ROLE OF THE CELL ADHESION MOLECULE CD166 IN HOMEOSTATIC MAINTENANCE AND REGENERATION OF THE INTESTINAL STEM CELL NICHE

by

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CERTIFICATE OF APPROVAL

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ABBREVIATIONS

ADCs	Antibody drug conjugates
ALCAM	Activated leukocyte cell adhesion molecule
APC/Apc	Adenomatous Polyposis Coli
APC	Allophycocyanin
Ascl2	Achaete-scute complex homolog 2
BMDC	Bone Marrow Derived Cell
BMI1/Bmi1	BMI1 polycomb ring finger oncogene
BMT	Bone Marrow Transplant
BrdU	5-Bromo-2-deoxyuridine
β-gal	beta-galactosidase
CBC	Crypt Base Columnar Cell
cDNA	Complementary Deoxyribonucleic Acid
CRC	Colorectal Cancer
Cre	Cre-recombinase
CSC	Cancer Stem Cell
CT	Computed Tomography
C-terminal	Carboxyl-terminal
Cy3	Indocarbocyanine
Cy5	Indodicarbocyanine
DAB	Diaminobenzidine
Dkk1	Dickkopf1
DNA	Deoxyribonucleic Acid
Dsh	Dishevelled
DSI	Distal Small Intestine
DTT	Dithiothreitol
E	Embryonic (day of gestation)
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced Green Fluorescent Protein
EMT	Epithelial-Mesenchymal Transition
EpCAM	Epithelial Cell Adhesion Molecule
ESA	Epithelial Surface Antigen
FACS	Fluorescence Activated Cell Sorting
FITC	Fluorescein
GALT	Gut-Associated Lymphatic Tissue
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green Fluorescent Protein
GI	Gastrointestinal
Gsk3	Glycogen Synthase Kinase 3

H^3	Tritiated Thymidine
H&E	Hematoxylin and Eosin
HBSS	Hank's Buffered Saline Solution
HSC	Hematopoietic Stem Cell
IHC	Immunohistochemistry
Lgr5	Leucine-rich-repeat-containing G-protein-coupled receptor 5
LRC	Label Retaining Cell
Mam	Mastermind
MIN	Multiple Intestinal Neoplasia
mRNA	Messenger Ribonucleic Acid
MSC	Mesenchymal Stem Cell
Msi1	Musashi1
Nicd	Notch intracellular domain
P	Postnatal (day)
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PI	Propidium Iodide
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RNA	Ribonucleic Acid
SEM	Standard Error of Mean
SI	Small Intestine
Tace	Tnf-α-Converting Enzyme
TA-cell	Transit Amplifying Cell
Tcf4	T-cell factor 4
WBM	Whole Bone Marrow
WT	Wild-type

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To my family and teachers
who have helped guide my enthusiasm for the unknown
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used in this research for their tissue.
Hundreds more bred to maintain colonies or were euthanized
without being directly used to answer research questions.

This dissertation is produced in full compliance with the government's regulations for conserving paper and other essential materials.

ABSTRACT

The epithelial lining of the intestine is one of the human body's most rapidly proliferating tissues, with complete renewal every 4-8 days. This expansive process is orchestrated by the intestinal stem cell and requires a fine balance between proliferation, differentiation, migration and cell death. Tight regulation of stem cell behavior is required to maintain the architecture and normal function of the tissue, and to prevent manifestation of diseases, such as cancer. The unique secondary structure of the intestinal crypt provides a regulatory niche for the stem cell. However, mechanisms coordinating signaling pathways to instruct stem cell behavior are poorly understood. Cell adhesion complexes are known to direct and regulate cell signaling and subsequent cellular behavior. Intriguingly, the cell adhesion molecule, CD166, was recently described as a cancer stem cell molecule in colorectal cancer. In the normal intestine, CD166 function is unknown. Based upon its relevance in colorectal cancer and its recently described expression within the hematopoietic stem cell niche, I hypothesize that CD166 is expressed within the intestinal stem cell niche and may participate in regulation of stem cell homeostasis. To directly test this hypothesis, we investigated the expression pattern of CD166 in the normal intestine and interrogated stem cell expression within the CD166-positive epithelial population. Further, to understand the normal function of CD166 within the intestinal stem cell niche we explored the impact of loss of CD166 on intestinal stem cell homeostasis. Intriguingly, we determined that CD166 expression within the intestinal crypt was tightly restricted to a domain

encompassing both Lgr5-positive progenitor cells and neighboring differentiated Paneth cells. This discrete expression domain is highly suggestive of an important regulatory relationship between the stem cell and neighboring niche cells. Consistent with this concept, we discovered that intestines from CD166-null mice harbored defects in proliferation, Wnt signaling and migration. These results were manifested, in part, by a reduction in stem cell numbers and crypt size. Radiation challenge to the intestine, as a means of testing activation of stem cell proliferation, revealed that CD166-null intestines harbored a delay in a proliferative response. Our findings support the notion that CD166 participates as a regulatory niche molecule in establishing a homeostatic set point in the intestine and in coordinating proliferative response. This important advance has implications for normal epithelial regeneration and cancer. Significantly, targeting CD166 may provide a novel mechanism for disrupting the interaction between the CD166-expressing cancer stem cell and its surrounding microenvironment.

CHAPTER 1

BACKGROUND AND INTRODUCTION

Structure and Function of the Mammalian Intestine

In mammals, the digestive tract is compartmentalized to optimize consumption of large amounts of food and permit efficient digestion, fermentation and nutrient absorption. Primary nutrient absorption occurs in the small intestine and is facilitated by the intestinal structure. The small intestine is coiled within the abdomen and organized into a long tube lined with simple columnar epithelium (Figure 1.1). The intestinal epithelium that forms a large contiguous sheet of cells organized into a complex secondary structure along the radial axis. Villi, large finger-like protrusions project into the lumen of the gut tube and are situated in between flask-like invaginations known as crypts of Lieberkühn (Figure 1.2A). Between the crypts that line the floor of the intestine, villi, large finger-like protrusions, project into the gut tube lumen (Figure 1.2A). This organization reflects the primary function of the intestine, nutrient absorption. To facilitate optimal nutrient absorption, organization of the intestine into crypts and villi increases the surface area of the intestine approximately 600-fold[1]. Surface area is further enhanced by micro-villi on the apical surface of absorptive epithelium[1]. To protect from the external environment, the intestinal epithelium forms an impermeable barrier facilitated by adhesive tight junctions and adherens junctions linking epithelial cells. In addition, the intestinal epithelium is completely renewed every 4-8 days, further preventing bacterial infection and providing fresh cells with maximal absorptive capacity[2].

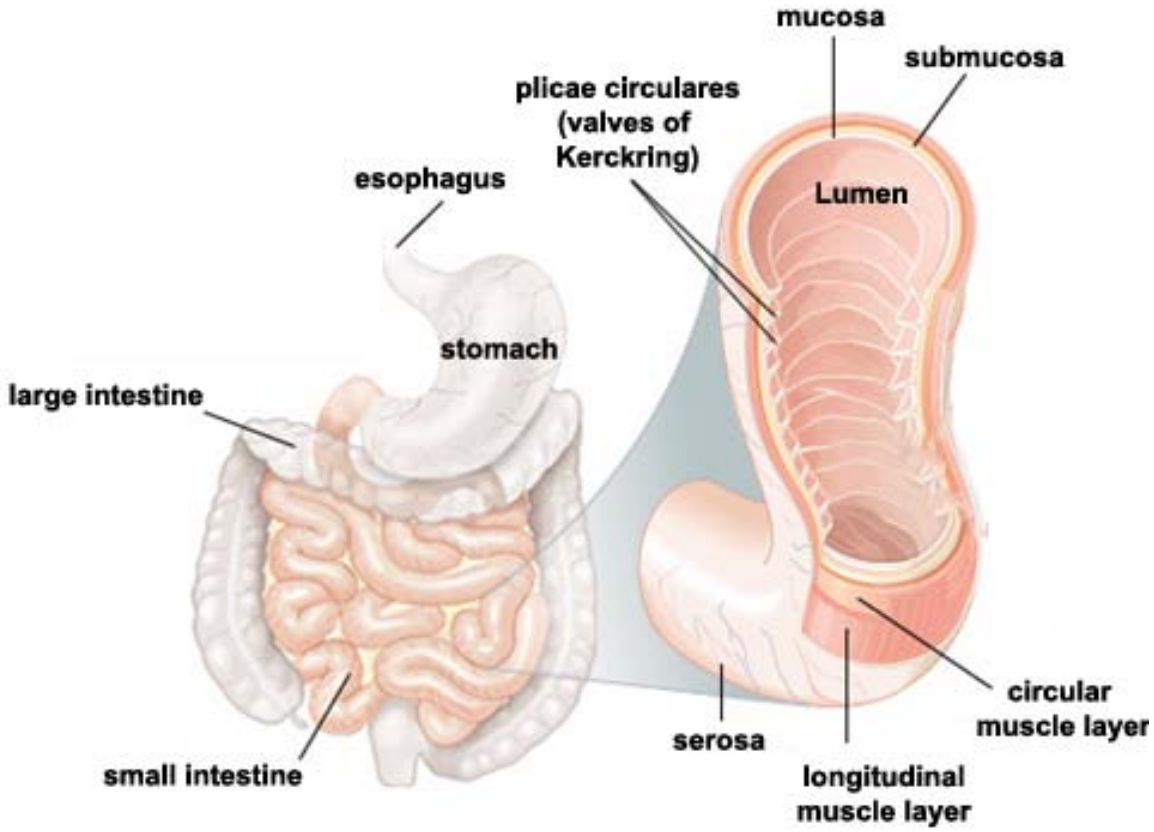

Figure 1.1 Organization of the human digestive tract. The small and large intestine are coiled within the abdomen. The lumen of the intestine is lined with a contiguous sheet of epithelium. © 2003 Encyclopedia Britannica, Inc. Reprinted in compliance with Britannica online services usage agreement.

Rapid renewal of the intestinal epithelium, facilitated by active proliferation, is coordinated by the intestinal stem cell. At least two multipotent stem cell populations exist in the small intestine, a rapidly dividing population of crypt base columnar cells and a rarely dividing population of cells located 4-5 cells from the base of the crypt (+4 population)[3]. While the relationship between these progenitors is not yet clear, both populations likely have unique function. Limited proliferation has been observed in the +4 population following targeted injury to the crypt base columnar cells suggesting this population may be responsible for tissue regeneration after insults which kill rapidly proliferating cells[4]. Conversely, the crypt-base columnar population, which divides rapidly during homeostasis, has been shown to primarily undergo cell death after radiation injury (unpublished data from our lab). Significantly, the unique response of these two progenitor populations after injury may help protect cells from the accumulation of genetic mutations which result from genotoxic stressors such as radiation. Stimulation of quiescent--and therefore resistant--cells to replace the dying damaged cells helps protect the integrity of the intestine and diseases like cancer are minimized.

During tissue homeostasis approximately 14 rapid cycling crypt-base columnar stem cells reside at the base of each crypt and give rise to progeny once per day[5]. Through this division, a subset of daughter cells are displaced out of the bottom of the crypt and become transit-amplifying cells (TA-cells). TA-cells rapidly divide every 12-16 hours[6] to amplify cell numbers for effective renewal of the epithelium. The progeny of the TA-cells then actively migrate up

and out of the crypt onto the villus and simultaneously differentiate into three primary epithelial cell lineages: nutrient-absorbing enterocytes, mucin-producing goblet cells, and hormone-producing enteroendocrine cells. A fourth common lineage, the anti-microbial producing Paneth cells, migrates downward into the base of the crypt where they reside adjacent to stem cells (Figure 1.2A, B)[7-11]. While the earliest description of intestinal stem cells was initially based on histological appearance and location[12], functional characterization of this diverse cell population has only recently occurred with the discovery of stem cell-specific molecular markers[5, 13-18].

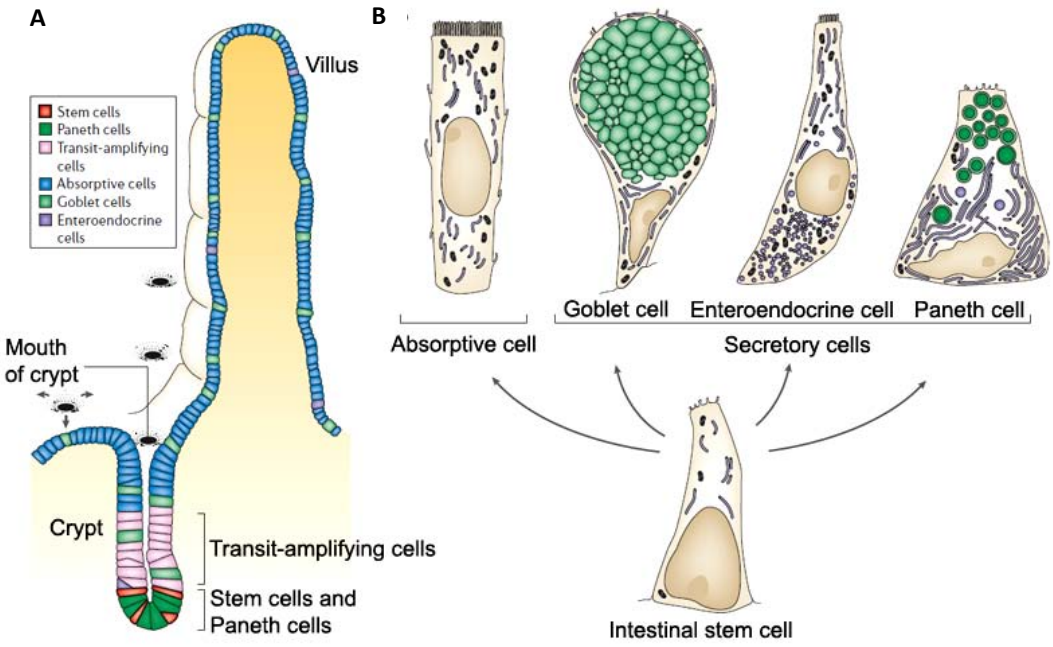

Figure 1.2 The structure and distribution of epithelial cell types within the small intestine. (A) The architecture of the small intestine is organized into flask-like invaginations called crypts and large finger-like projections called villi. Absorptive enterocytes, goblet and enteroendocrine cells are located on the villus while Paneth cells, stem cells and transit-amplifying cells are located in the crypt. **(B)** Multi-potent intestinal stem cells give rise to the differentiated lineages of the intestine: enterocytes, goblet cells, enteroendocrine cells and Paneth cells. Panel A reproduced with permission from The Royal Society ©1998[19]. Panel B reproduced with permission from John Wiley & Sons, Inc. ©1974[12]. Figure modifications originally published by Crosnier et al., 2006[20].

Identification of Intestinal Stem Cells

The earliest functional characterization of small intestinal stem cells were based on results obtained from DNA-label retaining studies of the mouse intestine using tritiated thymidine (H^3)[21,22]. In these long-term label retaining studies, mice were injected with H^3, which incorporates into cells actively copying DNA during cell division. The mice were then allowed to live for many months and up to a year before intestines were analyzed. This lengthy time course allowed differentiated and rapidly dividing H^3-labeld cells to migrate off the villus, only leaving labeled stem/progenitors to be retained in the intestine which divide less frequently. A rare label-retaining epithelial cell population was identified, often located at cell position 4-5 above the center base of crypts[21]. Around this same time (the mid 1970's) the crypt base columnar cell population was also first described and proposed as a potential progenitor. Through elegant short term H^3 label retention studies followed by electron micrograph analysis of epithelial phagosomes, Cheng and Leblond were able to lineage trace differentiated progenitors arising from crypt base columnar cells[12].

More recently, the intestinal stem cell field has been invigorated by the discovery and validation of numerous protein makers which label unique stem cell populations (Figure 1.3). Bmi1 polycomb ringfinger oncogene (Bmi1) and mouse Telomerase reverse transcriptase (mTert) have been shown to be expressed in the classical label retaining cells located at the +4 or +5 cell position (Figure 1.3)[13,18]. In separate studies, mTert or Bmi1 specific Cre-mediated lineage tracing induced global labeling of all downstream differentiated epithelial

cells, validating both Bmi1 and mTert populations as intestinal progenitor/stem cell populations. This group of progenitors is believed to behave like the traditionally defined stem cells, which are generally quiescenve and divide only occasionally in an asymmetric manner[13,18]. A second, now well-described, stem population marked by expression of Lgr5 and Sox9 is positioned within the base of the crypt and comprise the crypt-base columnar cell (CBC) population described in the 1970's by Cheng and LeBlond[5, 12, 14]. Histologically, these small wedge shaped cells are located at the base of the intestinal crypt interspersed between Paneth cells (Figure 1.3). Lgr5-promoter driven Cre-mediated lineage tracing has also demonstrated that this population is capable of giving rise to all of the differentiated intestinal epithelial lineages. This finding has most recently been supported by the development of a novel gut culture system in which isolated Lgr5-expressing stem cells are capable of giving rise to intestinal organoids *in vitro* that contain all lineages of the intestinal epithelium[23]. Unlike stem cells which undergo periods of quiescence, CBCs divide every 24 hours[5]. Further, using mathematical modeling, a recent study has suggested that these cells divide predominantly in a symmetric manner giving rise to two identical Lgr5-expressing stem cells. Then, in a seemingly stochastic process, a portion of these Lgr5-expressing daughter cells go on to become transit-amplifying cells[24].

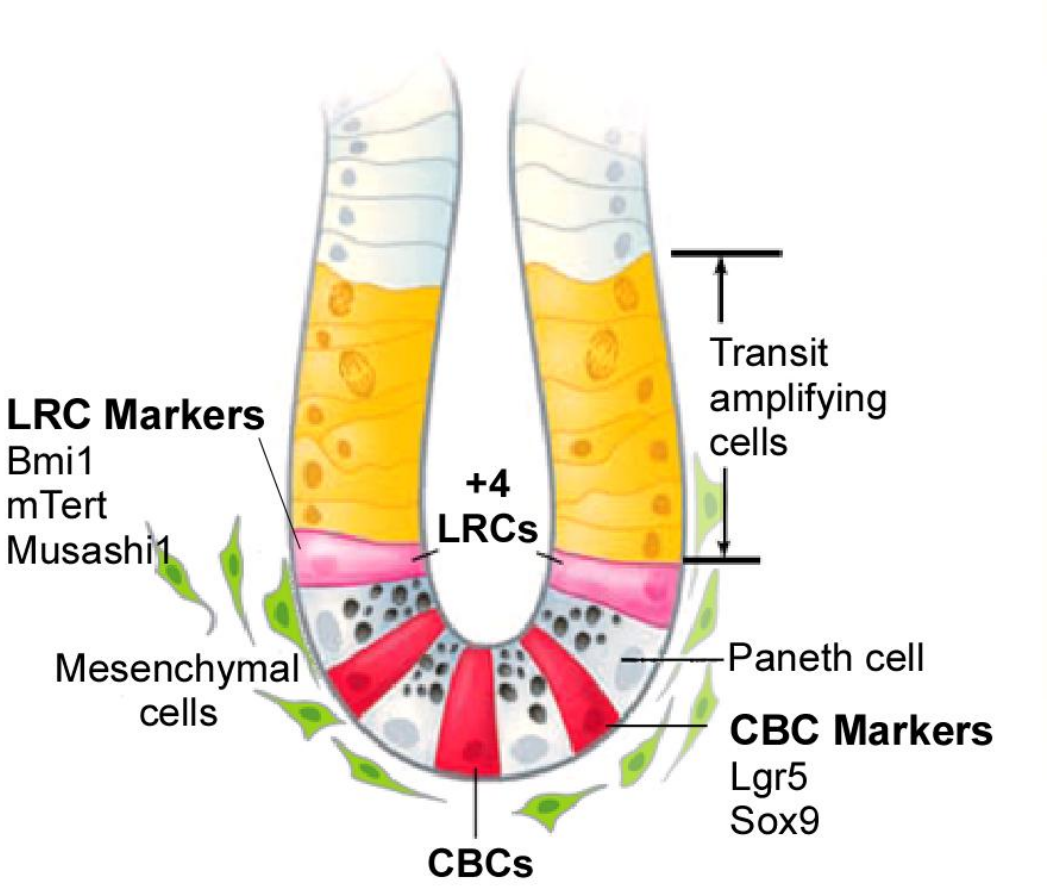

Figure 1.3 Stem cell location and markers in the small intestinal crypt. Crypt base columnar stem cells (CBCs) reside at the base of the crypt interspersed by differentiated Paneth cells. CBCs express Lgr5 and Sox9. Long term label retaining cells (LRCs) are located at the +4 cell position between the crypt base cells and transit-amplifying cells. LRCs have been described to express Bmi1, mTert and Musashi1. Figure modified and published from Li and Clevers 2010 with permission from AAAS[3].

The relationship between the actively cycling crypt-base columnar stem cell and the +4 slow-cycling stem cell has not been fully elucidated. It is clear that a number of these newly identified populations have stem or progenitor capacity. Further, it is also becoming apparent that these populations may be differentially regulated, as the Bmi1-expressing population appears not to respond to a Wnt signal like the Lgr5-expressing population (Calvin Kuo, personal communication)[25]. Therefore, understanding the ordered hierarchy among these stem/progenitor populations represents an important focus for interpreting intestinal disease origin and the corresponding effective therapeutic targets. Interestingly, a recent study suggests that Bmi1 cells are capable of giving rise to Lgr5 cells[4]. While the many factors which influence these different progenitor populations are not yet fully understood, the unique signaling and cellular microenvironment around each progenitor plays an important regulatory role. The function of the cellular microenvironment during gut development is well described, and many of the signaling pathways responsible for early intestinal formation continue to have important function in adult intestinal stem cells during homeostasis and disease[20].

Regulation of the Intestinal Stem Cell through Cellular Organization and Signaling

The stem cell's microenvironment, or niche, coordinates its maintenance and activation during development, homeostasis, regeneration and disease. The stem cell niche is comprised of two components, the adjacent epithelial cells and nearby non-epithelial cells of the mesenchyme including the endothelium, fibroblasts and cells of the Gut Associated Lymphatic Tissue[20]. Signaling between progenitors and the surrounding cellular environment is essential for establishing and maintaining intestinal morphology, cellular organization and behavior. Signaling between intestinal epithelial progenitors and mesenchyme prominently impacts early intestinal development[20]. In the adult intestine, signaling between stem cells and adjacent epithelial cells plays a significant role in homeostasis and disease (Figure 1.4).

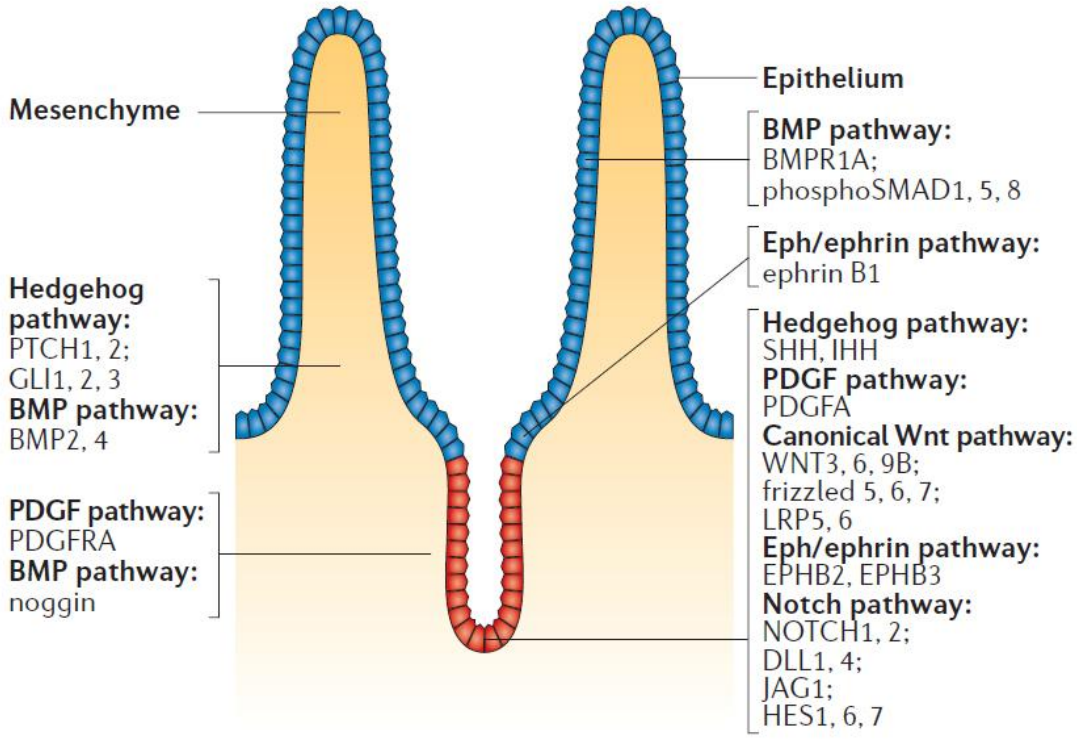

Figure 1.4 Signaling pathways in the intestine. Components of the Hedgehog, platelet-derived growth factor (PDGF) and bone morphogenetic protein (BMP) pathways mediate signaling between the mesenchyme and epithelium and play a major role in development of the intestine. The Wnt, Eph/Ephrin and Notch pathways have significant function in mediating signaling within adult epithelium. Expression of pathway components within intestinal architecture is tightly regulated and mediates function. Brackets denote the region of the intestine in which pathway components are expressed. Reprinted with permission © 2006 Nature Publishing Group[20].

In the adult intestine, Lgr5-positive stem cells reside in the base of the crypt interspersed between adjacent differentiated Paneth cells. In early studies to examine the relationship between these two cell types, Garabedian and colleagues selectively ablated Paneth cells to examine their function in maintaining crypt homeostasis[26]. Surprisingly, changes in the crypt structure and intestinal differentiation were not observed. However, there were a number of caveats with these studies including that the experimental design only targeted ablation of a subset of prominent Paneth cells leaving the possibility that minority Paneth cell populations could maintain sufficient signaling to stem cells. In addition, these studies were conducted prior to the availability of stem cell lineage markers, preventing a direct assessment of the Lgr5-expressing population within the short analytical timeframe. Recently, the influence of the Paneth cell on stem cell populations has been revisited in the intestinal organoid culture system[23,27]. In these studies, Sato and colleagues demonstrated that Paneth cells adherent to Lgr5 stem cells are necessary for efficient organoid formation in three-dimensional culture[23,27]. They went on to show that Paneth cells secrete the Wnt signaling ligand Wnt-3a, which promotes activation of Lgr5 cell division[27]. These studies exemplify the importance of the interaction between stem cells and adjacent Paneth cells, implicating the latter as an important modulator of the Wnt pathway.

Wnt signaling in intestinal epithelium regulates proliferation and differentiation

Components of the Wnt signaling pathway were orignially described in the fruit fly *Drosophila melanogaster* and subsequently as an oncogene in the mouse in 1982[28]. Since its original discovery, more than 50 proteins have been identified which are directly involved in Wnt signal transduction (see The Wnt Homepage, online at wnt.stanford.edu). With at least 19 ligands, 10 receptors, multiple co-receptors and inhibitors the Wnt pathway is a complex signal transduction network with tissue specific functions and regulation[29].

The most well-described function of the Wnt pathway is mediated through the downstream transcription factor β-catenin and is known as the canonical Wnt signaling pathway (Figure 1.5). When the Wnt pathway is in an inactive state, β-catenin is bound and phosphorylated by the Axin/Gsk3β/Ck1/Apc destrution complex which results in phosphorylation by Glycogen synthase kinase-3 (Gsk3β) and subsequent ubiquitination and degredation by the proteasome[30]. In order to activate the Wnt pathway, Wnt ligands are secreted from cells where they bind to the Frizzled and Lrp5/6 receptors in an autocrine or paracrine fashion[31-34]. Upon binding, the cytoplasmic protein Dishevelled (Dsh) is recruited to the receptor complex, interacts with Axin and Gsk3β, faciliating Axin's interaction with Lrp5/6[35,36]. Recruitment of Axin away from from the β-catenin destruction complex allows β-catenin to accumulate and translocate into the nucleus[37,38]. In the nucleus, β-catenin interacts with the T-cell factor (Tcf) and Lymphoid enhancing factor (LEF) transcription factors, displacing the inibitor Groucho and initiatiating Wnt-target gene transcription[39-41].

