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Notch receptor regulation of intestinal stem cell homeostasis and crypt regeneration

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ABSTRACT

The Notch signaling pathway regulates intestinal epithelial cell homeostasis, including stem cell maintenance, progenitor cell proliferation and differentiation. Notch1 and Notch2 receptors are expressed in the epithelium, but individual contributions to these functions are unclear. We used genetic deletion to define receptor roles on stem cell function, cell proliferation/differentiation, and repair after injury. Loss of *Notch1* induced a transient secretory cell hyperplasia that spontaneously resolved over time. In contrast, deletion of *Notch2* had no secretory cell effect. Compound deletions of *Notch1* and *Notch2* resulted in a more severe secretory cell hyperplasia than deletion of *Notch1* alone. Furthermore, only double deletion of *Notch1* and *Notch2* decreased cell proliferation, suggesting a low threshold for maintenance of proliferation compared to differentiation. Stem cells were affected by deletion of *Notch1*, with reduced expression of *Olfm4* and fewer LGR5$^+$ stem cells. Deletion of *Notch2* had no apparent affect on stem cell homeostasis. However, we observed impaired crypt regeneration after radiation in both *Notch1*- and *Notch2*-deleted intestine, suggesting that higher Notch activity is required post-injury. These findings suggest that Notch1 is the primary receptor regulating intestinal stem cell function and that Notch1 and Notch2 together regulate epithelial cell proliferation, cell fate determination, and post-injury regeneration.

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Introduction

The continuous renewal of the intestinal epithelium is fueled by active stem cells located at the crypt base. Although intestinal stem cells have been a topic of investigation for several decades, the discovery of LGR5 as a specific marker of the active stem cell population sparked a recent expansion of this field of study (Barker et al., 2007). LGR5$^+$ intestinal stem cells divide to produce daughters that can become stem or transit-amplifying (TA) cells depending on competition for open stem cell niche spots (Snippert et al., 2010). The TA cells are short-lived, highly proliferative progenitors that expand epithelial cell numbers and differentiate into the various epithelial cell types, including absorptive enterocytes, mucous-secreting goblet cells, antimicrobial peptide-secreting Paneth cells and hormone-secreting enteroendocrine cells. The molecular mechanisms that regulate intestinal stem cell number and function, and TA cell proliferation and differentiation to maintain overall tissue homeostasis are not well understood.

The Notch signaling pathway regulates several aspects of intestinal epithelial cell homeostasis. Notch plays a key role in regulating epithelial cell fate, with pathway activation leading to enterocyte differentiation (Fre et al., 2005; Jensen et al., 2000; Stanger et al., 2005), while pathway inhibition promotes secretory cell differentiation, including goblet, Paneth and endocrine cells (Pellegrinet et al., 2011; Riccio et al., 2008; van Es et al., 2005; VanDussen et al., 2012). Notch regulation of absorptive *vs.* secretory cell fate occurs by transcriptional repression of the secretory lineage transcription factor *Atoh1*. The expression of *Atoh1* has been shown to be both required (Shroyer et al., 2007; Yang et al., 2001) and sufficient (VanDussen and Samuelson, 2010) for concerted differentiation of all secretory cell types. Conversely, enterocyte differentiation results when *Atoh1* expression is repressed (Shroyer et al., 2007; Yang et al., 2001). Interestingly, the sole function of Notch to regulate cell fate appears to be through regulation of *Atoh1* and

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pathway activation is not required for enterocyte differentiation if *Atoh1* is genetically deleted (Kim and Shivdasani, 2011).

In addition to regulation of intestinal epithelial cell fate, Notch signaling regulates epithelial cell proliferation, with pathway activation resulting in increased proliferation (Fre et al., 2005; Stanger et al., 2005), while pathway inhibition leads to a marked reduction in overall cell proliferation in the intestine (Pellegrinet et al., 2011; Riccio et al., 2008; van Es et al., 2005; VanDussen et al., 2012). Moreover, we and others have shown that Notch signaling is necessary for stem cell proliferation and cell survival (Pellegrinet et al., 2011; VanDussen et al., 2012). Pathway inhibition resulted in reduced expression of the stem cell marker *Olfm4*, decreased numbers of LGR5$^+$ stem cells and reduced stem cell proliferation (VanDussen et al., 2012).

Expression of the 4 known Notch receptors (N1–4) and 5 ligands (Dll1, 3, 4 and Jag1 and 2) is temporally and spatially controlled for proper development and homeostasis of many tissues. N1 and N2 are both expressed in the adult intestinal epithelium (Fre et al., 2011; Sander and Powell, 2004; Schroder and Gossler, 2002), and lineage tracing studies suggest that both receptors are expressed in stem cells (Fre et al., 2011), but individual roles for each receptor are not well understood.

Previous studies investigating N1 and N2 functions utilizing humanized inhibitory antibodies suggested a role for N1 in regulating intestinal homeostasis, as anti-N1 treatment showed a mild secretory cell hyperplasia (Wu et al., 2010) and decreased intestinal proliferation, and toxicity when paired with irradiation damage (Tran et al., 2013). In contrast to these findings, genetic deletion studies reported that individual loss of N1 or N2 in the intestinal epithelium had no phenotype, and thus N1 and N2 were thought to be fully functionally redundant in the gut (Riccio et al., 2008). Due to the important therapeutic implications of intestinal Notch regulation, it is important to reconcile these disparate findings.

