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## Alteration of colonic stem cell gene signatures during the regenerative response to injury

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### Abstract

Since aberrant wound healing and chronic inflammation can promote malignant transformation, we determined whether dietary bioactive fish oil (FO)-derived n-3 polyunsaturated fatty acids (n-3 PUFA) modulate stem cell kinetics in a colitis-wounding model. *Lgr5*-LacZ and *Lgr5*-EGFP-IRES-creER<sup>T2</sup> mice were fed diets enriched with n-3 PUFA vs n-6 PUFA (control) and exposed to dextran sodium sulfate (DSS) for 5 days in order to induce crypt damage and colitis throughout the colon. Stem cell number, cell proliferation, apoptosis, expression of stem cell (*Lgr5*, *Sox9*, *Bmi1*, *Hopx*, *mTert*, *Ascl2*, and *DCAMKL-1*) and inflammation (*STAT3*) markers were quantified. DSS treatment resulted in the ablation of *Lgr5*<sup>+</sup> stem cells in the distal colon, concurrent with the loss of distal crypt structure and proliferating cells. *Lgr5*, *Ascl2* and *Hopx* mRNA expression levels were decreased in damaged colonic mucosa. *Lgr5*<sup>+</sup> stem cells reappeared at day 5 of DSS recovery, with normal levels attained by day 6 of recovery. There was no effect of diet on the recovery of stem cells. FO fed animals exhibited higher levels of phospho-STAT3 at all time points, consistent with a higher wounding by DSS in FO feeding. n-3 PUFA-fed mice exhibited a reduction in stem cell associated factors, *Ascl2*, *Axin2* and *EphB3*. These results indicate that rapidly cycling *Lgr5*<sup>+</sup> stem cells residing at position 1 in the colon epithelium are highly susceptible to DSS-induced damage and that dietary cues can impact stem cell regulatory networks.

### Keywords

*Lgr5*; dextran sodium sulfate; fish oil; Wnt signaling; colon

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Disclosures

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## 1. Introduction

“Adult” somatic stem cells of the colon are of particular interest because they sustain self-renewal and are target cells for cancer initiating mutations [1, 2]. Therefore, perturbations in stem cell dynamics are generally believed to represent the earliest step towards colon tumorigenesis. Exciting advances have been made in the identification of stem cells (approximately 6 per crypt), which replenish the intestinal epithelium every 3-5 days [3]. Although the exact identity of intestinal stem cells has proven controversial over the last 30 years, rapidly cycling crypt base columnar (CBC) *Lgr5*<sup>+</sup>/*GPR49* cells self-renew, actively divide, and give rise to all epithelial subtypes in the small intestine and colon [3-7]. Recent evidence indicates that intestinal crypt homeostasis results from neutral competition between symmetrically dividing *Lgr5*<sup>+</sup> stem cells [8]. By crossing stem-cell-specific *Lgr5*-EGFP-IRES-creER<sup>T2</sup> knockin mice to *Apc*<sup>flox/flox</sup> mice, it was unequivocally shown that crypt *Lgr5*<sup>+</sup> stem cells are the cells-of-origin of intestinal cancer [4]. Collectively, these findings lend support to the cancer stem cell concept, i.e., that in some circumstances, normal intestinal stem/progenitor cells can initiate colon tumorigenesis and drive cancer progression towards metastasis [9-11]. Emerging evidence indicates that both quiescent +4 cells and CBCs have cooperative functional roles, exhibiting bi-directional lineage relationships in the small intestine and to some degree in the colon [8, 12-16]. Hence, it is now possible for the first time to visualize stem cells and examine their behavior in the context of tissue remodeling and cancer chemoprevention.

There is substantial experimental, epidemiological and clinical evidence indicating that fish oil-containing diets rich in n-3 polyunsaturated fatty acids (PUFA), e.g. docosahexaenoic acid (DHA, 22:6<sup>Δ4,7,10,13,16,19</sup>) and eicosapentaenoic acid (EPA, 20:5<sup>Δ5,8,11,14,17</sup>), are protective against colon tumorigenesis [17-25]. In a major recent finding, it was demonstrated that EPA reduced rectal polyp number and size in patients with familial adenomatous polyposis (FAP) [25]. Most impressive was the fact that fish oil derived n-3 PUFA suppressed FAP to a degree similar to the selective COX-2 inhibitor celecoxib. Collectively, these data indicate that n-3 PUFA hold promise as chemoprevention agents for FAP and sporadic colon cancer. In addition, we have recently demonstrated that n-3 PUFA impact genes which modulate the colon stem cell niche and tumor evolution, e.g., *Wnt/TCF* signature, Ephrin B1, BMP-4, and PGE<sub>2</sub> [26, 27-29]. Despite the fact that aberrant wound healing/crypt regeneration can promote malignant transformation [27, 30,31], how the colonic stem cell population responds to environmental factors such as diet and chronic inflammation is not well understood. Therefore, we hypothesized that the chemoprotective effect of EPA and DHA may be attributed to a reduction in stem cell damage. Since little is known regarding homeostasis within the stem cell compartment in the large bowel, we examined the effect of dietary bioactive agents, i.e., EPA/DHA, on stem cell kinetics in a colitis-wounding model. These experiments are highly relevant because the effects of dietary cues in the context of DSS-induced damage on stem cell cytokinetics and regulatory networks in the large intestine have not been reported to date.

