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Characterization of the intestinal cancer stem cell marker, CD166/ALCAM, in the human and mouse gastrointestinal tract

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Abstract

Background & Aims—CD166 (also called activated leukocyte cell adhesion molecule, ALCAM) is a marker of colorectal cancer (CRC) stem cells; it is expressed by aggressive tumors. Although the presence of CD166 at the tumor cell surface has been correlated with shortened survival, little is known about its function and expression in normal intestinal epithelia.

Methods—We characterized the expression pattern of CD166 in normal intestinal tissue samples from humans and mice using immunohistochemistry, flow cytometry and quantitative reverse transcription PCR. Human and mouse intestinal tumors were also analyzed.

Results—CD166 was expressed on the surface of epithelial cells within the stem cell niche and along the length of the intestine; expression was conserved across species. In the small intestine, CD166 was observed on crypt-based Paneth cells and intervening crypt-based columnar cells (putative stem cells). A subset of CD166-positive, crypt-based columnar cells co-expressed the stem cell markers Lgr5, Musashi-1, or Dcamk1-1. CD166 was located in the cytoplasm and at the surface of cells within human CRC tumors. CD166-positive cells were also detected in benign adenomas in mice; rare cells co-expressed CD166 and CD44 or epithelial-specific antigen.

Conclusions—CD166 is highly expressed within the endogenous intestinal stem cell niche. CD166-positive cells appear at multiple stages of intestinal carcinoma progression, including benign and metastatic tumors. Further studies should investigate the function of CD166 in stem

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cells and the stem cell niche, which might have implications for normal intestinal homeostasis. CD166 has potential as a therapeutic target for CRC.

Keywords

colon cancer; intestinal stem cells; cancer stem cell

Colorectal cancer (CRC) is the third most prevalent cancer in the United States, with nearly 150,000 new cases diagnosed annually. Despite efforts to improve early detection and treatment, over one-third of patients die annually from this disease¹. The focus on cancer initiation and progression has dominated the effort to better understand disease pathology and guide therapeutic approaches. As such, the cancer stem cell (CSC) theory, which suggests that cancer is driven by cells harboring stem cell-like qualities, offers one explanation for why many current therapeutic approaches ultimately result in relapse of disease. In this model, some CSCs or cancer-initiating cells may be quiescent and, thus, evade eradication by standard cytotoxic therapies designed to target proliferating cells. These surviving cells can then proceed to support tumor growth and have potential to initiate recurrent or metastatic disease²⁻⁴. The reinvigoration of the CSC theory⁵⁻⁷ has led to identification, isolation and characterization of subsets of intestinal cancer cells that can recapitulate tumorigenesis in xenograft models⁸⁻¹². While some cell surface molecules, such as CD133 and CD44, have been shown to mark CSCs in multiple organs, an additional number of markers have shown promising CSC expression in intestinal cancer including Dcamk1-1, ESA and CD166^{10,11,13}.

CD166 or Activated Leukocyte Cell Adhesion Molecule (ALCAM) expression is pathologically correlated with aggressive disease in a variety of cancers including melanoma, prostate, breast, ovarian, esophageal, and bladder cancers¹⁴⁻²⁰. In human CRC, aberrant cell surface CD166 expression is strongly correlated with a 15 month shortened survival²¹. Further, isolation of CD166/CD44 or CD166/ESA double-positive cells from human CRCs cells can recapitulate tumorigenesis when xenografted at low numbers into immune-deficient mice¹⁰, a hallmark of a CSC population. Although these findings suggest that CD166 may have a role in the progression of CRC, little is known about its endogenous function and cellular localization within the intestine.