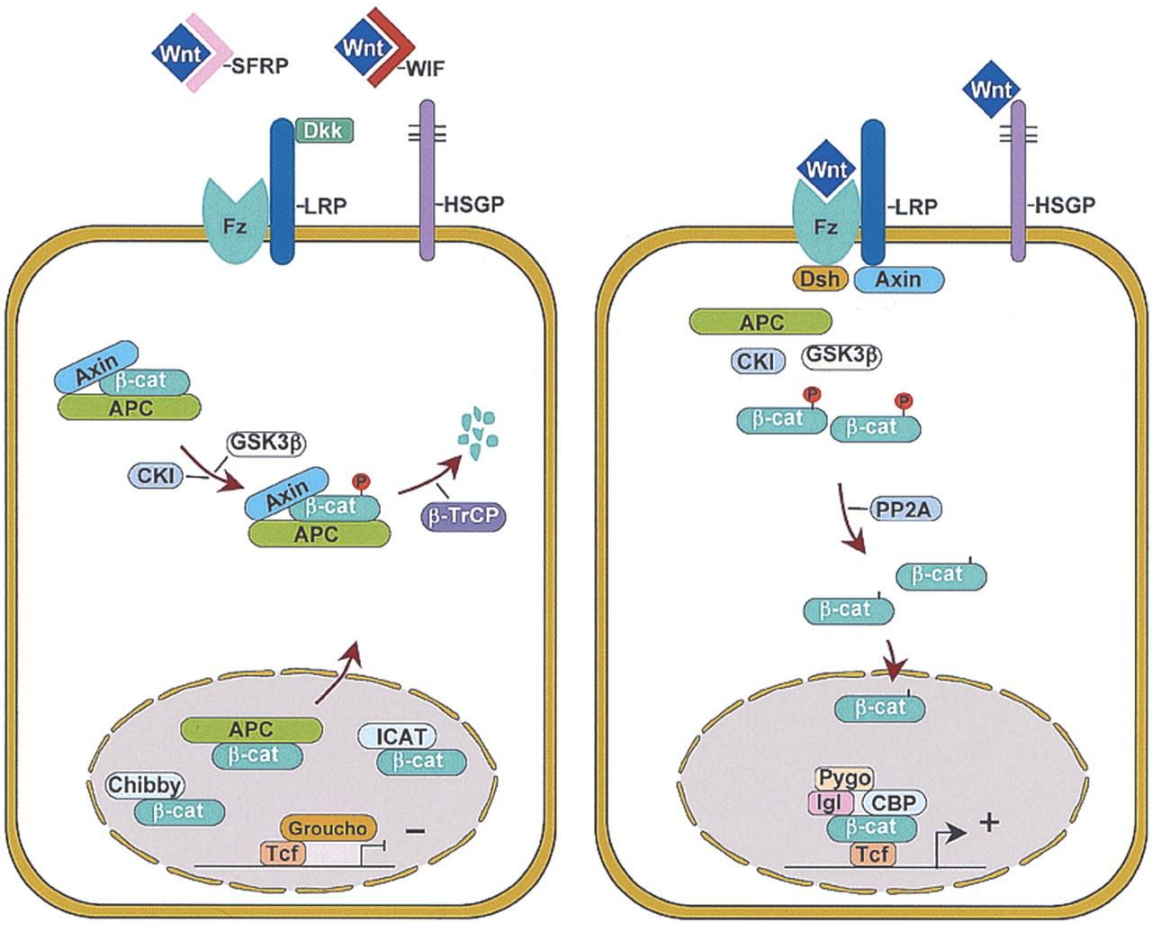

Figure 1.5 Primary components of the canonical Wnt signaling pathway. (*Left*) In the absence of Wnt stimulation, β-catenin levels are kept at a minimum through the destruction complex composed of Apc, Axin, Gsk3β, and Cki. In the nucleus, Tcf factors associate with transcriptional repressors to block target gene activation. (*Right*) In the presence of Wnt stimulation, the destruction complex is destabilized, and β-catenin accumulates in the nucleus to activate transcription of Tcf target genes. Reprinted with permission ©2005 Cold Spring Harbor Laboratory Press[42].

The canonical Wnt signaling pathway has well-described functions in maintenance of the intestinal stem cell niche and is mutated or dysregulated in >95% of colorectal cancers (CRC)[43]. Activation of the Wnt pathway plays a major role in regulating proliferation, differentiation and migration of the intestinal epithelium[44]. In the normal small intestine and colon, nuclear β-catenin is observed in crypt base stem cells which are also characterized by their expression of the Wnt target gene Lgr5[5,45,46]. Further, our lab has demonstrated that intestines from Wnt-reporter mice contain β-galactosidase-positive cells near the base of of normal crypts[47]. Taken together, these findings support the idea that intestinal stem cells require Wnt for maintenace of a stem state or activation of proliferation. This has been studied using genetic mouse models for both over activation and repression of Wnt signaling in the intestine.

Over activation of Wnt signaling in the adult mouse intestine by inducible deletion of *Apc* (a key component of the β-catenin destruction complex) leads to hyperproliferation of epithelial progenitors, expansion of cells within the crypt compartment and reduced differentiation[48,49]. Interestingly, germline mutation of a single *Apc* allele in mice leads to polyp formation in regions where the second wild-type copy is lost (Multiple Intestinal Neoplasia Mouse or $Apc^{Min/+}$)[50]. Loss of *APC* also appears to be a prominent initiating event in the majority of sporadic human CRC[51]. Further, germline mutations of *APC* cause hereditary familial adenomatous polyposis coli, a syndrome in which people develop hundreds of intestinal adenomatous polyps, which become invasive tumors if not removed[52]. Conversely, inhibition of Wnt signaling by deletion of the downstream Tcf4 in the

mouse intestine leads to absence of proliferating crypt cells, reduced villus height and animal death on the first day after birth[53]. In adult animals, inducible inhibition of Wnt by over expression of the Wnt pathway inhibitor Dickkopf-1 (Dkk1), resulted in loss of proliferation within the crypts[54,55]. Taken together, these findings demonstrate the importance of the Wnt signaling pathway for maintenance of normal tissue and tumorigenesis in the intestine.

A central question in intestinal biology is how the secondary architecture of crypts and villi are maintained. Regional segregation of proliferation (stem cell niche/crypt) and differentiation (functional epithelial cell types) must be balanced in the face of constant progenitor proliferation, differentiation, cell migration and cell death. Downstream targets of the Wnt signaling pathway are known to play an important role in maintenance of the intestine and lineage differentiation. For example, Wnt mediated β-catenin gene transcription induces expression of the tyrosine kinase receptors *EphB2* and *EphB3* and represses the expression of the ligand *Ephrin-B1*[45,46]. This Wnt-regulated expression results in restriction of the receptor *EphB3* to the crypt base, including Paneth and crypt base columnar cells, while the *Ephrin-B1* ligand is expressed in cells at the crypt villus junction. Eph-Ephrin signaling is a general mechanism for delineating tissue boundaries and organizing cell types[56,57]. Interestingly, genetic deletion of *EphB2* and *EphB3* in mice leads to aberrant migration of Paneth and proliferating cells out of the crypt onto the villus[45]. This finding suggests the Eph-Ephrin system generates tissue boundaries and modulates cell migration through a ligand-receptor repulsion mechanism. Significantly, loss of EphB receptors correlates with

invasive tumor behavior in humans and accelerates tumorigenesis in $Apc^{Min/+}$ mice[58]. Therefore, Wnt regulation of *Eph* genes further enforces the importance of this pathway on control of cellular migration and tissue architecture.

In addition to cellular migration and proliferation, differentiation of progenitors into the multiple intestinal epithelial cell types represents an important aspect of homeostasis. Wnt signaling may also play a role in cellular differentiation. Differentiation of progenitors into Paneth cells requires Wnt and increased differentiation occurs when Wnt signaling is over active[49,59]. Further, inhibition of Wnt signaling may compromise enteroendocrine lineage differentiation[53,55]. While Wnt signaling certainly plays a major role in regulating intestinal proliferation, it may also influence progenitor differentiation, though additional signaling pathways are clearly required to orchestrate this complex process.

Notch signaling regulates differentiation in the intestinal epithelia

Proper differentiation requires the integration of multiple signaling pathways. Notch signaling in progenitor populations represents a major regulator of lineage differentiation in multiple organ systems[60-64]. Further, the expression of Notch signaling components has been shown to be partially regulated by Wnt[65]. The Notch receptor and its ligands Delta and Jagged are transmembane receptors which mediate cell signaling through direct contact[60] (Figure 1.6). Upon contact with Delta/Jagged the Notch receptor is cleaved extracellularly by ADAM10 or ADAM17/TACE and intracellularly by γ-secretase releasing the notch intracellular domain. The notch intracellular domain then translocates to the nucleus where it acts as a transcription factor[66]. One major mechanism of regulation of this pathway is classic lateral feedback inhibition. When cells expressing the Notch receptor are activated by adjacent cells expressing the ligand Delta/Jagged they down regulate their own Delta/Jagged ligand expression. This feedback inhibition then deprives adjacent/lateral cells from Notch activation[67]. Originally described in drosophila, this mechanism allows for regulation of cell differentiation based on cellular position within a tissue.

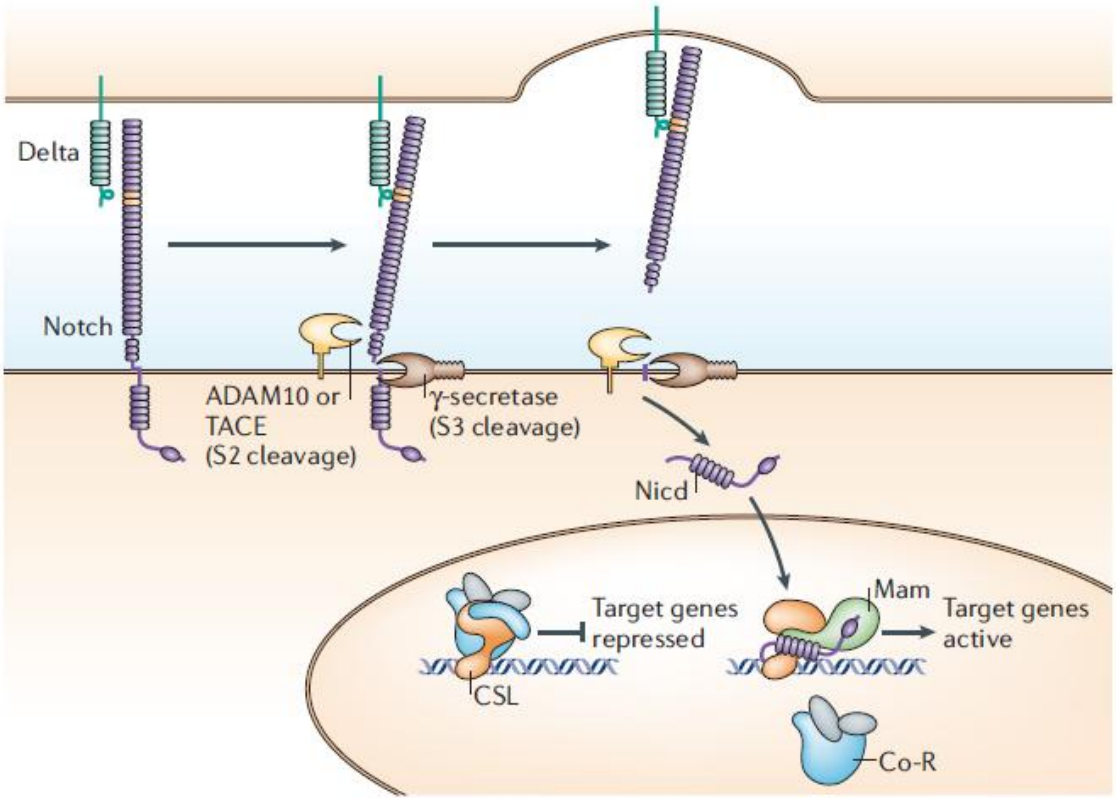

Figure 1.6 Core signaling components of the Notch pathway. Upon contact with Delta/Jagged (green) the Notch receptor (purple) is cleaved extracellularly by ADAM10 or ADAM17/TACE and then intracellularly by γ-secretase releasing the notch intracellular domain. The notch intracellular domain (Nicd) translocates to the nucleus where it acts as a transcription factor with the DNA-binding protien CSL (orange), recuiting Mastermind (Mam, green) and other transcriptional activators to Notch target genes. Transcriptional repressors (Co-R, blue and gray) are released from target genes. Reprinted with permission © 2006 Nature Publishing Group[66].

In the intestine, Notch signaling components are expressed in epithelial cells of the crypt[15,68]. Inhibition of the Notch signaling pathway in mice by deletion of *Hes1* (a Notch target gene and transcriptional repressor) results in increased numbers of goblet and enteroendocrine cells[69]. Stronger inhibition of Notch through treatment with a gamma-secretase inhibitor results in an intestine which is almost exclusively composed of goblet cells[65,70]. In the opposite situation where Notch is constitutively activated, a severe reduction in all secretory lineages is observed[64]. These studies suggest a model of intestinal differentiation where cells that express Delta and therefore avoid Notch activation give rise to secretory lineages. Conversely, those cells adjacent to Delta expressing cells which have Notch activated, subsequently express Hes1, and become enterocytes[71,72,73].

Appropriate signaling and dynamic interplay through the Notch and Wnt pathways is essential for normal tissue homeostasis. Significantly, it is these same pathways which become perturbed during acute injury and in tumorigenesis[72]. While dynamic modulation and acute up or down regulation of these pathways is necessary for efficient tissue regeneration after injury, a loss in regulatory control of these pathways is associated with tumor development. This dynamic interplay has been observed in proliferative regulation in the *Apc*$^{Min/+}$ mouse[50]. In these mice which spontaneously develop polyps from aberrant activation of the Wnt pathway, Notch also becomes ectopically activated. Further, when *Apc*$^{Min/+}$ mice are treated with a gamma-secretase inhibitor, proliferation is repressed in polyps[65]. This finding suggests that activation of both Notch and

Wnt pathways contribute to proliferation in Wnt driven polyps and may have therapeutic implication for the treatment of CRC. In addition, polyps in these mice treated with gamma-secretase inhibitor display an increased number of goblet cells suggesting aberrantly proliferating intestinal progenitors are still susceptible to differentiation[65]. While it is not yet clear whether Notch inhibition is a viable target in treatment of advanced human CRC, this concept is being actively pursued and clinical trials have been initiated in solid tumors[74-76].

Cell adhesion molecules integrate cellular microenvironments with signaling pathways

The complexity of signaling within the intestine supports a theory that cellular response varies with cellular context, signaling environment and cell type. If we are to apply this knowledge for treatment of human disease, we must develop a strong understanding of how both signaling pathways and cellular microenvironments interplay in normal tissue and are altered in disease states. One mechanism for integrating cellular environment and signaling is through adhesion complexes. Adhesion molecules permit cellular sensing of the surrounding microenvironment[77]. For example, adherens junctions, which contain cadherin adhesion molecules, associate with the Wnt transcription factor β-catenin[78]. In this context, β-catenin plays an important role in stabilizing the adhesion complex. Interestingly, formation of adherens junctions also modulates Wnt signaling by adjusting the amount of β-catenin available to participate in canonical Wnt signal transduction[79,80] (Figure 1.7). The interface between adhesion and signal modulation is a recently defined area of study with significant therapeutic potential. Adhesion molecules are druggable targets with known functions in tumor initiation and progression[81].

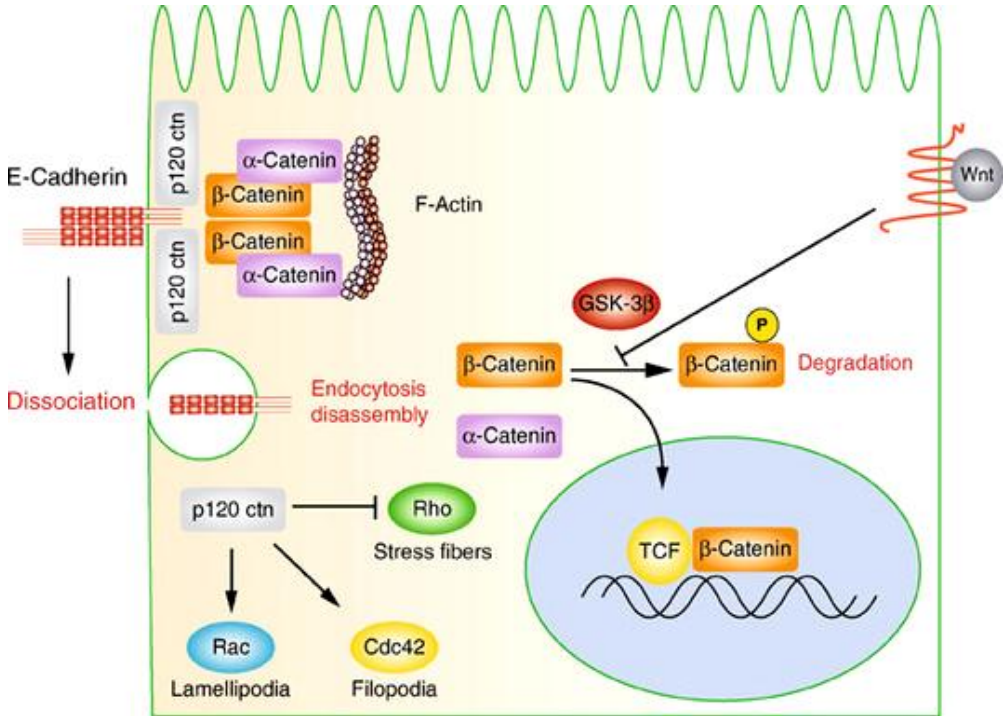

Figure 1.7 Signaling induced by loss of E-cadherin. Disruption of adherens junctions is caused by mutation or transcriptional repression of E-cadherin and growth-factor signaling. Dissociation of homophilic binding of E-cadherin promotes the endocytosis of E-cadherin and the disassembly of the catenins. $p120^{ctn}$ further promotes cell motility by activating Rac and Cdc42 to form lamellipodia and filopodia, and inhibits Rho activity that leads to stress-fiber formation. β-catenin dissociated from the E-cadherin and catenin complex accumulates in the cytoplasm. Part of β-catenin translocates to the nucleus and binds to TCF to activate transcription of key genes required for survival of detached cells, while the other part of β-catenin is modified by phosphorylation and ubiquitination, leading to proteosome degradation. The Wnt pathway promotes β-catenin signaling by repressing the phosphorylation of β-catenin mediated by GSK-3β. Reprinted from SpringerImages ©2008 accoding to subscriber terms of use.

CD166 is a Cell Surface Adhesion Molecule with Diverse Biologic Functions and a Potential Function in the Normal Intestine

CD166 function in tumorigenesis

CD166 is a cell adhesion molecule that has recently been described to define a population of CRC stem cells[82]. CD166-expressing CRC cells are capable of initiating tumors when transplanted into mice, generating tumors that recapitulate the histology of the tumor of origin. An important role for CD166 in modulating tumor behavior has become increasingly appreciated. Alterations in its expression pattern and intensity are pathologically correlated with aggressive disease in a variety of cancers including melanoma, prostate, breast, ovarian, esophageal, bladder and colorectal cancer[83-90]. In two clinical-correlative studies examining CD166 expression in CRC, high cell surface expression of CD166 was correlated with a shortened patient survival[90,91]. While intriguing, these findings remain controversial with one subsequent study suggesting that loss of cell surface CD166 expression correlates with higher tumor grade, invasion and worsened prognosis[92]. While the significance of CD166 as a prognostic marker is not yet conclusive, CD166 expression is enriched in colon cancer cells resistant to chemotherapy[93,94], and when inhibited by neutralizing antibodies, growth of colon cancer xenografts is reduced[95]. Although these findings suggest that CD166 may have a role in the progression of CRC, nothing is known about this molecule's normal expression and function in the intestine. Insight into its potential function in normal intestine can be gained by reviewing what is already known about CD166 in other tissues.

CD166 adhesion and function in extra-intestinal tissues

As a member of the immunoglobulin-like family of adhesion molecules, CD166 is known to bind homotypically to other CD166 molecules on adjacent cells (Figure 1.8), as well as having a high affinity interaction with CD6[96-99]. CD166 has diverse functions including roles in 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 cells[100-106]. Constitutive CD166 knockout mice are viable, and their analysis established a role for CD166 in axon fasciculation and path finding during embryonic development[107]. Further, CD166 has also been described to be expressed in hematopoietic progenitors. Within the hematopoietic system, it appears that CD166 regulates differentiation of the granulocyte lineage and participates in colony formation of CD34-positive hematopoietic progenitors *in vitro*[108,109]. Interestingly, CD166 expression has also been documented on mesenchymal stem cells, but its function in this population has not been explored[110,111]. The diverse function and specific expression of CD166 in progenitor populations supports the notion that CD166 mRNA and protein expression are tightly regulated and may be repressed in some differentiated cell types.

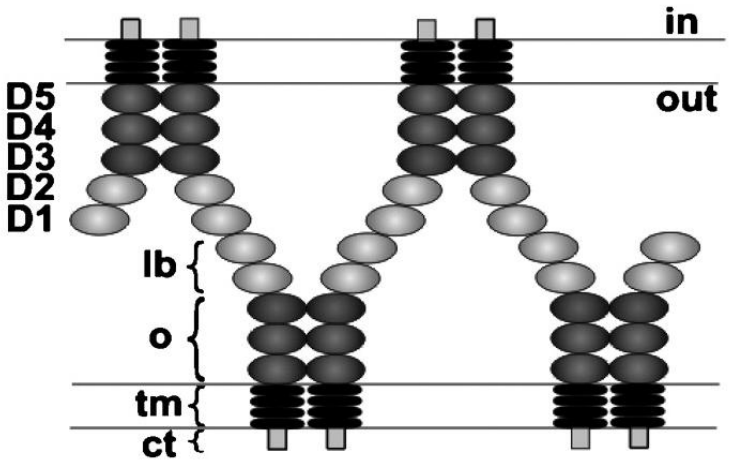

Figure 1.8 CD166 homotypic adhesion and domain structure. CD166 is a member of the immunoglobulin superfamily of adhesion molecules. CD166 has five extra-cellular immunoglobulin-like domains (D1-D5), a transmembrane domain (tm), and a short (32 amino acid) C-terminal tail (ct). Domains 1 and 2 are involved in ligand binding (lb) and have homology to immunoglobulin variable domains. Domains 3-5 form lateral oligomers (o) with adjacent CD166 molecules and have homology to immunoglobulin constant domains. Reprinted with permission © 2008 American Association for Cancer Research [112].

Genetic regulation of CD166 expression

The earliest description of CD166 transcriptional regulation was described by Zhang et al. where v-rel was over expressed in avian B-cells to transform them into lymphoma[113]. In this study CD166 expression was found to be highly induced in v-rel transformed cells while not expressed in myc transformed or normal B-cells. This study also showed that a CD166 specific antibody reduced proliferation in these cells. Significantly, these were the first studies to suggest that transcriptional members of the NF-kappa B pathway may be involved in regulation of CD166 expression. While these studies supported a correlation between over expression of v-rel and expression of CD166 they did not determine whether v-rel interacted directly with the CD166 promoter region, necessitating further study in this area. More recent chromatin immunoprecipitation followed by sequencing (ChIP-seq) studies, documented in the UCSC ENCODE database, have directly implicated the NF-kappa B pathway by identifying association of p65 with the promoter region of CD166 in multiple lymphoblastoid cell lines[114]. This gene regulation is consistent with CD166 function in immune cell response to inflammation, including activation of T-cells and transendothelial migration[100,101,105,106]. Significantly, direct transcriptional regulation of the CD166 promoter region by p65 has also been demonstrated in a hepatocellular carcinoma cell line (Hep2G) after serum deprivation[115] and in multiple breast cancer cell lines[116]. In addition to NF-kappa B regulation of CD166, Tcf4 and Tcf12 were also identified to occupy the CD166 promoter region in Hep2G cells[114]. This suggests that Wnt signaling may also regulate

CD166 expression. It is not yet known if regulation by the Wnt pathway is conserved in other epithelial cell types. In colorectal cancer, CD166 protein expression was shown to be highest in cells which have received a Wnt signal and are actively transcribing Wnt target genes[117]. Finally, these data are also supported by the finding that addition of Wnt3a to culture media of 3T3 cells induced greater than a 2-fold up regulation of CD166 transcript, further implicating the Wnt pathway as an important regulator of CD166[118].

In addition to a full length transcript, CD166 is also alternatively spliced into a secreted variant[102]. As originally described by Ikeda et al., this splice variant includes only the N-terminal immunoglobulin domain of CD166. Alternative splicing of CD166 was shown to be responsive to TNF-α treatment with enhanced levels of full length and soluble CD166 transcript. Interestingly, expression of the soluble splice variant was shown to directly antagonize CD166 mediated adhesion and enhance cell migration[102].

Further, genetic regulation of CD166 function includes silencing of the promoter region by methylation in a subset of CD166 non-expressing breast cancer cell lines[116]. CD166 transcript and protein levels can also be down regulated by microRNA-9 mediated inhibition of translation[115]. As with most genes, genetic regulation of CD166 expression is complex and multi-factorial. CD166 expression appears to be controlled by multiple signaling pathways likely in a cell-type dependent manner.

Regulation of CD166 protein function

CD166 is known to associate with the adherens junction and can engage in homotypic adhesion[119]. Trafficking of CD166 to the cell membrane requires α-catenin[120]. Significantly, prostate cancer cell lines which do not express α-catenin demonstrate cytoplasmic localization of CD166. Whether, a similar mechanism exists in human tumors is not known but this mechanism may help explain the heterogeneity observed in CD166 cellular localization in colorectal cancer tumors[90].

At the cell membrane, the CD166 c-terminus associates indirectly with the actin cytoskeleton, likely through α-catenin[121]. This interaction is dynamically mediated by protein kinase C and/or Tiam1/Rac and is required for homotypic adhesion and efficient formation of CD166 oligomerization at the cell membrane[121-123] (Figure 1.9). Once CD166 mediated homotypic adhesion is established between cells, it can be further regulated by proteolytic cleavage of the extracellular membrane domain by ADAM17/TACE and ADAM10[124,125] (Figure 1.9). In studies by Rosso et al., proteolytic cleavage was found to occur close to the transmembrane domain, releasing most the extracellular portion of CD166 and inhibiting CD166 adhesion[124]. In this same study they further demonstrated that cleavage of CD166 increased cell motility in ovarian carcinoma cells. These findings are consistent with results in melanoma where expression of a truncated form of CD166 in metastatic melanoma cell lines which express endogenous CD166 increased cell migration in vitro and the incidence of metastasis in mouse xenografts[126]. Interestingly, in this same study, they also

noted that primary xenografts expressing truncated CD166 did not grow as large as matched WT CD166 tumors, suggesting loss of CD166 function may reduce cellular proliferation.

Further inhibition of CD166 adhesion can be mediated through expression of a soluble CD166 splice variant which is proposed to act as an inhibitor of CD166 dependent and independent adhesion[102] (Figure 1.9). The influence of this CD166 secreted splice variant on cellular behavior has been documented in both endothelial and a melanoma cell lines[102,112]. Interestingly, ectopic expression of soluble CD166 in endothelium enhanced cellular migration[102] supporting the notion that soluble CD166 splice variant may modulate CD166 function with similar results to proteolytic cleavage. Significantly though, in another study, ectopic expression of soluble CD166 splice variant in a melanoma cell line decreased cellular migration[112]. Clearly, we have just begun to understand how CD166 protein function can be modulated. These findings highlight the need for further study of CD166 function in both normal cell types and tumors to understand potential reasons for these differential responses.