## 2. Materials and Methods

### 2.1 Animals and DSS treatment

All procedures involving animals followed guidelines approved by the Institutional Animal Care and Use Committee at Texas A&M University in accordance with EU Directive 2010/63/EU for animal experiments. *Lgr5*-EGFP-IRES-creER<sup>T2</sup> and *LacZ*-*Lgr5* mice, originally described by Barker et al [3, 5] (13-18 wk of age) were fed experimental diets for one wk prior to initiation of dextran sodium sulfate (DSS) treatment at 2.5% in drinking water, the only source of fluids during the treatment period. After 5 d of DSS treatment, plain water was provided during the DSS recovery period. Mice (n=3-5 per diet group and

time point) were terminated on days 1-7 of DSS recovery, with EdU injected 2 h prior to termination in order to assess cell proliferation as previously described [32]. Untreated control mice were provided with tap water for the entire study period.

## 2.2 Experimental diets

Diets varied only in type of lipid. Experimental diets consisted of (by weight): 20% casein, 42% sucrose, 22% corn starch, 6% cellulose, 3.5% AIN-76A salt mix, 1% AIN-76A vitamin mix, 0.3% DL-methionine, 0.2% choline chloride, and 5% fat. The corn oil (CO) diet contained 5% corn oil (Dyets, Bethlehem, PA) and the vacuum deodorized Menhaden fish oil (FO) diet contained 4% fish oil (Omega Protein, Houston, TX) plus 1% corn oil. All diet ingredients except oils were obtained from Bio-Serv (Frenchtown, NJ). Three fish oil fed/DSS treated mice died during the study and were replaced. Control animals (no DSS treatment) were also fed either the CO or FO diets.

## 2.3 Tissue samples

Upon termination, the colon was removed, cut open, cleaned in PBS, placed lumen side up and cut in half longitudinally. Each half was Swiss rolled, half being fixed in 4% PFA in PBS for 4 h at 4°C, embedded in paraffin and used for immunohistochemistry assays. The other half was fixed in 1% formaldehyde/0.2% glutaraldehyde/0.02% NP-40 in PBS for 2 h at 4°C for LacZ staining. On a separate set of mice (n=5) receiving the same diet and treatment regimen, colons were removed, cut open and cleaned in PBS, the mucosal layer was scraped with a microscope slide and total RNA was isolated using Ambion mirVana kit followed by DNase treatment with Ambion DNFree. RNA quality was assessed by Agilent Bioanalyzer and all samples had an RIN score greater than 9.0.

## 2.4 Immunohistochemistry

Cell proliferation was measured using the Click-IT EdU kit (Invitrogen, Carlsbad, CA) as per manufacturer's instructions on formalin fixed paraffin embedded (FFPE) sections. Apoptosis was assessed with the TACS 2 TdT TUNEL assay (Trevigen, Gaithersburg, MD) [33]. LacZ positive stem cells were detected with  $\beta$ -galactosidase staining as previously described [4]. Phospho-STAT3, Sox9 and DCAMKL1 were detected in FFPE sections after retrieval in 10 mM Tris base/1 mM EDTA (phospho-STAT3 and DCAMKL1) or 10 mM sodium citrate (Sox9) for 20 min in a sub-boiling bath. Primary antibodies were as follows: Phospho-STAT3 (Tyr705), Cell Signaling, 1:200 dilution; Sox9, Millipore, 1:1000 dilution; DCAMKL1, Abcam, 1:100 dilution. Prior to primary antibody incubation, Image-iT FX signal enhancer (Invitrogen) was used for phospho-STAT3 and Sox9 slides. Goat-anti-rabbit secondary antibody conjugated with Alexa-647 (Invitrogen) was used on all slides. For quantification of phospho-STAT3 expression, intensity of staining was measured using NIS Elements AR software on a Nikon Ti-E inverted microscope equipped with an X-cite 120 fluorescent microscopy illuminator using a 20X objective. Ten measurements were taken from each proximal or distal segment from each animal in order to determine average epithelial phospho-STAT3 expression.

## 2.5 Flow cytometry

Following tamoxifen induction, colonic crypts were isolated by the method of Sato et al [7] with minor modification. The intact colons were everted on a disposable mouse gavage needle (Instech Laboratories) and incubated with 20 mM EDTA in PBS at 37°C for 30 min. Following transfer to chilled Ca/Mg free HBSS, colons were vigorously vortexed to release crypts. The final crypt fraction was incubated with trypsin/DNase to produce a single cell suspension for flow cytometry of GFP positive cells using an Accuri C6 flow cytometer (BD Biosciences). In addition, for stem cell sorting, GFP<sup>high</sup> expressing stem cells and

GFP<sup>negative</sup> cells from the small intestine and colon were separately isolated using a BD FACSAria II cytometer/sorter (BD Biosciences).

## 2.6 Real Time PCR

Gene expression was quantified by TaqMan assay using an ABI 7900HT (Applied Biosystems) on control mice (no DSS treatment) and at 3 d of DSS recovery (n=5 per treatment and diet). Assays were tested for proportionality and efficiency prior to use and negative controls, omitting the reverse transcriptase enzyme, were performed for all genes. Gene expression was quantified for the following genes: *Ascl2*, *Axin2*, *Bmi1*, *Eph3*, *Hopx*, *Lgr5*, *mTert*, *Rspo1*, *Sox9*, *Stat3* and *18S* (for normalization). Because *Rspo1* was nearly undetectable, quantification was repeated on a Bio-Rad QX-100 Droplet Digital PCR system using the same TaqMan assay.

## 2.7 RNA Sequencing

RNA (80 ng) was converted to cDNA using the NuGEN Ovation 3'-DGE kit. Three micrograms of cDNA was then fragmented (Covaris). The fragmented sample was quantified with the Quant-IT Picogreen kit (Invitrogen) and evaluated for proper fragmentation of 150 to 200 bp on an Agilent Bioanalyzer DNA 1000 chip. Subsequently, 200 ng sheared double stranded cDNA was used for the Encore Library System I kit (NuGEN) to create the Illumina libraries as per manufacturer's instructions. Libraries were quantified by Quant-IT and run on an Agilent DNA Chip 1000 to confirm appropriate size and absence of adapter dimers. Samples were sequenced on an Illumina GAII with single end, 75 bp reads.

