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CD24 can be used to isolate Lgr5⁺ putative colonic epithelial stem cells in mice

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King JB, von Furstenberg RJ, Smith BJ, McNaughton KK, Galanko JA, Henning SJ. CD24 can be used to isolate Lgr5⁺ putative colonic epithelial stem cells in mice. Am J Physiol Gastrointest Liver Physiol 303: G443–G452, 2012. First published June 21, 2012; doi:10.1152/ajpgi.00087.2012.—A growing body of evidence has implicated CD24, a cell-surface protein, as a marker of colorectal cancer stem cells and target for anticancer therapy, although its presence in normal colonic epithelium has not been fully characterized. Previously, our group showed that CD24-based cell sorting can be used to isolate a fraction of murine small intestinal epithelial cells enriched in actively cycling stem cells. Similarly, we hypothesized that CD24-based isolation of colonic epithelial stem cells would generate a fraction enriched in actively cycling colonic epithelial stem cells (CESCs). Immunohistochemistry performed on mouse colonic tissue showed CD24 expression in the bottom half of proximal colonic crypts and the crypt base in the distal colon. This pattern of distribution was similar to enhanced green fluorescent protein (EGFP) expression in Lgr5-EGFP mice. Areas expressing CD24 contained actively proliferating cells as determined by ethynyl deoxyuridine (EdU) incorporation, with a distinct difference between the proximal colon, where EdU-labeled cells were most frequent in the midcrypt, and the distal colon, where they were primarily at the crypt base. Flow cytometric analyses of single epithelial cells, identified by epithelial cell adhesion molecule (EpCAM) positivity, from mouse colon revealed an actively cycling CD24⁺ fraction that contained the majority of Lgr5⁺ EGFP⁺ putative CESC s. Transcript analysis by quantitative RT-PCR confirmed enrichment of active CESC markers [leucine-rich-repeat-containing G protein-coupled receptor 5 (Lgr5), ephrin type B receptor 2 (EphB2), and CD166] in the CD24⁺ EpCAM⁺ fraction but also showed enrichment of quiescent CESC markers [leucine-rich repeats and immunoglobulin domains (Lrig), doublecortin and calmodulin kinase-like 1 (Dcamk1l-1), and murine telomerase reverse transcriptase (mTert)]. We conclude that CD24-based sorting in wild-type mice isolates a colonic epithelial fraction highly enriched in actively cycling and quiescent putative CESC s. Furthermore, the presence of CD24 expression in normal colonic epithelium may have important implications for the use of anti-CD24-based colorectal cancer therapies.

The epithelial lining of the mammalian colon is a dynamic structure, relying on a tight balance between proliferation, differentiation, senescence, and apoptosis to constantly renew itself in the face of homeostatic and pathological pressures. Colonic epithelial stem cells (CESC s) are thought to be the main regulators of this process, producing progeny that terminally differentiate into one of three lineages (colonocytes, mucus-secreting goblet cells, and enteroendocrine cells), and self-renew to propagate the pool of functional stem cells. Most current literature assumes that CESC s are located at the crypt base throughout the colon. However, this assumption is often extrapolated from what is known about the location of the well-characterized small intestine epithelial stem cell. Often overlooked are earlier studies calling into question such assumptions. These studies suggest that, at least in rodents, there may be regional variability in the location of the CESC zone, with its position at the crypt base in the distal colon but farther up the crypt in the proximal colon.

Advances in characterizing the exact location and function of CESC s have been hampered by the inability to specifically isolate this epithelial cell fraction of interest. While multiple potential CESC markers have been proposed (2, 7, 11, 14, 17, 18, 21), they remain plagued by nonspecificity, weak expression, and/or incompatibility with cell sorting. Recently, two promising CESC markers have been reported, namely, the Wnt target gene leucine-rich-repeat-containing G protein-coupled receptor 5 (Lgr5) and the transcription factor Sox9. For both of these, enhanced green fluorescent protein (EGFP)-engineered mice have allowed the generation of definitive data confirming their ability to identify CESC s. For Lgr5, the use of an in vivo lineage-tracing approach showed that Lgr5-expressing cells at the colonic crypt base are capable of self-renewal and differentiation into the three colonic lineages. For Sox9, the use of a Sox9-EGFP transgene showed highly expressing cells at the crypt base, which, when isolated by fluorescence-activated cell sorting (FACS), demonstrated self-renewal and differentiation in vitro. While these markers represent valuable advances in the study of CESC s, their application is limited by the need for specifically engineered mice.

