The Function of Lgr5+ Cells in the Gastric Antrum Does Not Require Fzd7 or Myc In Vivo

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Abstract: The extreme chemical and mechanical forces endured by the gastrointestinal tract drive a constant renewal of the epithelial lining. Stem cells of the intestine and stomach, marked by the cell surface receptor Lgr5, preserve the cellular status-quo of their respective tissues through receipt and integration of multiple cues from the surrounding niche. Wnt signalling is a critical niche component for gastrointestinal stem cells and we have previously shown that the Wnt receptor, Frizzled-7 (Fzd7), is required for gastric homeostasis and the function of Lgr5+ intestinal stem cells. Additionally, we have previously shown a requirement for the Wnt target gene Myc in intestinal homeostasis, regeneration and tumourigenesis. However, it is unknown whether Fzd7 or Myc have conserved functions in gastric Lgr5+ stem cells. Here we show that gastric Lgr5+ stem cells do not require Fzd7 or Myc and are able to maintain epithelial homeostasis, highlighting key differences in the way Wnt regulates homeostasis and Lgr5+ stem cells in the stomach compared to the intestinal epithelium. Furthermore, deletion of Myc throughout the epithelium of the gastric antrum has no deleterious effects suggesting therapeutic targeting of Myc in gastric cancer patients will be well tolerated by the surrounding normal tissue.

Keywords: Wnt; gastric stem cells; Frizzled-7; Fzd7; Myc; Lgr5

1. Introduction

The epithelium of the gastrointestinal tract encounters substantial chemical and physical stresses. One of the mechanisms that has evolved to help cope with these harsh conditions is the continuous turnover of the epithelium, in which new cells are generated from populations of stem cells. These new cells then differentiate and migrate towards the lumen and are eventually sloughed off so that any damaged cells are not retained, and new healthy cells are being produced constantly to replace them. Lgr5 was first identified as a Wnt target gene and a marker of highly proliferative stem cells located at the base of the intestinal crypts that fuel the constant turnover of cells [1]. It has since been confirmed as a stem cell marker in several epithelial tissues including the stomach [2,3], hair follicle [4], ovary [5], mammary gland [6] and kidney [7].
Lgr5 is a receptor for secreted Wnt agonist R-spondins, which acts to modify the strength of Wnt signalling in cells, including those of the intestinal crypt [8]. Wnt signalling regulates several cell functions including proliferation, migration, apoptosis and differentiation, and is critical during embryonic development, and the homeostasis of several tissues including the intestine, stomach, liver and mammary gland [9]. Wnts are a family of 19 glycoproteins that are modified during the secretion process by an O-acyltransferase called Porcupine, which can then bind to Frizzled receptors of which there are 10 in mammals [10]. The fatty acid modification of Wnt ligands is critical for their bind to the U-shape of Frizzled dimers, which also associate with other co-receptors, including Lrp5/6 to form a signalosome with cytoplasmic Dishevelled. This signalosome then inhibits the action of a multimeric degradation complex which allows the co-transcription factor β-catenin to escape degradation and translocate into the nucleus and associate with TCF/Lef transcription factors to regulate target genes [11].

In the intestine, Wnt signalling is most active at the base of the crypts, which contain the Lgr5+ stem cells and Paneth cells [1], with Wnt3a secreted from the Paneth cells [12], and Wnt2b from the underlying stroma [13]. We recently demonstrated that the deletion of Fzd7 in Lgr5+ intestinal epithelial cells was deleterious and triggered rapid repopulation with Fzd7 proficient cells, indicating its requirement for this population of stem cells [14]. Similarly, the deletion of Fzd7 throughout the antrum of the gastric epithelium also triggered repopulation, indicating that Fzd7 regulates a population of stem cells in the antrum [15]. Furthermore, Fzd7 was highly expressed in gastric tumours, and transmitted Wnt signalling to upregulate the transcription factor Myc, to promote tumour initiation and growth [16]. This is consistent with the role of Myc in the intestinal epithelium in which it is required for homeostasis [17], regeneration [18] and tumorigenesis [19].

As Lgr5 marks stem cells in several tissues it is important to understand how these populations of cells are regulated, and therefore here we asked if Fzd7 and Myc regulate Lgr5+ cells in the gastric antrum.