In other organ systems, CD166 has a myriad of functions. This conserved cell adhesion protein participates in physiologic processes including leukocyte intravasation across the blood brain barrier, monocyte migration across endothelial junctions, angiogenesis, capillary formation, protection against apoptosis in breast cancer cells, and T-cell activation by both antigen presenting and tumor cells²²⁻²⁸. Further, CD166 has been described as a ligand that binds to CD6 on thymic epithelium²⁹⁻³¹, acting in homophilic adhesion complexes between epithelial cells³², and as a cell surface marker for both a subset of hematopoietic progenitor cells^{33,34} and multipotent mesenchymal stem cells^{35,36}. Based upon the intriguing CD166 expression pattern in multiple stem cell populations, this molecule has a potential role in maintaining stem cells in both normal and disease states. However, the potential overlap between CD166 normal and tumorigenic physiologic function have not been defined because the normal intestinal expression pattern has not been reported. Further, based upon its multiple roles in tumor-related processes, it is possible CD166 plays an important role in tumor pathology.

Correlation of the CD166 expression pattern with aggressive disease has led to efforts for targeting this molecule as a cancer therapeutic. Treatment of cancer cells with a CD166-internalizing antibody conjugated to chemotherapy filled lipid vesicles was shown to effectively target and kill CD166-expressing ovarian cancer cells and prostate cancer cells *in*

vitro^{37,38}. While early results from these types of targeted cancer therapies appear promising, it necessitates an even more careful understanding of the endogenous expression pattern and function of CD166. In the current study, we analyzed CD166 expression in normal human and mouse intestine. We identified enriched cell surface CD166 expression in the colon and small intestine (SI) crypt-base. Interestingly in the SI, CD166 is expressed on the cell surface of the differentiated Paneth cell population and the intervening crypt-based columnar cells. Notably, both normal and tumor CD166 expression patterns were conserved in mice, highlighting the value of using a mouse model for studying CD166 function within the stem cell niche and in cancer. Further, we show that a subset of CD166-expressing cells residing in the stem cell niche co-express other putative stem cell markers, including Musashi-1 (Msi-1), Dcamk1-1 and Lgr5^{13,39-41}. We propose that CD166 defines the normal intestinal stem cell niche and encompasses both differentiated Paneth cells as well as stem cell and progenitor populations. A possible function for CD166 may be to maintain the epithelial microenvironment of the stem cell niche. Therefore, targeting this cell surface antigen in cancer therapy requires careful consideration of potential effects on normal tissues.

Material and Methods

Mice

Mice were housed in a specific pathogen-free environment under strictly controlled light cycle conditions, fed a standard rodent Lab Chow (#5001 PMI Nutrition International), and provided water *ad libitum*. All procedures were performed in accordance to the OHSU Animal Care and Use Committee. The C57Bl/6 and Apc^{Min/+} mice⁴² were purchased from The Jackson Laboratory (Bar Harbor, ME).

Immunohistochemical and histochemical analyses of intestinal tissue

Adult (>6 weeks) and embryonic [(E)14.5, 15.5, 16.5, 17.5, 18.5] mouse intestines were dissected and prepared for paraffin and frozen tissue analyses as we have previously described⁴³. Human SI and colonic tissue was fixed in 10% buffered formalin and embedded in paraffin or OCT. Five or 50µm tissue sections were stained with antibodies to CD166, Msi-1, Ki67, Serotonin, ChromograninA, ESA, CD44, Lysozyme and Laminin (Antibody information listed in Supplemental Table1). Antigen retrieval (10mM citrate buffer, pH=6 or 10mM Tris/1mM EDTA, pH=9 at 100°C for 20 minutes) was performed on paraffin embedded tissues. Visualization was performed using either fluorescent-conjugated species-specific secondary antibodies [Indocarbocyanine3 (Cy3), Indocarbocyanine5, Fluorescein isothiocyanate (FITC)] (1:500; Jackson ImmunoResearch), or brightfield diaminobenzidine (DAB) detection (Vectastain ABC kit; Vector) and Methyl green counter staining (Vector). Nuclear counterstaining with Hoechst dye (33258; Sigma; 0.1µg/ml) was performed for fluorescent analyses. For detection of CD166-positive Paneth cells, human SI tissue sections were incubated with antibodies to CD166, visualized with secondary Cy3-conjugated antibodies and images captured using a Leica DMR fluorescent microscope (Leica Microsystems). The tissue was then re-stained with Lendrum's Phloxine Tartrazine according to standard procedures⁴⁴, images recaptured and superimposed using Canvas X software (ACD). Confocal images were acquired as 0.5µm planes using an IX81 Inverted Microscope equipped with Fluoview FV1000-Spinning Disc Confocal (Olympus) scan head and FV10 ASW 1.7 software (Olympus).