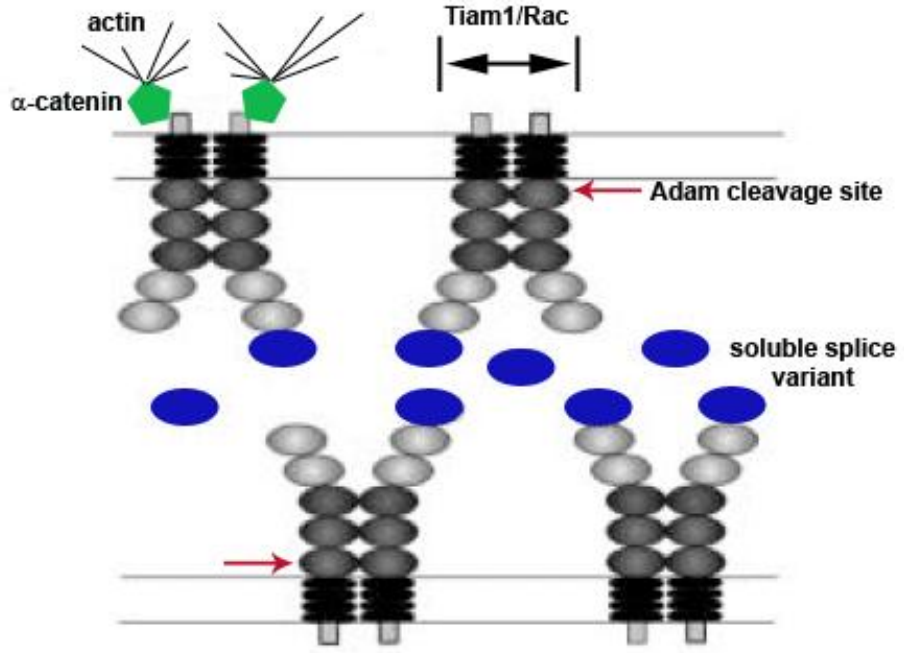

Figure 1.9. Regulation of CD166 protein function. α-catenin (green) is required for localization of CD166 to the membrane and likely facilitates CD166 interaction with actin. Activation of CD166 oligomerization and functional homotypic adhesion is regulated in part by Tiam1/Rac, potentially through cytoskeletal rearrangement. A soluble splice variant consisting of the n-terminal immunoglobulin domain is secreted from cells (blue) and is capable of antagonizing homotypic adhesion, although this variants effect on cellular motility varies with cell type assayed. CD166 is also susceptible to proteolytic cleavage by ADAM10 and ADAM17/TACE. Approximate location of the ADAM cleavage site is shown (red arrows). Modified and reprinted with permission © 2008 American Association for Cancer Research[112].

Taken together, studies of CD166 function and regulation suggest that CD166 can act as a potent modulator of cellular motility and proliferation. In the context of stem cell microenvironments, such as the bone marrow, proper regulation of both cellular motility and proliferation are crucial for maintenance normal tissue function. Therefore, it is not surprising that CD166 expression is also perturbed in cancers where cellular migration and proliferation are no longer properly regulated and contribute to progression of disease.

Based on the relatively small number of past studies one could propose a simple model in which active CD166 homotypic adhesion promotes proliferation and concurrently reduces cellular migration. Conversely, inhibition of CD166 adhesion by transcriptional mechanisms, proteolytic cleavage, expression of the soluble splice variant, or loss of expression/deactivation of other protein modulators increases motility/migration of cells but also decreases local proliferation. Clearly, CD166 function in modulating cellular behavior is cell type dependent. As previous studies have been conducted in a limited number of cell types and tissues it is clearly important to examine CD166 in additional tissues to determine how cellular context influences CD166 function. In the intestine, little is known about CD166 in normal tissue or disease states. As a tissue with a well defined architecture and regulation of stem cell dynamics, examining CD166 in the intestine may help better understand its function in progenitor populations. Ultimately study of CD166 in both stem cells and in cancer will help determine if CD166 is an active driver of disease progression or merely a bystander in colorectal carcinomas and clarify whether CD166 is a viable therapeutic target.

Experimental Rationale and Hypothesis

The intestinal epithelium is a highly proliferative and dynamic tissue dictated by continual renewal that is orchestrated by multiple stem cell populations. Regulation of these progenitors occurs through a surrounding microenvironmental niche, tightly controlled to prevent the development of disease states such as cancer. These processes are poorly understood. Signaling through cell adhesion molecules is one mechanism by which stem cells sense their surrounding environment and regulate cellular behavior. Intriguingly, the cell adhesion molecule, CD166, recently described as a cancer stem cell molecule in CRC, is also expressed on mesenchymal stem cells and has known function in hematopoietic progenitors. Yet, the function of CD166 in the intestine is unknown. My research goal is to determine the expression pattern of CD166 in the intestine and determine its function in this tissue.

Hypothesis: Based upon its relevance in CRC and its recently described expression within the hematopoietic stem cell niche, I hypothesize that CD166 is expressed within the intestinal stem cell niche and may participate in regulation of stem cell homeostasis. To test this hypothesis, I have addressed the following experimental aims:

1. Identify the intestinal expression pattern of CD166 and determine which intestinal progenitor populations express CD166.

2. Examine the function of CD166 in intestinal homeostasis by analyzing the impact of CD166 loss within the intestine.

3. Explore a role for CD166 in intestinal epithelial regeneration in response to radiation injury.

CHAPTER 2

Characterization of the Intestinal Cancer Stem Cell Marker CD166/ALCAM in the Human and Mouse Gastrointestinal Tract

Characterization of the Intestinal Cancer Stem Cell Marker, CD166/ALCAM, in the Human and Mouse Gastrointestinal Tract

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Author contributions: TGL, AEP, PSD, ADD, and MHW: conception and design, collection and/or assembly of data, manuscript writing, final approval of manuscript. ADS, ECA and JRS: collection and/or assembly of data.

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Abstract

Background & Aims: CD166 (also called activated leukocyte cell adhesion molecule, ALCAM) is a marker of 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 immunohistochemisty, 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, Musashi1, or Dcamkl1. 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 cells and the stem cell niche, which might have implications for normal intestinal homeostasis. CD166 has potential as a therapeutic target for CRC.

Introduction

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 disease[127]. 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 disease[128-130]. The reinvigoration of the CSC theory[131-133] has led to identification, isolation and characterization of subsets of intestinal cancer cells that can recapitulate tumorigenesis in xenograft models[82,134-137]. 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 Dcamkl1, ESA and CD166[17,82,136].

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 cancers[83-89]. In human CRC, aberrant cell surface CD166 expression is strongly

correlated with a 15 month shortened survival[90]. 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 mice[82], 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 cells[100-106]. Further, CD166 has been described as a ligand that binds to CD6 on thymic epithelium[97-99], acting in homophilic adhesion complexes between epithelial cells[96], and as a cell surface marker for both a subset of hematopoietic progenitor cells[108,109,138] and multipotent mesenchymal stem cells[110,111]. 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*[139,140]. 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 Musashi1 (Msi1), Dcamkl1 and Lgr5[5,16,17,23]. 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 $Apc^{Min/+}$ mice[141] 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 described[142]. 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, Msi1, Ki67, Serotonin, ChromograninA, ESA, CD44, Lysozyme and Laminin (Antibody information listed in Table2.1). 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 procedures[143], 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 (1µg/mL; R&D Systems) was co-incubated with the primary CD166 antibody (15µg/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 preparation[47], 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 (10^5) 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)

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 protocols[47]. 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 (Applied Biosystems Inc.). qRT-PCR was performed using a SYBR Green-based

assay, a 7900HT Sequence Detector and analyzed according to established protocols[47]. 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 Table1.1.

Table 2.1 Reagents. Antibodies and qRT-PCR primers used in these studies

Gene	**Primer (forward, 5'-3')**	**Primer (reverse, 5'-3')**
CD166	ACTGAAGCGTCTCCTGGAAT	ACACGCAACTTTCCTCCAC
CD166	AAACCGCGTCTACCTTGACT	ACACGCAACTTTCCTCCAC
Ascl2	CCGGTTCCTCGCGAGCACTTTT	TCCAGACGAGGTGGGCATGAGT
CD24a	AGCTTAGCAGATCTCCACTTACCGAAC	CGTGGGTAGGAGCAGTGCCAGA
CD44	TAGGAGAA GGTGTGGGCAGAA	GAGCTCACTGGGTTTCCTGTCTT
Klf5	TGCCAACGCAGCTTCTCCCG	CAGCGCTCGCTCGCTCAGTT
Lgr5	TTTGAGAAGCCTTCAATCCC	GACAGGGACGTCTGTGAGAG
Lrig1	GGGTCCACGGCTATCATCAGCTC	CCGCCTCTGAGGACTGAAAACGC
Gapdh	CACTGCCACCCAGAAGACTGT	GGAAGGCCATGCCAGTGA
Fabpl	GGTCTGCCCGAGGACCTCAT	CCAGTTCGCACTCCTCCCCC
Antibody	**Working Concentration**	**Manufacturer**
CD166	15ug/ml	R&D Systems
Msi1	1:100	Chemicon International
Ki67	1:250	Abcam
Serotonin	1:500	Incstar
ChromograninA	1:400	Abcam
ESA	1:100	US Biological
CD44	1:400	CalTag
Lysozyme	1:50	Abcam
Laminin	1:500	Abcam
Lgr5	1:100	GeneTex
CD45-APC	1:100	BD Pharmingen

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 CRC[90], 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 base[90]. 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 (Figure 2.1A, 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 (Figure 2.1A-B,E; arrowheads) and colon (Figure 2.1C-D,F; arrowheads). Peptide competition with the CD166 antibody supported the specificity of the CD166 antibody recognition pattern (Figure 2.2). Importantly, in contrast to previous report[90], CD166 expression appeared strongest on the cell surface, and was more robustly expressed on cells that resided in the base of the crypt.

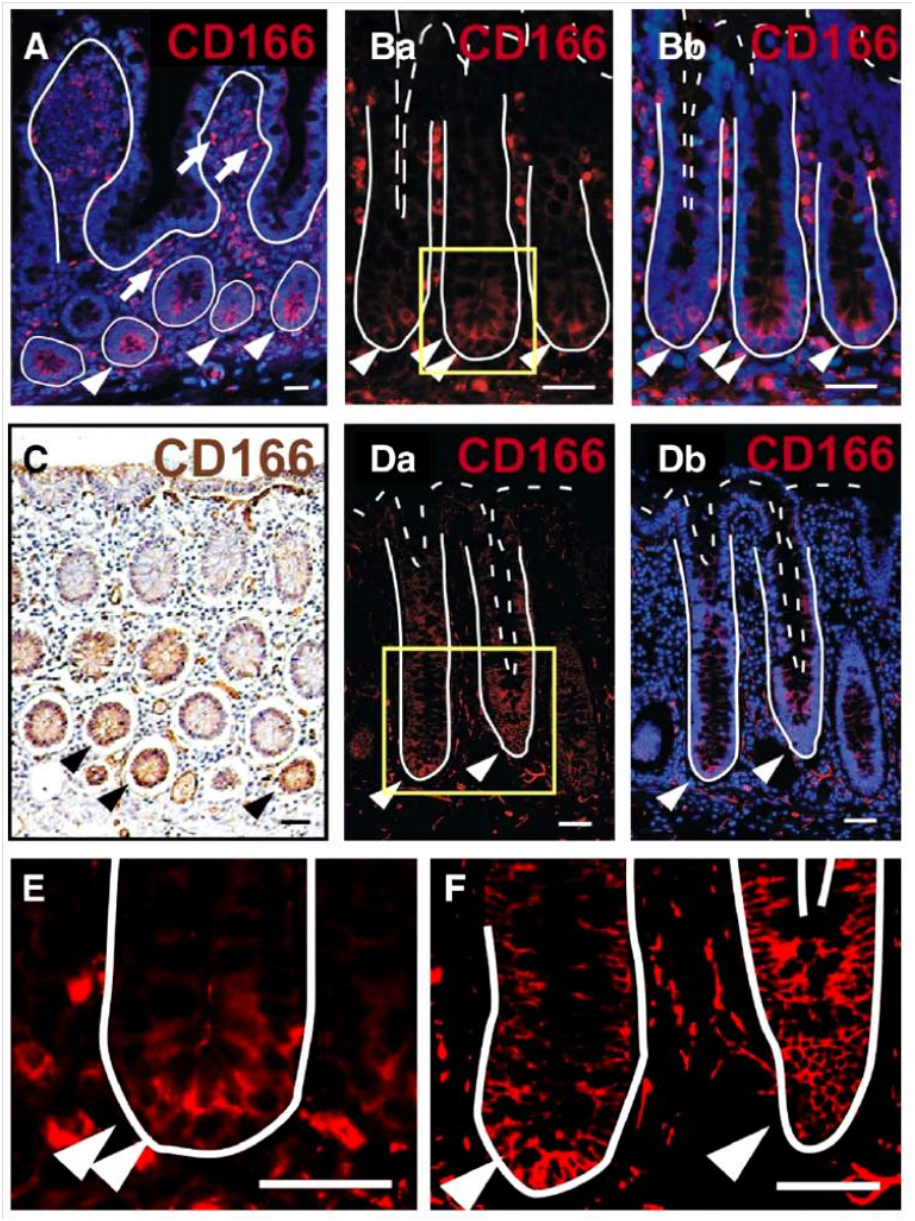

Figure 2.1 CD166 expression pattern in the human small intestine and colon. (A-C) Human small intestine stained with antibodies to CD166 (red) and counterstained with Hoechst (blue). (A) CD166-positive cells are located within the mesenchymal (arrows) and epithelial compartments (arrowheads). (Ba-Bb) 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=25μm.

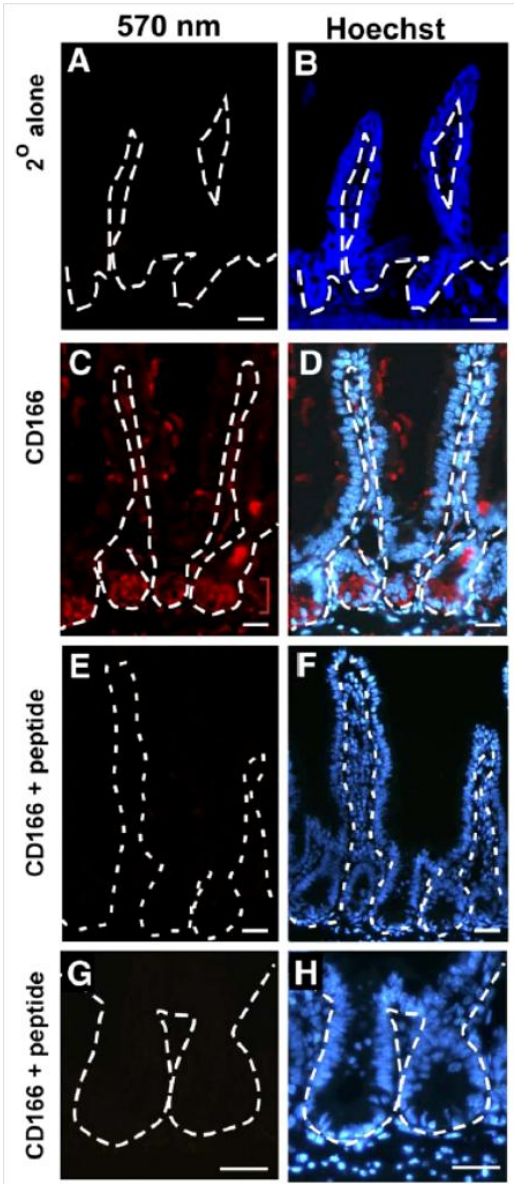

Figure 2.2 CD166 antibody controls. (A-B) Mouse intestine stained with secondary antibody alone as an isotype control. (B) Hoechst-stained (blue) image provides crypt villus distinction. (C-D) Mouse intestine stained with CD166 antibodies (red), Hoechst nuclear counterstain (blue) as a positive control for peptide blocking. (E-H) CD166 antibodies pre-incubated with recombinant mouse CD166 peptide, then subsequently incubated on intestinal tissue sections prevents antibody recognition of CD166-expressing cells. Dashed white lines denote epithelial-mesenchymal border. All images visualized at 570 nm were captured at the same exposure time. Bar = 25 μm.

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 (Figure 2.3). Importantly, the cell surface localization and increased expression levels on cells within the crypt-base were more readily appreciated (Figure 2.3A-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 (Figure 2.4B). 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 Bmi1[13]. 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 (Figure 2.3B). Low levels of cell surface CD166 were also detected on the small intestinal villus when sectioned on a tangential plane and by qRT-PCR (Figure 2.4A, B). Although it is apparent that CD166-expressing cells reside in the mesenchyme, a subpopulation of CD166-expressing epithelial cells was also present within the intestine;

CD166/ESA double-stained cells are apparent within the intestinal epithelial crypt base (Figure 2.3C).

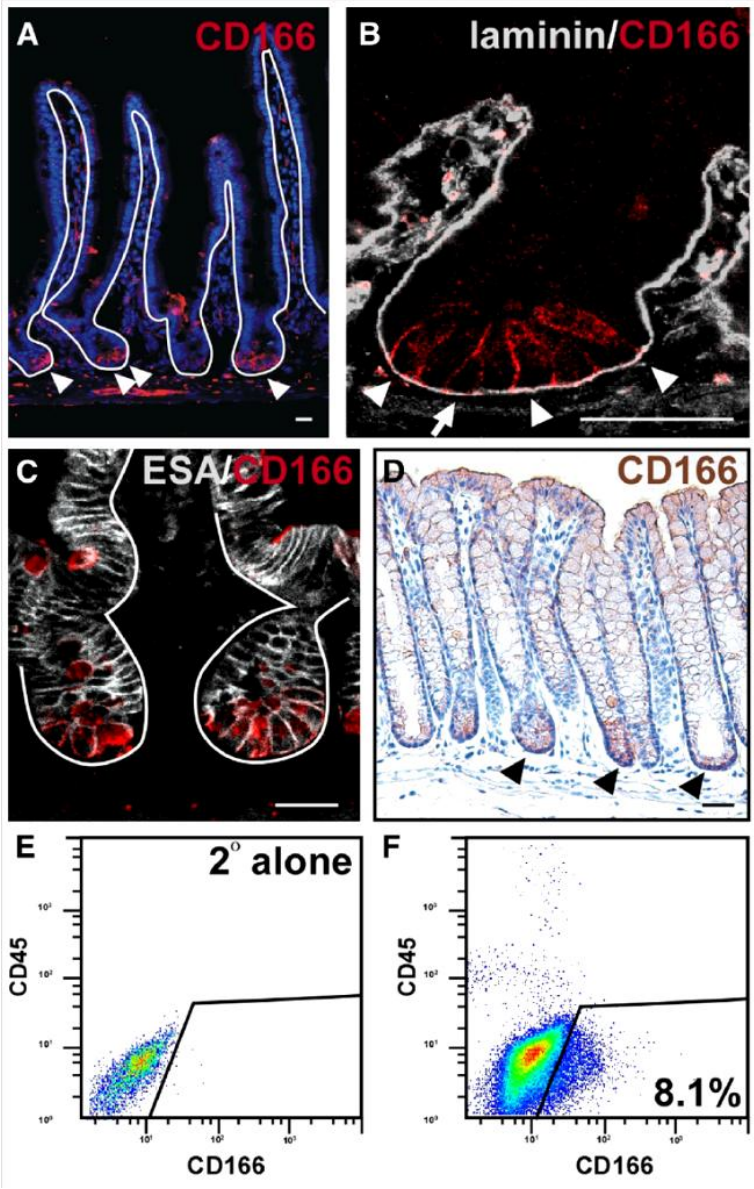

Figure 2.3 CD166 expression pattern in the mouse small intestine and colon. (A-B) Mouse small intestine stained with antibodies to CD166 (red) and (A) counterstained with Hoechst (blue) or (B) co-stained with antibodies to laminin (gray) in higher magnification of the crypt. Arrowheads mark CD166-expressing 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.

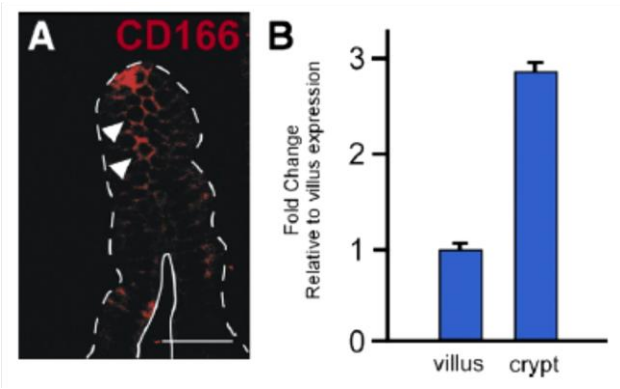

Figure 2.4 CD166 is expressed at different levels in crypt and villus epithelial cells. (A) Mouse small intestine (SI) stained with antibodies to CD166 (red). The tangential section at the tip of the villus allows for appreciation of low levels of CD166 expression. (B) qRT-PCR assay of CD166 mRNA levels in differentially isolated villus and crypt mouse epithelium. Assay was performed twice with triplicate samples in each run. CD166 expression was normalized to the internal reference gene Gapdh and calibrated to the expression in the villus sample.

To confirm cell surface expression on epithelial cells, we isolated the intestinal epithelium using a method that disrupts epithelial cell adhesion complexes[47], then performed FACS to isolate CD166-positive epithelium. Enriched populations of differentiated, villus epithelium and undifferentiated crypt-based epithelium were isolated (Figure 2.3E-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) (Figure 2.5A). 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 cytospun (Figure 2.5B) and subsequently analyzed for marker expression to determine if they were Paneth cells or stem cells (Figure 2.5C-F). A subset of FACS-isolated CD166-expressing cells stained with Phloxine Tartrazine, an established Paneth cell histochemical stain (Figure 2.5B-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 (Figure 2.6A-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 (Figure 2.6D-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; Figure 2.7).

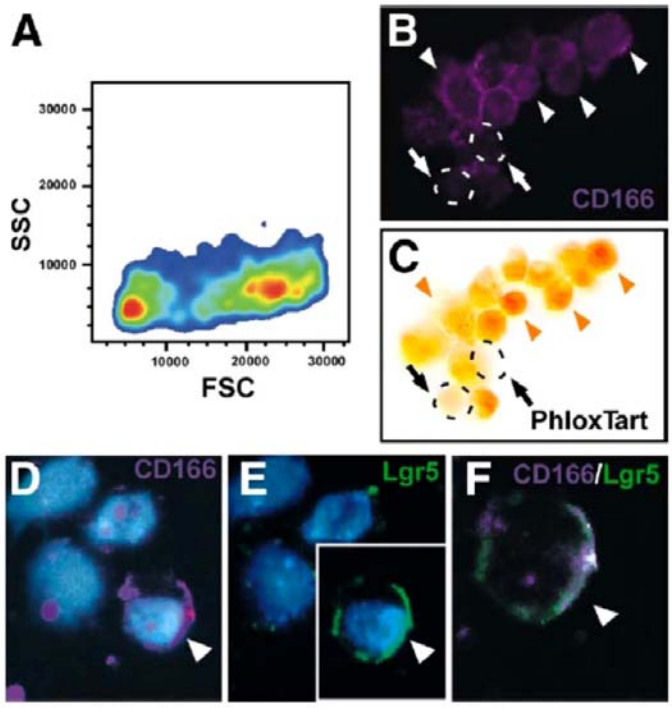

Figure 2.5 FACS-isolated CD166 epithelial cells express Paneth cell granules or Lgr5. (A) Forward (FSC) and side (SSC) scatter analysis of CD166-expressing crypt epithelial cells display two distinct populations of cells (orange centers). (B-C) FACS-isolated, cytospun CD166-expressing mouse crypt cells (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.

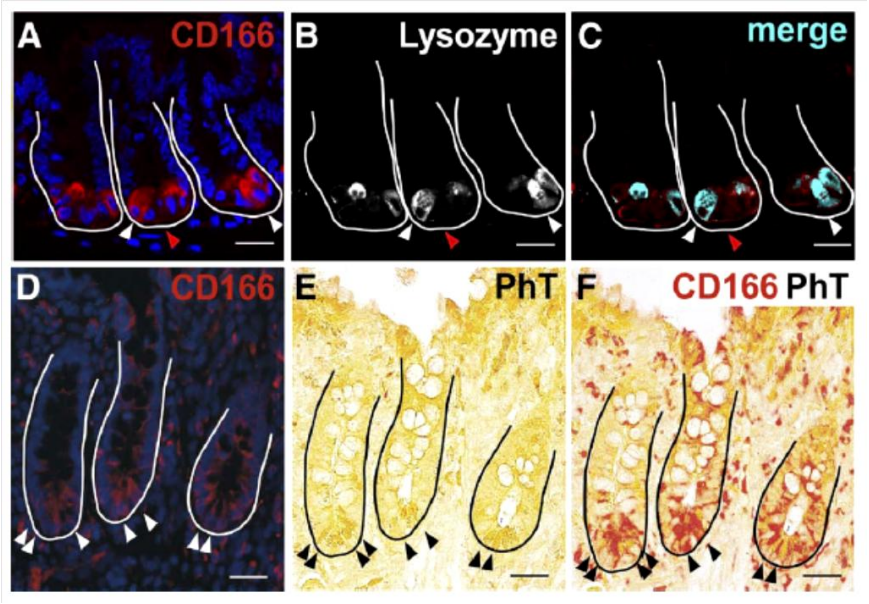

Figure 2.6 Small intestinal Paneth cells express CD166. (A-C) Mouse small intestinal tissue sections were stained with antibodies to (A) CD166 (red) and (B) lysozyme (white). (A) Nuclei are stained with Hoechst dye (blue). (C) Co-expressing CD166 and lysozyme cells are light blue (white arrowhead). Red arrowhead points to a CD166-positive, lysozyme-negative cell. (D) CD166-expressing crypt-base cells (red, arrowheads) exist in the human small intestine. The intestine is counterstained with the nuclear dye Hoechst (blue). (B) The same section is subsequently stained with the histochemical stain Phloxine Tartrazine (PhT), identifying Paneth cells (yellow stain, black arrowheads). (C) Digital overlay of images from D and E indicate that these stains identify the same cell population (arrowheads). Bar = 25 μm.

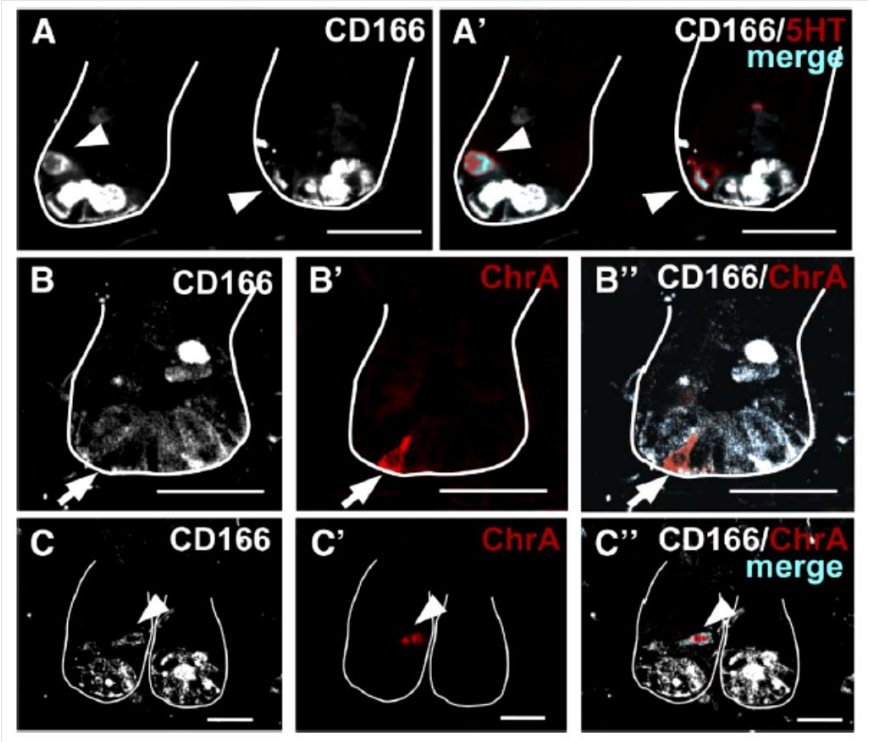

Figure 2.7 Crypt-based enteroendocrine cells express CD166.

(A-A') A subset of differentiated small intestinal enteroendocrine cells, marked by expression of serotonin (5-HT, red), also express CD166 (white), marked by arrowhead and depicted as light blue in overlay (A'). (B-B") Small intestinal tissue stained with CD166 (white) and Chromogranin A (red). A subset of crypt-base Chromogranin A-expressing enteroendocrine cells do not express CD166 (arrow). (C-C") A subset of Chromogranin A-expressing cells express CD166. (C") Co-expression is apparent in the merged image (light blue, arrowhead). White lines mark epithelial mesenchymal boundary. Bar = 25 μm.

Interestingly, there was a population of FACS-isolated CD166-positive cells that did not co-stain with Phloxine Tartrazine (Figure 2.5B,C; arrows) or enteroendocrine markers (Figure 2.7). To determine if this subset of cells co-expressed putative stem cell markers, FACS-isolated CD166-expressing cells were cytospun then analyzed for Lgr5 expression using antibodies. Lgr5-positive cells represented a small fraction of CD166-positive crypt-based cells (Figure 2.5D-F; arrowheads). Additional putative stem cell markers, Msi1[16] and Dcamkl1, also shared overlapping expression with a subset of CD166-expressing cells (Figure 2.8A,C), but extended into the adjacent CD166-negative zone (Figure 2.8B,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 (Figure 2.8E); this includes Ascl2[144], Lgr5[23], CD24[145,146], Klf5[147], CD44[82,148], Lrig1[149]. As expected, expression of an intestinal epithelial differentiation gene, Fabpl[150], is decreased in the $CD166^+$ cell population.

