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ORIGINAL RESEARCH

Foxl1-Expressing Mesenchymal Cells Constitute the Intestinal Stem Cell Niche

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SUMMARY

Forkhead box l1+ cells are a small subset of mesenchymal subepithelial fibroblasts. These cells are a critical component of the intestinal stem cell niche as shown by using 2 separate models to ablate these cells.

BACKGROUND & AIMS: Intestinal epithelial stem cells that express leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) and/or B cell specific Moloney murine leukemia virus integration site 1 (Bmi1) continuously replicate and generate differentiated cells throughout life. Previously, Paneth cells were suggested to constitute an epithelium-intrinsic niche that regulates the behavior of these stem cells. However, ablating Paneth cells has no effect on the maintenance of functional stem cells. Here, we show definitively that a small subset of mesenchymal subepithelial cells expressing the winged-helix transcription factor forkhead box l1 (Foxl1) are a critical component of the intestinal stem cell niche.

METHODS: We genetically ablated Foxl1+ mesenchymal cells in adult mice using 2 separate models by expressing either the human or simian diphtheria toxin receptor under Foxl1 promoter control.

CONCLUSIONS: Killing Foxl1+ cells by diphtheria toxin administration led to an abrupt cessation of proliferation of both epithelial stem- and transit-amplifying progenitor cell populations that was associated with a loss of active Wnt signaling to the intestinal epithelium. Therefore, Foxl1-expressing mesenchymal cells constitute the fundamental niche for intestinal stem cells. *(Cell Mol Gastroenterol Hepatol 2016;2:175–188; http://dx.doi.org/10.1016/j.jcmgh.2015.12.004)*

Keywords: Intestinal Stem Cell Niche; Wnt; Mesenchyme.

Adult multipotent stem cells replenish the gut epithelium both during homeostasis and after injury throughout life. The mammalian intestinal epithelium undergoes complete regeneration every 3–5 days; this renewal is supported by multipotent intestinal stem cells.[1–6] Epithelial stem cell populations reside in 2 zones: at the crypt base and at the +4 position, 4–6 cell widths above the crypt base. Cells in both zones express markers associated with stem cell behavior, such as CD24 and Lrig1.[4,5] In addition, there are zone-specific markers. For the small intestine, the crypt base cells are marked by leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5)[1] and Olfm4,[7] and the +4 position cells express Bmi-1, HopX, and mTert.[2,3,8] These epithelial stem cells divide to produce progenitor (or transit-amplifying) cells that proliferate rapidly and then differentiate into Paneth cells, which are located at the crypt base, or into goblet cells, enteroendocrine cells, M cells, and absorptive enterocytes that are located closer to the gut lumen.

Stem cell niches are defined as local microenvironments that provide physical support and/or molecular signals necessary for proper stem and progenitor cell replication and differentiation. Wnt signaling has been established as the major driver of intestinal stem and progenitor cell proliferation, as evidenced, for instance, by a rapid loss of proliferation when the secreted Wnt inhibitor Dickkopf-1 is overexpressed in the epithelium.[9,10] However, the cellular identity of the intestinal stem cell niche has remained controversial. Sato et al[11] concluded that epithelial Paneth cells constitute the niche for Lgr5+ stem cells in intestinal crypts, based on the fact that Paneth cells elaborate important signaling molecules such as Wnt3 and EGF and on the observation that in vitro organoid formation by Lgr5+ stem cells was enhanced by co-culture with a Paneth cell–enriched population. However, complete and permanent absence of Paneth cells in mice deficient for the

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Abbreviations used in this paper: **BAC, bacterial artificial chromosome; cDNA, complementary DNA; Foxl1, forkhead box l1; hDTR, human diphtheria toxin receptor; iDTR, inducible diphtheria toxin receptor; mRNA, messenger RNA; Myh11, rabbit myosin heavy chain 11; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; YFP, yellow fluorescent protein.**

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transcription factor Math1 (Atoh1) had no impact on intestinal stem cell maintenance and proliferation.[12,13] The latter studies support earlier work by Garabedian et al[14] who used 2 independent methods to ablate mature Paneth cells and concluded that "stemness in the crypt is not defined by instructive interactions involving the Paneth cells." Moreover, epithelial-specific deletion of Wnt3 had no effect on intestinal stem cells in mice, suggesting the presence of other Wnt source(s).[15] These findings point to the existence of an extraepithelial source of Wnt and other signaling molecules necessary to maintain epithelial homeostasis.

Materials and Methods

Derivation of Forkhead Box l1–Human Diphtheria Toxin Receptor Mice

All protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. The human diphtheria toxin receptor (*hDTR)* coding sequence (NM_001945.1) was introduced into the coding region of the mouse forkhead box l1 (*Foxl1*) gene in the bacterial artificial chromosome (BAC) RP23-446J14 by means of BAC recombineering as described previously.[16] The targeting primers were as follows:

forward: GGGGCAAAGTCCTTAGGACTCCCCGGTGGAGCG GAGAGGCTGCTGTCGCCGAATTCGGCACGAGGGCTACGCGGG; reverse: AGGCCCCTCAGTGCACGACTTTGGCCGGCACGGGTAC GCTGCTCCAAACC AGCTCCACCGCGGTGGCGGCCGCTC.

