid cultures described in A (*= p<0.05, mean ±SEM, n=3 biological replicates, Mann-Whitney).
E. qRT-PCR for Fzd receptors on organoid cultures described in A. Expression of Fzd shown as Log₂ ratio.

Figure 6. Deletion of Fzd7 rescues gastric adenoma formation following Apc truncation.
A. Representative whole mount and IHC (PCNA) on wild-type (Tff1Cre;Apc^{fl/fl}), Apc mutant (Tff1Cre⁺;Apc^{fl/fl}), and Apc/Fzd7 mutant mice (Tff1Cre⁺;Apc^{fl/fl};Fzd7^{fl/fl}) 30 days following tamoxifen. Black arrows indicate gastric adenomas in top panels. Scale bars = 100µm.
B. Weights of gastric adenomas from harvested mice described in A (**= p<0.005, mean ±SEM, n=7 mice, Mann-Whitney).
C. Quantification of PCNA⁺ cells in adenoma sections from mice described in A (***= p<0.001, mean ±SEM, n=3 mice, Mann-Whitney).
D. Conventional PCR for recombined Fzd7 (Fzd7Δ) and Apc (ApcΔ) alleles in mice described in A.
E. qRT-PCR for Wnt target genes on tamoxifen-treated mice described in A (**= p<0.005, mean ±SEM, n=4 mice, Mann-Whitney).