2. Materials and Methods

2.1. Mice

The Tff1CreERT2 [20], Fzd7fl/fl [14], c-Mycfl/fl [21], Rosa26LacZ [22] and Lgr5CreERT2 [1] mice 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 (1513488, approved on 18th May 2015).

2.2. Treatments

For short-term labelling, mice received a single daily intraperitoneal (IP) injection of 2 mg of tamoxifen. For long-term (>14 days) labelling, mice received single IP injections of 2 mg tamoxifen over three consecutive days.

2.3. 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, as previously described [14,23,24]. List of antibodies used available upon request.

2.4. β-Galactosidase (X-gal) Staining

Mouse stomachs were prepared and stained for X-gal as previously described [15].

2.5. Gland Isolation, Cell Dissociation and Organoid Culture

Antral stomachs isolated from experimental mice were prepared for organoid culture as previously described [24]. Antral glands isolated for flow cytometry were prepared, as previously described [3].
2.6. Genomic Recombination PCR

Conventional PCR to detect the Fzd7 and c-Myc mutant alleles following recombination in genomic DNA extracted from compound transgenic mice was performed, as previously described [14,19].

2.7. RNA Extraction and Analysis

Whole antral glands and FACS-isolated single antral cells 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 \(2^{-\Delta \Delta C_t}\) were performed as previously described [25].

2.8. 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 [14,15].

2.9. Statistical Analysis

Data are expressed as mean ± SEM, where the mean represents the number of mice (≥3 per genotype) or number of independent experiments (≥3). Statistical tests used were two-way ANOVA with Prism7 (GraphPad software) where \(p\) values of ≤0.05 were considered significant.

3. Results

To determine the requirement for Fzd7 in antral Lgr5\(^+\) cells we deleted Fzd7 specifically in Lgr5\(^+\) cells using Lgr5Cre\(^{ERT2}\), Fzd7\(^{fl/fl}\), LacZ\(^{LSL}\) mice and performed lineage tracing via X-gal staining. At 3 days post tamoxifen induction recombined cells of Fzd7 proficient Lgr5Cre\(^{ERT2}\), Fzd7\(^{+/+}\), LacZ\(^{LSL}\) mice can be seen at the base of the antral crypts where the Lgr5\(^+\) cells are located, and 30 days after tamoxifen many of the crypts are composed entirely of lineage traced cells indicating the variegated Lgr5 locus gives rise to entire gastric units in the antrum as previously reported [2] (Figure 1A). Surprisingly, lineage tracing also proceeded in the Fzd7 deficient Lgr5Cre\(^{ERT2}\), Fzd7\(^{fl/fl}\), LacZ\(^{LSL}\) mice indicating that Fzd7 loss is not deleterious to Lgr5\(^+\) cells in the gastric antrum, and these stem cells can function without Fzd7. To confirm robust deletion of Fzd7 we performed PCR for the recombined product which gave a very strong band 3 and 30 days after tamoxifen induction in Lgr5Cre\(^{ERT2}\), Fzd7\(^{fl/fl}\), LacZ\(^{LSL}\) mice, whilst in Lgr5Cre\(^{ERT2}\), Fzd7\(^{+/+}\), LacZ\(^{LSL}\) mice the recombined product was undetectable as expected (Figure 1B). The Lgr5Cre\(^{ERT2}\) locus also has an EGFP cassette and therefore we performed immunohistochemistry (IHC) for GFP which demonstrated no difference in the number of Lgr5\(^+\) cells in the gastric antrum after Fzd7 deletion (Figure 1C,D). Together these data demonstrate that, in contrast to the intestinal epithelium, Fzd7 is not required for the activity of Lgr5\(^+\) stem cells in the gastric antral epithelium. Indeed, RT-qPCR for Fzd genes in cells FACS sorted for high GFP expression from the antrum epithelium of Lgr5Cre\(^{EGFP-ERT2}\) mice revealed that Fzd7 expression was undetectable (Figure 1E), thus supporting our in vivo observations that Lgr5\(^+\) cells in the gastric antrum are regulated differently from those in the intestinal epithelium, and do not require Fzd7.
Figure 1. Conditional deletion of Fzd7 from Lgr5+ gastric stem cells does not perturb homeostasis. (A) X-gal stained tissue sections from Lgr5Cre+; LacZ and Lgr5Cre+; Fzd7fl/fl; LacZ mice 3 and 30 days following tamoxifen. Scale bars = 100 µm. (B) Conventional PCR for recombined Fzd7(Fzd7Δ) alleles in mice described in (A). (C) Representative IHC staining for GFP, which permits detection of Lgr5GFP+ stem cells, on sections from mice described in A at 30 days post tamoxifen. Scale bars = 100 µm. (D) Quantification of GFP+ cells in sections from mice described in A (mean ± SEM, n = 3 mice, at least 30 glands scored/mouse, Unpaired student t-test). (E) qPCR for Fzd receptors in FACS-isolated GFP+ (Lgr5hi) cells. Expression was normalized to Lgr5lo cells.