For broader application, the identification of a membrane marker for CESC s would be of significant value. A promising candidate in this arena is CD24, a heavily glycosylated mucin-like adhesion molecule that also participates in signal transduction and influences cell turnover in various tissues by enhancing proliferation and inhibiting apoptosis. Previous studies have shown that CD24 is robustly expressed in the stem cell zone of the murine small intestine and that cell sorting with
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a commercially available CD24 antibody isolates a fraction of epithelial cells that includes actively cycling stem cells (10, 30). One limitation of using CD24 as a stem cell marker in the small intestine is that it marks not only stem cells but, also, differentiated Paneth cells. In the normal colon, this is not an issue, as Paneth cells are only present in cases of chronic inflammation (5, 19, 32). There does, however, appear to be a largely uncharacterized mucin-producing goblet-like cell (GLC) (1, 16, 22) at the colonic crypt base that may serve a role in maintenance of the stem cell niche similar to the role of Paneth cells in the small intestine (25).

The importance of studying CD24 expression in the normal colon is magnified because of the growing body of literature implicating CD24 as a marker of colon cancer stem cells. In human studies, expression of CD24 in colorectal tumors is associated with higher tumor stage/grade, as well as increased systemic and/or nodal metastases (4, 33). One retrospective study has shown that cytoplasmic expression of CD24 in colorectal cancers is associated with decreased median patient survival (33). Furthermore, overexpression of CD24 has been shown to induce cancer cell growth in vitro and in vivo (31), while underexpression of CD24 leads to a milder phenotype (23). These data have spawned further studies investigating the potential anti-tumor effects of monoclonal antibodies against CD24 (23, 26), despite the fact that implications for such therapy in normal intestinal physiology are unknown.

Our current study characterizes CD24 expression in the normal murine colon and describes regional differences in the localization of the stem cell zone in the proximal and distal colon. On the basis of our prior work with CD24 and small intestine epithelial stem cells, we hypothesized that a commercially available CD24 antibody could be used to sort a population of wild-type (WT) mouse colonic epithelial cells that is enriched in actively cycling CESCs. Using Lgr5-EGFP expression as a validated marker of murine CESCs, we further hypothesized that our CD24+ fraction would contain the majority of Lgr5-EGFP-expressing cells, thus obviating the need for genetically engineered mice to study CESCs. Lastly, drawing parallels to the expression of CD24 in the small intestine, we hypothesized that the CD24+ colonic epithelial fraction would be heterogeneous and may include the GLCs of the colonic crypt base.

MATERIALS AND METHODS

**Mice.** Adult male WT C57BL/6J mice and heterozygote breeder pairs of Lgr5-EGFP-IRES-creERT2 (Lgr5-EGFP) mice (Jackson Laboratories, Bar Harbor, ME) were housed under a 12:12-h light-dark cycle in facilities approved by the American Association for Accreditation of Laboratory Animal Care. All animals were used within the age range of 6–12 wk. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

**Immunohistochemical analyses.** Adult WT mouse colonic tissue was dissected and flushed with Mg2+-/Ca2+-free HBSS. Segments (4 mm) of midproximal and middistal colon were fixed in 4% PFA followed by 30% sucrose and embedded in Tissue-Tek optimal cutting temperature (OCT) embedding medium for frozen tissue specimens (Sakura Finetek). Sections (7 μm) were incubated with Protein Block solution (Dako) for 1 h and then with purified rat anti-mouse CD24 primary antibody (1:50 dilution; catalog no. 557436, BD Bioscience) at 4°C overnight. After they were washed, the samples were blocked with 3% peroxide in methanol at room temperature for 10 min, washed, and incubated in biotinylated mouse anti-rat IgG2b,κ secondary antibody (1:200 dilution; catalog no. 553987, BD Bioscience) at room temperature in a humidity chamber for 1 h. Then the samples were washed and incubated in avidin-biotin complex (1:50 dilution; Vectastain Elite ABC Kit, Vector Laboratories) at room temperature in a humidity chamber for 30 min. The samples were washed again, and diaminobenzidine (Zymed Kit, Invitrogen) was applied to the slides. After completion of the reaction, the slides were placed in distilled water for 4 min and then counterstained with hematoxylin. Slides were then dehydrated, coverslips were applied using DPX mountant (Crown Scientific), and the slides were allowed to dry overnight and analyzed by light microscopy. For quantification of CD24 expression as a proportion of crypt length, 20 well-oriented crypts per animal (n = 5) were measured for total crypt length and length of CD24 expression.