The resulting *Foxl1–hDTR* BAC was linearized and microinjected into the pronucleus of C57Bl/6 mice. The positive transgenic founders were identified by genomic polymerase chain reaction (PCR) and crossed to C57Bl/6 mice for at least 5 generations. Animals were euthanized at 2–6 months of age for subsequent experiments.

Generation of Foxl1–Cre;Rosa–iDTR/YFP, Foxl1–Cre;Rosa–YFP, and Foxl1–Cre; Rosa–mT/mG Mice

Foxl1–Cre mice were generated and characterized previously.[16] *Foxl1–Cre* mice were crossed to *Rosa*–inducible diphteria toxin receptor (*iDTR*)/yellow fluorescent protein (*YFP*) mice (Jackson Laboratories, Bar Harbor, ME) to obtain *Foxl1–Cre;Rosa–iDTR/YFP* mice. *Foxl1–Cre;Rosa–*membrane-targeted dimer tomato protein (*mT*)/membrane-targeted green fluorescent protein (*mG*) mice, *Foxl1–Cre* mice resulted from crossing to *Rosa–mT/mG* mice (Jackson Laboratories). Animals were killed at 2–6 months of age for subsequent experiments.

Diphtheria Toxin Treatment

For *Foxl1–hDTR* mice, diphtheria toxin (Sigma-Aldrich, St. Louis, MO) dissolved in 0.9% sodium chloride was administered intraperitoneally at 20 ng/g body weight. Mice were injected on days 0 and 2 and killed on day 3. *Foxl1–Cre;Rosa–iDTR/YFP* mice and their control littermate (*Rosa–iDTR/YFP*) mice were administered diphtheria toxin at 22 ng/g body weight, twice daily from days 0–3, and killed on day 4.

Anti-Foxl1 Antibody Production

A Foxl1–pUC57 codon-optimized plasmid containing amino acids 150–255 of mouse Foxl1 (avoiding regions of similarity with other Fox proteins, especially focusing on non–winged-helix domains with good antigenicity prediction and likely subdomain folding) with flanking BamHI/HindIII sites was generated (GenScript USA, Inc, Santa Clara, CA). The BamHI/HindIII fragment was inserted into pGexKG,[17] confirmed by sequencing, and used to produce soluble GST-Foxl1 fusion protein from bacteria. GST-Foxl1 was dialyzed against phosphate-buffered saline (PBS) and used as an antigen in goat and guinea pig (SDIX, LLC) or chicken (Aves Labs). Raw sera/antibodies were used to purify Foxl1-specific antibodies via GST/*Escherichia coli*–extract depletion and GST-Foxl1 matrix-based affinity purification. GST-Foxl1 protein production and antibody purification were performed as described previously.[18]

Fluorescent Activated Cell Sorting, RNA Isolation, and Sequencing Library Preparation

Isolation of Foxl1+ cells using fluorescent-activated cell sorting was performed using *Foxl1–Cre;Rosa–YFP* mice. Small intestines were dissected and washed thoroughly with PBS. Intestinal villi were scraped using a coverslip and the remaining tissue was incubated in 30 mmol/L EDTA plus 1.5 mmol/L dithiothreitol and Hank's balanced salt solution on ice for 20 minutes and subsequently incubated in 30 mmol/L EDTA at 37° for 8 minutes to completely remove the epithelium. After vigorous washes, the remaining mesenchymal fraction was collected and cut into small pieces. The mesenchymal tissue was collected by centrifugation and resuspended in 7 mg/mL Dispase II/0.05% trypsin solution (Sigma-Aldrich, St. Louis, MO) at 37° until the solution became cloudy and the mesenchyme was dissociated. A single-cell suspension was obtained by collecting the supernatant and washing with Hank's balanced salt solution before cell sorting using a BD influx instrument (BD Biosciences, San Jose, CA). For RNA isolation, YFP+ cells were lysed and total RNA was isolated by column purification (Agilent Technologies). Messenger RNA (mRNA) was isolated using Poly(A) mRNA isolation magnetic beads and an mRNA sequencing library prepared using the NEBNext RNA library prep kit (New England BioLabs, Inc, Ipswich, MA). RNA sequencing was performed on an Illumina HiSeq instrument.

RNA Isolation and Library Formation From Crypt Cells

To isolate RNA from intestinal crypts after diphtheria toxin injection, *Foxl1–hDTR* and control mice were injected with diphtheria toxin at 20 ng/g body weight on day 0 and euthanized on day 3. Dissected intestines were washed in PBS to remove the luminal content, incubated in 5 mmol/L