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 (Figure 2.9, A). However, at the onset of villus formation, E16.5, CD166 expression became localized to both the villus (arrows) and intervillus region (arrowheads; Figure 2.9, B), and by post-natal (P) development, expression was localized in the intervillus region (P4; arrowhead; Figure 2.9, C).

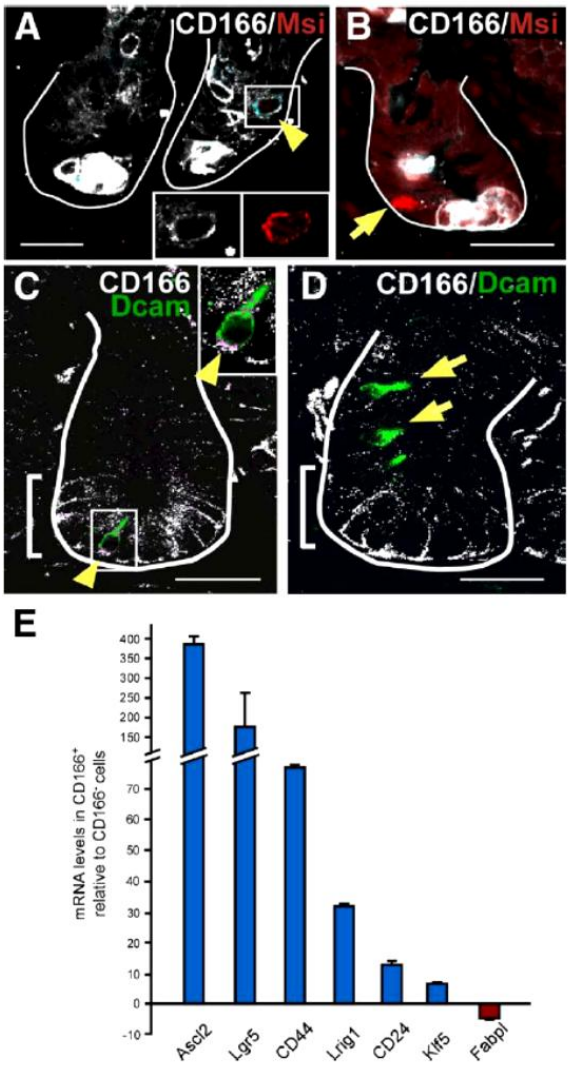

Figure 2.8 CD166 is expressed in a subset of cells expressing stem cell markers Musashi1 and Dcamkl1. (A,B) Mouse small intestinal tissue co-labeled with antibodies to CD166 (white) and the putative stem cell marker, Musashi1 (Msi, red). (A) White box and yellow arrowhead designates a CD166/Msi co-expressing 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, Dcamkl1 (green). (C) Yellow arrowhead and box designates a CD166/Dcamkl1 co-expressing cell, with the co-expressing cell surface in purple. A higher magnification is provided in the upper right inset. (D) CD166-negative, Dcamkl1-positive cells are designated by arrowhead. Brackets mark CD166-expressing region. White lines mark epithelial-mesenchymal boundary. Bar=25μm. (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.

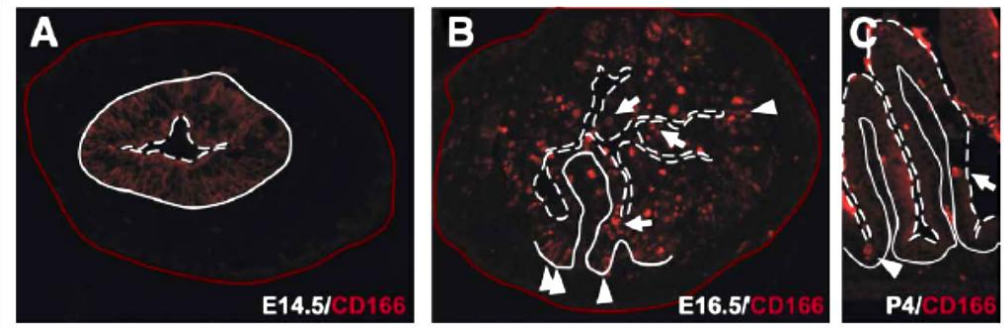

Figure 2.9: CD166 expression in the developing mouse intestine.
(A) CD166 is ubiquitously expressed in the epithelium at embryonic day (E) 14.5. (B) At the onset of villus formation, E16.5, CD166 expression is localized to both the villus (arrow) and intervillus region (arrowheads). (C) By post-natal (P) day 4, CD166 expression was detected in the intervillus region (arrowhead) and sporadically on the villus (arrow). Solid red line marks the outside of the intestinal tube. Dashed line marks the intestinal lumen. Solid white line designates the epithelial mesenchymal boundary.

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 (Figure 2.10 A-C). Human tumors were decidedly heterogeneous in their CD166 expression. While some tumor samples exhibited only cell surface expression (Figure 2.10 A), others exhibited cytoplasmic expression (Figure 2.10 B). CD166-positive cells generally appeared within clustered regions of epithelium. Interestingly, CD166 expression within liver metastasis was also heterogeneous, shown here as cytoplasmic expression (Figure 2.10 C).

Tumors in a mouse model for intestinal tumorigenesis, the $Apc^{Min/+}$ mouse[141], displayed a strikingly similar CD166 expression pattern compared to human colorectal tumors (Figure 2.10 D). 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 (Figure 2.11). 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 transit-amplifying cell population (Figure 2.11 B, 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 CRC[82]. We found that CD44 was detectable on most human tumor epithelial cells (Figure 2.10Ea), but that the overlapping expression region with CD166 was somewhat more restricted (Figure 2.10Eb, 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 (Figure 2.10Eb, 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 (Figure 2.10Fa-Fc, arrow).

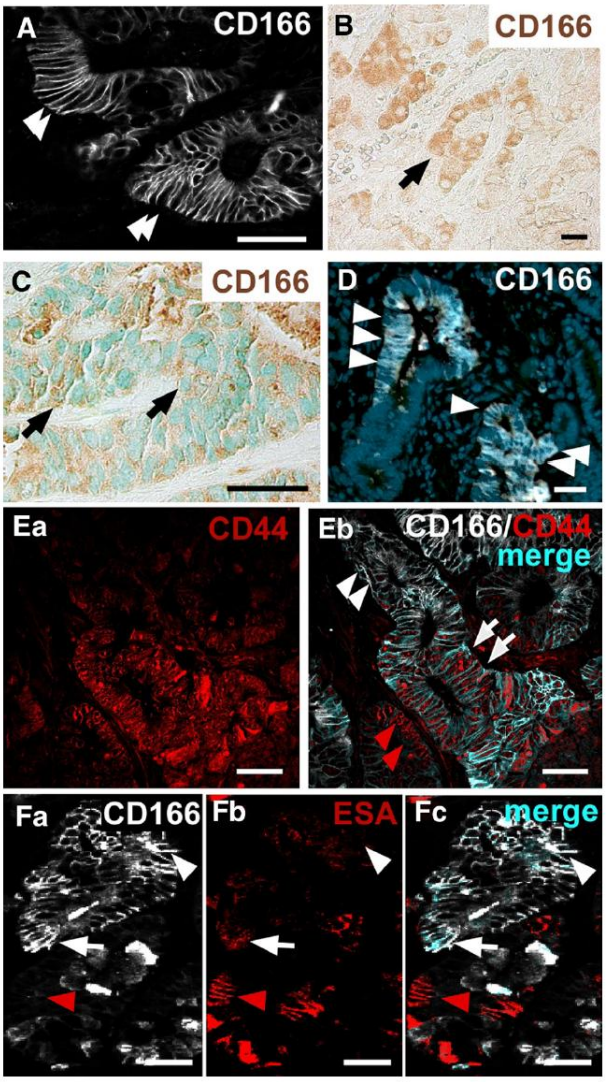

Figure 2.10 CD166 expression of human colorectal cancer is recapitulated in mouse colorectal adenomas. (A-B) Human primary colorectal adenomas labeled with antibodies to CD166 show (A) cell surface expression (white, arrowheads) or (B) cytoplasmic expression (brown, arrow). (C) Human colorectal liver 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=25μm.

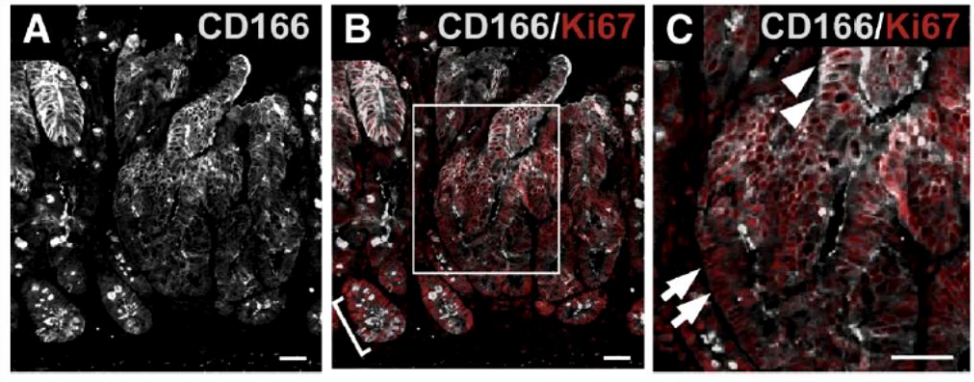

Figure 2.11 A subset of CD166-expressing mouse tumor cells are proliferating.

(A) Mouse tumor from an $Apc^{Min/+}$ mouse is stained with antibodies to CD166 (white) and (B) Ki67 (red). Ki67-positive cells (red) are scattered throughout the mouse intestinal tumor and clustered in the base of the crypt structures marking the transit-amplifying cells (white bracket). The boxed region in (B) is magnified in (C). A subset of CD166-expressing cells (light blue) also express Ki67 (red), and are marked by arrowheads. Arrows mark CD166-negative, Ki67-poistive tumor cells. Bar = 25 μm.

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 ($Lgr5^+$, $Msi1^+$, $Dcamkl1^+$), 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 division[151].

A number of intestinal stem cell markers (Figure 2.8) were co-expressed in CD166-expressing crypt cells. In contrast, the putative stem cell marker Msi1 was often expressed in a single crypt cell within a zone of CD166-positive cells. Interestingly, Msi1 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 humans[82] 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 CSCs[82]. Along with this observation and its history in cancer progression as a marker for aggressive disease[90], 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.

CHAPTER 3

Function of CD166 in Maintenance and Regeneration of the Intestinal Stem Cell Niche

Function of CD166 in Maintenance and Regeneration of the Intestinal Stem Cell Niche

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Author Contributions: TGL, NS and MHW designed experiments, TGL and NS executed experiments and performed quantification of results. TGL, NS, MHW compiled data for figures and TGL, MHW prepared the manuscript.

To be submitted.

Abstract

Background & Aims: CD166 is a marker for colorectal cancer stem cells and is highly expressed by aggressive tumors. While important in cancer, nothing is known about its function in normal intestinal epithelia. We have demonstrated that CD166 is expressed within the stem cell niche of the intestine. This expression domain encompasses multiple crypt-base progenitor populations and adjacent differentiated Paneth cells. As an adhesion molecule, CD166 may facilitate communication between intestinal progenitors and adjacent epithelia thereby regulating progenitor behavior. Methods: We explore the function of CD166 in the intestinal stem cell niche by investigating crypt homeostasis and regeneration in response to injury. These studies were performed in mice lacking CD166. Results: CD166 knockout mice displayed reduced proliferation and suppressed activation of Wnt signaling within the cells of the intestinal crypt. Lgr5-expressing stem cell numbers were reduced compared to that in wild-type intestines. Further, activation of epithelial proliferation in response to injury was delayed. Consistent with a defect in the Wnt signaling pathway, a subset of differentiated Paneth cells displayed aberrant migration. Conclusions: Functional studies of CD166 suggest its involvement in the regulation of the Wnt signaling pathway by facilitating progenitor interaction with surrounding intestinal epithelium. A CD166-mediated stimulation of the Wnt signaling pathway may also be conserved in CRC where elevated CD166 expression is associated with shortened patient survival. These findings support future investigation of therapeutic inhibition of CD166 in cancer.

Introduction

The intestinal epithelium is one of the most proliferative tissues in the human body, necessitating highly active and tightly regulated stem cells. Regulatory signals are facilitated by the stem cell microenvironment, which includes fibroblasts, endothelium, immune cells and adjacent epithelium. Signaling between intestinal stem cells and their complex cellular niche is necessary for homeostatic maintenance and prevention of disease. One prominent mechanism for coordinating signaling between stem cells and the cells of their microenvironment occurs through cell adhesion complexes[152].

Interestingly, we have shown that the adhesion molecule CD166 is located in a restricted expression domain at the base of the stem cell niche, encompassing the Lgr5-positive stem cell population as well as adjacent differentiated Paneth cells[153]. Consistent with the possibility that CD166 may function to coordinate cell signaling in the stem cell niche, its expression has previously been associated with activated Wnt signaling[117, 118]. Wnt pathway induction of adhesion molecules may provide a feedback mechanism to permit the surrounding cellular environment to either enhance or repress activation of the Wnt signal. This potential regulatory mechanism supports the idea that CD166 may function to coordinate cell adhesion and Wnt signaling between Lgr5-expressing stem cells and adjacent Paneth cells. Previous studies have implicated the Paneth cell as an important source of Wnt-3a for propagation of the Lgr5-expressing stem cell population[27]. These observations support the notion that CD166 may facilitate this regulatory interaction. Currently, the

physiologic function of CD166 within the intestine is not understood, however enhanced expression in CRC is well established.

High cell surface expression of CD166 in CRC correlates significantly with shortened patient survival[90,91]. Further, isolation of CD166/CD44 or CD166/ESA double-positive cells from human CRC can initiate tumorigenesis when xenografted into immune-deficient mice, with as few as 300 cells[82]. In this same study, when CD166-negative tumor cells were xenografted, a significantly larger number of cells was required (>100,000) to initiate tumors. This finding suggests that CD166 expression marks a population of cancer cells with increased virulence and tumor-initiating capacity. The function of this molecule in tumor initiation is supported by another study, in which growth of colon cancer xenografts were inhibited when mice were treated with a CD166 neutralizing antibody[95]. These results suggest that CD166 may function to modulate cellular survival and proliferation. Importantly, expression of CD166 in stem cell populations of other tissues implies that CD166 may also modulate survival and proliferation in normal intestinal progenitor populations[*108-111*]. This raises concerns about whether CD166 could be safely targeted therapeutically without severe side effects to normal tissue. Clearly, a better understanding of CD166 function in cancer and normal tissue is needed.

We have recently determined that CD166 is expressed in a restricted domain within the intestinal stem cell niche which encompasses a subset of intestinal stem cells and adjacent niche cells[153]. Based upon this initial finding we explored its physiologic function in the intestines of CD166 knockout mice[107].

Interestingly, absence of CD166 resulted in reduced proliferation and Wnt signaling in cells of the intestinal crypts. To assess the impact of CD166 loss on stem cell numbers, CD166 null mice were crossed onto Lgr5-GFP stem cell reporter mice. Stem cell numbers and crypt size were reduced in the intestines of resulting progeny. Finally, stem cell activation was tested by challenging the intestine with radiation exposure. After irradiation, CD166 null intestines regenerated more slowly and were less able to activate a proliferative response. These findings suggest that CD166 plays an important role in establishing a homeostatic set point in the intestine for both Wnt signaling and stem cell numbers.

Regulation of proliferation, Wnt signaling and response to radiation by CD166 supports the investigation of its inhibition in CRC and may explain this molecule's association with aggressive disease. The viability of CD166 knockout mice in conjunction with our findings suggests that although targeting this molecule in cancer may affect normal stem cell behavior, a viable therapeutic window with limited toxicity may still exist. To date, targeting the Wnt pathway in CRC has been challenging due to limited druggable candidates. CD166 may provide a novel Wnt pathway target in certain phases of disease or treatment reliant on positive feedback through cell adhesion.

Materials 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. $CD166^{-/-}$ mice were obtained as a kind gift from Dr. Josh Werner (University of Iowa) and from The Jackson Laboratory (Bar Harbor, ME)[107]. C57Bl/6 and Lgr5-eGFP mice were obtained from The Jackson Laboratory[5]. Lgr5-eGFP and $CD166^{-/-}$ mice were crossed in our facility to generate Lgr5-eGFP; $CD166^{-/-}$ mice.

Histochemical and immunohistochemical analyses of intestinal tissue

Adult mouse intestines were dissected and prepared for frozen or paraffin tissue embedding and analyses, as we have previously described[142]. Briefly, for frozen blocks, intestines were dissected and divided into thirds. Each third was flushed with ice cold PBS followed by 4% paraformaldehyde. Intestines were cut open longitudinally along the mesenteric attachment line, pinned out flat on a black wax plate, incubated in 4% paraformaldehyde for one hour at room temperature, washed three times in PBS, and incubated in 30% sucrose overnight at 4ºC. Tissues were then blocked in frozen Optimum Cutting Temperature Media (Sakura Finetek, Torrance, CA). For paraffin blocks, after paraformaldehyde fixation, tissue was dehydrated in 70% ethanol overnight.

Intestinal strips were embedded in 2% agarose, positioned within a tissue cassette and then embedded in paraffin wax.

For histochemical analsys, tissue sections (5µm) were stained with hematoxylin and eosin (Vector Laboratories, Burlingame, CA) or incubated with antibodies to GFP (1:500; Invitrogen, Carlsbad, CA), lysozyme (1:300; The Binding Site, Birmingham, UK), and Ki67 (1:500; Abcam, Cambridge, MA) overnight at 4°C. Visualization of antigen was facilitated by species-specific secondary antibodies conjugated to Fluorescein isothiocyanate (FITC) (1:500; Jackson ImmunoResearch) or Indocarbocyanine (Cy3, 1:500; Jackson ImmunoResearch). Nuclei were counterstained with Hoechst dye (33258, 0.1µg/ml in PBS; Sigma; St. Louis, MO). Images were captured using a Leica DMR fluorescent microscope (Leica Microsystems) or by confocal microscopy. Confocal images were acquired as 0.5µm planes using an IX81Inverted Microscope equipped with Fluoview FV1000-Spinning Disc Confocal (Olympus) scan head and FV10 ASW 1.7 software (Olympus). To quantify Ki67 staining cells, the number of positive cells (Ki67 signal which co-localized with nuclear Hoechst staining) was counted in a minimum of 50 well-oriented crypts per animal. An n=3-5 $CD166^{-/-}$ and WT mice were analyzed for each.

Wholemount confocal imaging of crypt-base cells

Wholemount confocal imaging of the Lgr5-GFP-expressing cells within the intestinal crypt base was performed. Intestinal tissue from the 5 centimeters

proximal to the cecal junction was dissected as described above and fixed in 4% buffered formalin for 24 hours, washed and stored in PBS for imaging. Prior to analysis, tissue was stained in a Hoechst solution for 1 hour and placed on a glass slide, villus side down. The tissue was then mounted in 4% n-propyl gallate in glycerol and PBS such that the muscularis was adjacent to the glass coverslip. Adhesive silicone spacers (CoverWell chamber gasket, Invitrogen) were used to maintain spacing between the slide and cover glass to prevent tissue distortion. Twenty-five micron confocal stacks were acquired with a 0.5µm step size. The diameter of each crypt and number of Lgr5-GFP^{+} cells in each crypt were quantified, a minimum of 50 crypts were analyzed by confocal microscopy for each mouse. An n=3-4 mice were analyzed for each mouse strain.

Whole body irradiation

Mice were subjected to a single 8.4 Gy dose of whole-body irradiation from a calibrated cesium-137 source, administered at 1.548 Gy/min. Intestines were dissected, processed and analyzed at 0, 12, 24, 48, 96 and 172 hours after exposure. A total of n=4 mice were analyzed for each genotype at each time point.

Analyses of isolated intestinal epithelial cells

The undifferentiated (crypt) epithelial cells of the mouse SI were isolated using a modified Weiser preparation[153], stained using a novel antibody developed in our lab, clone 6A6. Clone 6A6 specifically stains villus epithelium and is used by our group to positively isolate villus or negatively isolate crypt epithelium. Cells were sorted using a Cytopeia Influx to collect crypt epithelia from CD166 null and WT mice. Briefly, SI was dissociated using 30 mmol/L EDTA followed by incubation with 0.3 U/mL dispase to reach single cell suspension. Cells were resuspended in modified Hank's buffered saline solution (HBSS) and gently filtered through a 70µm filter followed by incubation on ice for 20 minutes with antibodies against CD45 conjugated to allophycocyanin and 6A6, followed by incubation with secondary antibodies conjugated to fluorescein isothiocyanate for 6A6 detection. Cells were resuspended in modified HBSS with 5 µg/mL propidium iodide (PI)/1% bovine serum albumin and sorted using a 150-µm nozzle, 4.5 psi, and Spigot software (BD Biosciences, San Jose, CA). To ensure sorting and analysis of single cells, a doublet discriminator was used to exclude cells based on pulse width. Fluorescent activated cell sorting (FACS) data were analyzed using FCS Express Version 3 Research Edition (DeNovo Software, Los Angeles, CA). 6A6-negative, CD45-negative, PI-negative cells were collected as the crypt fraction and RNA was extracted as described below for gene expression analysis.

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)

qRT-PCR was used to evaluate gene expression in FACS-isolated crypt epithelium from CD166 null and WT mice. Epithelial cells were isolated as described above. Total RNA was isolated from crypt CD166 null and WT FACS isolated epithelium using an RNAqueous kit (Ambion, Austin, TX) and cDNA generated using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA). qRT-PCR was performed using a SYBR Green-based assay, a 7900HT Sequence Detector (Applied Biosystems), and analyzed according to established protocols[47]. Each cDNA sample was analyzed in triplicate and reported as an average of two independent runs. Data were normalized to Gapdh. CD166-null crypt gene expression is presented as a fold difference relative to gene expression in WT crypt epithelium. Primers are listed in Table 3.1 on the following page.

Table 3.1 qRT-PCR Primers Used in Study

Gene Name	Primer (Forward 5'-3')	Primer (Reverse 5'-3')
Ascl2	CCGGTTCCTCGCGAGCACTTTT	TCCAGACGAGGTGGGCATGAGT
CD24a	AGCTTAGCAGATCTCCACTTACCGAAC	CGTGGGTAGGAGCAGTGCCAGA
CD44	TAGGAGAA GGTGTGGGCAGAA	GAGCTCACTGGGTTTCCTGTCTT
Dll1	GGACGATGTTCAGATAACCC	CCACATTGTCCTCGCAGTA
Dll4	CAGTGTGCCTGCGATGA	GGAGACAGGTGCAGGTAT
EphB2	ACCTCAGTTCGCCTCTGTGAA	GGACCACGACAGGGTGATG
EphB3	TCTGACACTCAGCTCCAACGA	CCAGGCATCCAAAAGTCCA
EphrinB1	AGGTTGGGCAAGATCCAAATG	AGGAGCCTGTGTGGCTGTCT
Klf5	TGCCAACGCAGCTTCTCCCG	CAGCGCTCGCTCGCTCAGTT
Lgr5	TTTGAGAAGCCTTCAATCCC	GACAGGGACGTCTGTGAGAG
Lrig1	GGGTCCACGGCTATCATCAGCTC	CCGCCTCTGAGGACTGAAAACGC
Fabpl	GGTCTGCCCGAGGACCTCAT	CCAGTTCGCACTCCTCCCCC
Gapdh	CACTGCCACCCAGAAG ACTGT	GGAAGGCCATGCCAGTGA
Hes1	GTGCATGAACGAGGTGACCC	GTATTAACGCCCTCGCACGT
Math1	TACAGATGGCCCAGATCTACATCA	TGGTCATTTTTGCAGGAAGCT
Notch1	CCAGCTTGCACAACCAGACA	ACGGAGTACGGCCCATGTT
Wnt3a	CAAGCACAACAATGAAGCAGGC	TCGGGACTCACGGTGTTTCTC

Results and Discussion

Proliferation is reduced in CD166$^{-/-}$ mouse intestines

The role of adhesion molecules in maintaining the location of stem cells within a niche, regulating differentiation, and proliferation is a well-described mode of regulation[154]. For example, conditional deletion of β1-integrin in the mouse intestine results in a dramatic increased proliferation of progenitors, loss of Hedgehog signaling and subsequent lethality[155]. This demonstrates that adhesion molecules can play an important role in regulating intestinal stem cell signaling and behavior. The unique crypt base expression of the adhesion molecule CD166[153], and its association with the Wnt signaling pathway[117,118], suggests a potential role for CD166 in regulating stem cell behavior.

Although CD166 is expressed in a number of adult stem cells[108-111,138], its function in maintaining homeostasis has not been explored. We recently showed that CD166 is robustly expressed in human and mouse intestine in a discrete subset of cells within the intestinal stem cell niche[153]. Importantly, this expression domain encompasses intestinal progenitors, as well as epithelial niche cells and may play an important role in coordinating interactions between these populations. Therefore, to understand the function of CD166 within the stem cell niche we analyzed the effects of CD166 loss on intestinal homeostasis. Significantly, CD166 null mice are viable and readily reproduce, suggesting that CD166 is not essential for development and survival[107]. The CD166$^{-/-}$ intestine was grossly normal. However, the overall villus height was shortened along the entire length of the intestine compared to age-matched WT mice (Figure 3.1A).

Therefore, to determine if decreased proliferation within the intestinal stem cell niche could account for the shortened villus phenotype, tissue sections from CD166$^{-/-}$ intestines were analyzed for proliferative capacity by Ki67 antibody staining (Figure 3.1B). Ki67 is detectible in cells actively engaged in the cell cycle and reflects the overall proliferative capacity of a tissue. The number of Ki67-positive cells per crypt were quantified within the distal third of the small intestine in both CD166$^{-/-}$ and WT mice (Figure 3.1C). This analysis revealed a significant reduction in the number of proliferating cells per crypt in the CD166$^{-/-}$ intestines (13±0.70 vs. 18.4±0.63). Interestingly, CD166 expression was associated with Wnt activation in CRC cells[117] and Wnt signaling is known to play an important role in maintaining proliferation in the intestinal stem cell niche[43]. In order to determine if loss of CD166 modulates the Wnt signaling pathway, expression of Wnt pathway components and target genes were compared between isolated crypt cells from CD166$^{-/-}$ and WT distal small intestines by qRT-PCR. CD166 null mice had a significant 2-3 fold down-regulation of multiple Wnt pathway components including Wnt3a, Axin-2 and Ascl2 (Figure 3.1D).

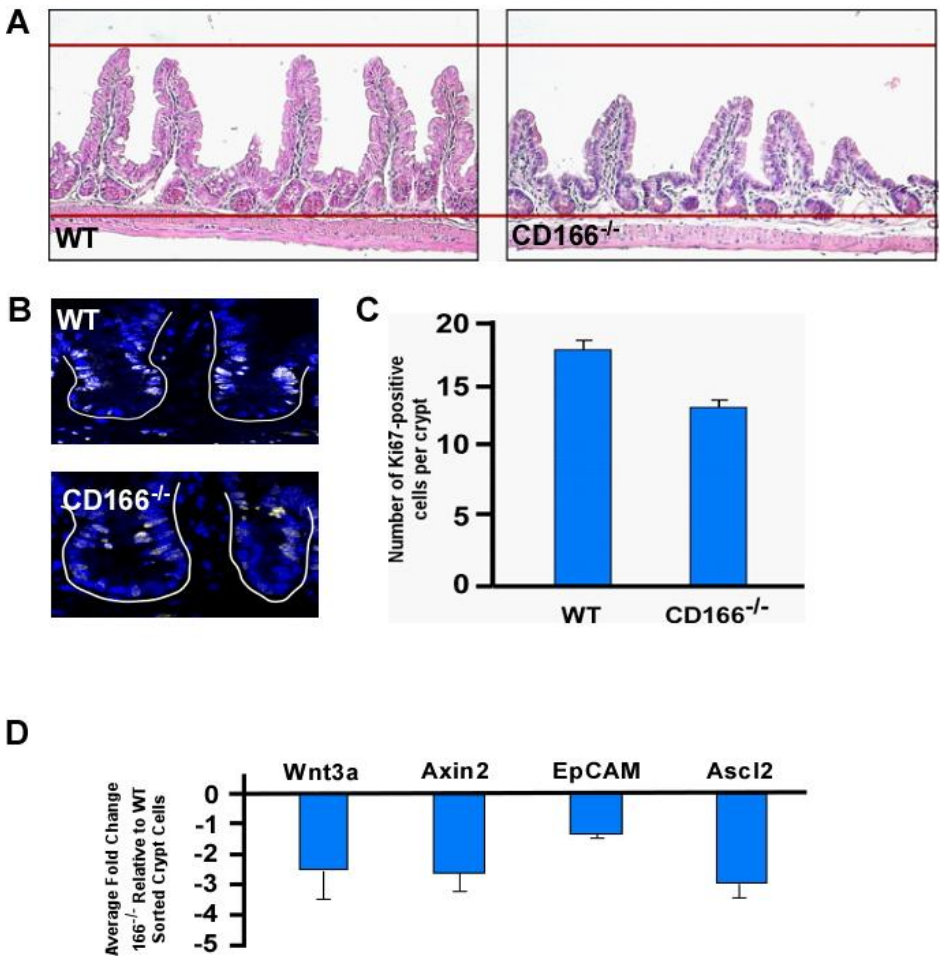

Figure 3.1 Proliferation and Wnt signaling is reduced in CD166 null crypts. **(A)** Height comparison of WT (left panel) and CD166 null (right panel) intestinal villi, both images acquired in the 1cm proximal to the ileal-cecal junction. Villus height is reduced by approximately one-third in CD166 null mice. **(B)** Staining of the cell cycle proliferation-associated protein Ki67 in WT (top panel) and CD166 null (bottom panel) crypts. Ki67 (orange) is overlayed with the nuclear stain Hoechst (blue). **(C)** Quantification of Ki67 staining in crypts. The median number of proliferating cells per crypt is significantly reduced from approximately 18 in WT to 13 in CD166 null crypts ($p < 0.000001$) **(D)** Gene expression represented as fold change in CD166 null crypts relative to WT. Crypt cells were enriched and gene expression of Wnt genes was assessed by qRT-PCR. Wnt3a, Axin2 and Ascl2 are all down regulated greater than 2-fold in CD166 null crypts. Error bars represent standard error.