We have previously shown that deletion of Myc in the intestinal epithelium phenocopies deletion of Fzd7 and results in rapid repopulation with Myc proficient cells [17]. Furthermore, we recently demonstrated that Wnt regulates Myc expression, via Fzd7, to control tumour initiation and growth in the stomach [16], regeneration in the intestine [14] and homeostasis in the stomach [15]. To investigate
if Lgr5+ cells in the antrum require Myc, we deleted Myc in Lgr5+ cells in vivo. Surprisingly, and similar to deletion of Fzd7, fully lineage traced gastric units were observed in the antrum of Lgr5CreERT2; Mycfl/fl; LacZLSL mice 30 days after tamoxifen induction (Figure 2A), with no difference in the number of Lgr5+ cells between Lgr5CreERT2; Mycfl/fl and Lgr5CreERT2; Myc+/+ mice (Figure 2B,C). These data demonstrate that Lgr5+ antral cells do not require Myc for their stem cell activity. However, there are several stem cell populations identified in the antrum based on expression of distinct maker genes including Lrig1 [26], Sox2 [27] and CCK2R [28] and therefore Myc could be required for activity of one of these populations of stem cells which would have been missed in the analysis of our Lgr5Cre mice.

Figure 2. Cont.
Together these data demonstrate that the epithelium of the gastric antrum does not require Myc epithelium can function in the absence of Myc deletion has occurred in at least one population of antral stem cells as previously published [15]. Remarkably, deletion of Myc does not adversely affect gastric organoids [15].

To investigate if Myc is required generically in the antral epithelium we deleted Myc from the gastric epithelium does not trigger epithelial repopulation. X-gal stained tissue sections from Lgr5Cre; LacZ and Lgr5Cre; Mycfl/fl; LacZ mice 3 and 30 days following tamoxifen. Scale bars = 100 µm. (B) qRT-PCR for c-Myc on gastric epithelial cells isolated from Lgr5Cre; Myc+/+ and Lgr5Cre; Mycfl/fl mice following tamoxifen at 3 and 30 days post tamoxifen as indicated (* p < 0.05, mean ± SEM, n = 3 mice, Two-way ANOVA). (C) Representative IHC for GFP, which permits detection of Lgr5GFP+ stem cells, on sections from mice described in A. Scale bars = 100 µm. (D) Quantification of GFP+ cells in sections from mice described in A (mean ±SEM, n = 3 mice, at least 30 glands scored/mouse, unpaired student t-test). (E) X-gal stained tissue sections from Tff1Cre+; LacZ and Tff1Cre+; Mycfl/fl; LacZ mice 3 and 30 days following tamoxifen. Scale bars = 100 µm. (F) qRT-PCR for c-Myc on gastric epithelial cells isolated from Tff1Cre+; Mycfl/fl and Tff1Cre+; Mycfl/fl mice following tamoxifen (* p < 0.05, mean ± SEM, n = 3 mice, two-way ANOVA). (G) Conventional PCR for recombed Myc (Mycflox) alleles in mice described in (E). (H) Representative DIC images of gastric organoids derived from Tff1Cre+; Mycfl/fl mice 5 days after treatment with vehicle (EtOH) or tamoxifen (4-OHT). Scale bars = 100 µm. (I) MTT viability assay performed on organoid cultures described in (G) (mean ±SEM, n = 3 biological replicates, unpaired student t-test). Individual experiments were repeated twice. (J) Conventional PCR for recombed Myc (Mycflox) alleles on organoid cultures described in (H).
4. Discussion