To test specificity of CD166 antibodies, recombinant mouse CD166 (1 µg/mL; R&D Systems) was co-incubated with the primary CD166 antibody (15 µg/ml; R&D Systems) at room temperature for 30 minutes. Tissue sections were then incubated with the antibody peptide solution and visualized as described above.

Analyses of isolated intestinal epithelial cells

The differentiated (villus or colon cuff) and undifferentiated (crypt) epithelial cells of the mouse SI and colon were independently isolated using a modified Weiser preparation⁴⁵, stained with antibodies to CD166, and sorted using a Cytopeia Influx to collect CD166⁺ SI villus or colonic cuff epithelia and CD166⁺ SI and colonic crypt epithelia, or crypt CD166⁻ epithelia. Briefly, SI villus cells were isolated from 1mM EDTA washes and SI/colon crypt cells were isolated with subsequent 10mM EDTA washes at 4°C. Cells were resuspended in modified HBSS and gently filtered through a 45µm filter, then incubated on ice for 20 min with antibodies against CD45 conjugated to Allophycocyanin (APC) and CD166, followed by incubation with secondary antibodies conjugated to FITC for CD166 detection. Cells were resuspended in modified HBSS/5µg/ml propidium iodide/1% bovine serum albumin and sorted using a 150 µm nozzle, 4.5 psi and Spigot software. To insure sorting and analysis of single cells, a doublet discriminator was used to exclude cells based on pulse width. FACS data was analyzed using FCS Express Version 3 Research Edition (DeNovo Software). CD166⁺, CD45⁻, PI⁻ cells (10⁵) were collected and spun onto glass slides using a Shandon Cytospin 4 (Thermo Electron) and subsequently analyzed for expression of Paneth cell markers or expression of Lgr5 as described in the previous section.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) CD166⁺ populations

qRT-PCR was used to evaluate gene expression in isolated crypt and villus epithelium or in CD166⁺ FACS-isolated cells. Epithelial cell isolated described above. For crypt and villus epithelial expression, total RNA was isolated using RNeasy Mini kit (Qiagen) and cDNA was then generated using standard protocols⁴⁵. For stem cell marker expression total RNA was isolated from CD166⁺ and CD166⁻ FACS isolated cells using an RNAqueous kit (Ambion) and cDNA generated using a High Capacity cDNA Reverse Transcription kit (ABI). qRT-PCR was performed using a SYBR Green-based assay, a 7900HT Sequence Detector and analyzed according to established protocols⁴⁵. Each cDNA sample was analyzed in triplicate, along with triplicate samples of the endogenous reference gene, Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and reported as an average of n=2 runs. Data was normalized to Gapdh, then for CD166 expression, calibrated against levels within the villus epithelium, or for stem cell genes, calibrated against CD166⁻ expression. Primers listed in Table1.