CD166 may facilitate adhesion between the stem cell and its niche, while coincidentally regulating Wnt signaling between these populations. In this fashion, CD166 may participate in establishing a homeostatic set point in the gut where proliferation and stem cell activity is reduced. This idea is supported by our observation that gene expression of other stem cell markers is reduced in the crypts of CD166$^{-/-}$ mice (Figure 3.2C), suggesting that overall capacity of the stem cell niche is suppressed. While stem cell markers expressed within the CD166 expression domain were significantly down regulated (Lgr5, CD24), progenitor markers, which encompass a broader expression domain, were reduced but not to significant values (CD44, Lrig1, Klf5). Consistently, the differentiation maker Fabpl was up-regulated in the epithelial crypt cells of CD166 null mice. These changes may, in large part, be due to the observed reduction of Wnt signaling in CD166 null mice. As mentioned above, we show that loss of CD166 results in an approximate 3-fold down-regulation of Wnt-3a messenger RNA (Figure 3.1D). Recent work has shown that Paneth cells secrete the ligand Wnt3a, which is necessary for proliferative activation of the adjacent Lgr5 stem cell population.

To determine if loss of CD166 and the subsequent reduction in crypt-base Wnt signaling specifically affects the number of cells in the Lgr5 stem population, CD166 null mice were crossed to Lgr5-eGFP reporter mice. Numbers of GFP positive stem cells per crypt and crypt size were quantified. Confocal microscopy was used to image through the muscularis to view the base of the intestinal crypts of Lieberkühn. Interestingly, CD166$^{-/-}$ mice have statistically fewer Lgr5

expressing stem cells per crypt (on average 10 vs. 14, Figure 3.2A, B), without a significant change in crypt size (data not shown). These findings suggest that CD166 is an important regulator of the Lgr5 stem cell population and crypt homeostasis. While Lgr5 stem cell numbers appear reduced, a down-regulation of stem cell and Wnt associated genes may also reduce stem cell activation after injury.

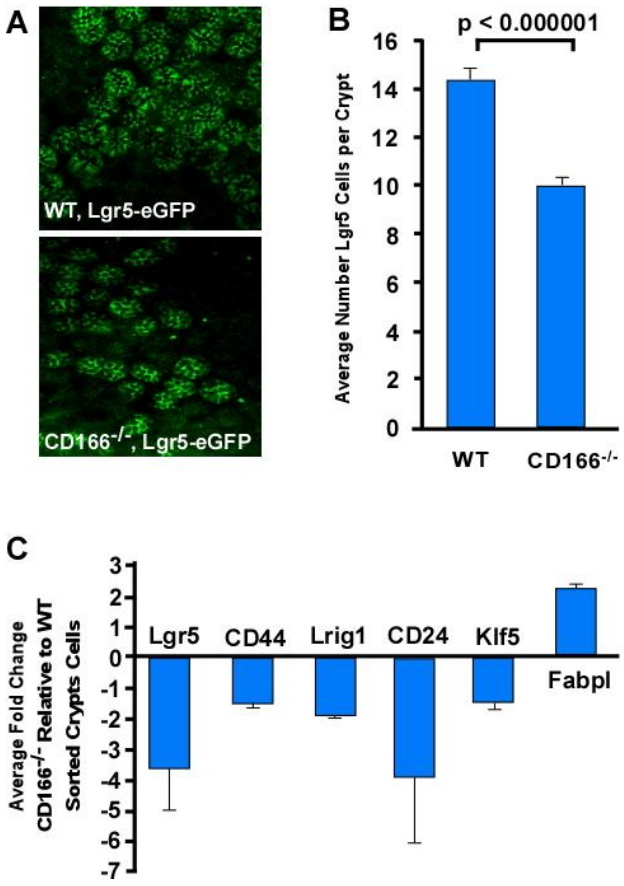

Figure 3.2 Stem cell numbers and gene expression are reduced in CD166 knockout mouse intestines. (A) Confocal micrograph of the base of crypts imaged through intestinal muscularis. Top panel, WT Lgr5-eGFP$^{+/-}$ reporter mouse. Bottom panel, CD166$^{-/-}$; Lgr5-eGFP$^{+/-}$ mouse. (B) Average number of Lgr5-eGFP cells per crypt in WT and CD166$^{-/-}$ mice. (C) Average fold change of gene expression in CD166$^{-/-}$ crypts relative to WT crypts. Error bars represent standard error.

Regeneration after injury is delayed in CD166 null mice

A reduction in Wnt signaling and lower numbers of Lgr5 stem cells within the CD166 null intestine may compromise its ability to regenerate after injury. To test the influence of CD166 loss on stem cell activation and regeneration, CD166 null and WT mice were first irradiated with sub-lethal whole-body radiation. Their intestines were then analyzed for cell proliferation by Ki67 staining at 0, 6, 12, 24, 48, 72 and 96 hours after irradiation. The number of Ki67 cells in each crypt was quantified as a measure of proliferative capacity after injury. No change in proliferation was apparent immediately following radiation (0 hours) when compared to unirradiated controls. However at 12 and 24 hours, significant reduction in proliferation was observed in CD166 null mice compared to the traditional proliferative increase within WT intestines (Figure 3.3B). By 72 hours, the level of proliferation in CD166 null mice increased and neared the proliferative level in WT intestines. Significantly, the dramatic inability of the CD166 null mice to mount a rapid intestinal proliferative response suggests they are less capable of activating stem cells into proliferative expansion after injury. This is in addition to a reduction in proliferation and stem cell numbers during homeostasis, as described above (Figure 3.1A, B). Interestingly though, a late wave of proliferation at 72 hours still persists in CD166 null mice after irradiation. This finding suggests that multiple post-injury mechanisms may exist to activate proliferation, some of which are *not* dependent on CD166. In future studies, it will be informative to examine cell death by caspase-3 cleavage or TUNEL staining to determine if reduced proliferation and delayed regenerative response are also due to increased radiation sensitivity and death of stem cells.

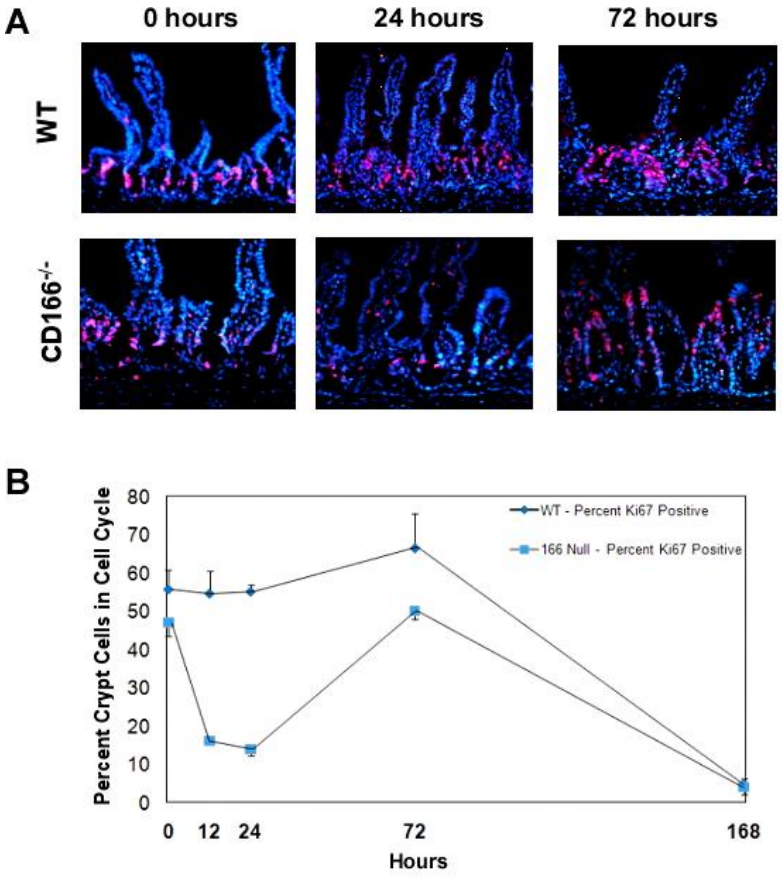

Figure 3.3 Delayed proliferative stem cell activation in CD166 null intestinal crypts. (A) Representative images of Ki67 staining (red) overlaid with nuclear Hoechst stain (blue) at 0, 25 and 72 hours after irradiation. Top row WT mice, bottom row CD166$^{-/-}$. (B) Average percent of crypt cells in cell cycle as determined by Ki67-positivity at 0, 12, 24, 72 and 168 hours. WT quantification represented by dark blue diamonds; CD166$^{-/-}$ quantification represented by light blue squares. Error bars represent standard error.

Paneth cell migration is disrupted in CD166 null mice.

Lineage differentiation and cell migration within the intestine is known to be influenced by multiple cell signaling pathways, including the Notch and Wnt pathways[20]. Previous studies have shown that CD166 is associated with the canonical Wnt signaling pathway in cancer and its expression is regulated in part by Wnt3a[117,118]. In addition, our analysis of CD166 null crypts reveals a significant reduction in Wnt3a (Figure 3.1D). Further, loss of the of the canonical Wnt targets EphB2 and EphB3 have been shown to play an important role in Paneth cell and cancer cell migration[45,58,156]. In CD166 null intestines, we observe granule containing lysozyme-positive cells located in the transit-amplifying or crypt-villus junction region, in approximately 10% of crypts when analyzed by cross-section (Figure 3.4 A-D). It is not clear whether these are Paneth-goblet cell precursors or aberrant migration of differentiated Paneth cells from the base of the crypt. Defects in differentiation of Paneth-goblet precursors have been observed in Notch pathway knockout mice, including Notch1 and Math1 null mice while Paneth cell migration defects have been observed in mice with Wnt signaling defects[45,71].

To determine if the Notch pathway was also perturbed in CD166 null crypts, we compared the crypt-base expression of Notch1, Hes1, Math1, Dll1 and Dll4 in CD166 null and WT crypt epithelium by qRT-PCR. Interestingly, Notch related genes did not show significant changes within the crypt epithelium from CD166 null and WT intestines (Figure 3.4E). Further, co-staining for Paneth and goblet cell markers did not show significant co-localization (data not shown). This

suggests that Notch mediated differentiation of secretory lineages may be unperturbed in the CD166 null intestine. Consistent with a Paneth cell migration defect and potential Wnt involvement, EphB3 is down-regulated 2.0 fold (Figure 3.4F). This finding supports the idea that lysozyme-positive, granule containing cells, in the TA-cell region may be the result of abnormal Paneth cell migration.

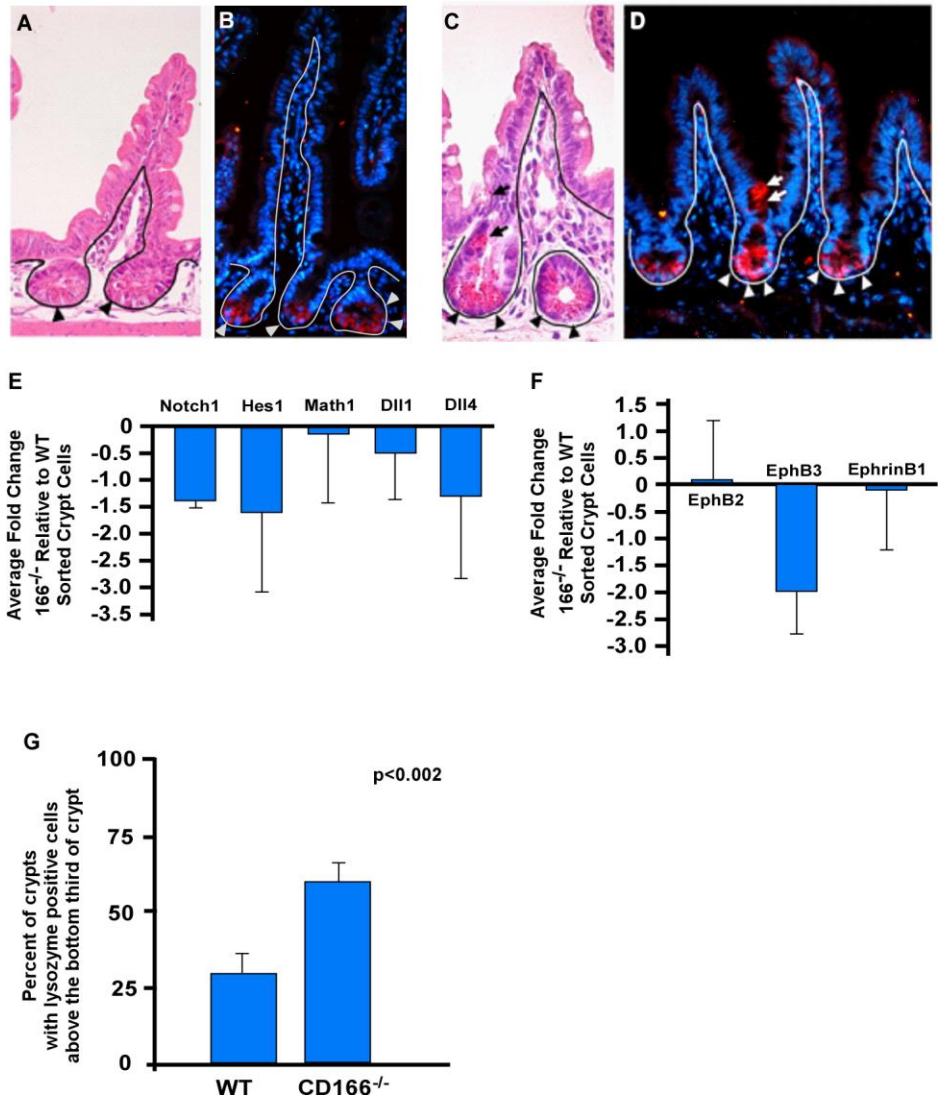

Figure 3.4 CD166 knockout mice display a Paneth cell migration defect. Hematoxylin and eosin stained intestine from a WT mouse (A) and from a CD166$^{-/-}$ mouse (C). Paneth cell granules are hyper eosinic and are denoted with black arrow heads. Aberrantly migrated Paneth cells denoted with a black arrow in (C). Lysozyme antibody staining labels Paneth cells in WT (B) and CD166$^{-/-}$ mice (D). Crypt base paneth cells denoted with white arrow heads, aberrantly migrated cells denoted with white arrows. (E) Average fold change in Notch pathway gene expression in CD166$^{-/-}$ relative to WT crypts. No genes were expressed at equal or greater than a 2-fold change. (F) Average fold change in Eph-Ephrin receptor gene expression in CD166$^{-/-}$ relative to WT crypts. A significant 2.0-fold down regulation of EphB3 was observed. (G) Quantification of lysozyme positive cell positioning in WT and CD166$^{-/-}$ intestine. The percent of crypts with lysozyme cells found above the bottom third were quantified and found statistically significant between groups. Error bars represent standard error.

Conclusion

While CD166 expression has been observed in hematopoietic, mesenchymal and now intestinal progenitor populations, its molecular function in these progenitors has not previously been described [108-111, 138, 153]. Here we provide evidence that CD166, expressed within the epithelial stem cell niche of the intestine, participates in modulating proliferation and Wnt signaling during tissue homeostasis. Absence of CD166 expression resulted in reduced Lgr5 stem cell numbers, and decreased activation of progenitor proliferation after injury. As a stem cell niche marker, it is possible that CD166 facilitates adhesive interactions between stem cells and adjacent Paneth cells, thereby enhancing efficient dispersal of the Paneth cell Wnt-3a ligand to neighboring Lgr5-expressing stem cells. Alternatively, like the adherens junction molecules, E-cadherin and β-catenin, CD166 may also influence associations of Wnt receptors and adhesion complexes into signaling nodes at the cell surface. For example, CD166 is known to be expressed in adherens junctions[120], which also regulate Wnt signaling though dynamic regulation of β-catenin[79]. Loss of CD166 may influence adherens junction formation thereby modulating the amount of β-catenin available for transducing Wnt signals. Another intriguing possibility is that CD166 may have an independent outside-in signaling mechanism in which it acts as a positive feedback mechanism for enhancing Wnt signaling. This concept is supported by the finding that in 3T3 cells, Wnt3a significantly upregulates CD166 gene expression. Finally, CD166 is known to influence the activation of MMP8[157] and is itself susceptible to proteolytic cleavage by Adam10/17[124]. While the

specific modulation of cell migration by Adam10 has only recently been described in the intestinal crypt[158], this is one additional mechanism by which CD166 may influence signaling and subsequent cell migration within the stem cell niche. Through cleavage of E-cadherin at the EphB/Ephrin-B junction, ADAM10 promotes differential adhesion between crypt and differentiated villus cells and helps to localize Paneth cells to the base of the crypt[158]. Our observation that Paneth cells are mislocalized in CD166 knockout mice supports the idea that ADAM10 regulation of CD166, in addition to E-cadherin, may be necessary for proper localization of Paneth cells.

Deregulation of the Wnt pathway is a hallmark of CRC, with detection of Wnt pathway mutations occuring ubiquitously in patients with CRC[43]. While the role of the Wnt signaling pathway in the pathogenesis of CRC is well established, direct targeting and inhibition of this pathway has proven challenging due to a lack of viable drug targets[159]. Intriguingly, our data suggest that CD166 may provide a novel target for modulation of Wnt signaling in cancer. Absence of CD166 expression reduces the mRNA levels of a number of Wnt signaling components at multiple levels of the pathway, without overt physiologic toxicity. Further, CD166 expression is enriched in cells resistant to chemotherapy[93, 94]. Whether CD166 is functionally involved in conferring resistance is not yet known, but interestingly, over-expression of CD166 is reported in a subset of CRC patients and correlates with shortened survival[90,91]. In these patients, CD166 may be a promising therapeutic target. While direct CD166 inhibitory antibodies have been described[95], they have not been examined extensively in CRC. These

studies and our findings support the notion that inhibition of CD166 enhances Wnt signaling, proliferation and chemo-sensitivity in cancer and support a rationale for further investigation of CD166 as a therapeutic target.

CHAPTER 4

CONCLUSION AND FUTURE DIRECTIONS

CONCLUSION

Stem cell biology has entered a golden era of discovery. In the intestine, the recent identification of new stem cell makers has created a research environment primed for understanding mechanisms of stem cell activation in regeneration from injury and their dysregulation in disease states, such as cancer[5,13-17]. Signaling between stem cells and the cells of their microenvironment is a primary mechanism of regulation. These important regulatory interactions are coordinated, in many cases, through adhesion molecules[154]. Junctional complexes provide the context for coordination of signaling nodes between cells which control important cellular processes such as proliferation, cell migration, differentiation and cell death. Intriguingly, the cell adhesion molecule, CD166, was recently described as a cancer stem cell molecule in colorectal cancer[82]. But in the normal intestine, CD166 function is not well characterized. Based upon its relevance in CRC and its described expression within the hematopoietic stem cell niche[109], we set out to explore the contribution of CD166 function in normal intestine. Interestingly, we determined that CD166 is expressed at low levels on all of the epithelium. Even more striking, it was robustly expressed in a discrete domain at the base of the intestinal crypt. This distinct expression domain encompassed both Lgr5-expressing stem cells and adjacent differentiated Paneth cells, supporting the possibility that CD166 participates in regulating the Lgr5-expressing stem cell population. Phenotypic defects observed with loss of CD166 expression included suppressed proliferation, reduced stem cell numbers, aberrant Paneth cell

migration, suppressed Wnt signaling and delayed proliferative response after injury. These findings support a function for CD166 in enhancing Wnt signaling between stem cells and adjacent Paneth cells.

A positive feedback model between Wnt and CD166 in the intestine

Our findings suggest that CD166 plays an important role in establishing a homeostatic set point in the intestine by enhancing Wnt signaling between stem cells and adjacent Paneth cells. One potential mechanism by which this could occur would be through a positive feedback loop in which Wnt signaling transcriptionally regulates CD166, up regulating its expression (Figure 4.1). Upon assembly into the adherens junction, CD166 would engage in homotypic adhesion and may reduce the need for E-cadherin/β-catenin based adhesion, freeing a pool of β-catenin to positively feedback on the Wnt pathway (Figure 1.7, Figure 4.1B). This is corroborated by the observation that active Wnt signaling directly inhibits transcription of E-cadherin[160,161], supporting the need for alternative adhesive mechanisms in the presence of active Wnt and freeing of the adherens junction pool of β-catenin to participate in Wnt signaling. Interestingly, disassembly of cadherin based adhesion can activate Rac1[162] which is required for nuclear translocation of β-catenin[163] but is also directly involved in activation of CD166 homotypic adhesion[121]. This further supports the notion of complex cross talk between Wnt, E-cadherin disassembly, and activation of CD166.

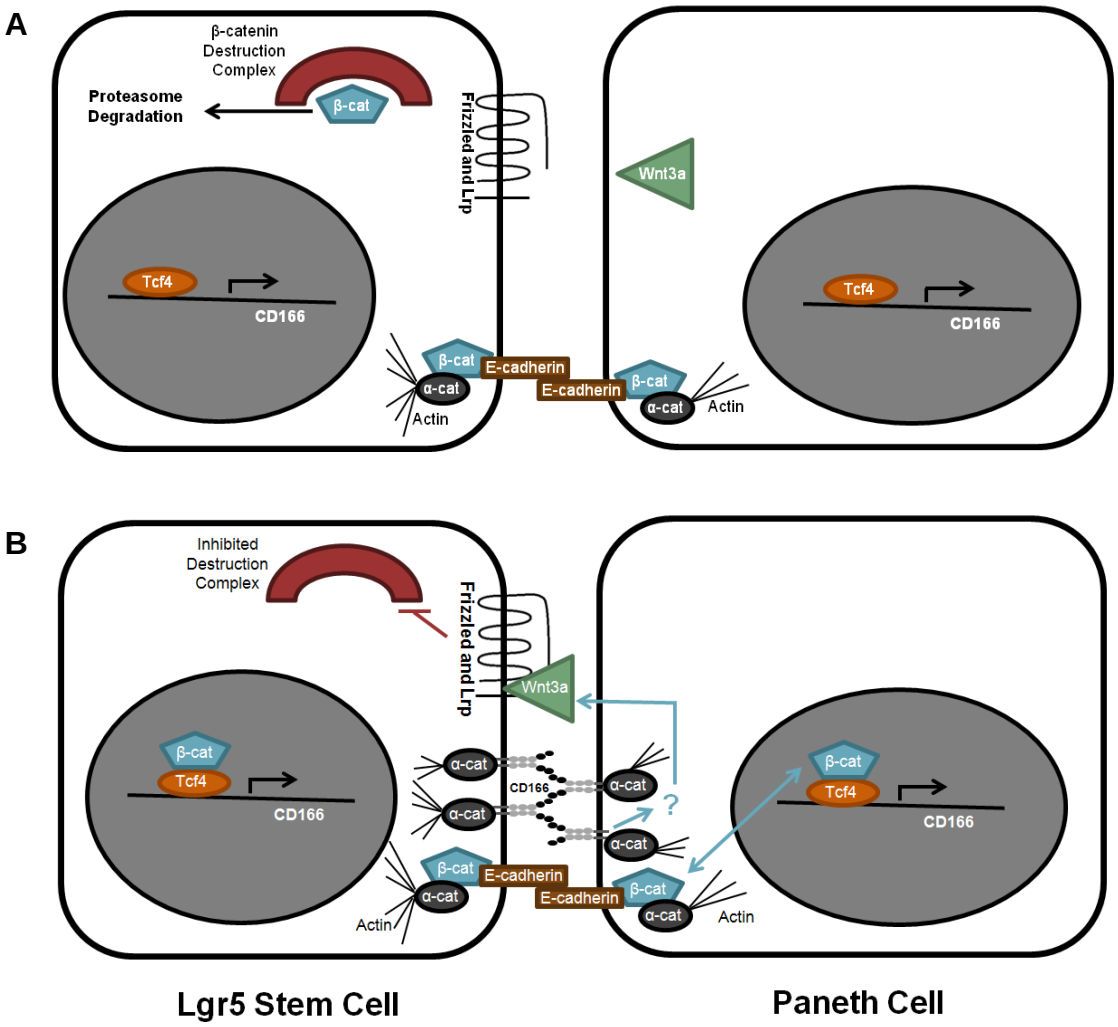

Figure 4.1. A positive feedback model between Wnt and CD166 in the intestine. (A) In the absence of Wnt signaling CD166 is not expressed in the intestinal crypt and E-cadherin based adhesion is dominant. (B) Paneth cells secrete Wnt3a which acts in an autocrine and paracrine (shown here) fashion. Binding of Wnt3a to Frizzled and Lrp recruits Dishevelled (not shown) to the membrane which subsequently inhibits/disrupts the β-catenin destruction complex (further detail in chapter 1). Upon cytoplasmic stabilization, β-catenin translocates to the nucleus where it activates CD166 transcription with its Lef/Tcf4 binding partner. CD166 is localized to the adherens junction where it potentially reduces the need for cadherin based adhesion, freeing up a pool of β-catenin to enhance Wnt signaling. Alternatively, CD166 directly signals through unknown mediators to enhance Wnt signaling by an alternative mechanism.

This model serves as a basis for future study of CD166 function within the intestinal stem cell niche. To test this model, future studies should confirm that E-cadherin becomes down regulated in the intestinal stem cell niche upon Wnt stimulation. In light of our finding that CD166 expression marks the stem cell niche, we now have a tool to enrich for Wnt activated cells by flow cytometry and compare E-cadherin gene expression relative to Wnt-inactive villus cells. Further, it will be informative to determine whether CD166 expression can directly displace or down regulate E-cadherin mediated adhesion. CD166 over expression studies will nicely complement our CD166 knockout analysis. Using a cell culture system we could directly monitor E-cadherin and adherens junction associated β-catenin in the presence of CD166 over expression. This system would also allow us to measure Wnt activation and further test our hypothesis that CD166 enhances Wnt signaling. These over expression experiments could also be performed in the presence or absence of Wnt agonists to determine whether Wnt status influences the ability of CD166 to enhance Wnt signaling. Finally, it would be very interesting to test the influence of CD166 expression and inhibition in the context of activating Wnt pathway mutations to determine if CD166 is capable of modulating Wnt signaling downstream of common oncogenic mutations.

Clearly, understanding intestinal homeostasis provides important insights for therapeutic applications to enhance intestinal regeneration and treat intestinal diseases, such as cancer. Based on the knowledge gained regarding the function of CD166, it is possible that this protein may also modulate proliferation and

radiation-sensitivity in cancer. CD166 may define a regulatory niche within tumors to modulate enhanced proliferative Wnt signaling, aberrant cancer cell migration and therapeutic sensitivity. While the Wnt pathway is known to play a driving role in CRC, targeting the Wnt pathway has not yet been successful due to a lack of easily druggable targets. Because there are no apparent viability issues in the CD166 knockout mouse nor a phenotype that threatens viability[107], and targeting of CD166 through inhibitory antibodies has had preclinical success in mouse models[95,100], targeting CD166 may be a safe way to modulate Wnt signaling and radiation sensitivity in intestinal cancers.