Fzd7 is required for intestinal stem cell activity during homeostasis, regeneration and survival of cultured organoids [29]. Here we show for the first time that deletion of Fzd7 does not inhibit the capacity of Lgr5+ cells to lineage trace full gastric units in the antral epithelium illustrating a substantial difference for Fzd7 in regulating Lgr5+ stem cells in the intestine compared to the antrum. We also show that the number of Lgr5+ cells is maintained in the Fzd7 deficient antrum at similar levels to that of Fzd7 proficient mice, demonstrating that lineage tracing is not due to a small population of Lgr5+ cells that were resistant to Fzd7 deletion, but rather that Fzd7 deletion has not affected Lgr5+ survival and activity. We also observe that other Fzd genes are expressed in Lgr5+/+ cells in the antrum, with Fzd3 and Fzd4 the highest, suggesting one of these may be transmitting Wnt signalling in antral cells. Interestingly, the expression pattern for Fzd3 seems to be consistent with that of Lgr5 in the gastric antrum whilst Fzd4 is expressed broadly throughout the gastric units [30]. However, Fzd3 is significantly upregulated when Fzd7 is deleted in gastric antrum organoids, but this increased expression is unable to compensate for the loss of Fzd7 in vitro and Fzd7-deficient organoids undergo apoptosis [15]. Thus, the exact Fzd receptors required for antral Lgr5+ cell activity will require additional functional studies in the future including analysis of Wnt pathway activity. We have previously shown that the deletion of Fzd7 throughout the epithelium of the gastric antrum is deleterious and triggers rapid repopulation [15]. This demonstrates that Fzd7 is required for at least one population of stem cells in the gastric antrum, but this population has yet to be identified, and our data here illustrate it is not Lgr5+ antral stem cells. A possible candidate for this population is Axin2+/Lgr5- cells which are located in a similar location as Fzd7 expressing cells in the lower half of the antral glands [30]. Furthermore, the Axin2+/Lgr5- population in the antrum expands in response to Helicobacter pylori infection via upregulation of Rspo3 in the underlying myofibroblasts [30], whilst inhibition of Fzd7 can suppress H. pylori-induced Wnt signalling and proliferation [31]. These data suggest Fzd7 may be regulating Axin2+/Lgr5- cells in the antrum, although this is yet to be confirmed.

We have previously shown that Myc is required for all the tumourigenic phenotypes following truncation of Apc in the intestine [19], and also for homeostasis [17], regeneration [18] and apoptosis [32] in this tissue. Furthermore, we recently demonstrated that tumour initiation and growth in the gastric antrum requires Fzd7 dependant upregulation of Myc [16]. Myc is upregulated in many cancers and thus represents a potential target for therapy which has been the subject of intense research for several years. However, Myc is not required in all the adult tissues it is expressed in, for example, both liver zonation and Apc loss induced hepatomegaly are regulated by Wnt signalling via Myc independent mechanisms [33,34]. Given its differential requirement between different tissues it is important to gain a full insight into how the loss of Myc affects separate tissues. To our surprise, Myc deletion did not inhibit stem cell activity in Lgr5+ stem cells of the gastric antrum or when genetically deleted throughout the entire epithelium of the gastric antrum. Furthermore, organoids cultured from the antrum were able to thrive and showed no difference in viability when Myc was deleted compared to Myc proficient organoids. These data demonstrate that Myc is not only dispensable for antral Lgr5+ stem cells, but the entire antral epithelium.

These data identify a distinct difference in the way Wnt regulates homeostasis and stem cells in the gastric antrum compared to the intestinal epithelium. As Myc is required for gastric tumour growth, it also informs future clinical trials with putative Myc inhibitors that the gastric epithelium can tolerate the loss of Myc. Future investigations into the differences between the intestinal and gastric epithelium could help uncover why the intestine is more sensitive to deregulated Wnt, and explore if Wnt inhibitors elicit a different response from these two tissues thus identifying new oncogenes/tumour suppressors in both cancer types.

Intestinal-type gastric tumours undergo metaplasia to become more intestinal-like, with upregulation of intestinal specific genes and morphological features including tubular structures. This could explain why gastric adenomas are sensitive to deletion of Myc, whilst the normal gastric epithelium is not, as the adenoma has acquired features of the intestinal epithelium which is sensitive
to Myc loss. However, the exact molecular mechanism behind this observation has yet to be identified and requires further comparative experiments to delineate the role of Wnt/Fzd/Myc in both the normal and transformed tissue of the stomach and intestine.

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