## 2.8 Statistics

Data analyses were performed using either the exact Wilcoxon two-sample test or one-way ANOVA with Tukey's adjustment for multiple comparisons using SAS 9.2.

## 3. Results

DSS treatment resulted in the ablation of LacZ positive-Lgr5<sup>+</sup> stem cells in the distal colon (**Figure 1A**), concurrent with the extensive loss of distal crypt structure. In contrast, the proximal colon was protected from DSS damage, maintaining ~4-6 stem cells per crypt after the first day of DSS recovery, similar to untreated control animals (**Figure 1A&B**). Similar results were observed using flow cytometric analysis of Lgr5 EGFP<sup>+</sup> colonic epithelial cells (**Figure 1C**). Stem cells in the distal colon, almost completely absent from days 1-4 of DSS recovery, reappeared at day 5, with higher than normal levels attained by days 6-7 of recovery. There was no difference in recovery of stem cells between the n-3 and n-6 PUFA groups (**Figure 1B**).

Since early alterations in cell proliferation and apoptosis occur following damage to the colonic epithelium [34], experiments were conducted to document the effects of DSS-induced wounding on crypt cell cytokinetics. The percentage of crypts with proliferating cells, i.e., crypts with EdU<sup>+</sup> cells, remained relatively constant at 50-70% in the proximal colon region during all 7 days of recovery, compared to 85% of crypts containing proliferating cells in control animals. In contrast, in the distal colon, 5-20% of the crypt area contained proliferating cells during days 1-2 of recovery, increasing to 60-70% by day 5 (**Figures 2A&B**). These data indicate that, in general, the epithelial cell cycle has been arrested. In complementary analyses, apoptosis was measured using the dUTP nick end-labeling (TUNEL) assay. DSS exposure elevated the apoptotic index equally in the proximal and distal colon (**Figure 3**). Overall, diet had no effect on crypt cell cytokinetics.

To better understand the impact of inflammation and dietary bioactive agents on the colonic epithelial progenitor niche during the regenerative response to colitis, we examined important regulators of intestinal homeostasis after induction of wounding injury. Phospho signal transducer and activator of transcription (pSTAT3) levels, a well-accepted marker of mucosal inflammation, wound healing and crypt cell survival [35, 36], were significantly elevated compared to untreated animals for days 1-5 of recovery, after which expression approached control levels (**Figure 4**). In general, STAT3 phosphorylation was localized primarily to damaged and regenerating crypts (**Supplemental Figure 1**). Fish oil fed animals exhibited higher levels of pSTAT3 compared to corn oil fed mice at all time points, consistent with a higher wounding by DSS in fish oil feeding [37] (**Figure 4**). Sox9, a marker of cell proliferation, self-renewal and repair in the intestine [38, 39], was strongly upregulated in recovering crypts, and was correlated to the regeneration of the damaged tissue and the reestablishment of Lgr5<sup>+</sup> stem cells in the distal colon (**Figure 5**). For comparative purposes, an additional putative stem cell marker (DCAMKL1) was assessed by IHC (**Supplemental Figure 2**). In general, DCAMKL1 expression was dispersed throughout the crypt and was not associated with damaged and regenerating crypts.

The continuous cell renewal of the intestinal epithelium is regulated by a number of homeostatic pathways, including Wnt signaling [6]. Therefore, initially in control animals (no DSS or n-3 PUFA exposure), we assessed the transcriptome profile of stem cells (GFP high population) as compared to GFP negative cells (non stem cell population) in the small intestine and colon using next generation sequencing (**Table 1**). Both small intestine and colon transcript GFP positive cells were enriched in genes associated with Wnt signaling (Lgr5, BMP4) [40], and stem cell renewal (EPHB3, HES1, SNAI2, SOX9) [38, 41-43]. Interestingly, the “master” intestinal transcription factor (CDX2) [44] was only enriched in colonic Lgr5 positive cells. We subsequently sought to determine the combined effect of wounding injury and an n-3 PUFA-enriched diet on the relative expression of Wnt signaling activators; R-spondin (Rspo-1); R-spondin receptor (Lgr5) [45]; Wnt signaling inhibitor, Axin2 [46] and intestinal stem cell associated factors, Achaete Scute-Like 2 (Ascl2), Ephrin type-B receptor 3 (EphB3) [6], mouse telomerase reverse transcriptase (mTERT) [47], and HOP homeobox (Hopx) [13]. At 3 days of recovery from DSS, a time when very few Lgr5<sup>+</sup> stem cells are detectable (**Figure 1B**), Lgr5 mRNA was significantly ( $P<0.05$ ) suppressed relative to control mice (no DSS treatment) (**Table 2**). Other stem cell markers, Ascl2 and Sox9, were similarly down regulated by DSS exposure. Interestingly, Axin2 was suppressed, consistent with an enhancement of Wnt signaling during the recovery phase. Consistent with the expression of pSTAT3 (**Figure 4**), STAT3 mRNA levels were also increased following DSS exposure.

Since dietary FO alters the levels of genes which modulate both intestinal inflammation [27, 48, 49] and the stem cell niche, e.g., Wnt/TCF signature, Ephrin B1, BMP4, and PGE<sub>2</sub> [8, 27-29], it was noteworthy that n-3 PUFA-fed mice exhibited a significant ( $P<0.05$ ) reduction of Ascl2, Axin2 and EphB3 expression (**Table 2**).