**Fluorescent image analyses.** Lgr5-EGFP mouse colonic tissue was dissected, fixed, and frozen as described above. Tissue blocks were sectioned at 7 μm, and coverslips were applied using Vectashield mounting medium with 4',6-diamidino-2-phenylindole (Vector Laboratories) and sealed. For assessment of proliferative status, adult WT mice were euthanized 60 min following intraperitoneal injection with 100 μg of the thymidine analog ethynyl deoxyuridine (EdU). Proximal and distal colonic tissue was dissected, fixed, and frozen as described above. Tissue blocks were sectioned at 7 μm and processed using the Click-iT Alexa Fluor 594 EdU Imaging Kit according to the manufacturer’s protocol (Invitrogen). Slides were analyzed by fluorescence microscopy (Olympus IX81). For quantification of EdU expression by cell position, 100 well-oriented half-crypts were counted per animal (n = 5). Cell position was recorded by counting up from the crypt base (cell position 1). In addition, single colon epithelial cells prepared from Lgr5-EGFP mice were stained with CD24-Pacific Blue (PB) antibody and placed on a thin-bottomed glass dish for confocal microscopic analysis (Olympus FV1000). Images at a magnification of ×60 were taken at focal planes 0.5 μm apart to generate an image stack of the cell suspension. The image stack was analyzed using National Institutes of Health ImageJ software to exclude doublets.

**Dissociation of colon epithelial cells.** A modification of the method described by Ramalingam et al. (21) was used to prepare single colon epithelial cells from mouse colonic tissue. Mouse colons were flushed with HBSS, sliced longitudinally to expose the crypts, washed in HBSS, and then incubated in 10 ml of a solution containing high-glucose DMEM (DMEM-H), 750 U of collagenase XI (catalog no. C9407, Sigma), 0.16 U of dispase (catalog no. 17105-041, Gibco), 5 mM CaCl2, and 1% FBS at 37°C for 1 h. After the tissue was washed in 15 ml of DMEM-H followed by 15 ml of PBS, it was incubated in 10 ml of a solution containing PBS, 60 mM EDTA, and 3 mM DTT at 4°C for 30 min. Immediately following this incubation, the tube was vigorously shaken for 2 min, and shed cells were collected in a separate 50-mL conical tube. This shaking-and-cell collection step was repeated twice after additional 15-min incubations of the remaining tissue in 10 ml of the PBS-EDTA-DTT solution. The residual intestinal tissue was discarded, and the isolated cells were centrifuged at 650 g for 5 min at 4°C. After removal of the supernatant, the pellet was resuspended in 10 ml of HBSS containing 13.5 U of dispase, 0.2 mg of DNase I, and 3 mM DTT and incubated at 37°C for 10 min, with gentle shaking every minute to avoid precipitation of the DNA. The sample was then passed successively through 70- and 40-μm filters and centrifuged at 650 g for 5 min at 4°C. The supernatant was removed, and the pellet was resuspended in PBS for cell counting and viability testing using Trypan blue staining. Live cells accounted for 90.8 ± 2.7% (n = 6) of the total dissociated cells.