Results

CD166 protein expression is enhanced in the base of the human and mouse small intestinal and colonic crypt epithelium

CD166 expression has been documented in human CRC²¹, but extensive evaluation of its expression pattern in normal tissue has not been performed. A previous study localizes CD166 to the cytoplasm of normal colonic epithelial cells within the crypt base²¹. When we stained normal human SI and colonic tissue sections with antibodies to CD166, we detected a different intestinal staining pattern. Consistent with its function in immune cells, CD166-expressing cells were detected within the intestinal mesenchyme (Fig.1A, arrows). In the epithelial compartment, detection with fluorescent and brightfield immunohistochemistry revealed enriched cell surface expression of CD166 protein in epithelial cells at the base of the crypts in both the SI (Fig.1A-B,E; arrowheads) and colon (Fig.1C-D,F; arrowheads). Peptide competition with the CD166 antibody supported the specificity of the CD166 antibody recognition pattern (Supplemental Fig.1). Importantly, in contrast to previous report²¹, CD166 expression appeared strongest on the cell surface, and was more robustly expressed on cells that resided in the base of the crypt.

Optimal immunohistochemical expression patterns and *in vivo* molecular manipulations required to define functional properties are not readily obtainable within human tissues. Therefore, to validate the mouse as a viable model organism for future studies examining the role of CD166 in normal intestinal physiology and in tumorigenesis, we extended our analysis of CD166 to the mouse intestine. Because the mouse intestine is easily dissected, oriented and manipulated, it allowed for a more in-depth analysis of CD166 intestinal epithelial expression. As expected, the mouse expression pattern of CD166 recapitulated that of the human expression pattern (Fig.2). Importantly, the cell surface localization and increased expression levels on cells within the crypt-base were more readily appreciated (Fig.2A-D). Further, high expression levels on cells of the stem cell niche was confirmed on the RNA level using a differential isolation of villus and crypt epithelial cells followed by qRT-PCR and primers for CD166 (Supplemental Fig.2B). Interestingly, crypt-based expression did not vary down the length of the SI, which has not been the case for other putative stem cell markers such as Bmi1⁴⁶. Because the protein expression was more readily detectable in the mouse, increased resolution of the distinct expression domain in the small intestinal crypt was apparent. CD166 expression appeared to be predominantly on the cell surface of the lower crypt-base cells (Fig.2B). Low levels of cell surface CD166 were also detected on the small intestinal villus when sectioned on a tangential plane and by qRT-PCR (Supplemental Fig.2A,B). Although it is apparent that CD166-expressing cells reside in the mesenchyme, a subpopulation of CD166-expressing epithelial cells were also present within the intestine; CD166/ESA double-stained cells are apparent within the intestinal epithelial crypt base (Fig.2C).

To confirm cell surface expression on epithelial cells, we isolated the intestinal epithelium using a method that disrupts epithelial cell adhesion complexes⁴⁵, then performed FACS to isolate CD166-positive epithelium. Enriched populations of differentiated, villus epithelium and undifferentiated crypt-based epithelium were isolated (Fig.2E-F). The crypt epithelium contained a sizable CD166-expressing population (8.1%). Reanalysis of the CD166-expressing crypt cell population revealed two distinct populations based upon forward scatter (FSC, cell volume) and side scatter (SSC, inner complexity including type of cytoplasmic granules) (Fig.3A). Isolation of these intact crypt-based CD166-expressing cells allowed for a closer examination of cell identity.

Both differentiated Paneth cells and intestinal stem cells reside in the region marked by CD166-expressing cells. Isolated CD166-expressing epithelia were cytopun (Fig.3B) and subsequently analyzed for marker expression to determine if they were Paneth cells or stem cells (Fig.3C-F). A subset of FACS-isolated CD166-expressing cells stained with Phloxine Tartrazine, an established Paneth cell histochemical stain (Fig.3B-C; arrowheads). This approach bypasses non-specific cross-reactivity of antibodies with Paneth cell granules on cut tissue surface because intact cells are analyzed. For completeness, *in vivo* co-localization of CD166 expression and Paneth cells, co-staining of mouse small intestinal tissue with antibodies to CD166 and lysozyme was performed. As predicted, these two markers co-localized within the crypt base (Supplement Fig.3A-C). The human expression pattern was also consistent with the mouse pattern in the SI as determined by sequential staining of human tissue with antibodies to CD166 and the histochemical stain, Phloxine Tartrazine (Supplemental Fig.3D-F, arrowheads). Further, a subset of CD166-expressing crypt-based cells also co-expressed markers for differentiated or differentiating enteroendocrine cells (5-HT and Chromogranin A; Supplemental Fig.4).