#### 4. Discussion

Recent findings lend support to the concept that in some circumstances, inflammation-induced wound healing/intestinal crypt regeneration can perturb homeostasis within the stem cell compartment and promote malignant transformation [10, 27, 30, 31]. Therefore, it is important to know precisely how intestinal stem cell populations respond to environmental factors in the context of inflammation. Since little is known regarding the homeostatic mechanisms within the stem cell compartment in the large intestine, in this study we used the *Lgr5-LacZ* and *Lgr5-EGFP-IRES-cre knockin* mouse models to examine the adult colon stem cell response to DSS exposure and a putative therapeutic diet (fish oil).



Exposure to DSS represents a wounding model that induces mucosal injury and subsequent inflammation via the recruitment and activation of inflammatory cells and mediators, ultimately leading to the development of colitis [50]. As expected, acute DSS exposure ablated colonic crypts in the distal colon (**Figure 1**) [51]. The simultaneous abrupt reduction in crypt DNA synthesis (**Figure 2**) and enhanced deletion of damaged cells (**Figure 3**) represents a classical manifestation of damage to the colonic epithelium [34, 52]. Since colon stem cell kinetics have not been reported in the context of a colitis/wounding model, in the current study we also investigated how the DSS model impacts the colonic stem cell compartment. Rapidly cycling  $Lgr5^{+}$  stem cells were also completely eliminated in the distal colon and returned by day 5 of recovery. The elimination of stem cells and their subsequent recovery in response to acute DSS exposure was confirmed by (i) the disappearance of  $Lgr5$ -GFP and LacZ-positive cells and (ii) the absence of EdU at the base of crypts at positions 1-4. These data indicate that the rapidly cycling CBC stem cell ( $Lgr5^{+}$ ) compartment located at the base of the colonic crypt is highly susceptible to damage-induced intestinal injury. It is possible that  $Lgr5$  gene expression is selectively turned off in response to damage, however, there is no evidence to date that this receptor is reversibly responsive to environmental insult.

Although it is not clear why the proximal colon is resistant to the effects of DSS, our findings extend a previous report indicating that following  $\gamma$ -irradiation,  $Lgr5$  expressing cells are eliminated [53]. Interestingly,  $Lgr5^{+}$  cells in the small intestine are not susceptible to Doxorubicin-induced cell death [54]. With regard to the reestablishment of  $Lgr5^{+}$  cells, it is possible that a small number of rapidly cycling stem cells survive insult in the crypt base and repopulate the crypts. There is some evidence indicating that the small intestine contains a quiescent stem cell population at 4 cells above the base of the crypt (+4 position), e.g., *Hopx*, *Bmi1*, *Lrig1*, and *mTert* expressing cells [13, 15, 47, 55]. These slowly cycling stem cells are capable of interconverting with more rapidly cycling  $Lgr5$  stem cells at the crypt base [13, 15]. Therefore, a putative intestinal model of stem cell renewal and crypt regeneration incorporates a bi-directional lineage relationship between active and quiescent stem cells in their niches. However, recent data imply that *Bmi1*, *Hopx*, *mTert* and *Lrig1* cannot be used as markers for a quiescent reserve stem cell population [56]. This small intestinal model is further complicated by the fact that during regenerative responses to injury, stromal stem cells, including activated macrophages [57, 58], as well as bone marrow derived cells [59], transmit regenerative signals to neighboring colonic epithelial progenitors. Since many of the experiments focusing on stem cell identity and location have focused on the small intestine, additional work is needed in order to confirm these relationships in the colon.

Examination of the  $Lgr5$  positive cell global transcriptome in the small intestine and colon revealed a significant enrichment in genes related to Wnt signaling and stemness (**Table 1**). These data are consistent with previous targeted qPCR analyses [60]. With respect to the DSS-induced colitis and crypt regeneration model, we showed that both  $Lgr5$  and *Hopx* mRNA expression levels were decreased in damaged colonic mucosa (**Table 2**). Other Wnt targeted stem/progenitor cell markers, *Ascl2* and *Sox9*, were also down regulated at the mRNA level by DSS exposure. This was unexpected given that Wnt signaling is activated during intestinal regeneration [6, 61], and may be attributed to the time point sampled (3 d DSS recovery). It was not surprising to see the inverse mRNA expression of *Axin2* (suppression of Wnt signaling inhibitor) and *Rspo-1* (enhancement of Wnt signaling activator) following DSS exposure. These two mediators reciprocally regulate Wnt signaling [45, 46].

To our knowledge, this is the first study to utilize  $Lgr5^{+}$  reporter mice to determine the impact of diet on intestinal stem cell survival and gene expression. Dietary fish oil,

containing long chain n-3 PUFA, has been shown to attenuate intestinal inflammation [27, 37, 48, 49], and impact genes which modulate mediators of the colon stem cell niche, e.g., Wnt/TCF signature, Ephrin B1, BMP-4, and PGE<sub>2</sub>, in animal models of intestinal inflammation/carcinogenesis [26, 27-29]. Therefore, we hypothesized that the protective effect of n-3 PUFA may be partially attributed to a reduction in stem cell damage. To our surprise, there was no difference in the recovery of stem cells between the n-3 and n-6 PUFA groups (**Figure 1B**), although there was a modest elevation in the number of stem cells at recovery days 6 and 7 in FO fed mice. Interestingly, STAT3 activation (**Figure 4**) was highest in DSS exposed, FO-fed mice. This is noteworthy, because STAT3 mediates immune homeostasis in the gut by promoting mucosal wound healing and stem cell survival [35, 36]. The enhancement of STAT3 by n-3 PUFA is generally consistent with a higher wounding by DSS. Indeed, we have unambiguously demonstrated that FO feeding disrupts mucosa repair during the acute phase of DSS exposure in part due to the inhibition of NF- $\kappa$ B activity in the colonic mucosa [37].