**Flow cytometric analysis of isolated colon epithelial cells.** The isolated WT and Lgr5-EGFP mouse colonic epithelial cells, at a concentration of 106 cells/100 μL, were incubated for 30 min on ice

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with the following fluorescently labeled antibodies: CD24-PB (0.25 µg/10^6 cells; catalog no. 101820, BioLegend), epithelial cell adhesion molecule (EpCAM)-FITC (0.5 µg/10^6 cells; catalog no. 118207, BioLegend), or EpCAM-Alexa Fluor 647 (0.5 µg/10^6 cells; catalog no. 118211, BioLegend) and/or CD45-FITC (0.5 µg/10^6 cells; catalog no. 553080, BD Phamingen). The following isotype controls were used: rat IgG1 (catalog no. 12-4301-81, eBioso) and rat IgG2b (catalog no. 12-4031-81, eBioso). Post-EDU-injection WT mouse colonic epithelial cells were processed using the Click-iT Alexa Fluor 647 EdU Imaging Kit (Invitrogen) after labeling with the CD24-PB antibody. Cells were then washed and resuspended in PBS for analyses using a Dako Cytometry flow cytometer (Dako/Cytomation). Flow cytometry data were analyzed using Summit 4.3 software.

**Sorting and analysis of colonic epithelial cells for mRNA markers.** Proximal and distal colonic tissue from six WT mice was pooled for digestion, staining, and FACS, as described above. A MoFlo FACS machine (Dako/Cytomation) was used to collect EpCAM^+ single cells from the CD24^- and CD24^+ fractions. RNA was isolated using the RNeasy Micro Kit (Ambion), and cDNA was generated from these fractions, as well as intact proximal and distal colon, using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative RT-PCR (qRT-PCR) was conducted for each sample in triplicate. TaqMan probes [Actb (Mm0047937_5), Car2 (Mm00456116_m1), CD166 (Mm00515616_m1), CD44 (Mm0044950_m1), EphB2 (Mm01186021), Lgr5 (Mm00438991_m1), Lrig1 (Mm00456116_m1), Muc2 (Mm00458299_m1), and mTert (Mm00436931_m1)] were obtained from Applied Biosystems and used according to the manufacturer’s protocol. β-Actin RNA was used as an internal control, and cycle threshold (ΔC_T) values were calculated to obtain fold changes vs. intact colon (proximal or distal).

**Statistical analyses.** The proportion of total crypt length expressing CD24 in the proximal and distal colon was compared using a repeated-measures regression with an unstructured correlation matrix to account for correlation of repeated observations within animals. Numbers of EdU^- epithelial cells at each cell position for the proximal and distal colon were compared using a repeated-measures regression with EdU^- cells per 100 crypts as the response and site (proximal or distal) and cell position as predictor variables. A term for interaction between site and cell position was included in the model to examine whether the response across cell positions was different for the two sites. An autoregressive correlation matrix was modeled to account for the correlation across cell positions within a given mouse and site. A second-order interaction between site and cell position proved statistically significant, so it was also included in the model. For each gene in our qRT-PCR data, the distribution of fold changes for the CD24^+ and CD24^- fractions in relation to intact proximal or distal colon was examined for normality. Since the distributions did not violate the normality assumption, a t-test was used to compare mean fold changes in gene transcripts in the CD24^+ vs. CD24^- fractions of isolated single epithelial cells [2-sided t-test for carbonic anhydrase (Car2) and chromogranin A (Chga) and 1-sided t-test for CD166, cKit, doublecortin and calmodulin kinase-like 1 (DCAMKL-1), ephrin type B receptor 2 (EphB2), Lgr5, leucine-rich repeats and immunoglobulin domains (Lrig), mucin (Muc2), and murine telomerase reverse transcriptase (mTert)]. P < 0.05 was considered statistically significant. Analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC).

**RESULTS**

**Localization of CD24 expression in colonic tissue.** Although no evidence has precisely verified the location of CESC’s, in the mouse they are generally thought to reside at the colonic crypt base. However, the possibility of the CESC’s being in different locations in the proximal vs. distal colon, as is the proliferative zone (12, 24, 27), has been raised (8). To assess the location of CD24 expression in colonic crypts, sections from proximal and distal colon were subjected to immunohistochemical staining with CD24 antibody. Figure 1, A and B, shows expression of CD24 to be restricted to epithelial cells in the lower half of the crypt in the proximal colon, as well as to cells within the lamina propria. The high-power image (Fig. 1B) indicates that CD24 is expressed on the crypt base columnar cells and intervening GLCs. In the distal colon (Fig. 1, C and D), CD24 expression is more limited to the base of the crypt, while still present in the lamina propria. Although there are fewer secretory cells in the crypt base of this region, just as in the proximal colon, CD24 appears to be expressed on the crypt base columnar cells and the GLCs. The differential distribution of CD24 staining was verified by quantification of CD24 expression as a proportion of total crypt length (Fig. 1E).