Interestingly, there was a population of FACS-isolated CD166-positive cells that did not co-stain with Phloxine Tartrazine (Fig.3B,C; arrows) or enteroendocrine markers (Supplemental Fig.4). To determine if this subset of cells co-expressed putative stem cell markers, FACS-isolated CD166-expressing cells were cytopun then analyzed for Lgr5

expression using antibodies. Lgr5-positive cells represented a small fraction of CD166-positive crypt-based cells (Fig.3D-F; arrowheads). Additional putative stem cell markers, Msi-1³⁹ and Dcamkl-1, also shared overlapping expression with a subset of CD166-expressing cells (Fig.4A,C), but extended into the adjacent CD166-negative zone (Fig. 4B,D). Further expression analysis of putative stem cell markers reported in the intestine, other organ systems, or in cancer stem cells showed high relative expression levels in CD166⁺ versus CD166⁻ isolated crypt-based cell populations by qRT-PCR (Fig.4E); this includes *Ascl2*⁴⁷, *Lgr5*⁴¹, *CD24*^{48,49}, *Klf5*⁵⁰, *CD44*^{10, 51}, *Lrig1*⁵². As expected, expression of an intestinal epithelial differentiation gene, *Fabpl*⁵³, is decreased in the CD166⁺ cell population.

Interestingly, in contrast to the adult intestine, analysis of the developing mouse intestine revealed that CD166 expression was ubiquitously expressed in the epithelium at embryonic day (E)14.5 (Supplemental Fig.5A). However, at the onset of villus formation, E16.5, CD166 expression became localized to both the villus (arrows) and intervillus region (arrowheads; Supplemental Fig.5B), and by post-natal (P) development, expression was localized in the intervillus region (P4; arrowhead; Supplemental Fig. 5C).

CD166 is highly expressed in human colon adenocarcinoma and liver metastases

To further characterize the expression patterns of CD166 during intestinal tumorigenesis, we stained human adenocarcinoma and liver metastases with antibodies to CD166. We identified both cell surface and cytoplasmic expression in primary tumors and metastases (Fig.5A-C). Human tumors were decidedly heterogeneous in their CD166 expression. While some tumor samples exhibited only cell surface expression (Fig.5A), others exhibited cytoplasmic expression (Fig.5B). CD166-positive cells generally appeared within clustered regions of epithelium. Interestingly, CD166 expression within liver metastasis was also heterogeneous, shown here as cytoplasmic expression (Fig.5C).

Tumors in a mouse model for intestinal tumorigenesis, the *Apc*^{Min/+} mouse⁴², displayed a strikingly similar CD166 expression pattern compared to human colorectal tumors (Fig.5D). Both predominant cell surface staining and diffuse cytoplasmic expression was detected. Interestingly, only a subset of the CD166-expressing tumor cells was in the cell cycle, as determined by co-expression of the proliferative marker Ki67 (Supplemental Fig.6). This might reflect the possibility that at any one time, only a subset of CSCs were actively cycling. Supporting this notion, in crypt-like regions of the *Apc*^{Min/+} mouse intestine, Ki67 generally marked the transient-amplifying cell population (Supplemental Fig.6B, bracket), but also marked a rare subset of CD166 crypt-base columnar epithelial cells.