FO feeding modulated the expression of genes known to regulate the stem cell niche, e.g., *Ascl2*, *Axin2* and *Eph3* (**Table 2**). Furthermore, we have recently demonstrated that dietary FO modulates a subset of non-coding microRNAs (miR 19b, 26b, 200c and 203) and their target genes (mRNAs) implicated in the regulation of the colon stem cell niche and tumor evolution [29]. Collectively, these results suggest that select dietary cues can impact stem cell regulatory networks. Additional research is needed in order to elucidate the biological relevance of the FO-induced changes in genes which define intestinal stem cells.

The present study has defined the impact of wounding/inflammation and dietary n-3 PUFA on the colonic epithelial progenitor niche during the regenerative response to colitis. Since transformation of adult stem cells is an extremely efficient route towards initiating intestinal cancer [4], including the transition from colitis to cancer [62], it is imperative that we understand how environmental factors modulate colonic stem cell survival and self-renewal in response to chronic inflammation and carcinogen exposure.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

<b>CBC</b>	crypt base columnar cell
<b>CO</b>	corn oil
<b>FO</b>	fish oil
<b>DSS</b>	dextran sodium sulfate
<b>PUFA</b>	polyunsaturated fatty acids

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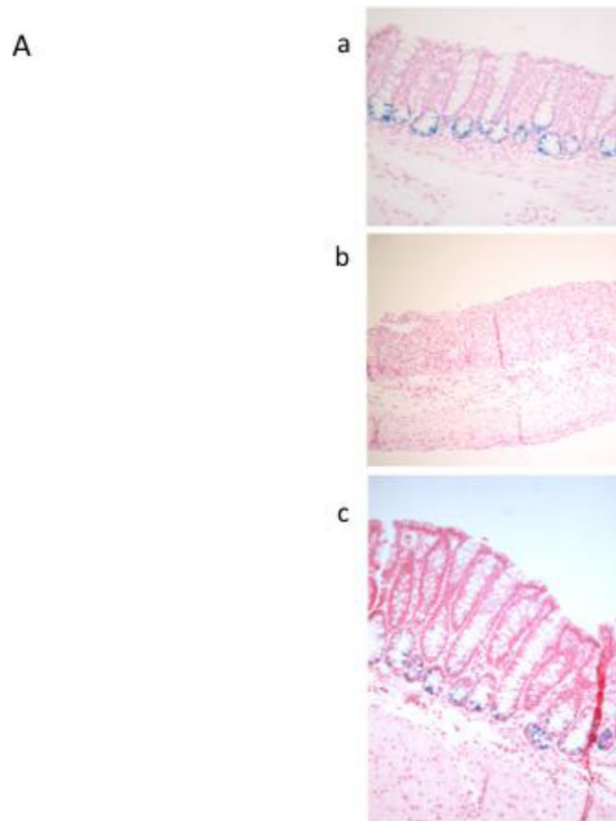
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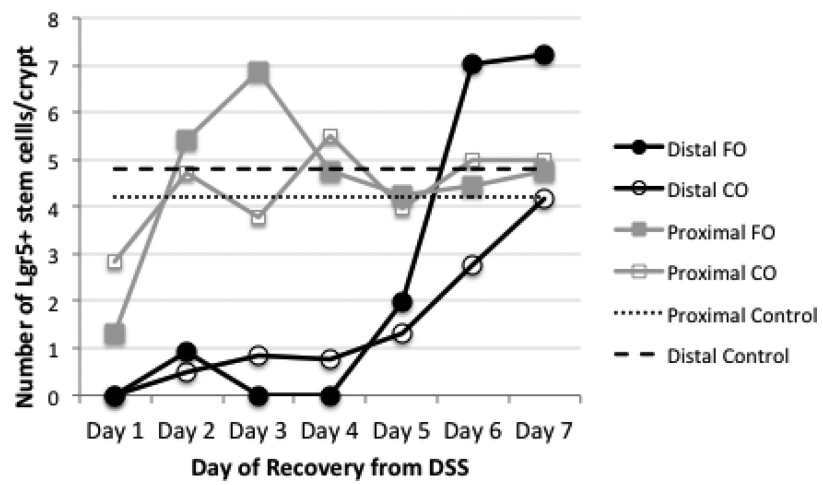
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### Highlights

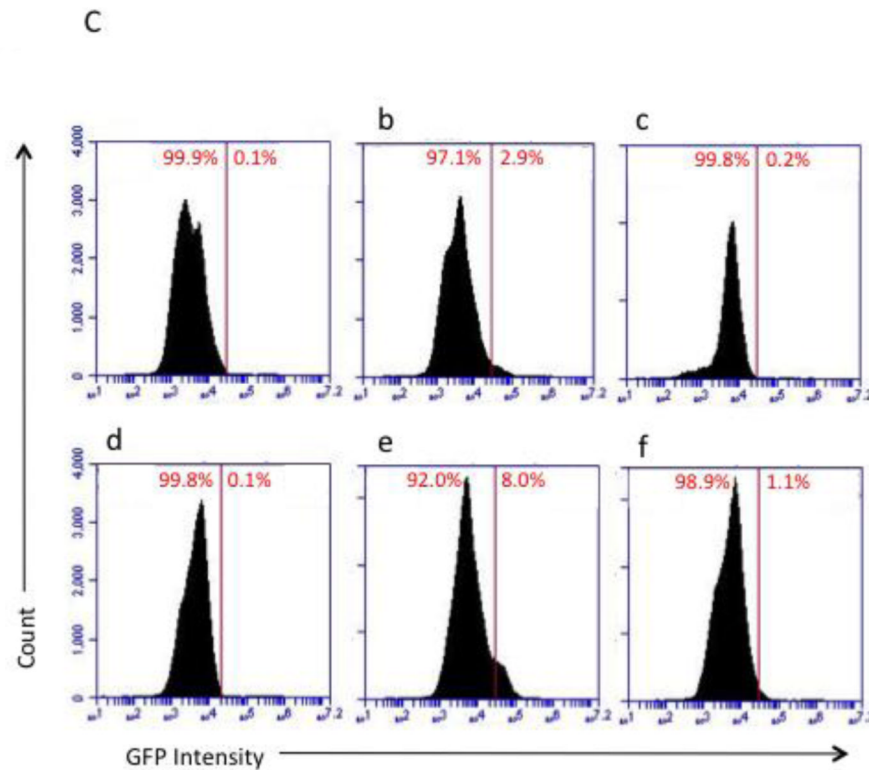
- Colon stem cell kinetics are altered in the colitis/wounding model
- Lgr5+ stem cells in the colon are highly susceptible to DSS-induced damage
- Diet modulates stem cell associated genes, Ascl2, Axin2 and Eph3