**Flow cytometric identification of a CD24^+ epithelial fraction.** Because the colonic epithelial preparation involves enzymatic digestion and mechanical disruption of the epithelial layer, too vigorous a digestion theoretically could contaminate the epithelial isolate with cells from the underlying lamina propria. Therefore, to validate the purity of our epithelial preparation, we first used flow cytometric analysis of isolated cells stained with an antibody to the pan-epithelial marker EpCAM and the pan-leukocyte marker CD45. This analysis showed the dissociated cells to be largely epithelial (97.7 ± 0.8% EpCAM^+; n = 6; Fig. 2B) and devoid of hematopoietic cells (99.4% CD45^-; Fig. 2D). Next, we quantified epithelial CD24 expression by flow cytometry. The CD24^+ fraction accounted for 22.7 ± 2.9% (n = 6) of all dissociated cells (Fig. 2F) and 21.0 ± 4.2% (n = 6) of the EpCAM^+ epithelial cells (Fig. 2G). The vast majority (97.2%) of the CD24^- cells were CD45^- (Fig. 2H). Once the flow cytometric characteristics of our cell population (pooled from proximal and distal colon) were demonstrated, we quantified CD24 expression for each colonic region separately in light of differential CD24 expression seen on immunohistochemistry in the proximal vs. distal colon. The CD24^- fraction accounted for 26.0 ± 0.6% (n = 3) of all dissociated cells in the proximal colon and 24.6 ± 3.1% (n = 3) in the distal colon.

**EdU incorporation identifies a fraction of actively cycling CD24^- cells.** As noted in Fig. 1, CD24 appears to mark a heterogeneous population of epithelial cells in the murine colonic crypt; therefore, we wanted to verify that this population contained the actively cycling cells that would be candidate CESC’s. Fluorescence microscopic analyses of colonic tissue harvested after EdU injection showed cycling cells to be located primarily toward the midcrypt in the proximal colon (Fig. 3, A and B) and at the base of the crypt in the distal colon (Fig. 3, C and D). While the expression of CD24 (Fig. 1, C and D) overlaps closely with EdU positivity in the distal colon, in the proximal colon CD24 expression includes a portion of noncycling cells, particularly in the bottom third of the crypt. When quantified by cell position (Fig. 3E), the proximal colon was found to be EdU^- at higher cell positions than the distal colon. Moreover, there was a distinct difference in the pattern of distribution of the EdU^+ cells, with proximal colon showing a broad peak at cell position 4–9 and distal colon showing an increasing gradient toward the crypt base.

Analysis of CD24 and EdU staining by flow cytometry showed that, in the proximal colon, 7.4 ± 0.8% (n = 3; Fig. 4A) of the total isolated epithelial cells are actively cycling while, in the
distal colon, 4.6 ± 0.8% (n = 3; Fig. 4B) are actively cycling. Approximately one-quarter (26.4 ± 3.2, n = 3; Fig. 4A) of these cycling cells in the proximal colon and over one-half (58.4 ± 1.7, n = 3; Fig. 4B) of cycling cells in the distal colon are also CD24+. Conversely, only a small proportion of the CD24+ epithelial cells [14.3 ± 3.9 (proximal) and 8.2 ± 0.9 (distal), n = 3 for both; Fig. 4] was actively cycling at a given time.

CD24+ fraction of colonic epithelial cells is enriched in Lgr5+ cells. Since Lgr5 has been demonstrated via lineage tracing to be a marker of CESCs, our next goal was to compare expression of Lgr5 with CD24 using the Lgr5-EGFP transgenic mouse. Fluorescence microscopic analyses of colonic tissue showed Lgr5-EGFP to be localized to the lower third of the crypt in the proximal colon (Fig. 5, A and B) and the crypt base in the distal colon (Fig. 5, C and D), both within the zone of CD24 staining described previously (Fig. 1). As in the small intestine (30), occasional EGFP+ cells were observed in the upper crypts. Although they have not been definitively identified in the small or large intestine, on the basis of position and morphology, they probably represent enteroendocrine cells. Figure 5 also illustrates the mosaicism of Lgr5-EGFP expression seen throughout the colon.