Therapeutic modulation of CD166 function

Modulation of CD166 adhesion for therapeutic purpose must be considered carefully. Based on CD166 literature and these current studies, CD166 can promote cellular proliferation and enhances cell adhesion/reduces cell migration. While inhibition of cellular proliferation may be therapeutically beneficial and limit tumor growth, enhanced cellular migration upon CD166 inhibition could potentially promote metastatic spread of disease. Further study of CD166 in cancer should determine the effects of CD166 inhibition in a wide array of CD166 expressing cancer cell lines to assess whether dependence on CD166 for both proliferation and migration is universal or specific to certain cancers. Interesting observations by G.W. Swart's group in the Netherlands suggest that the proliferative and migratory functions of CD166 may be uncoupled depending on how CD166 function is inhibited, supporting the concept that it may be

possible to inhibit CD166 dependent proliferation without enhancing cell migration[112,126]. Clearly though, these early studies must be confirmed and expanded to a greater number of cell lines. If only a small subset of CD166 expressing cancers demonstrate dependence on CD166 for regulation of growth and migration it will be important to determine what cellular and genetic factors predict this dependence. For example, do cancer cells which express high levels of cadherin adhesion molecules rely on the relatively weaker CD166 adhesive interaction for modulating cell adhesion but still respond to CD166 signaling for regulation of proliferation? This question could be addressed in future experiments analyzing response to CD166 inhibition in cancer cells where E-cadherin is present or absent. These types of questions highlight the importance of carefully choosing the correct cancer subtypes for CD166 targeted therapy and understanding how CD166 functions within unique cellular contexts.

One potential mechanism for inhibition of CD166 function is through targeting by inhibitory antibodies. The design of inhibitory antibodies against CD166 should take into account the domain structure of this protein (Figure 1.5). CD166 contains five immunoglobulin-like domains. The three most N-terminal domains (domain 1-domain 3) are involved in lateral oligomerization, while the two most C-terminal domains are involved in facilitating homotypic and heterotypic adhesion[164]. It may be possible to generate specific antibodies that inhibit one adhesive interaction while sparing the other. This may be beneficial as CD166-CD6 interaction is required for efficient T-cell maturation and antigen presentation to T-cells by granulocytes[99,105,106,165]. Importantly, CD166-CD6 T-cell

activation function may provide survival benefit to cancer patients, while the CD166 homotypic interactions in tumor cells promote cell survival and proliferation. Antibodies designed to target the oligomerization domains could also inhibit formation of adhesive signaling nodes and result in the dysregulation of the interaction between CD166-expressing cancer stem cells and the instructive cells of the microenvironment. Ultimately, while intriguing data exists to establish a role for CD166 in cancer[166], a better understanding of its function and the degree to which it is an active driver of cancer pathology is required for establishing CD166 as a therapeutic target. One of the more elegant studies using CD166 inhibitory antibodies was not in studying cancer, but in the setting of stroke[100]. In these mouse studies, CD166 inhibitory antibodies were found to have a dramatic effect on reducing inflammation and brain swelling, after stroke, by inhibiting migration of lymphocytes across the blood brain barrier. This further highlights the diverse function of CD166 in mammalian biology and the need to understand its diverse functions in different cell types.

In addition to inhibitory antibodies, recent publications have described the generation of CD166 internalizing antibodies conjugated to chemotherapy filled lipid vesicles[139,140,167]. These antibody drug conjugates (ADCs) are designed to increase the therapeutic window between killing of cancer cells and toxicity to normal tissues. While the ADC therapeutic paradigm shows some promise in breast cancer and non-Hodgkin lymphoma[168,169], the selection of antibody targets is of upmost importance. An ideal target would be expressed highly on tumors and expressed at low levels (or not at all) on normal tissues to permit selective

delivery of drug to the cancer[170]. While CD166 is expressed highly in many types of cancer it is also expressed in an important subset of healthy progenitor cells, including hematopoietic progenitors, mesenchymal stem cells and intestinal stem cells, as well as granulocytes, lymphocytes and endothelium[100,105,108-111,138,153]. The targeting of ADCs to CD166 would likely also deliver toxic chemotherapy to these important cell populations. Relative levels of CD166 expression between normal and cancer cells may still provide a sufficient window for dosing if cancers express CD166 at sufficiently high levels relative to endogenous progenitor populations. Another interesting mechanism for delivery of ADCs specifically to tumors could utilize glycosylation-specific antibodies. CD166 has been shown to be differentially glycosylated in cancer[171,172] and antibodies may be generated against cancer-specific CD166 glycosylation isoforms. A greater understanding of CD166 glycosylation in normal tissues and across cancer patient subtypes may provide a novel mechanism for selectively targeting ADCs to cancer cells while sparing healthy tissue.

While downstream components of CD166 signaling are not well understood, modulation of CD166 at the cell surface has previously been investigated[173,174]. An mRNA splice variant of CD166 that codes for the extracellular domain was originally discovered and characterized in endothelial cells[102]. This soluble splice variant is secreted from cells and has been hypothesized to act as a dominant negative, inhibiting adhesion between full length CD166. Further, in melanoma cell lines, expression of the soluble splice variant attenuated melanoma cell migration[112]. Future studies should be

performed to confirm that the soluble splice variant directly interacts with full length CD166 and that modulation of cellular behavior occurs through this direct interaction. Heterotypic interaction between CD166 and CD6 suggest the potential for interactions between soluble CD166 and other cell surface molecules.

Additional regulation of CD166 at the cell membrane can occur through proteolytic cleavage. The extracellular domain of CD166 is susceptible to proteolytic cleavage by ADAM17/TACE and ADAM10[124,125]. Interestingly, ADAM10 is regulated by EphB/Ephrin-B signaling, which has also been shown to regulate Paneth cell cell migration in the intestine[158]. Through cleavage of E-cadherin at the EphB/Ephrin-B junction, Adam10 promotes differential adhesion between crypt and differentiated villus cells and helps to localize Paneth cells to the base of the crypt[158]. Significantly, in CD166 knockout mice, we observe aberrantly migrated Paneth cells similar to that observed in EphB3 and Adam10 knockout mice[45,158]. Interestingly, CD166 has also been described to associate with E-cadherin in adherens junctions[120], suggesting that Adam10 may simultaneously regulate both E-cadherin and CD166 to instruct Paneth cell localization. Further study into the potential interactions between ADAM10 and CD166 in the intestine may provide insight into regulation of cell migration and establishment of tissue boundaries.

CD166 is clearly an important regulator of normal homoeostasis and disease states. However, functional signaling mechanisms attributed to CD166 have yet to be explored. In CRC, why is CD166 dysregulated in some tumors but

not in others? Why do some tumors display high cytoplasmic localization while others display high cell surface localization? And is this localization regulated in part by ADAM10/17 proteases or CD166 soluble splice variants? How does CD166 modulate proliferation, survival and cell migration in different molecular and histologic subtypes of colorectal cancer? Does CD166 expression, in combination with other markers, define a true cancer stem cell population in colorectal cancer, and what is the significance of this? And do cancer stem cell populations vary between tumors, stage of disease and treatment status? And finally, what is the influence of inhibiting CD166 in these various disease states? These are just some of the questions that must be addressed.

The findings I have described in this dissertation support the notion that CD166 participates as a regulatory niche molecule in establishing a homeostatic set point in the intestine and in coordinating proliferative response after injury. This important advance guides our thinking regarding both normal epithelial regeneration and cancer therapy, implicating CD166 as a potential enhancer of Wnt signaling, and ultimately an intriguing targetable molecule for future investigation.

FUTURE DIRECTIONS

Future experiments to improve understanding of CD166 function in the stem cell niche

These studies implicate CD166 in enhancing Wnt signaling, proliferation, Lgr5 stem cell numbers, and maintaining proper tissue localization of Paneth cells. Significantly, these conclusions rely on analysis of CD166 expression pattern in WT mice and analysis of a constitutive CD166 knockout mouse. As with any constitutive knockout mouse, compensatory mechanisms may evolve during embryonic development for cellular processes which would normally be regulated by CD166. To detect potential CD166 function which may be masked by compensatory mechanisms, development of a tissue specific-inducible knockout mouse would be useful. For example, if a cre-inducible CD166 knockout mouse line were crossed to a tamoxifin inducible villin-cre expressing mouse line, one would expect to be able to knock down CD166 specifically in the intestinal epithelium in a tamoxifin dose dependent manner. This experiment would allow for the examination of the immediate effect to the stem cell niche from loss of CD166. Alternatively, CD166 inhibitory antibodies could also be used to determine the acute effects of loss of CD166 on the stem cell niche. These experiments may more closely resemble a therapeutic scenario where CD166 would be acutely inhibited but may also result in inhibition/internalization of other cell surface molecules which closely interact with CD166. For these reasons, using both genetic and protein inhibition methods would complement each other well to specifically determine the effect of loss of CD166.

While our preliminary analysis of the CD166 knockout mouse implicates CD166 in the positive regulation of progenitor proliferation, further studies should be conducted in this area to clarify these results. BrdU pulse-chase studies would complement our Ki67 analysis to provide a higher resolution description of proliferative dynamics in the intestinal crypts of CD166 knockout mice. In response to intestinal injury it will be important to clarify whether the delayed proliferative response is primarily due to attenuated Wnt signaling after injury or increased susceptibility of progenitors to apoptosis. To answer this question it will be necessary to analyze gene expression of Wnt pathway components at multiple time points after radiation injury. Further analysis of apoptosis by analyzing cleaved caspase-3 staining or TUNEL positivityat early time points (0-6 hours after radiation) may be informative. A more focused analysis of the response of specific progenitor populations after injury may also better define CD166 function in maintaining discrete populations of intestinal stem cells and their activation after tissue damage.

In light of recent studies demonstrating that the intestine can maintain homeostasis in the absence of an Lgr5 stem cell population through Bmi1 progenitor expansion[4], it is also important to look at how different progenitors expand or contract in CD166 knockout mice. Indeed, our observation that there are fewer Lgr5 stem cells per crypt in the CD166 knockout mouse supports the need for this analysis. Unfortunately though, these types of studies have been hindered by a lack of good antibodies against intestinal stem cell markers (such

as Bmi-1 and Tert) and may require the crossing of the CD166 knockout mouse onto additional stem cell reporter mouse lines.

In addition to further loss of function analysis, over expression of CD166 could be very informative. Earlier in this chapter, I discussed how over expression analysis in cell lines is necessary to validate my proposed model for CD166 as an enhancer of Wnt signaling. These types of studies could also be performed in mice to more specifically address the effect of CD166 over expression on the intestinal stem cell niche. Generation of a CD166 transgenic line where CD166 was over expressed off an intestinal stem cell promoter (such as Lgr5) could be informative. If my proposed model is accurate, I may expect over activation of Wnt and a phenotype similar to genetic models for loss of Apc with increased tumor incidence in adults or potential embryonic defects. If this mouse did not present with gross intestinal abnormalities it may also be interesting to cross this mouse to other mouse lines susceptible to intestinal tumorigenesis.

Future experiments to improve understanding of CD166 function in cancer and clarify its potential as a therapeutic target

Perhaps the single largest barrier to understanding the function of CD166 in cancer is that the cellular effects resulting from CD166 inhibition and over expression have not been assessed in a wide variety of cancer types/cell lines and its role in tumor cell signaling has not been well defined. One further complication in studying the function of CD166 in tumorigenesis is that standard

2-dimensional culture methods may not adequately define the function of CD166. For example, CD166 expression and Wnt signaling are significantly enhanced in 3-dimensional colonosphere culture of HCT-116 cells[175]. Findings like these suggest that more sophisticated culture or animal models may be necessary to fully appreciate CD166 function in cancer.

To address the lack of understanding of CD166 signaling function, experiments should be performed using a systems biology approach. This approach would use high throughput genomic and proteomic analysis in multiple tumor cell lines after knockdown or over expression of CD166. Further these studies would ideally be conducted using 3-dimensional culture systems. Genomic and proteomic data should then be computationally analyzed to correlate experimental conditions and changes in cellular behavior and signaling. These types of studies will help define which signaling pathways CD166 has the greatest influence upon and may also define cancer subtypes most susceptible to CD166 inhibition. Ultimately, these types of comprehensive "-omics" approaches will help to determine whether CD166 acts as a primary driver in tumorigenesis or whether it is merely a bystander.

APPENDIX 1

Gut-associated Lymphatic Cell Homing After Whole Body and Abdomen-Focused Irradiation Injury: An *In Vivo* Model to Study Mechanisms of Response to Radiation Enteritis

Gut-associated Lymphatic Cell Homing After Whole Body and Abdomen-Focused Irradiation Injury: An *In Vivo* Model to Study Mechanisms of Response to Radiation Enteritis

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Abstract

Gastrointestinal radiation toxicity, also known as radiation enteritis, is a primary side effect of radiation therapy in cancer and a primary toxicity of environmental or occupational exposure. Although a number of promising drug candidates are in development, there are currently no approved drugs for the treatment of radiation enteritis. A major gap in the development of therapeutics is the lack of animal models that closely mimic radiation doses to the intestine, analogous to that in human patients. Here, we present a new approach for targeted-abdominal irradiation in mice to facilitate precise dosing to the intestine. Intestinal response to injury was compared between this targeted irradiation approach and a traditional whole body irradiation method. Further, we provide evidence that trafficking of circulating or transplanted bone marrow-derived cells differentially home to the intestine within the first week post-irradiation. This may suggest differences in dynamics of regenerative cell recruitment to the intestine under specific damage contexts. Together, these observations and this novel experimental mouse model have the potential to vertically impact the development of future cell-based therapies for treatment of radiation enteritis due to environmental exposure or radiation therapy for cancer.

Introduction

Gastrointestinal (GI) radiation toxicity is a primary side effect of radiation therapy in cancer, utilizing targeted doses. Further, it represents a primary toxicity due to environmental or occupational exposure, resulting in whole body irradiation. While the actual incidence of radiation-induced injury is not well documented, it is thought to occur in a significant portion of cancer patients receiving pelvic or abdominal radiation[176-179] and contributes to chronic radiation enteritis and associated pathologies in approximately 5% of these patients[176,180]. Clearly, radiation exposure poses a significant health concern[181]. Dosage and extent of the radiation field impacts the magnitude of injury and the ability for functional tissue regeneration. Unfortunately, few therapies exist for treatment of GI radiation toxicities and no drugs are currently approved to treat extreme radiation sickness[182].

Stem cell therapeutic strategies possess the potential to effectively treat radiation toxicities, such as radiation enteropathy. The cells of the bone marrow are well-characterized modulators of epithelial regeneration[183-186] and have been shown to “home” to sites of injury or inflammation[187-195]. However, the impact of whole bone marrow cells (WBM) on intestinal regeneration after injury is not well documented. Additionally, a gap in knowledge exists for in the differential mechanisms regulating the dynamics of cell trafficking after injury induced by targeted-radiation damage compared to whole body exposure. This distinction is important to elucidate in order to effectively exploit the therapeutic uses of WBM or mesenchymal stem cell (MSC) transplantation in mediating intestinal epithelial

repair, and to ultimately prevent or diminish radiation enteropathy in occupational exposure, nuclear disaster or cancer therapeutic settings.

Radiation therapy is a mainstay of cancer treatment with approximately 70% of cancer patients receiving radiation as part of curative and/or palliative care. Patients receiving radiation to the thoracic, abdomen or pelvic areas are at risk for intestinal-associated toxicities[176,178]. Although recent advances have allowed for the delivery of more complex radiation beam arrangements to directed sites, the potential for intestinal epithelial injury remains, thereby limiting the ability to achieve an optimal therapeutic ratio. Post-irradiation intestinal epithelial ulceration and dysfunction is a primary side effect of radiation therapy afflicting approximately 1.5-2 million patients annually in the United States[176]. The acute effects of radiation result from intestinal cell death in healthy proliferating epithelial progenitor populations, compromising barrier function and promoting immune infiltration. Endothelial cell death in the intestinal lamina propria may also contribute to acute intestinal injury, although the actual levels of gut-associated endothelial cell death after radiation exposure remain somewhat controversial[196-201]. Despite this, the role for endothelial dysfunction and sclerosis in long-term radiation bowel disease is well documented[180,201]. In some patients, radiation exposure can induce chronic inflammation resulting in extracellular matrix deposition in the mucosa, submucosa, muscularis and subserosa of the intestine[177]. This can result in long-term and debilitating effects including ulceration, enteric nerve damage, reduced intestinal motility, intestinal narrowing, and obstruction[176].

On a cellular level, the effects of whole body irradiation in the intestine are also well established[202,203]. In the mouse small intestine, cell death (apoptosis) peaks around 3-4 hours after low dose (0.01-1 Gy) radiation exposure. This effect is primarily restricted to actively dividing cells within the intestine, known as the progenitor population that resides near the base of the intestinal crypt (or stem cell compartment)[202,204]. At higher doses (greater than 7.5Gy, delivered in a single dose), increased levels of apoptosis have been reported to extend beyond 24 hours[203]. While the intestinal stem cell is thought to be radio-resistant, massive cell death among the rapidly dividing progenitor population results in a net loss of epithelial cells resulting in a compromised stem cell compartment and a breach in the intestinal epithelial cell barrier[203,205]. It is likely that recruited bone marrow-derived cells capable of stimulating epithelial and endothelial regeneration play a significant role in the ultimate severity of radiation-induced intestinal toxicity.

Our group and others have shown that after radiation-mediated intestinal injury, bone marrow-derived cells traffic to the mesenchymal compartment of the small intestine and cluster around the intestinal crypts[184,194,206]. Interestingly, this has been shown to influence epithelial regeneration through paracrine signaling as well as though cell-cell fusion events resulting in nuclear reprogramming[195,206]. Moreover, cell transplant after radiation represents one potential mechanism to modulate the acute and long-term effects of radiation damage to normal tissues. Most research has focused on transplantation of *in vitro* generated MSCs or hematopoietic stem cells (HSCs) after radiation or inflammatory damage. These

studies have demonstrated varying levels of trafficking to sites of injury and subsequent effects on intestinal regeneration[186-188,190,192,193]. Experimental radiation schemes vary, including whole body irradiation and focused-irradiation utilizing lead shielding, which may unintentionally include bone marrow harboring tissues[187,207]. A clear mechanistic understanding of injury-mediated bone marrow-derived cell trafficking to the intestine represents a critical gap in knowledge for identification of novel therapies with potential to promote regeneration and reduce the long-term effects of radiation damage.

Radiation enteritis clearly poses a fundamental barrier in delivery of effective therapeutic doses for cancer treatment. Studies to model abdomen-focused radiation toxicities in mice have previously relied upon shielding of limbs and pelvis or shielding and surgery to expose a gut loop to mimic clinical delivery[205]. Here we provide a novel use of three-dimensional computed tomography (CT)-guided radiotherapy planning to directly irradiate the intestine of a mouse. Further, we present comparative analyses of the dynamic trafficking of bone marrow-derived cells to the intestine after abdomen-focused external beam irradiation or whole body irradiation, and document response of intestinal epithelial cell proliferation and death.

Abdomen-directed radiation induced dramatic damage and resulted in areas of crypt loss and epithelial ulceration. Interestingly, this epithelial damage did not elicit a rapid recruitment of bone marrow-derived cell trafficking when compared to that in whole body irradiated intestines. These findings suggest that damage in the bone marrow compartment that accompanies whole body

irradiation may modulate the inflammatory response in distant immune organs such as the intestine. Further, we demonstrate that bone marrow-derived cells transplanted into lethally irradiated mice interact with host bone marrow, resulting in cell fusion with local populations. While the underlying physiologic relevance of cell fusion in this compartment is unclear, it is possible that the fusion event protects cells from radiation-induced death and preserves populations of educated immune cells. This may be a novel mechanism for bone marrow-derived response to injury and promote tissue regeneration. The interplay between epithelial cell death, regeneration and cell recruitment to promote these responses, is an intricate and highly dynamic process. This study describes a novel experimental mouse model as a foundational step toward understanding the complex cellular interactions during regeneration and thus may provide a useful model to test therapeutics for treating radiation-induced intestinal injury.

Materials 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, RFP[208], Tie2Cre[209], CMV-Cre[210] and R26R-YFP[211] mice were purchased from The Jackson Laboratory (Bar Harbor, ME). OsbYO1-GFP (green fluorescent protein) mice[212] were bred in-house.

Histochemical and immunohistochemical analyses of intestinal tissue

Mouse intestines were dissected and prepared for frozen tissue analyses as we have previously described[142]. Briefly, intestines were dissected and cut into thirds. Each third was flushed with ice cold PBS followed by 4% paraformaldehyde. Intestines were cut open longitudinally along the mesenteric attachment line, pinned out flat on a black wax plate, incubated in 4% paraformaldehyde for one hour at room temperature, washed three times in PBS, and incubated in 30% sucrose overnight at 4ºC. Tissues were blocked in frozen Optimum Cutting Temperature Media (Sakura Finetek, Torrance, CA). Tissue sections (5µm) were incubated with antibodies to GFP (1:500; Invitrogen, Carlsbad, CA). Visualization was performed using species-specific secondary antibodies conjugated to Fluorescein isothiocyanate (FITC) (1:500; Jackson ImmunoResearch) and nuclei were counterstained with Hoechst dye (33258;

Sigma; 0.1µg/ml). Images were captured using a Leica DMR fluorescent microscope (Leica Microsystems) or by confocal microscopy. 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).

Histology and quantification of acute radiation-induced intestinal damage

Tissue sections (5µm) of irradiated distal small intestine were stained with Hematoxlyn and Eosin (Vector Laboratories, Burlingame, CA). A minimum of 50 representative crypt villus units were imaged in each mouse (range of n=2-4 per experiment). Crypt height and villus depth were measured in each unit and the ratio between crypt height and villus depth (Crypt/villus) was calculated. Results are presented as an average of all measurements taken for each condition and time point analyzed. Error bars are presented as standard error. Student t-tests were used to determine statistical significance between treatment groups. Crypt/villus ratio for abdomen-focused irradiation at 96h could not be calculated due to large areas of crypt loss and ulceration.

CellProfiler quantification of donor-derived cell trafficking

Quantification of fluorescent immunohistochemical data was performed by analyzing a minimum of 5 images from distinct fields of view demonstrating radiation-associated histological changes. A minimum of 2,000 total mesenchymal cells were counted, as detected by their Hoechst-stained nuclei.

CellProfiler (http://www.cellprofiler.org/), an automated open source image quantification program was used to ensure consistent image analysis[213]. E-cadherin positive-cells were identified using local intensity maxima. The thresholds for E-cadherin were calculated using the Robust Background method with a threshold correction factor of 0.6, and upper and lower threshold bounds of 0.005 and 1.

Using the identified E-cadherin cells as a negative selection mask, nuclei within the mesenchymal compartment only (E-cadherin negative) were identified using local intensity maxima. Thresholds were calculated using Otsu's method with an adjustment factor of 1 with upper and lower threshold bounds of 0.005 and 1. Additional size criteria were used and objects outside of the range of 5 to 25 pixel units were discarded. E-cadherin negative mesenchymal cells were then identified using the identified nuclei as seeds for the Distance function. GFP positivity of mesenchymal cells was determined by local intensity maxima. Thresholds for GFP were calculated using the Robust Background method with a threshold correction factor of 0.75, and upper and lower threshold bounds of 0.004 and 1. Relative trafficking of transplanted cells was determined by calculating the percent of GFP positive cells relative to total mesenchymal cells.

Whole body irradiation and bone marrow transplantation

Whole bone marrow (WBM) transplantation was carried out as we have previously described with some modifications[184]. Briefly, 6-week-old recipient C57Bl/6 or RFP[208] mice received whole-body irradiation from a calibrated

cesium-137 source (a single 12 Gy dose). Immediately following, mice were retro-orbitally administered 1.5 x 10^7 WBM cells harvested from 8- to 12-week-old donor GFP^{212} mice as previously described[184]. Prior to injection, WBM was filtered to obtain a single-cell suspension and resuspended in Hank's balanced salt solution supplemented with 3% fetal bovine serum and 10 mM HEPES. Mice were sacrificed and analyzed at 6h, 12h, 24h, 96h and 1week after exposure to radiation.

To accurately determine the radiation dose to the intestine after whole body irradiation, two mice were sacrificed and three lithium fluoride thermoluminescent dosimeters (TLD) were placed in the intestinal region of the abdomen in each mouse. Mice were irradiated using the 12 Gy whole body protocol as described above. A dose-response curve, generated by irradiating calibration TLDs with the calibrated Cesium-137 source (Figure A1.3A) was used to convert measured TLD readings to dose, facilitating the calculation of the average dose received by the intestinal TLDs in each mouse (Figure A13B).

Abdomen-focused irradiation

Semi-solid spray foam (Dow Chemical) was custom molded to the shape of a 25g mouse in a prone position and allowed to set (Figure 1B, insert). A reference mouse, anesthetized in the prone position on the mold, underwent computed tomography (CT) simulation on a 16-slice helical big-bore simulator (Philips Medical Systems, Cleveland, OH, USA) with 1 mm slice thickness. A dosimetric plan was generated using a radiotherapy treatment planning system

(Eclipse v8.6, Varian Medical System, Inc., Palo Alto, CA, USA) for use on all other 25g mice (Figure 1B). The dosimetric plan consisted of bilateral fields, each with a collimation setting of 3.0 cm x 3.2 cm to encompass the entire region of interest (intestine). A half-beam block was used to exclude the spinal column from the primary irradiation beam. The dosimetric plan was computed for a single dose of 14Gy to the abdomen using a 6-MV photon beam for delivery on a clinical linear accelerator (Varian Trilogy, Varian Medical Systems Inc, Palo Alto, CA, USA). The impact of tissue density differences on dosimetric computation was taken into account. Prior to irradiation, each mouse underwent general anesthesia by isoflurane inhalation. Once adequate anesthesia was confirmed, each animal was set up on the irradiation table using laser guides, as per CT simulation for dose administration. Mice were transplanted with WBM immediately after radiation and analyzed at 6, 12, 24, and 96 hours (as described above).

Detection of bone marrow-derived cell fusion after bone marrow transplant.

To detect cell-cell fusion after irradiation and bone marrow transplant, RFP[208] mice were transplanted with GFP[212] whole bone marrow as described in the primary methods. In this transplant scheme, cell-cell fusion was detected by the co-localization of both donor and host fluorescent markers by confocal microscopy as described in primary methods. To further validate cell fusion in the gut associated lymphatics, R26R-YFP mice[211] were transplanted with Tie2Cre[209] or CMV-Cre[210] whole bone marrow. In this transplant scheme, fusion was

detected by expression of the Cre-repoter gene YFP. In these mice, YFP is only expressed when a *loxP*-flanked stop codon has been deleted by cre-recombinase. This only occurs after bone marrow transplant when donor cre-recombinase is expressed in the same cell as the recipient YFP reporter genetic construct by a cell fusion mediated mechanism. YFP expression was detected by confocal microscopy as described in primary methods. To insure no auto-fluorescence or spurious reporter gene expression, non-transplanted R26R-YFP mice served as negative controls. Alternatively, bone marrow combined from two donor mice (Tie2Cre and R26R-YFP or CFP and RFP) was transplanted into lethally irradiated recipient hosts in an attempt to enhance the incidence of this rare fusion event (Figure A1.7 E-H).

Detection of fusion in a blood cell lineage.

The blood cell lineage contributing to fusion hybrids was determined by staining mouse intestine with antibodies against CD4, CD8, or F4/80 (BD Biosciences, Sparks, MD; 1:500), as described in primary methods with either Indocarbocyanine3 (Cy3) or Indocarbocyanine5 (Cy5) as fluorescent secondary antibody detection. Co-localization of lineage and fusion markers was determined using confocal microscopy as described in primary methods. Importantly, single color controls and secondary antibody only controls were used to ensure no spectral bleed through or non-specific secondary antibody interactions.

Results and Discussion

Intestinal response to whole body vs. abdomen-focused irradiation exposure.