**B**

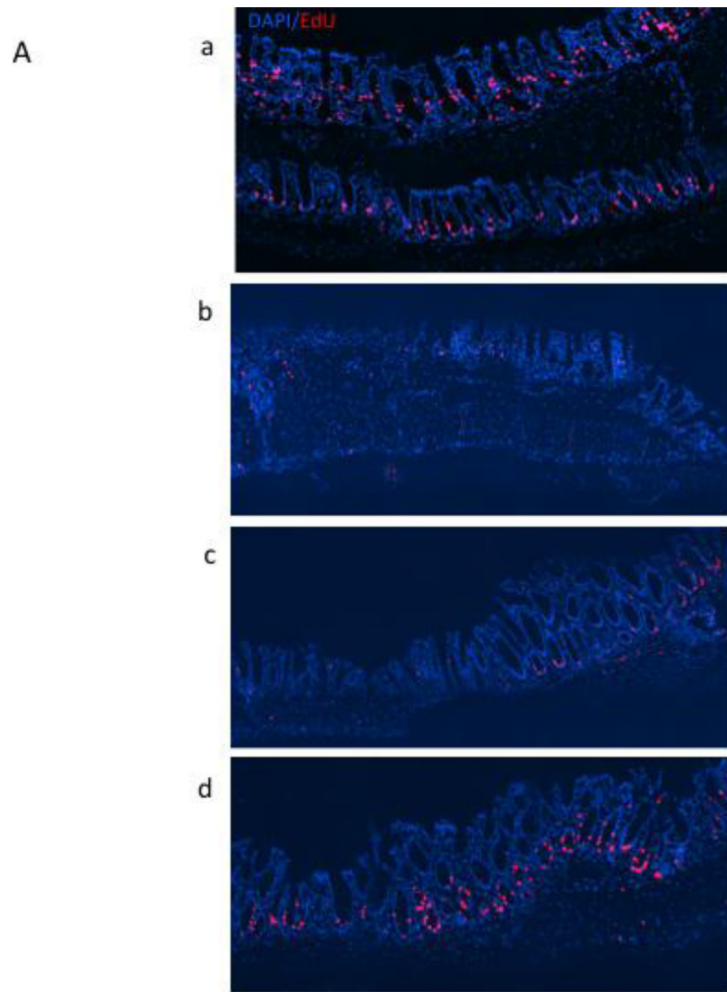




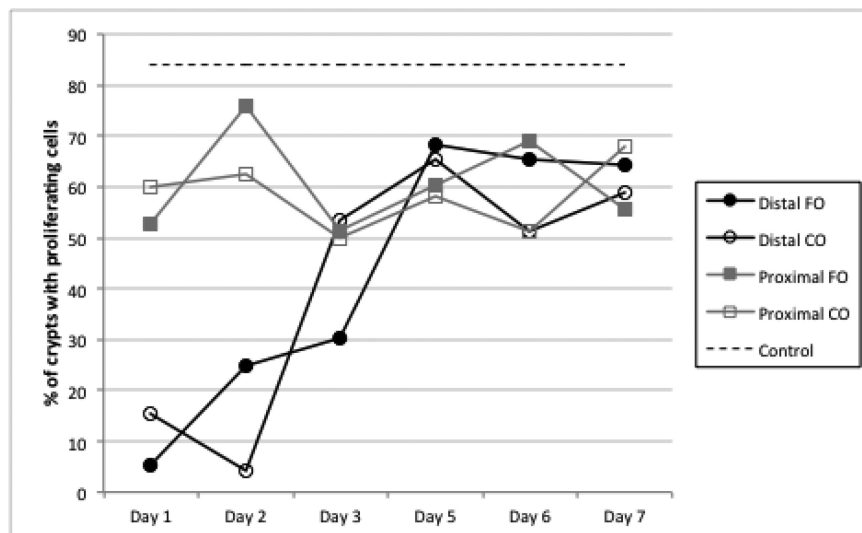


**Figure 1.**

Detection of Lgr5<sup>+</sup> stem cells during recovery from DSS treatment. Animals were treated with DSS for 5 d to induce colitis and terminated at daily intervals during the “recovery” phase. A, representative photomicrographs depicting  $\beta$ -galactosidase staining of Lgr5<sup>+</sup> stem cells in distal colon in (a) untreated control, (b) DSS treated, 2 d recovery, (c) DSS treated, 7 d recovery,  $\times 100$ -200. B, mean number of Lgr5<sup>+</sup> stem cells per crypt during DSS recovery (at least 20 crypts each from  $n=3$  mice per diet group and time point. However, during days 1-4 of recovery, many animals had no scoreable crypts in the distal colon.) There were no significant differences ( $P>0.05$ ) between CO and FO fed mice. C, representative flow cytometric analysis of Lgr5 GFP<sup>+</sup> colonic epithelial cells. a-c: distal colon; d-f: proximal colon. a and d are from wild type mice (no GFP) and were used for gating. b and e represent control animals displaying the expected levels of GFP<sup>+</sup> cells, c and f are from DSS treated mice, d 1 recovery. FO, fish oil fed; CO, corn oil fed mice.