In this study we used a genetic deletion model to definitively show that N1 is the predominantly active Notch receptor in the intestinal epithelium, as N1 receptor deletion led to secretory cell hyperplasia and reduced stem cell number. Furthermore, we investigate the dynamic regulation of lost N1 signal and expand on the understanding of how N1 and N2 function together to regulate proliferation and differentiation in the intestine. Finally, we uncover an unexpected sensitivity to loss of either N1 or N2 in irradiated intestine with implications on therapeutic use of Notch receptor blockade.

Materials and methods

Mice

Floxed-*Notch1* (N1$^{F/F}$) (Yang et al., 2004) (Jackson Lab, no. 007181), floxed-*Notch2* (N2$^{F/F}$) (McCright et al., 2006) (Jackson Lab, no. 010525), floxed-*Rbpjκ* (*Rbpj*$^{F/F}$) (Tanigaki et al., 2002) (gift from T. Honjo), *Villin-CreER*^{T2} (el Marjou et al., 2004) (gift from S. Robine) and *Lgr5-GFP-IRES-CreER*^{T2} (*Lgr5-GFP*) (Barker et al., 2007) (Jackson Lab, no. 008875) alleles were verified by PCR genotyping with the primers listed in Supplementary Table 1. All mouse strains were on a C57BL/6 strain background. Adult mice of both sexes aged 2–4 months were analyzed for all experiments except where indicated that juvenile mice aged 10 days were used. Mice were housed in ventilated and automated watering cages with a 12-h light cycle under specific pathogen-free conditions. Protocols for mouse usage were approved by the University of Michigan Committee on Use and Care of Animals.

Animal treatment protocols and tissue collection

To activate CreER^{T2}-mediated recombination, mice were injected intraperitoneally with 100 mg/kg tamoxifen (10 mg/ml in 5% ethanol and 95% corn oil, Sigma) or corn oil once per day for 5 days and tissue was collected on day 6, unless otherwise noted. Corn oil-treated control animals were found to have no background goblet cell hyperplasia (data not shown). Juvenile mice were injected at 10 days of age *via* the same protocol except with 20 mg/ml tamoxifen to achieve 100 mg/kg. To induce intestinal injury, animals were exposed to one dose of 12 Gy whole body irradiation from a ^{137}Cs source. Animals were fasted overnight and injected intraperitoneally with 25 mg/kg 5-ethynyl-2′-deoxyuridine (EdU) (Life Technologies) 2 h prior to tissue collection. Intestinal tissue was harvested and fixed in 4% paraformaldehyde in PBS (PFA) overnight before paraffin processing as previously described (VanDussen et al., 2012). Tissue prepared for frozen sections was fixed in PFA for 1 h and placed in 30% sucrose overnight before embedding in OCT (Tissue-Tek).

Immunohistochemistry

Paraffin sections (5 μm) were stained with Periodic acid Schiff and Alcian Blue (PAS/AB) (Newcomer Supply) to visualize mucin-containing goblet cells. EdU-Click-it (Life Technologies) was used to evaluate proliferating cell number. Immunostaining with rat α-MMP7 (1:400, Vanderbilt Antibody and Protein Resource), rabbit α-MUC2 (1:200, Santa Cruz), rabbit α-CHGA (1:200, Abcam), rat α-PROM1 (1:100, eBioscience) and rabbit α-Ki67 (1:200, Thermo) was performed as described (Lopez-Diaz et al., 2006). For AB/CHGA co-staining, tissues were stained with α-CHGA (1:100, Abcam) and visualized with the DAB substrate kit (VectorLabs) per manufacturer's instructions. Slides were then stained in AB for 30 min and counterstained in neutral red for 1 min. Transgenic LGR5-GFP was directly imaged on frozen sections without antibody staining. Images were captured on a Nikon E800 microscope with Olympus DP controller software.

In situ hybridization

Olfm4 cDNA plasmid (IMAGE clone 9055739) was linearized and sense and antisense probes were made with DIG labeling kit according to the manufacturer's instructions (Roche). Probes were purified using the Min-Elute Gel Extraction kit (Qiagen). Slides were deparaffinized, hydrated, and treated with 0.2 N HCl for 15 min at room temperature. Tissues were treated with Proteinase K (30 μg/mL, Roche) for 30 min at 37 °C, post-fixed in 4% PFA for 10 min, acetylated for 10 min in 13.4% triethanolamine, 0.2 M HCl and 2.5% acetic anhydride, incubated with hybridization buffer (50% deionized formamide (Invitrogen), 10% dextran sulfate (Millipore), 2% Denhardts (Sigma), 1 mg/mL yeast tRNA, 0.2 M NaCl, 0.1 M Tris–HCl, pH 7.5, 0.1 M phosphate buffer, pH 6.8, 5 mM EDTA, pH 8 in DEPC-treated H_2O) for 1 h, followed by incubation with *Olfm4* probe diluted in hybridization buffer at 68 °C overnight. Tissue sections were then washed, incubated in blocking solution (20% heat-inactivated serum, 0.02 g/mL blocking reagent (Roche)) in buffer (0.1 M Tris–HCl, pH 7.5, 0.15 M NaCl, 0.1% Tween 20 in sterile H_2O) for 1 h, and anti-DIG antibody (1:2500, Roche) overnight at 4 °C. Slides were washed and developed with NBT/BCIP solution (1:100, Roche) in 0.1 M Tris–HCl, pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$, 0.5 mg/mL levamisole (Sigma) in sterile H_2O. Minimal signal was detected with *Olfm4* sense probe control.

Quantitative morphometric analysis

All observers were blinded to slide identity for cell counting. Goblet cell hyperplasia was measured as the number of crypts that displayed increased goblet cells over total crypts per section. EdU morphometrics was achieved by counting the total number of epithelial EdU$^+$ cells per well-oriented crypt and averaged per

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