To further characterize the expression domain of CD166 in intestinal tumors, we performed double staining with CD166 and either CD44 or ESA on human or *Apc*^{Min/+} mouse intestinal tumor sections. CD44 and ESA were previously used in combination with CD166 to identify and isolate a CSC population in human CRC¹⁰. We found that CD44 was detectable on most human tumor epithelial cells (Fig.5Ea), but that the overlapping expression region with CD166 was somewhat more restricted (Fig.5Eb, arrows). Interestingly, CD166 expression was generally lost in the aberrant crypt structures and, therefore, CD166 and CD44 were primarily expressed in mutually exclusive cell populations. However, there was a small subset of dual-expressing cells (Fig.5Eb, arrows). In contrast, ESA was lost on large clusters of tumor cells, but a discrete double-labeled population of cells existed within both human and mouse tumors, shown here on *Apc*^{Min/+} tumors (Fig.5Fa-Fc, arrow).

Discussion

The elucidation of CD166 expression within the stem cell niche of the small intestine and colon suggests an intriguing and potentially important role for this molecule in the intestinal stem cell niche. Although we show that CD166 was expressed at low levels in differentiated intestinal cells, it is robustly expressed at high levels on the cell surface of cells within the stem cell niche at the base of the crypt. In the SI, CD166 was distinctly present on both putative stem cell populations comprised of the crypt-based columnar epithelial cells (Lgr5⁺, Msi-1⁺, Dcamkl-1⁺), as well as the crypt-based differentiated Paneth cell population. In light of its previously described role in cell adhesion and its capacity to form homodimers across adjacent cell membranes, it is intriguing to postulate that CD166 may have an important function in anchoring the stem cell within the intestinal stem cell niche, or in instructing stem cell behavior. In support of this, a precedent exists for the participation of adhesion molecules in establishing cell polarity or directing asymmetric stem cell division⁵⁴.

A number of intestinal stem cell markers (Fig.4) were co-expressed in CD166-expressing crypt cells. In contrast, the putative stem cell marker Msi-1 was often expressed in a single crypt cell within a zone of CD166-positive cells. Interestingly, Msi-1 was also expressed in CD166-negative regions. The current number of putative stem cell markers clearly highlights the complexity of stem and progenitor cell dynamics within the intestinal crypt. While CD166 may be shown, in the future, to mark stem cells, it is more likely, based upon its broader expression pattern, that it is actually a stem cell niche marker, expressed on both the stem cell and the surrounding supporting epithelial cells. In this context, the understanding of how niche cells influence stem cell behavior during homeostasis, tissue regeneration and disease can be examined in this newly defined cluster of crypt-based cells.

CD166 may possess multiple functions within the intestinal epithelium. This is suggested by its multi-faceted expression pattern in subsets of fully differentiated Paneth and enteroendocrine cells juxtaposed to its expression in a putative stem population. Future exploration of CD166 differential function and regulation in intestinal epithelium will contribute to a better understanding of whether its dysregulation contributes to disease progression in intestinal cancer, and may provide insight into whether CD166 has active function on CRC stem cells, or whether it is merely a “coincidental” marker.

Consistent CD166 expression in both human and mouse tumors demonstrates that the mouse provides a viable model for studying the function and expression of CD166 in tumorigenesis. Interestingly, CD166 was highly expressed in early adenoma formation in the Apc^{Min/+} mouse. Further, we confirmed that CD166 expression was retained within human CRC and metastatic disease, and that both a cell surface and cytoplasmic expression pattern was apparent. These findings, in particular an alteration in cellular localization of CD166, support a potential functional role for this molecule in tumorigenesis in either a cell adhesion or signaling capacity. Our analyses extend these initial findings and show that the observed expression patterns are also retained in metastatic lesions.