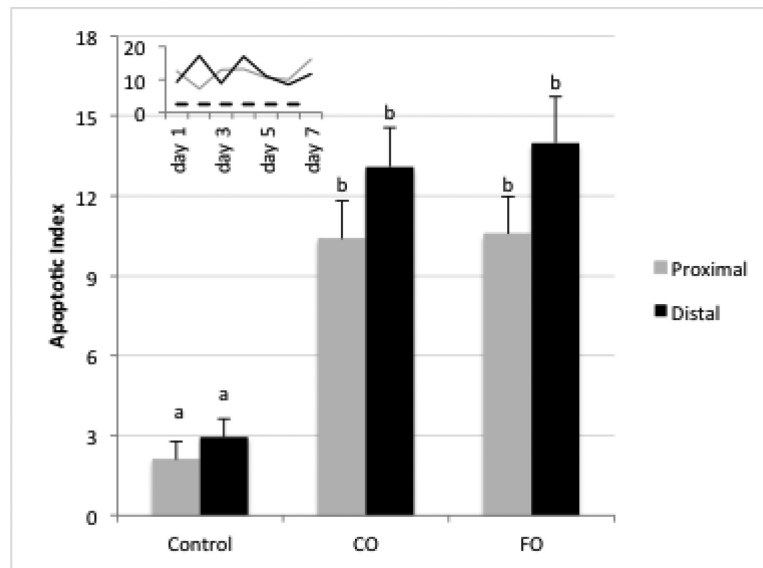


**B**

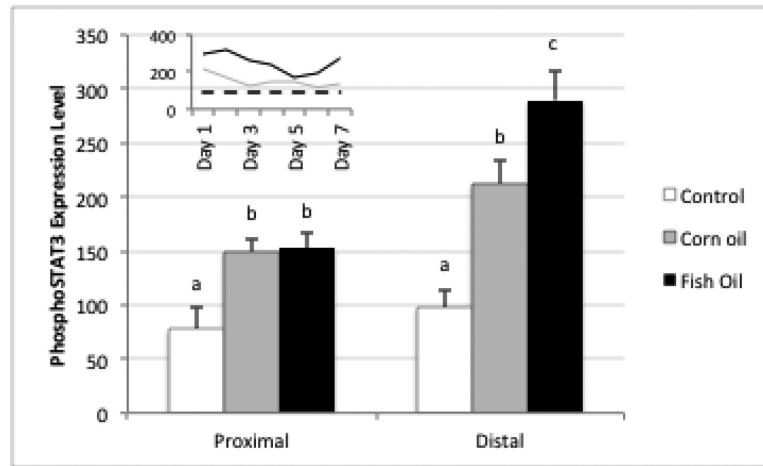


**Figure 2.**

Detection of proliferating (EdU<sup>+</sup>) cells following DSS exposure. A, representative photomicrographs ( $\times 100$ ) of distal colonic crypts stained for EdU (red) with all nuclei stained with DAPI (blue), (a) untreated control, (b) DSS treated, d 2 recovery, (c) DSS treated, d 3 recovery, (d) DSS treated d 7 recovery. B, mean percentage of crypts with proliferating cells during DSS recovery. Control represents non-DSS treated mice. There was no significant difference between CO and FO fed mice at any time point ( $p > 0.05$ ).

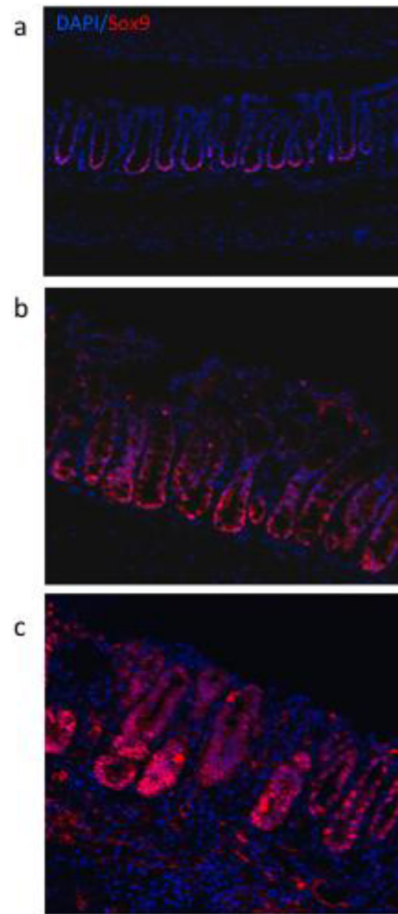


**Figure 3.** Apoptotic index during recovery from DSS treatment. Data represent average apoptotic index within diet groups over the 7 d DSS recovery period (all days combined). At least 20 crypts each from n=3 mice per diet group and time point were scored for TUNEL positive (apoptotic) cells and expressed as apoptotic index (number of apoptotic cells per 100 crypts). Bars with different letters are statistically different ( $p < 0.05$ ). Inset: Apoptosis on individual days in proximal (grey) and distal (black) colon for both diets combined. Dotted line: untreated control mice.