The lack of potential treatments for GI-toxicity from widespread nuclear radiation exposure or side effects from radiotherapy, such as radiation enteritis, highlights the need to better understand intestinal-mediated damage and the process that mediates epithelial repair. Importantly, the recruitment of regenerative cells in these contexts has important implications for potential therapies designed to guard against radiation toxicity or to enhance tissue regeneration. To determine how the intestine responds to whole body vs. abdomen-focused irradiation exposure, we compared the overall morphology of the mouse small intestine after whole body (12Gy) or abdomen-focused (14Gy) radiation exposure in the presence or absence of subsequent bone marrow transplantation (Figure A1.1). Our abdominal irradiation scheme relied upon a novel use of CT guided conformational radiation to directly irradiate the intestine of a mouse, while sparing bone-marrow harboring structures, closely mimicking highly conformal radiotherapy in human patients. Interestingly, mice receiving 14 or 17Gy abdomen-directed irradiation suffered gross morphologic intestinal damage, but surprisingly did not die. This suggests that previous reports[186, 193] of death in mice receiving focused abdominal irradiation may have been due to bone marrow failure from inadvertent exposure. Therefore, this indicates that our experimental paradigm can specifically target the GI tract, thereby providing the ability to more accurately study the GI effects from high dose radiation akin to that delivered for cancer therapy. One of the limits in our approach is that our

single fraction approach does not directly mirror the daily fractioned (1.8-2.0 Gy) schedule in the common clinical setting. Nevertheless, we chose the single large fraction approach since most *in vivo* experiments of whole body irradiation were carried out in such a fashion.

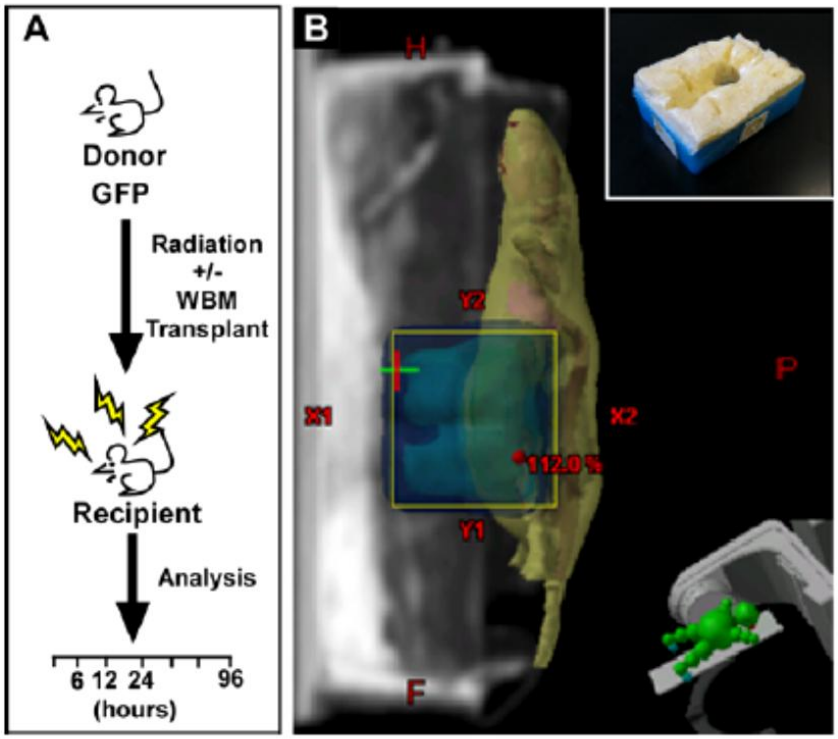


Figure A1.1: Experimental design
(A) Recipient mice were irradiated with whole body or abdomen-focused irradiation and underwent subsequent whole bone marrow transplant or not. Mice were analyzed at 6, 12, 24, and 96 hours after irradiation. **(B)** Computed tomography (CT) image of an anesthetized 25g mouse in a custom designed mold to accurately position the mouse for uniform targeted irradiation. CT imaging permits the development of a computer-guided irradiation protocol, which specifically targeted the abdomen with 14Gy, sparing the bone marrow compartments in the femur and spine (yellow box). Precise positioning of mice of equal weight in the mold and laser guided positioning of the mold via positioning beebes (photo insert) allowed for reproducible and precise targeting of the intestine with radiation. The mouse prone position relative to the positioning of the linear accelerator source is shown in the bottom right corner (green figurine). Radiation was administered using two laterally opposing fields by moving the radiation source while maintaining the mouse position fixed.

Gross analysis of the mouse intestine was performed at 6h, 12h, 24h, 48h (not shown), 96h, and 7d, on hematoxylin and eosin (H&E)-stained tissues from the ileum (distal small intestine). Notably, there was little difference in overall intestinal structure at 6h (not shown), 12h, and 24h post-irradiation (Figure A1.2). However, by 96h, in whole body irradiated mice, a statistically significant increase in the Crypt/villus ratio was observed ($p=0.0014$) in mice who received cell transplant relative to mice which did not receive a transplant. This is reflected by elongated crypts in the transplanted intestines, which is consistent with enhanced regeneration (Figure A1.2D). Interestingly, in abdomen-focused irradiated mice, there was a loss of tissue architecture; crypt loss was prevalent, as was shortened villi, which did not support quantification of the Crypt/villus ratio (Figure A1.2C, right panel). In our system, the intestine received 14Gy in the abdomen-focused irradiated approach compared to ~10Gy from a 12Gy whole body irradiation (Figure A1.3). Distinct differences in epithelial response within the proliferative stem cell zone clearly highlight differential responses from radiation dose. However, these differences may also reflect systemic effects due to immune system modulation elicited from whole body irradiation, providing a mechanism for regulation of local regeneration, proliferation and death.

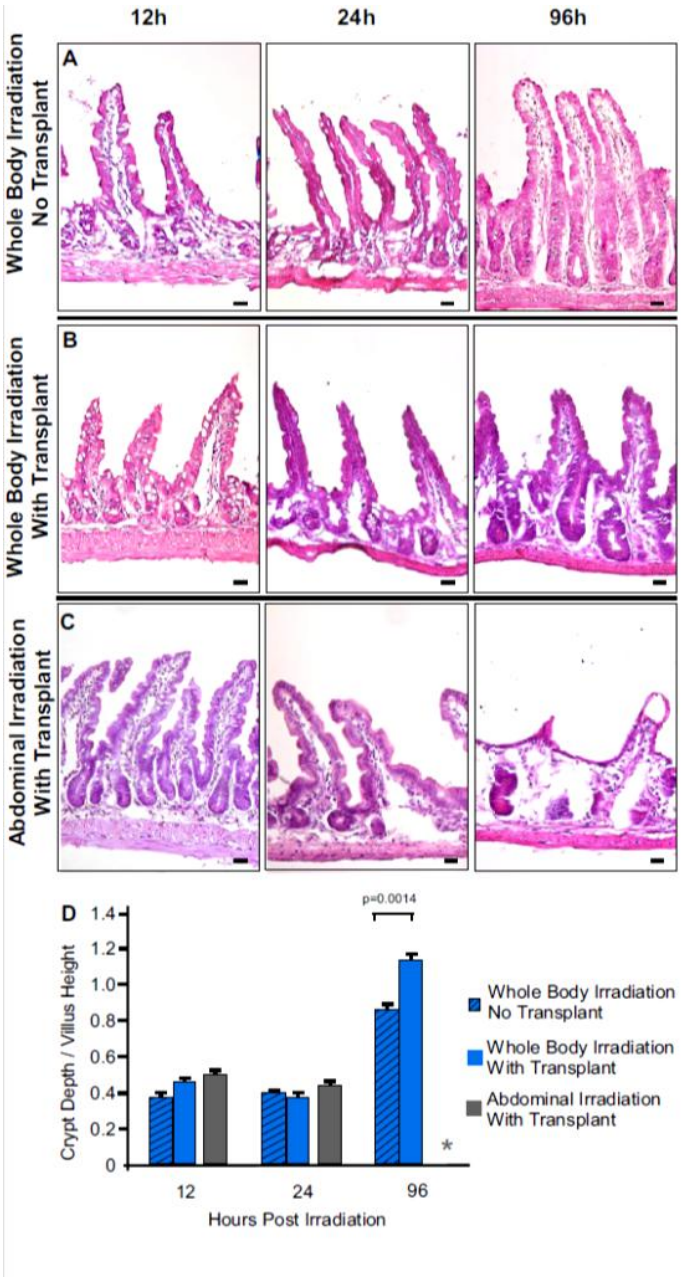
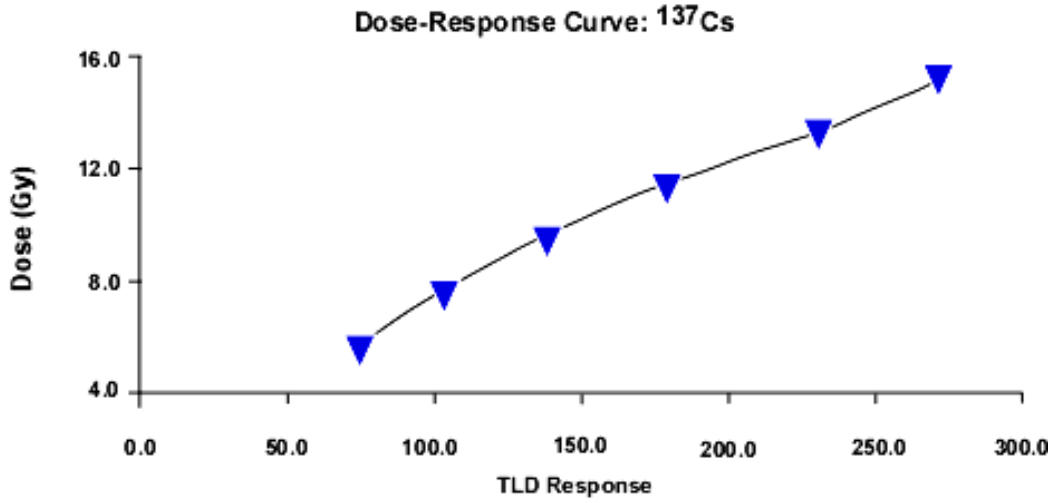


Figure A1.2: Temporal analyses of morphologic intestinal change in response to irradiation.
Hematoxilyn-Eosin staining of irradiated intestines. A time course analysis of intestine analyzed at 12, 24 and 96h post-irradiation is depicted for three conditions, **(A)** after whole body irradiation without subsequent transplantation, **(B)** after whole body irradiation followed by whole bone marrow transplantation, and **(C)** after abdomen focused irradiation followed by whole bone marrow transplantation. **(D)** Crypt/villus ratios measured for each radiation condition. Asterisk denotes condition where crypt/villus ratio could not be calculated.

A



B

Abdominal radiation dose in whole body irradiation

Site	Mean TLD RDG	Mean Dose (Gy)
Intestine: Mouse 1	131.1	9.9
Intestine: Mouse 2	140.0	10.5

Figure A1.3: (A) TLD Dose-response curve was created by placing TLDs in a calibrated Cesium-137 irradiator for defined radiation exposure and then reading TLD response after 24 hours. **(B)** Mean TLD response and calculated radiation dose for intestine localized TLDs.

The intestinal epithelial proliferative balance differs when exposed to whole body vs. abdomen-focused irradiation.

Elongation of crypt structures, as observed in whole body-irradiated intestines, suggests an increase in active proliferation or a reduction in cell death. To determine if there were differences in the proliferative capacity of the intestinal crypts between treatment groups, we performed expression analysis of Ki-67, a well-described approach for identifying cells in interphase (Figure A1.4A-C). In agreement with our morphological observations on H&E-stained tissues, the extent of Ki-67-positive cells was increased at 96h in whole body irradiated mice (regardless of bone marrow transplant status) relative to abdomen-focused irradiated transplanted intestines. Further, early damage resulting in cellular apoptosis as determined by cleaved caspase-3 antibody staining (Figure A1.4D-F) did not appear to be different between whole body and abdomen-focused irradiated intestines that were transplanted with bone marrow-derived cells. Activated caspase-3 was not detected at elevated levels in later time points compared to non-irradiated controls (data not shown). This suggests that the resulting alteration in intestinal tissue morphology in whole body irradiated mice is due to an imbalance of proliferation and apoptosis. Interestingly, in abdomen-focused irradiated intestines there is less of a proliferative response but similar apoptosis, likely the cause of crypt structure loss.

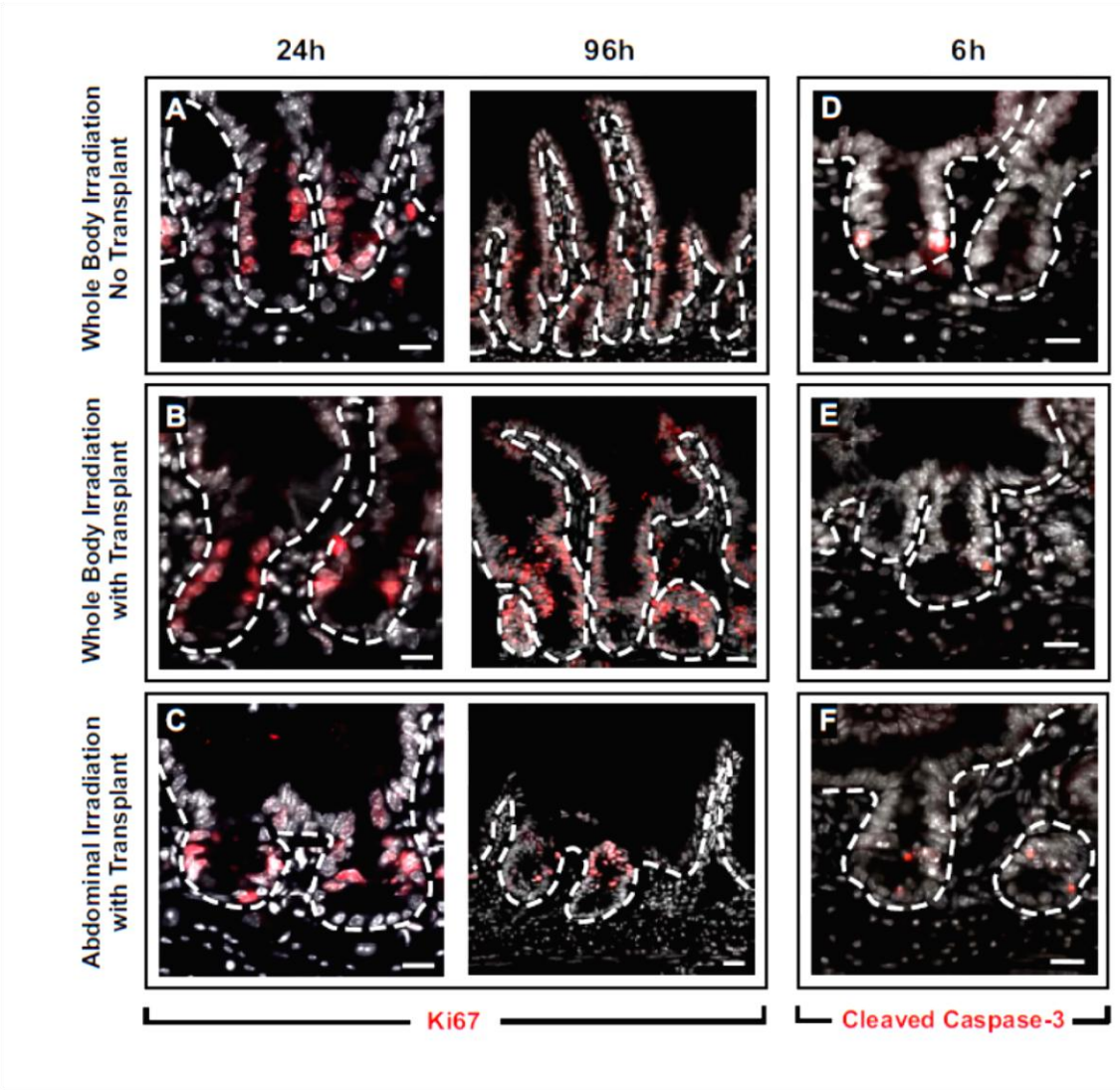


Figure A1.4: Intestinal epithelial proliferative and cell death response to irradiation.

(A-C) Ki67 antibody staining (red) and nuclei countered-stained with Hoechst (grey) on intestinal tissue sections from three different radiation paradigms: **(A)** after whole body irradiation without subsequent bone marrow transplant, **(B)** after whole body irradiation followed by whole bone marrow transplant, and **(C)** after abdomen-focused irradiation followed by whole bone marrow transplant. Representative images at 24 hours (left) and 96 hours (right). **(D-F)** Analysis of apoptosis by staining with antibodies for cleaved caspase-3 (red) and counterstained with Hoechst (grey), 6h post-irradiation. White dashed lines demark epithelial-mesenchymal boundary. Bars=25μm.

Circulating bone marrow-derived cells home to the intestine within 24 hours after exposure to whole body irradiation.

We hypothesized that one major difference in the apparent regenerative up-regulation in epithelial proliferation at the 96h time point in whole body vs. abdomen-focused irradiated intestines might be due to differences in recruitment of bone marrow-derived cells to the injury site. Bone marrow-derived cells, including HSCs, MSCs and a number of immune cells, have been described to have regenerative properties[183,185]. Therefore, to determine the response time for cell trafficking to sites of radiation-induced injury, mice were exposed to whole body (12Gy) or abdomen-focused (14Gy) irradiation and subsequently transplanted with green fluorescent protein (GFP)-expressing whole bone marrow (WBM) cells (Figure A1.1A). Analysis of trafficked GFP-expressing cells within the intestine was performed at 6, 12, 24, 96 hours post-WBM transplantation using immunohistochemical detection of the GFP protein. Trafficking of GFP-expressing bone marrow-derived cells occurred preferentially to the intestinal crypt (compared to villus or muscularis) as early as 12 hours (Figure A1. A, B, left panels) for both whole body and abdomen-focused irradiated intestines. However, for whole body-irradiated intestines, an apparent accumulation or increased homing of GFP-expressing bone marrow-derived cells occurred with time, culminating in clusters of GFP-expressing cells around the crypt-base at 96h post-irradiation (Figure A1.5A right panel) and near complete replacement of host-derived cells by 7 days (not shown)[195]. This observation was in contrast to abdomen-focused irradiation, which lacked increased

recruitment of GFP-expressing cells by 96h (Figure 1.5B, right panel) and at 7days (data not shown). Differences in recruitment of GFP-expressing cells between whole body and abdominal irradiation was statically significant at both 24 and 96h (Figure A1.5C, p=0.002, 0.003 respectively). It is possible that trafficking of bone marrow-derived cells to the intestine modulates epithelial regeneration (Figure A1.6), supporting the possibility for cell transplant therapies in promoting epithelial repair.

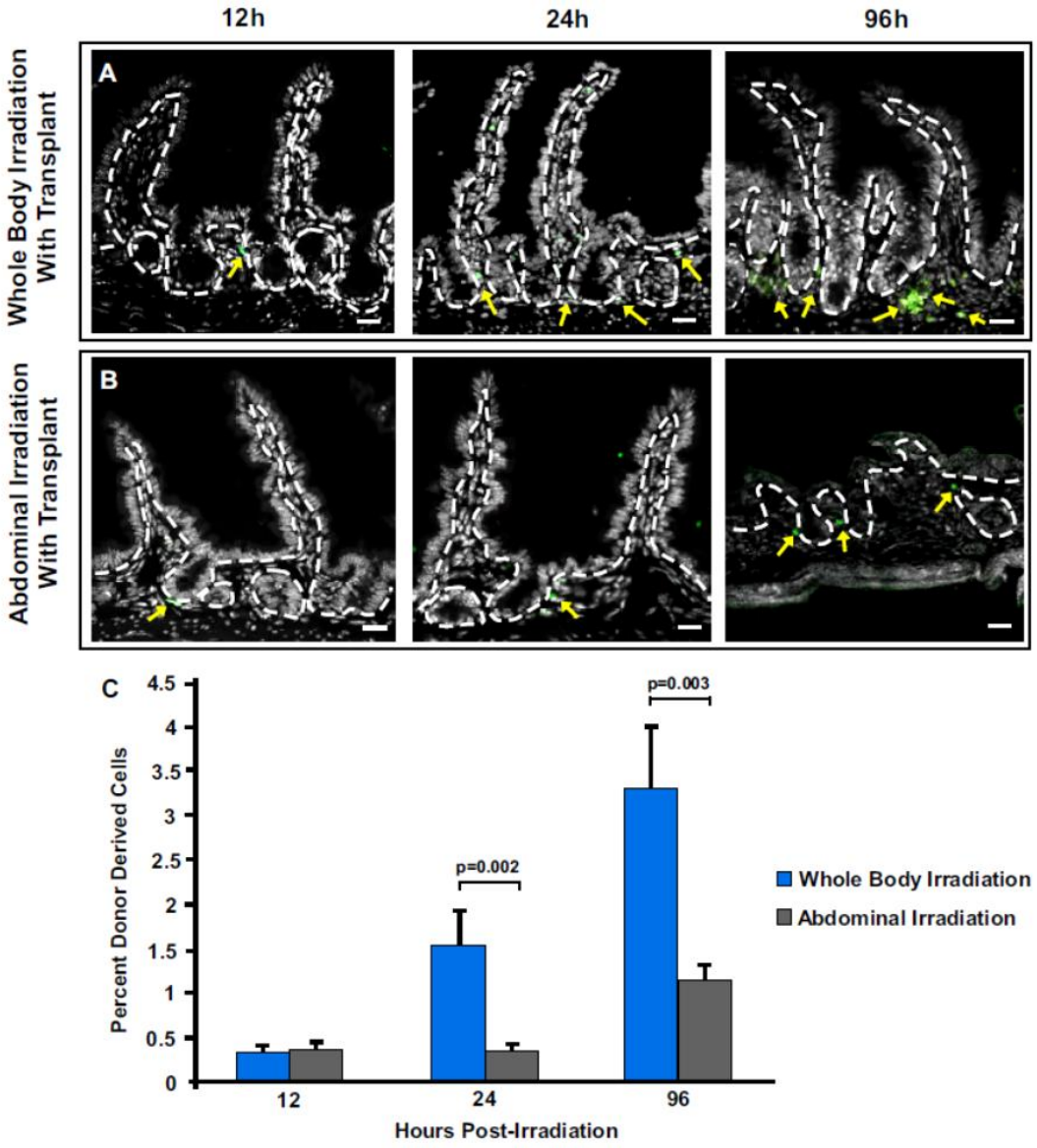


Figure A1.5: Trafficking kinetics of transplanted bone marrow-derived cells in the mouse small intestine.
Irradiated mice were transplanted with GFP-expressing whole bone marrow. **(A-B)** GFP-expressing donor bone marrow-derived cells (green) and nuclei stained with Hoechst (grey) were detected at 12, 24 and 96h post-irradiation in **(A)** whole body irradiated or **(B)** abdomen-focused irradiated mice. White dashed lines demark epithelial-mesenchymal boundary. Yellow arrows mark GFP+ cells. Donor derived cells increase with time and cluster around crypts by 96h in whole body irradiated intestines. **(C)** Quantification of bone-marrow derived cells in the mesenchymal intestinal compartment. The percentage of GFP-expressing cells is represented, +/- standard error. Bars=25μm

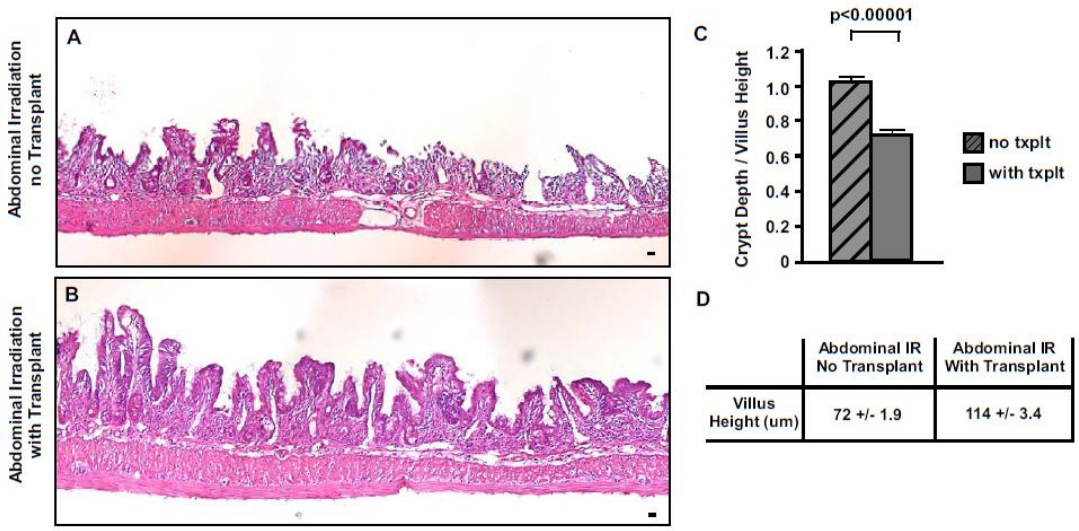


Figure A1.6: Irradiation injury in the presence or absence of whole bone marrow cell transplantation

(A-B) Intestinal regeneration after abdomen-focused irradiation in the absence (A) or presence (B) of whole bone marrow cell transplantation analyzed at 7 days post-transplantation. (A) Representative image of small intestine after abdominal irradiation without subsequent transplant. Areas of crypt loss and ulceration are observed. (B) Representative image of small intestine after abdominal irradiation followed by whole bone marrow transplant. Intestinal villi appear taller and a greater number of intact crypt villus units are present. (C-D) Quantification of intestinal architecture. Crypt/Villus ratio 7 days after irradiation. Villus height is greater than crypt depth in mice which received transplants.

Our data suggests that trafficking of bone marrow-derived cells participates in epithelial regeneration. Further, we noted that a small number of these trafficked cells fuse with endogenous intestinal cells in the lamina propria (Figure A1.7). Interestingly, cell fusion between transplanted GFP-expressing cells and endogenous host cells occurs in at least three populations of the immune system: macrophages, T and B cells (Figure A1.8). It is possible that fusion among these populations reflects rescue of a damaged cell population and may provide a novel mechanism to enhance tissue regeneration mediated by trafficking of endogenous or transplanted bone marrow-derived therapeutic progenitor cells. Importantly, our work represents a basis for stem cell therapy efficacy in the treatment of radiation toxicity.

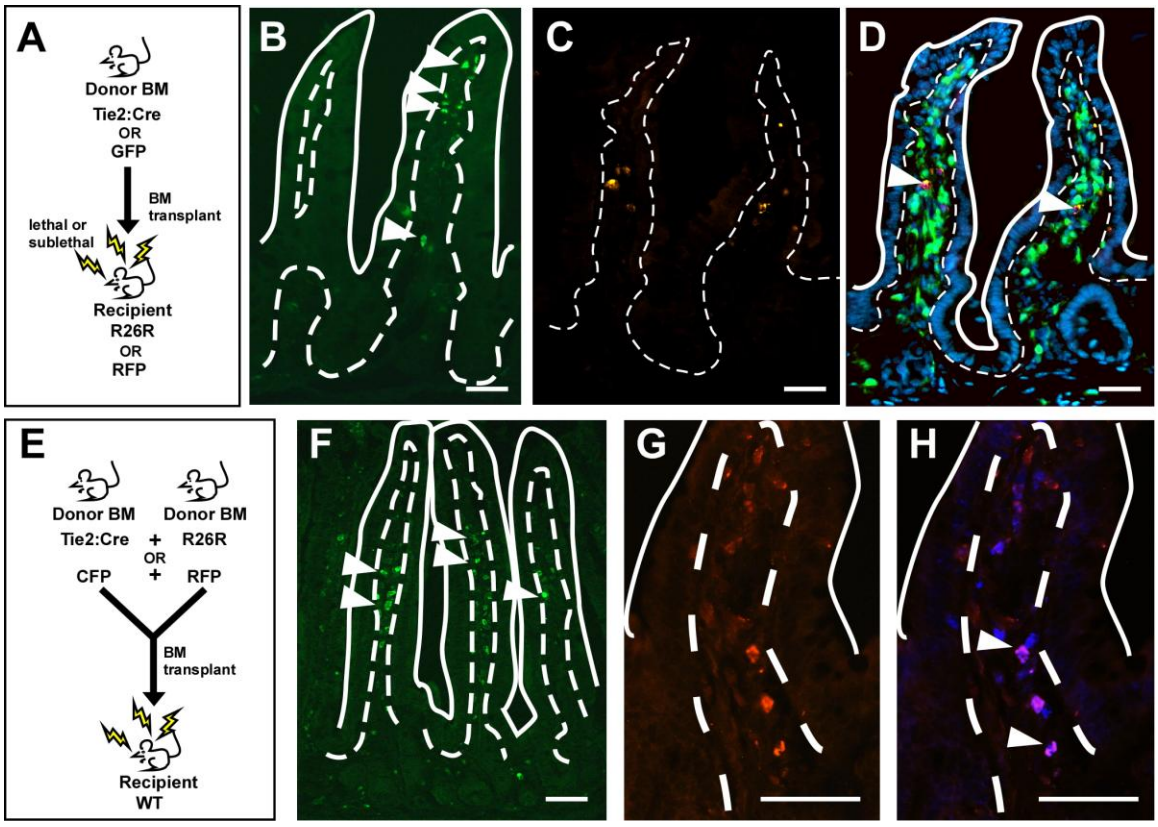


Figure A1.7 Cell-cell fusion occurs in the intestinal mesenchyme after irradiation damage:

(A) Bone marrow transplantation scheme to enable detection of cell-cell fusion. Donor bone marrow expressing the transgene for Tie2-Cre or GFP was transplanted into R26R-YFP or RFP mice, respectively. Fusion was detected by expression of the Cre-reporter YFP or by co-localization of GFP and RFP in a nucleated cell. (B) Intestine from the first approach with YFP-expressing cells detected in green (arrowheads). (C-D) Intestine from a transplanted recipient mouse expressing (C) RFP (red), (D) merge of RFP-expressing (red) and GFP-donor (green) cells, with co-expressing cells seen as yellow (arrowhead). Counterstained with nuclear stain Hoechst (blue). (E) A modified bone marrow transplantation scheme relying on mixed bone marrow populations to enhance detection of cell-cell fusion. Bone marrow cells from two donors were injected into a lethally irradiated wild-type (WT) recipient mouse. Fusion was observed by Cre-mediated YFP reporter gene expression (green, arrowheads, F) or through co-localization of donor markers (CFP and RFP, shown in G-H). (G-H) Confocal micrograph of RFP-expressing cells in the intestinal mesenchyme. (H) Overlay of both donor markers, CFP expression (blue) and RFP (red), co-expressing cells are designated by arrowheads. Bars=25µm. Dashed white lines demark epithelial-mesenchymal boundary. Sold lines demark epithelial lumen boundary.