CD166 expression relative to other CSC and proliferative markers in the Apc^{Min/+} mouse recapitulated previous findings in humans¹⁰ that cells positive for both CD166, CD44 and ESA constitute a small subpopulation of total tumor mass. By analyzing the expression pattern of these markers in the Apc^{Min/+} mouse, a model of pre-neoplastic intestinal cancer, our findings suggest that mere co-expression of these markers may not be sufficient to promote invasive tumorigenesis. Alternatively, the presence of cells harboring these markers might suggest that these benign tumors have the potential for metastatic advance. Analysis of CD166 expression in Apc^{Min/+} polyps found that crypt-like structures near the muscularis tend to be low or lack expression of CD166 although they are high in CD44 and Ki67

expression. While the significance of this observation is not known, it is possible that loss of CD166 cell surface expression is a precursor for tumor progression. While our study does not evaluate in depth the percentage of CD166/ESA/CD44 triple positive cells within a benign tumor, we do not rule out the possibility that early or benign tumor states may harbor few numbers or lack cells capable of acting as CSCs. Importantly, our data reveals robust cell surface expression of CD166 within the endogenous intestinal stem cell niche, suggesting that as a cell adhesion molecule, it may play an important role in maintaining the integrity of the stem cell niche, or in directing cells to the crypt base. Inarguably, future studies examining the epithelial function of CD166 within the stem cell niche remain a critical focus for understanding the importance of this molecule in homeostasis and in disease; studies in cell lines and in enterospheres will facilitate this understanding. Ultimately, however, based upon the robust expression of CD166 within the stem cell niche, functional inhibition of this molecule has the potential to disrupt the maintenance of the stem cell niche and thereby compromise the epithelial barrier. Our data provides caution for therapeutically targeting CD166 for cancer.

Conclusions

Cell surface antigen expression of CD166 was recently identified as an important marker on human intestinal CSCs¹⁰. Along with this observation and its history in cancer progression as a marker for aggressive disease²¹, CD166 has been proposed as an intriguing molecule for therapeutic targeting in the treatment of cancer. For effective targeting of any cell surface antigen, its endogenous expression pattern must first be elucidated. Here, we report a broad range of CD166 expression patterns in the human and mouse intestine. We show that CD166 is expressed on a number of intestinal cells, including putative stem cells and differentiated crypt-based cells. This discovery provides important implications for future targeting of CD166 in disease therapy and, significantly, provides insight into the potential functional role of this critical molecule.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

5-HT	5-hydroxytryptamine
ALCAM	Activated leukocyte cell adhesion molecule
APC	Allophycocyanin
APC/Min mouse	Adenomatous polyposis coli, multiple intestinal neoplasm mouse
CRC	Colorectal cancer
CSC	Cancer stem cell
Cy3	Indocarbocyanine-3

DAB	Di-aminobenzidine
Dcamkl-1	Doublecortin and calmodulin kinase-like 1
E	Embryonic day
EDTA	Ethylenediaminetetraacetic acid
ESA	Epithelial specific antigen
FACS	Fluorescent-activated cell sorting
FITC	Fluorescein isothiocyanate
HBSS	Hanks buffered saline solution
Msi-1	Musashi-1
SI	Small intestine

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Figure 1. CD166 expression pattern in the human small intestine and colon

(A-C) Human small intestine stained with antibodies to CD166 (red) and counterstained with Hoechst (blue). (A) CD166-positive cells are located within the mesenchymal (arrows) and epithelial compartments (arrowheads). (Ba-Bb) Enlarged view of small intestinal crypts. (C-Da,Db) Human colon stained with antibodies to CD166, visualized with DAB (brown) in (C) and with fluorescence (red) and Hoechst counterstain (blue) in (Da-Db). Arrowheads point to cell surface epithelial expression. (E,F) larger magnification views of the crypt base boxed in yellow from B and D, respectively. Solid lines demark epithelial-mesenchymal boundary and dashed lines mark the apical epithelial surface. Bar=25 μ m.