Flow cytometric analyses of colonic epithelial preparations from Lgr5-EGFP mice showed that Lgr5-EGFP+ cells account for 5.2 ± 1.2% (n = 3) of total epithelial cells in the proximal colon (Fig. 6A) and 4.0 ± 0.6% (n = 3) of total epithelial cells in the distal colon (Fig. 6B). Costaining with anti-CD24 antibody revealed the majority of these Lgr5-EGFP+ cells also to be CD24+ (58.1 ± 2.5% proximal and 54.1 ± 3.7% distal, n = 3 for both; Fig. 6, A and B) in both regions of the colon. Given the mosaic expression of EGFP in this mouse model, calculating the reverse, percentage of CD24+ cells that also express Lgr5-EGFP, would greatly underestimate the coexpression of CD24 and intrinsic Lgr5.

To verify that the CD24+ Lgr5-EGFP+ fraction seen by flow cytometry was not reflective of doublets, isolated single colonic epithelial cells from an Lgr5-EGFP mouse were stained with CD24-PB antibody and evaluated by confocal microscopy. Figure 6C shows representative images of CD24+Lgr5-EGFP−, CD24−Lgr5-EGFP+, and CD24+Lgr5-EGFP+ single cells.

CD24 positivity identifies a fraction of colonic epithelial cells enriched in stem cell transcripts. Although the flow cytometry data show that the CD24+ fraction enriches for Lgr5+ epithelial stem cells in the transgenic Lgr5-EGFP mouse, to confirm enrichment of CESCs in the CD24+ fraction from WT mice, we performed qRT-PCR for Lgr5, EphB2, and CD166, which mark actively cycling CESCs (2, 11, 14), and Lrig, DCAMKL-1, and mTert, which mark quiescent CESCs (17, 18, 20). Given the staining pattern of CD24 in the colon (Fig. 1), we also wanted to determine the degree to which the CD24+ fraction enriched for Muc2 (a goblet cell and GLC...
marker) and cKit (a GLC marker). Lastly, we quantified transcripts of the other differentiated colonic cells in the sorted fractions to determine whether using CD24 positivity as a sorting criterion included or excluded enteroendocrine cells (Chga) and differentiated colonocytes (Car2). Figure 7 shows the CD24^+ fractions to be significantly enriched for all three active CESC markers (Lgr5, EphB2, and CD166) in the proximal colon compared with intact proximal colon and for Lgr5 and EphB2 in the distal colon compared with intact distal colon. All three quiescent CESC markers (Lrig, DCAMKL-1, and CD24^-)}
and mTert) were enriched in the CD24+ fractions in the proximal and distal colon compared with intact colon. The CD24+ fractions were also highly enriched in Muc2, cKit, and Chga mRNA, showing that CD24 indeed does mark a heterogeneous cell population, as in the small intestine (30). Transcripts for the colonocyte marker Car2 were deenriched in the CD24+ fraction vs. the CD24− fraction in the proximal and distal colon, although this only reached statistical significance in the distal colon. Transcripts for the myofibroblast marker Acta2 were not detectable in proximal or distal colon, further confirming the data in Fig. 2 showing these cell preparations to be essentially pure epithelium.

Fig. 3. A–D: mouse proximal and distal colon labeled with 4',6-diamidino-2-phenylindole (DAPI) and ethynyl deoxyuridine (EdU) at low (×10) and high (×20) power. E: quantification of EdU+ colonic epithelial cells by cell position. Values (means ± SE) represent mean number of EdU+ cells at each position per 100 colonic crypts (n = 5). Mathematical modeling of data showed significant differences in distribution pattern (P < 0.0001), with proximal colon showing a peak between cell position 6 and 7 and distal colon showing a gradual decline from the crypt base.

