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Using a new Lrig1 reporter mouse to assess differences between two Lrig1 antibodies in the intestine

Emily J. Poulin[a,1], Anne E. Powell[b,1], Yang Wang[b,1], Yina Li[b], Jeffrey L. Franklin[a], Robert J. Coffey[a,b,c,*]

[a] Department of Cell and Developmental Biology, Vanderbilt University Medical Center, Nashville, TN 37232, USA
[b] Department of Medicine, Vanderbilt University Medical Center, Nashville, TN 37232, USA
[c] Department of Veterans Affairs Medical Center, Nashville, TN 37232, USA

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Abstract Lrig1 is an intestinal stem cell marker important for epithelial homeostasis. However, the position of the Lrig1$^+$ population in the intestinal crypt has been debated, largely due to discrepant staining patterns using two Lrig1 antibodies. Here, we set out to decipher the differences between these Lrig1 antibodies to clarify their use for Lrig1-related studies. We confirmed that the commercially available Lrig1-R&D antibody stained the bottom third of the colonic crypt, whereas an independently generated Lrig1-VU antibody recognized a subset of anti-Lrig1-R&D$^+$ cells. Biochemically, we found that anti-Lrig1-VU recognized a non-glycosylated form of Lrig1; in contrast, anti-Lrig1-R&D recognized both glycosylated and non-glycosylated forms of Lrig1. In addition, we generated a reporter mouse (*Lrig1-Apple*) as an independent readout of *Lrig1* transcriptional activity. Flow cytometry of isolated colonic epithelial cells from *Lrig1-Apple* mice demonstrated anti-Lrig1-R&D recognized mostly RFP-hi cells, while anti-Lrig1-VU recognized cells that were largely RFP-mid. Of note, by qRT-PCR, *Lgr5* was expressed in the RFP-hi population, but not in the RFP-mid population. We conclude that anti-Lrig1-R&D appears to recognize all Lrig1$^+$ cells, while anti-Lrig1-VU recognizes a subpopulation of Lrig1$^+$ cells.

Introduction

Identification of adult intestinal stem cell markers has accelerated in recent years, following the discovery of the first *bona fide* marker, Lgr5, by Barker et al., 2007 (Barker et al., 2007). Powell et al. identified leucine-rich repeats and immunoglobulin-like domains protein 1 (Lrig1) as an intestinal stem cell marker in 2012 (Powell et al., 2012). At the same time, Wong et al. demonstrated that Lrig1 was important for intestinal homeostasis (Wong et al., 2012).

Abbreviations: Lrig1, leucine-rich and immunoglobulin-like domains protein 1; RFP, red fluorescent protein; FACS, fluorescence-activated cell sorting; PCR, polymerase chain reaction; qRT-PCR, quantitative real-time PCR.

* Corresponding author at: Epithelial Biology Center, Vanderbilt University Medical Center, 2213 Garland Ave., 10415-F MRB IV, Nashville, TN 37232-0441, USA. Fax: +1 615 343 1591.
E-mail address: robert.coffey@vanderbilt.edu (R.J. Coffey).
[1] Authors contributed equally.

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While both groups demonstrated that Lrig1 marks cells in the intestinal epithelial stem cell zone, discrepant observations of Lrig1 protein distribution in the intestinal crypt were observed.

Wong and colleagues, focusing on the small intestine, demonstrated that Lrig1 transcript and protein are expressed in the progenitor cell zone of the crypt base using in situ hybridization and immunofluorescent analysis. Using flow cytometry, they showed that 30% of intestinal epithelial cells express Lrig1 and these Lrig1$^+$ cells express intestinal stem cell marker transcripts (Wong et al., 2012). Our group—focused on the colon—demonstrated that Lrig1 marks a *bona fide* intestinal stem cell population that gives rise to all differentiated intestinal epithelial cell types using lineage tracing studies. Additionally, we showed that Lrig1 protein is expressed in select cells in the colonic crypt base, rather than in a broad pattern. Flow cytometry demonstrated that only 4.8% of colonic epithelial cells express Lrig1; RNA-Seq analysis of this Lrig1$^+$ flow-sorted population also revealed enrichment of intestinal stem cell marker transcripts (Powell et al., 2012). The relationship between different stem cell populations and between stem cells and committed progenitors, as well as studies of stem cell behavior, are marker-based. Therefore, it is essential to clarify the Lrig1 expression discrepancy to facilitate Lrig1-related studies.