**Figure 4.** Detection of phospho-STAT3 (Y705) following DSS exposure. Mean fluorescence intensity of proximal versus distal colon during DSS recovery (n=3 mice per diet group, all time points combined). Inset: mean fluorescence intensity across time from pooled proximal and distal colon for corn oil (grey) and fish oil fed mice (black). Dotted line: untreated control mice. Bars with different superscripts are significantly different a p<0.05.





**Figure 5.** Effect of DSS exposure on Sox9 crypt localization. Representative photomicrographs ( $\times 100$ ) of Alexa-647 fluorescently labeled distal colonic crypt cells. (a) untreated control, (b) DSS treated, d 1 recovery, (c) DSS treated, d 3 recovery.

**Table 1**

Gene expression by RNA sequencing of colonic and small intestinal GFP<sup>High</sup> stem cells and non-stem cell (GFP negative) populations. Values are expressed as RPKM (reads per kilobase per million mapped reads). Cells were sorted from two pooled mice.

<b>Colon</b>			
<b>Gene</b>	<b>Expression in stem cell population (GFP high)</b>	<b>Expression in non stem cell population (GFP negative)</b>	<b>Fold Change (GFP high/negative)</b>
Adam9	18.665	5.991	3.116
Ascl2	0	0.132	0
Axin2	1.646	2.023	0.814
Bmi1	0	1.783	0
Bmp4	0.758	0.016	47.844
Cdx2	44.720	7.527	5.941
Dclk1	0	0.282	0
Ephb3	21.991	0.622	35.344
Foxn1	1.224	0.077	15.948
Hes1	46.953	5.296	8.866
Hopx	0.317	2.477	0.128
Hoxc8	1.230	0.024	51.831
Igf1	0.028	0.004	7.974
Lgr5	2.599	0.862	3.015
Lgr6	0	0	0
Lrig1	0.856	5.031	0.170
Lrig2	0.020	2.548	0.008
Lrig3	0.527	4.684	0.112
Notch4	0.106	0.067	1.595
Omlf4	0	0.041	0
Prom1	6.541	40.642	0.161
Rspo-1	0	0	0
Runx3	0.042	0.007	5.980
Smoc2	10.571	1.452	7.280
Snai2	0.588	0.052	11.296
Sox4	4.910	2.816	1.744
Sox9	51.631	4.288	12.040
Stat3	0.347	3.722	0.093
Tert	0.017	0.216	0.077
Tgfbr2	4.761	2.454	1.940

<b>Small Intestine</b>			
<b>Gene</b>	<b>Expression in stem cell population (GFP high)</b>	<b>Expression in non stem cell population (GFP negative)</b>	<b>Fold Change (GFP high/negative)</b>
Adam9	1.448	2.858	0.506
Ascl2	5.776	1.064	5.426
Axin2	27.925	16.477	1.695

<b>Small Intestine</b>			
<b>Gene</b>	<b>Expression in stem cell population (GFP high)</b>	<b>Expression in non stem cell population (GFP negative)</b>	<b>Fold Change (GFP high/negative)</b>
Bmi1	2.333	3.054	0.764
Bmp4	0.070	0	0
Cdx2	7.731	8.247	0.938
Dclk1	0.360	0.212	1.696
Ephb3	13.987	2.026	6.904
Foxn1	0.012	0.100	0.122
Hes1	42.973	8.090	5.312
Hopx	9.810	9.202	1.066
Hoxc8	0.053	0.024	2.198
Igf1	0.004	0	0
Lgr5	13.870	3.575	3.880
Lgr6	0	0	0
Lrig1	7.571	8.813	0.859
Lrig2	0.710	2.207	0.321
Lrig3	3.226	3.230	0.999
Notch4	0.108	0.103	1.051
Olfm4	647.212	678.583	0.954
Prom1	36.511	29.596	1.234
Rspo-1	0.020	0	0
Runx3	0	0.057	0
Smoc2	38.483	12.751	3.018
Snai2	0.327	0.105	3.114
Sox4	17.097	6.821	2.506
Sox9	15.853	8.702	1.822
Stat3	1.511	4.083	0.370
Tert	1.551	0.512	3.027
Tgfbr2	1.441	1.219	1.182

**Table 2**

mRNA expression profiles of stem cell mediators in colonic mucosa from control and DSS treated mice - 3 days of recovery.

Gene	Diet	Expression Level (% of control)	pvalue vs control	pvalue CO vs FO
Ascl2	CO	61.3	<0.001	
	FO	24.1	0.002	0.016
Axin2	CO	53.1	<0.001	
	FO	28.8	0.002	0.016
EphB3	CO	65.8	<0.001	
	FO	43.7	0.002	0.032
STAT3	CO	168.8	<0.001	
	FO	161.3	<0.001	0.111
Hopx	CO	70.0	0.006	
	FO	64.7	0.002	0.191
Lgr5	CO	43.8	0.008	
	FO	24.1	0.002	0.191
Sox9	CO	59.9	<0.001	
	FO	50.8	<0.001	0.310
Rspo-1	CO	114.6	0.550	
	FO	128.0	0.413	0.342
mTERT	CO	86.1	0.243	
	FO	71.6	0.122	0.421
Bmi1	CO	127.3	0.222	0.548
	FO	113.2	0.030	

Real-time PCR was performed using Taqman probes (Applied Biosystems) and expression levels were normalized to ribosomal 18S. Values are means, n=5 mice per group. TaqMan gene expression assay IDs were as follows: Ascl2, Mm01268891\_g1; Axin2, Mm00443610\_m1; Bmi1, Mm03053308\_g1; EphB3, Mm00802553\_m1; Hopx, Mm00558630\_m1; Lgr5, Mm00438890\_m1; mTert, Mm00436931\_m1; Rspo1, Mm00507077\_m1; Sox9, Mm00448840\_m1; Stat3, Mm01219775\_m1; 18S, Hs99999901\_s1.