Fig. 4. Flow cytometric identification of a CD24+EdU+ fraction from colonic epithelium. A and B: cells from proximal and distal colon labeled with CD24-PB and EdU-Alexa 647. Values represent mean percentage of events falling within their respective quadrants (n = 3).
**DISCUSSION**

Studying the location and behavior of murine CESC has largely depended on genetically engineered Lgr5-EGFP (2) and Sox9-EGFP mice (21). In addition, other putative murine CESC markers (EphB2, CD166, DCAMKL-1, and mTert) have been proposed on the basis of crypt base staining patterns but have not been validated by cell sorting or Lgr5 or Sox9 enrichment (11, 14, 17, 18). Recently, using a proprietary Lrig1 antibody, Powell et al. (20) demonstrated that Lrig1 marks a predominantly quiescent murine CESC population through lineage tracing and postradiation colonic epithelial regeneration. In humans, two putative CESC markers, /H9252 integrin and EphB2, have been used to enrich for colonic epithelial cells able to proliferate in culture (7, 13). To our knowledge, the present study is the first report of a commercially available antibody to a cell surface marker, CD24, being used to isolate a population of colonic epithelial cells enriched in CESC from WT mice.

Evidence that our CD24-based sorting method generates a fraction enriched in actively cycling CESC has been demonstrated in several ways. First, we have shown that CD24-expressing murine colonic epithelial cells are located in the same region of the colonic crypt as the EdU actively cycling cells (Figs. 1 and 3). Furthermore, we have demonstrated by flow cytometry a fraction of CD24+ colonic epithelial cells that are actively cycling at any given time (Fig. 4). Because these actively cycling CD24+ cells could represent CESC or transit-amplifying cells, we next showed that CD24 expression is present in the majority of cells expressing the Lgr5-EGFP transgene (Fig. 6), representing CESC. Lastly, sorting with antibodies to CD24 revealed a CD24+ fraction of colonic epithelial cells that was enriched for the active CESC markers Lgr5, EphB2, and CD166 (Fig. 7). Interestingly, our qRT-PCR data also revealed significant enrichment for the quiescent CESC markers Lrig and DCAMKL-1.

Since CD24 is known to be expressed on white blood cells and endothelial cells, we assessed the purity of our epithelial...
cell dissociation method by labeling with anti-CD45, a pan-leukocyte marker, and EpCAM, a pan-epithelial marker. As seen in Fig. 2, B and D, our epithelial cell preparation was almost entirely composed of epithelial cells and contained rare hematopoietic cells. However, within the epithelial compartment, our CD24⁺ fraction was heterogeneous, containing putative CESC as well as mucin-producing cells and enteroendocrine cells. Although transcripts of Muc2 were highly enriched in our CD24⁺ fraction, particularly in the distal colon, this likely does not reflect the absolute number of contaminating Muc2-expressing cells. The critical point here is that increased levels of mRNA do not always correlate with an increase in cell number, particularly in secretory cells, where secreted products, such as Muc2, may have very high numbers of transcripts per cell. In contrast, in stem cells, Lgr5 mRNA is recognized as being a relatively rare transcript. At the time we initiated these studies, further purification of our CD24⁺ CESC fraction was limited by the lack of a surface marker for mucin-producing cells, which would allow antibody-based FACs. While this work was in progress, Rothenberg et al. (22) reported an elegant study in which they showed that the GLCs in the bases of murine colonic crypts are marked by expression of cKit. Flow cytometry showed that cKit⁺ cells had a higher mean fluorescence for CD24 than cKit⁻ cells; however, there was extensive overlap of the two populations. Thus this remains an area ripe for further investigation.

In the small intestine, it has been suggested that the CD24⁺ fraction typically obtained by FACS might actually represent doublets of CD24⁻ stem cells with CD24⁺ Paneth cells, instead of single CD24⁺ stem cells (25). As GLCs may serve a stem cell niche function in the colon similar to that served by Paneth cells in the small intestine (22), we verified that our CD24⁺Lgr5⁺ fraction indeed consists of single epithelial cells, rather than doublets of CD24⁺Lgr5⁻ CESC with CD24⁺Lgr5⁺ GLCs (Fig. 6C). The CD24⁺Lgr5⁺ population likely represents crypt base GLCs, while the CD24⁺Lgr5⁻ population likely represents the bright Lgr5⁺ cells toward the top of the crypt (Fig. 5), which appear morphologically to be enteroendocrine cells. In addition, we have verified that our gating strategy for doublet discrimination, using forward scatter vs. pulse width, is rigorous and reliable (data not shown).