These two independent studies utilized different anti-Lrig1 antibodies to assess Lrig1 protein expression. Wong et al. used a commercial goat polyclonal anti-Lrig1 antibody from R&D Systems™, raised against nearly the entire ectodomain of mouse Lrig1 (#AF3688; hereafter anti-Lrig1-R&D) (Wong et al., 2012), while in collaboration with Covance (Denver, PA), Powell et al. generated a rabbit polyclonal peptide antibody to a sequence (KILSVDGSQLKSY) in the ectodomain of mouse Lrig1 (hereafter anti-Lrig1-VU) (Powell et al., 2012). Using a new Lrig1 reporter mouse (*Lrig1-Apple*), we set out to further characterize these antibodies to clarify their use for future Lrig1-related studies. We show that anti-Lrig1-R&D appears to recognize all Lrig1$^+$ cells, while anti-Lrig1-VU recognizes a subset of Lrig1$^+$ cells, likely expressing a non-glycosylated form of Lrig1.

Materials and methods

Mice

The *Lrig1-mAppleC1* construct was made by BAC recombineering using the *129 Lrig1* BAC clone from the Sanger Institute (bMQ291-E18). The Apple red fluorescent protein variant excites at 568 nm and emits at 592 nm. The 5′ and 3′ oligonucleotide probes were generated by PCR; the primers used for their generation are listed in Supplemental Table 1. The Transgenic Mouse/ES Cell Shared Resource at Vanderbilt University performed ES cell electroporation and blastocyst injections. ES cell clones were screened by Southern blotting to identify *Lrig1-mAppleC1* integration. Chimeras were generated and individuals with germline transmission were identified by PCR genotyping of tail DNA (oligonucleotide primers listed in Supplemental Table 1). Germline-transmitted *Lrig1-mAppleC1* chimeras were intercrossed with *FlpE* mice (B6.SJL strain) to eliminate the FRT-flanked PGK-neo cassette. Genotyping PCR identified wildtype and *Lrig1-Apple*/+ mice. Loss of the PGK-neo cassette was detected by PCR (oligonucleotide primers listed in Supplemental Table 1). All animal protocols were approved and performed in accordance with the Vanderbilt University Medical Center Animal Care and Use Program. Mice were fed standard rodent chow and water ad libitum and housed under controlled light cycle conditions.

Cloning of Lrig1-EGFP and transfection

Full-length mouse *Lrig1* cDNA (#MG50511-M, Sina Biological Inc.) was cloned into the pEGFP-N1 plasmid (#6085-1, Clonetech), resulting in the Lrig1-EGFP C-terminal fusion protein. Lrig1-pEGFP-N1 (Lrig1-EGFP) and pEGFP-N1 (EGFP) were transiently transfected into human HEK293T cells using Metafectene (Biontex, Germany) according to the manufacturer's instructions.

Isolation of colonic epithelium for western blotting, cell lysis, and immunoprecipitation

Intestinal tissue was freshly dissected and crypts were isolated as previously described (Powell et al., 2012; Whitehead et al., 1987). Isolated crypt epithelium was lysed as previously described (Powell et al., 2012). Protein concentrations were determined using a microBCA assay kit (Pierce, Rockford, IL). Thirty milligrams of lysates were resolved on a 7.5% SDS-PAGE gel and western blotting was performed according to a standard western blotting protocol. Autoradiography visualization was performed with ECL reagents (Perkin Elmer, Waltham, MA). The following primary antibodies were used for western blotting: anti-Lrig1-R&D 1:300 (#AF3688, R&D Systems, Minneapolis, MN); anti-Lrig1-VU 1:300 (made in collaboration with Covance, Denver PA (Powell et al., 2012)); anti-green fluorescent protein (GFP) 1:1000 (#A11122, Invitrogen, Grand Island, NY); and anti-α-tubulin 1:10,000 (#CP06, Calbiochem, San Diego, CA). Species-specific HRP-conjugated secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). Transfected HEK293T cells were lysed with RIPA buffer (50 mM Tris pH 7.2; 150 mM NaCl; 1% NP-40; 0.5% deoxycholic acid; 0.1% SDS) containing protease inhibitors (#P2714, Sigma, St. Louis, MO). Lysates were centrifuged to remove the insoluble pelleted fraction and the supernatant was used for immunoprecipitation. Five hundred micrograms of cell lysates were immunoprecipitated with anti-Lrig1-R&D, anti-Lrig1-VU, or anti-GFP, and recovered using Dynabeads® Protein A (#10001D, Invitrogen) or Dynabeads® Protein G (#10009D, Invitrogen). Immunoprecipitates were resolved with SDS-PAGE and western blotting as above.

Peptide blocking

Wildtype mouse colonic crypt lysates were resolved by SDS-PAGE. Twenty-fold molar excess of anti-Lrig1-VU blocking peptide was added to anti-Lrig1-VU and anti-Lrig1-R&D and incubated at 4°C for 4 h. The antibody-blocking peptide solutions were diluted in 5% BSA-TBST as above and western blotting was performed.