Figure 2. CD166 expression pattern in the mouse small intestine and colon

(A-B) Mouse small intestine (SI) stained with antibodies to CD166 (red) and (A) counterstained with Hoechst (blue) or (B) co-stained with antibodies to laminin (gray) in higher magnification of the crypt. Arrowheads mark CD166-expressing cells. Arrows mark columnar crypt-based cell in B. (C) Mouse small intestinal crypt co-stained with CD166 (red) and the pan-epithelial ESA (white). (D) Mouse colon stained with antibodies to CD166 (brown), demonstrating enhanced expression in the crypt base (black arrowheads). Solid lines denote the epithelial-mesenchymal boundary. Bar=25μm. (E) Flow cytometry isotype control on isolated intestinal epithelial crypt cells. (F) Flow cytometry analysis of isolated crypt epithelial cells stained with CD166 antibodies. Box denotes CD166-positive, CD45-negative cells.

Figure 3. FACS-isolated CD166 epithelial cells express Paneth cell granules or Lgr5
 (A) Forward (FSC) and side (SSC) scatter analysis of CD166-expressing crypt epithelial cells display two distinct populations of cells (orange centers). (B-C) FACS-isolated, cytopun CD166-expressing mouse crypt cells (B; purple) stained with (C) Phloxine Tartrazine. White arrowheads designate CD166-positive cells; orange arrowheads designate CD166-positive, Phloxine Tartrazine-positive cells. Arrows and dashed circles designate CD166-positive, Phloxine Tartrazine-negative cells. (D-F) Cytospun, isolated CD166-positive cells (purple) co-stained with antibodies to Lgr5, a putative stem cell marker (green). Arrowhead designates a double-labeled cell.

Figure 4. CD166 is expressed in a subset of cells expressing stem cell markers Musashi-1 and Dcamkl-1

(A,B) Mouse small intestinal tissue co-labeled with antibodies to CD166 (white) and the putative stem cell marker, Musashi-1 (Msi, red). (A) White box and yellow arrowhead designates a CD166/Msi co-expressing cell with cell surface co-expressing in light blue. Bottom right insets depict higher magnification of singly stained cell, CD166 (white) and Msi (red). (B) A Msi-positive, CD166-negative cells indicated with arrow. (C,D) Mouse intestinal tissue co-labeled with antibodies to CD166 (white) and the putative stem cell marker, Dcamkl-1 (green). (C) Yellow arrowhead and box designates a CD166/Dcamkl co-expressing cell, with the co-expressing cell surface in purple. A higher magnification is provided in the upper right inset. (D) CD166-negative, Dcamkl-1-positive cells are designated by arrowhead. Brackets mark CD166-expressing region. White lines mark epithelial-mesenchymal boundary. Bar=25 μ m. (E) Quantitative RT-PCR analysis of stem cell markers within the CD166-positive cell population relative to the adjacent CD166-negative population. Triplicate samples of n=2 runs, and S.E.M.

Figure 5. CD166 expression of human colorectal cancer is recapitulated in mouse colorectal adenomas

(A-B) Human primary colorectal adenomas labeled with antibodies to CD166 show (A) cell surface expression (white, arrowheads) or (B) cytoplasmic expression (brown, arrow). (C) Human colorectal liver metastasis that has both cell surface and cytoplasmic expression of CD166 (brown, arrow). Methyl Green nuclear counterstain (green). (D) Benign mouse intestinal tumor labeled with antibodies to CD166 (white, arrowheads) and Hoechst nuclear counterstain (blue). (Ea-Eb) Human primary colorectal adenoma co-stained with antibodies to CD44 (red) and CD166 (white). (Eb) Subpopulations of tumor cells express both CD44 and CD166 (merged, light blue, white arrows), or only CD44 (red staining, red arrowheads). Some tumor cells do not express CD44 but express CD166 (white staining, white arrowheads). (Fa-Fc) Mouse adenoma stained with antibodies to CD166 (white) and ESA (red). Subpopulations of cells express CD166 and ESA (merged, light blue, white arrow), ESA alone (red arrowhead) or CD166 alone (white arrowhead). Bar=25 μ m.