Prior work by our group and others (10, 30) has shown that, in the murine jejunum, epithelial CD24 expression is largely confined to the crypt base, the region containing the small intestinal epithelial stem cells. Although it is widely assumed that CESC also reside at the base of the colonic crypt, we have provided evidence supporting former claims that the location of the stem cell zone, instead, varies along the length of the colon (8, 12, 24, 27). In particular, CD24 and Lgr5-EGFP expression track about halfway up the proximal colonic crypt while being confined to the crypt base in the distal colon (Figs. 1 and 5). This differential pattern of expression is mirrored by EdU incorporation into actively dividing cells, with the proliferative zone being in the midcrypt in the proximal colon and at the crypt base in the distal colon (Fig. 3), just as has been reported previously using titrated thymidine and bromodeoxyuridine incorporation in the proximal and distal rat and mouse colon (8, 12, 24, 27).

The reason for this differential pattern of CESC localization is not understood. One potential explanation is the existence in the proximal colon of a large number of mucin-producing GLCs at the crypt base, which reduces the amount of space available for the CESC. Therefore, the CD24-expressing CESC are effectively crowded out and, instead, reside farther up the crypt. Another explanation may involve the role of CD24 in signal transduction pathways. The proximal colon is where partially digested material deposited from the small intestine first interacts with the colonic microflora. As a result, a large number of metabolic products, e.g., cytokines, supportive or destructive to the colonic epithelium, are elaborated and join the milieu of luminal contents. This makes sense from an evolutionary perspective, as luminal sensors, such as CD24, on the surface of CESC may allow stem cells to react more quickly and more reliably to the constantly changing contents of the colonic lumen.

An unexpected and thought-provoking finding was the enrichment of DCAMKL-1 and Chga in the CD24⁺ fraction in the proximal and distal colon. In light of the recent publication by Van Landeghem et al. (29), our data suggest the presence of a subset of CD24⁺ cells in the colon that express the enteroendocrine marker Chga and could have quiescent CESC properties or the ability to gain CESC-like function in the setting of damage, such as the Sox9-EGFP⁻Chga⁺ epithelial cells in the small intestine (29). Furthermore, the DCAMKL-1-expressing cells could represent quiescent CESC (17) or colonic tuft cells (9). These findings warrant the reconsideration and reevaluation of the conventional model of four distinct cell types (CESC, goblet cells, enteroendocrine cells, and differentiated colonocytes) being present in the colon.
The incomplete understanding of the functional role of CD24 in normal murine colon physiology raises potential issues regarding the growing body of literature in which anti-CD24 therapies are used to directly target putative colorectal cancer stem cells (23, 26). Most of these studies have reported in vitro experiments or tumor xenograft models using human-specific anti-CD24 monoclonal antibodies to show inhibition of colon cancer tumor growth. Although no host effects have been observed in xenograft models, this is to be expected, as the human anti-CD24 monoclonal antibody is species-specific and does not cross-react with mouse. Therefore, before such promising anticancer therapies can translate into widespread clinical application, the potential negative implications to normal colon homeostasis must be completely understood. Our studies suggest that any CD24-based cytotoxic therapy will also destroy the CESC's if delivered locally to the colon and could impair the CESC's and small intestinal stem cells if delivered systemically. Although no overt colonic phenotype has been reported in CD24 knockout mice, Duckworth et al. (6) presented data showing that CD24 knockout mice are more adversely affected (increased apoptosis and decreased capacity for crypt regeneration) than WT mice when subjected to radiation and develop more severe colitis when challenged with dextran sodium sulfate. Clearly, further work is needed to directly assess these possibilities and to determine if there are molecular variants of CD24 that differ between tumors and normal tissue (e.g., due to distinct glycosylation patterns), thus reopening the potential for tumor-specific CD24-based therapy.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.B.K., R.J.v.F., and S.J.H. are responsible for conception and design of the research; J.B.K., B.J.S., and K.K.M. performed the experiments; J.B.K., R.J.v.F., J.A.G., and S.J.H. interpreted the results of the experiments; J.B.K., R.J.v.F., and B.J.S. prepared the figures; J.B.K. drafted the manuscript; J.B.K., J.A.G., and S.J.H. edited and revised the manuscript; J.B.K., R.J.v.F., B.J.S., K.K.M., J.A.G., and S.J.H. approved the final version of the manuscript.

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