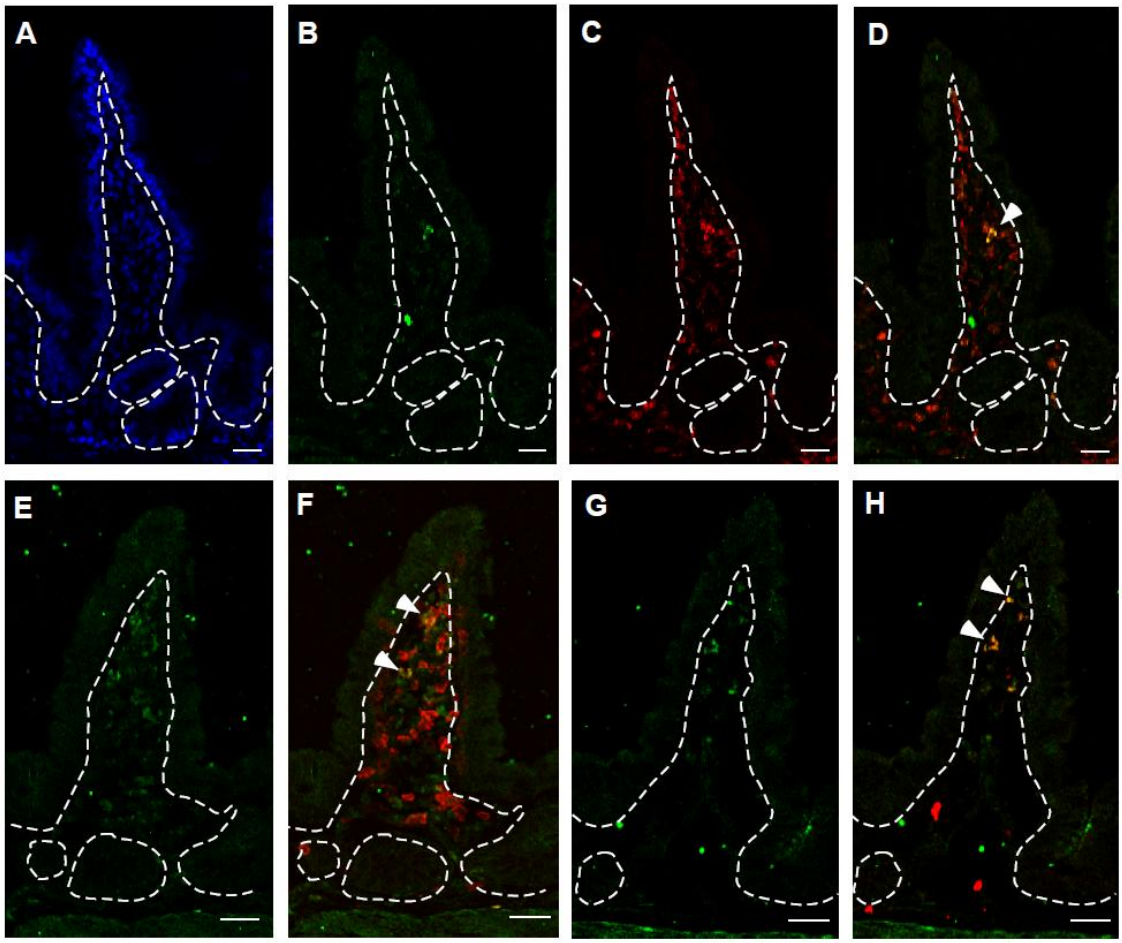


Figure A1.8: Fusion hybrids express macrophage, T-cell and B-cell lineage markers.

(A-H) Confocal micrographs of distal small intestine of a WT mouse transplanted with CMV-Cre and R26R-YFP whole bone marrow. **(A)** Nuclear staining by Hoechst (blue). **(B)** YFP expression indicating a Cre-expressing cell and reporter YFP cell fusion (green). **(C)** Mouse macrophage marker F4/80 expression (red) **(D)** Merge of YFP expression (green) and F4/80 staining (red), colocalization (yellow, marked with arrowhead). **(E-F)** Expression of fusion hybrid marker YFP (green) and T-cell markers CD4 and CD8 staining (red), colocalization (yellow). **(G-H)** Expression of fusion hybrid marker YFP (green) and B-cell marker B220 staining (red), colocalization (yellow). Co-expressing cells are designated by arrowheads. Bars=25µm. Dashed white lines demark epithelial-mesenchymal boundary.

Here we show that the intestinal response to radiation damage and its subsequent regeneration is a highly complex and dynamic process. Interestingly, we suggest that unique radiation exposures, namely whole body (occupational or nuclear disaster) or organ-targeted (radiation oncology) result in measurable differences in response of the intestinal epithelium. Further, we provide evidence that transplanted cells from whole bone marrow may participate in the regenerative process of the intestinal epithelium after radiation injury. This exciting observation provides a basis for potential stem cell-based therapy, in a climate where few therapies exist for treatment of GI radiation toxicity. Insight from novel mouse models for targeted-radiation will provide an essential foundation to better determine clinical benefit and mechanism of regenerative therapies. Further research into the mechanisms of bone marrow and MSC promoted repair holds significant promise for the development of new therapies in GI radiation syndrome.

APPENDIX 2

Review: The Role of the Colorectal Cancer Stem Cells

In Metastatic Disease and Therapeutic Response

Review: The Role of Colorectal Cancer Stem Cells in Metastatic Disease and Therapeutic Response

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Abstract: Colorectal cancer is the third-leading cause of cancer-related mortality in the United States. The intricate molecular mechanisms involved in the regenerative process of the normal intestine and the identity of putative somatic intestinal stem cells have become clear. In parallel with this, experiment evidence has emerged supporting the century old hypothesis that solid tumor initiation, progression, chemoresistance and recurrence is the result of a small population of cancer cells with self-renewal and pluripotency capabilities. These “cancer stem cells” (CSCs) present a unique opportunity to better understand the biology of solid tumors in general, as well as targets for future therapeutics. In this review, we will summarize the current understanding of intestinal stem cell biology and translate it to colorectal CSCs to provide a basis for understanding chemoresistance, cancer recurrence and metastasis. A more complete understanding of the biology of colorectal CSCs will translate into the development of better chemotherapeutic and biological agents for the treatment of colorectal cancer.

Keywords: colon cancer; metastatic, cancer stem cell

Introduction

Colorectal cancer (CRC) is a leading cause of morbidity and mortality in the United States and worldwide. Although the underlying molecular events leading up to the development of primary CRC are well understood and advances in early detection have led to an overall decrease in the number of deaths, advanced and metastatic CRC is rarely curable. Over the past 15 years, evidence has emerged to suggest that cancers, including CRC, can be considered a stem cell disease. The cancer stem cell (CSC) theory posits that both primary and metastatic tumors develop from a small population of cancer cells possessing the characteristics of self-renewal and pluripotency and are responsible for initiation and maintenance of tumors. Additionally these CSCs can give rise to a wide variety of more “differentiated” cancer cells which comprise the bulk of the tumor and provide the basis of tumor heterogenity. While the source of CRC stem cells remains to be completely elucidated, it is clear that because these cells behave in a manner similar to endogenous stem cells, a better understanding of the somatic intestinal stem cell and its niche will further our knowledge of the function – and dysfunction – of CSCs in the colon. In this review, we describe the role of CSCs in CRC with a focus on their role in metastatic disease. We illustrate that a basic understanding of the normal intestinal structure, function and stem cell niche lends insight into the initiation and progression of CRC. We then integrate the CSC theory and the role of CSCs in this process and extend it to metastatic spread of disease. Finally, we discuss therapeutic implications for the existence of CSCs.

Intestinal structure and the stem cell niche

The primary function of the gastrointestinal tract is to facilitate nutrient absorption and act as a barrier to the external environment. As such, the colonic lining has optimally evolved to accommodate both functions, by maximizing absorptive surface area and maintaining continual renewal of a barrier-tight sheet of epithelium. To accommodate these diverse functions, the colonic epithelium is organized as a contiguous layer of columnar epithelia arranged along the radial axis into distinct crypt-like structures. Within the base of the crypts, the intestinal stem cell provides continual renewal of the diverse epithelial subtypes. Four differentiated cell lineages reside within the colonic crypt and surface cuff epithelium: colonocytes, the primary absorptive cell; goblet cells, the mucin secreting cell; enteroendocrine cells, the hormone-producing population; and in the ascending colon—anti-microbial secreting Paneth cells. Differentiated cells rapidly migrate up the intestinal glands and die or are sloughed into the lumen within 4-8 days[2]. These cells are continually repopulated by a long-lived, intestinal epithelial stem cell that resides in the base of the crypt (Figure A2.1). The stem cell is capable of self-renewal and also gives rise to the transit-amplifying (TA) cell population located near the lower portion of the crypt, which functions to rapidly expand epithelial renewal and initiate lineage differentiation. The resulting differentiated progeny migrate upward along the colonic crypt to the crypt opening. These intricate details and cellular relationships were first elucidated in the small intestine using BrdU and ^{3}H-thymidine label retention studies along with electron microscopy[9-12,21,214]. Interestingly, although the

location of intestinal progenitor cells has been known for some time, specific markers for these cells have only recently been characterized[22].

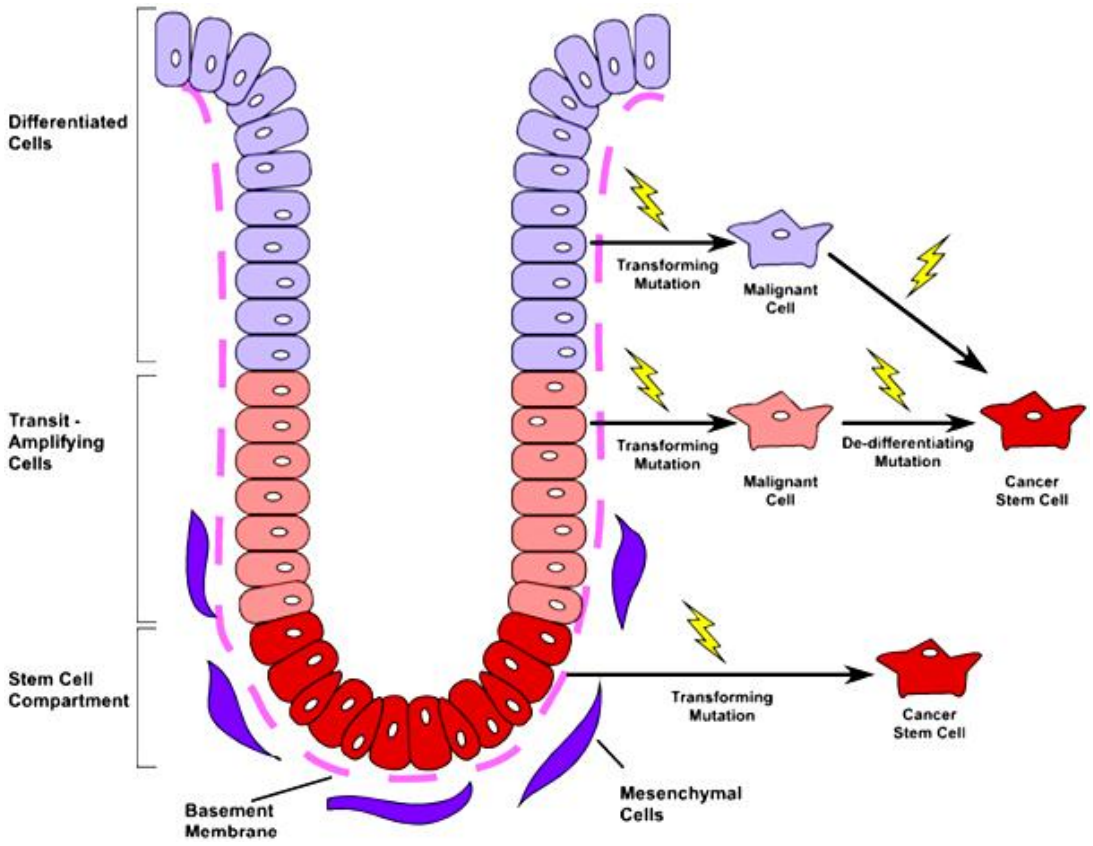

Figure A2.1. Colon crypt anatomy and models of CSC development

Diagram of the human colonic crypt structure. (Left) The stem cell compartment resides at the base of the crypt. Rapidly dividing transit-amplifying (TA) cells arise from this population and differentiate into the functional cells of the colon. (Right) The source of the colon CSC remains controversial. A single transforming mutation in a somatic intestinal stem cell could give rise to a CSC, while two mutations (one transforming and one de-differentiating) would be required to change a TA or differentiated colonic cell into a CSC.

Insights into the identification of the intestinal stem cell have mainly focused on the small intestine rather than the colon. However, in both regions, the leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) protein is expressed in a crypt-base, progenitor population capable of giving rise to all of the differentiated lineages within the intestine[5]. Further, this population has been shown to initiate intestinal organoid growth in a three-dimensional culture system when isolated from the mouse small intestine[23]. Culture conditions for both human tissues and mouse colonic cells are currently being investigated by a number of laboratories. While not all identified stem cell populations have been validated by both lineage tracing and *in vitro* assays, a number of other protein markers for the epithelial stem cell/progenitor cells have been identified. These include: BMI1 polycomb ring finger oncogene (Bmi1)[13], Musashi1 (Msi1)[16], DCAMKL1[17], CD133[215] and Activated Leukocyte Adhesion Molecule (ALCAM/CD166) which marks a broader stem cell region as a niche marker[153]. While the epithelial function for many of these proteins has yet to be elucidated, continued understanding of the populations that express them is certain to shed important insight into epithelial homeostasis, regeneration, and disease.

Currently, it is unclear if a hierarchical lineage relationship exists among the various progenitor cells of the intestine. It has been proposed that the Wnt-responsive gene Lgr5 exclusively marks actively dividing intestinal stem cells[22]. It is possible that a more dormant or quiescent population of stem cells is at the apex of the stem cell hierarchy and gives rise to the rapidly cycling Lgr5 progenitors in a similar fashion as the well-described hematopoietic and neuronal

stem cell hierarchies. This type of relationship may help explain how the intestine regenerates after radiation exposure and chemotherapy, which target actively cycling cells (likely Lgr5-expressing populations)[205]. Solid tumors which develop resistance to these therapies may use a similar mechanism, in which a subset of cells capable of repopulating a tumor are in a dormant (protected) state during dosing of cytotoxic therapeutics. A progenitor cell hierarchy may also exist among the TA population where lineage restriction is initiated, resulting in generation of specific cell types[216]. Interestingly, dysregulation of these progenitor pools may be reflected in cancers where single cell types dominate the tumor, such as mucinous adenocarcinoma. A better understanding of differences between normal intestinal progenitors and their progeny will lead to greater insight into the various initiating cells within a cancer and has great potential to lead to novel therapeutic approaches for eradicating disease.

Colorectal cancer and metastatic disease

CRC will account for approximately 150,000 new cases and 56,000 deaths in the United States this year, making it the third most commonly diagnosed cancer, as well as the third-leading cause of cancer-related mortality[217]. The incidence of CRC has declined over the last two decades with the advent and implementation of routine screening colonoscopy, which allows for early detection and removal of adenomatous polyps before they progress to invasive cancer. Early detection and treatment is the key to better survival. Patients diagnosed with early stage CRC have a 5-year survival rate of greater than 90% compared to 11% for those diagnosed with locally advanced or metastatic disease. Furthermore, patients with metastatic CRC have a median survival of only 2 years despite multiple available treatment modalities, including surgical resection, chemoradiation, monoclonal antibodies to tumor growth factors, and liver-directed therapies for metastatic disease. Unfortunately, only a small subset of metastases are sensitive to these therapies and fewer still are cured, highlighting our lack of knowledge regarding the biological underpinnings of this most deadly phase of CRC.

A major challenge in treating metastatic CRC is the inability to predict tumor behavior and response to therapy *a priori*. In part, this is due to the complexity of molecular mutations that evolve within each individual cancer. The early pathway to CRC tumorigenesis has been well elucidated by Vogelstein and colleagues. Tumorigenesis is initiated when a single colorectal epithelial cell acquires a mutation in the tumor suppressor APC gene that controls the Wnt/β-

catenin signaling pathway[218]. Mutations in the KRAS and BRAF genes enable growth into a clinically significant adenoma with a diameter > 1cm. Additional mutations in TGF-β, PIK3CA, and TP53 further drive clonal expansion and transformation from a benign adenoma to a carcinoma that now has the potential for invasion and metastasis. These mutations that cause an adenoma to transform into an advanced carcinoma occur over a long period of time, 15-20 years on average. However, cells within a carcinoma quickly acquire the potential to metastasize, as the average interval to liver metastases is approximately two years following diagnosis of an advanced carcinoma[218]. Despite an understanding of the mutations that give rise to a primary colorectal tumor, the molecular basis for the development of metastatic CRC remains largely unknown and clearly differs from that of primary tumorigenesis. The unique signatures displayed in metastatic CRC impart different functional behaviors and, interestingly, are also exemplified in a form of CRC seen within the young adult population (<50 years of age). In this younger population, the disease is much more aggressive with a shorter time to metastasis. Because screening is not routinely recommended, the incidence of CRC within the young adult population is actually increasing by 2% per year[217]. These two aggressive forms of CRC clearly exemplify the lack of understanding of the basic tumor biology driving this disease.

It is not surprising that there are few effective targeted therapies for aggressive metastatic CRC. With the exception of K-ras mutations with anti-EGF-R therapy and 5-fluorouracil treatment in microsatellite unstable tumors[219,220], the

response of any individual tumor to a specific therapy must be determined empirically. New, potentially more effective therapies are evaluated only after traditional treatments fail. This also highlights the fact that the biology of primary and metastatic tumors differs in clinically important ways. This is not surprising, as metastatic tumor cells must evolve to escape the primary tumor niche, migrate and establish a new niche in a potentially hostile cellular environment. Whether these differences are due to molecular differences as a result of the accumulation of additional genetic mutations or a change in the cellular profile of the tumor (through epigenetic changes or post-translational regulation of tumor cells) remains to be determined (Figure A2.2). Therefore, a better understanding of tumor biology will provide valuable clues to therapeutic resistance as well as offer new targets for the development of novel chemotherapeutic and biological agents for the treatment of advanced and metastatic CRC.

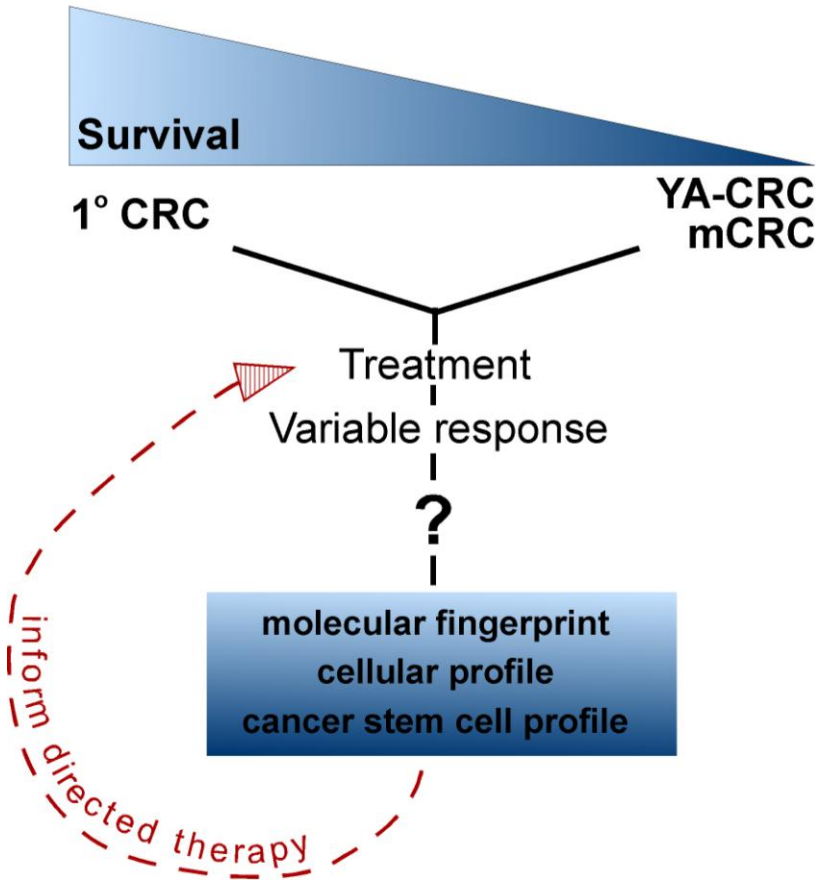

Figure A2.2. Clinical Spectrum of Colorectal Cancer

Colorectal cancer (CRC) has better survival odds than metastatic CRC (mCRC) or young-adult CRC (YA-CRC). The difference in disease response to the current state-of-the-art treatment reflects a gradient of disease with the early staged primary CRC (1^{o}CRC) responding more favorably than late stage CRC, YA-CRC or mCRC. The variability in treatment response is likely dependent upon differences in molecular and cellular characteristics among the disease spectrum. The current challenge is to understand these differences to inform targeted therapy with the ultimate goal of cancer eradication.

There is a growing – although somewhat controversial – body of evidence suggesting that heterogeneous tumors harbor a specialized population of tumor-initiating cells that have been compared to endogenous stem cells. While these tumor-initiating cells may or may not truly be considered stem cells, it is clear that this specialized sub-population of tumor cells is able to recapitulate the heterogeneous tumor for all solid tumors examined to date, including CRC. As with somatic stem cells, these CSCs possess the ability to initiate and sustain tumor growth and have been shown to be resistant to damage and death after exposure to standard chemotherapeutic agents[221].

Given this new understanding of the mechanisms of tumor initiation and maintenance through the CSC, it is clear that a better understanding of the somatic stem cell and its niche will provide insight into the development of CRC in both its primary and metastatic environments.

The cancer stem cell theory

It has been long recognized that tumors are composed of a heterogeneous population of cells with various levels of cellular differentiation and morphologic features. At the same time, most tumors are believed to be monoclonal in origin[222,223], supporting the notion that the originating tumor must be capable of giving rise to various cell types that make up the tumor. Interestingly, for several decades, selection of mutant subpopulations derived from a common progenitor (clonal evolution), as well as microenvironmental

influences, have been the predominant explanations for how a complex and heterogeneous tumor develops from a single cell. In addition, these selective pressures have been thought to provide the driving force for tumor growth and progression[131].

Portions of this model have recently been challenged by increasing evidence that tumor growth and progression are supported by a small population of tumor cells with stem-like properties, and the reinvigoration of the CSC theory. While most normal tissues are supported by a small population of slowly cycling and self-renewing stem cells, the CSC theory proposes the existence of a similar tumor cell hierarchy with a CSC residing at the apex[224]. In this model, the self-renewing CSC divides to give rise to tumor cell subpopulations with more limited replicative ability that generally comprises the bulk of the tumor. Because of the difference in replicative capacity, the tumorigenic supporting abilities are thought to be exclusive to the CSC, while tumor growth and expansion is attributed to the rapidly dividing progeny. This critical point is the departure from previous models of tumorigenesis which support the notion that each tumor cell should be capable of tumor formation[225] (Figure A2.3).

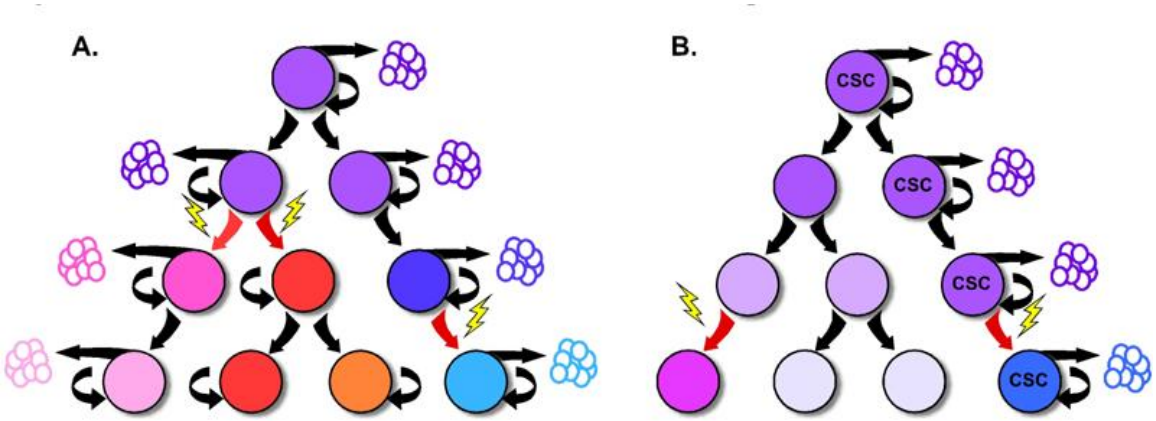

Figure A2.3. Hierarchical and stochastic models of tumor development

(A) Classical hierarchical model of tumorigenesis where any tumor cell has the potential and capacity to recapitulate the tumor, thus giving rise to tumor heterogeneity. (B) In the cancer stem cell (CSC) model of tumorigenesis, only CSCs have the potential to recapitulate the tumor. All other tumor cells are "differentiated." Tumor heterogeneity arises as the result of mutations of the CSC and differentiation of its progeny.

While increasing evidence supports the existence of the CSC, the origins of this cell remain uncertain. Genetic or epigenetic changes may render a normal tissue stem cell cancerous, or may confer stem-like abilities on a progenitor or differentiated cell[226]. Because of this uncertainty, the terms "cancer-initiating cell" or "tumor-initiating cell" are often used interchangeably with "cancer stem cell." The true definition of a CSC, however, is based upon its function—namely the capacity for self-renewal and the ability to give rise to the heterogeneous lineages of cancer cells that comprise a tumor[225].

The CSC theory of tumorigenesis, while receiving a great deal of attention recently, is based on concepts that have existed for over 150 years. As early as 1855, Rudolph Virchow proposed that tumors develop from residual embryonic nests (reviewed in[226]). Over the last century, this idea has been revisited multiple times. In the 1960s, evidence supporting the notion that not all tumor cells have an equal capacity for tumorigenesis was highlighted in quantitative tumor autotransplantation assays. In this study, tumor cell suspensions derived from patients with disseminated malignancy were injected subcutaneously into patients' own thighs. Based upon the high number of cells required for tumor growth, the authors speculated that the entire tumor cell population might be derived from a single CSC[226,227].

CSCs were first identified from the blood of patients with acute myelogenous leukemia (AML), by John Dick and colleagues in the 1990s[228,229]. Using xenotransplantation assays in NOD/SCID mice, they showed that tumorigenic potential resided with only a small subset of leukemic cells,

characterized by high CD34 and low CD38 cell surface expression. Furthermore, when this population of leukemic cells was transplanted into immunocompromised mice, they developed AML that was phenotypically similar to the subtype of AML present in the patient from which the cells were originally derived.

Several years later, Clarke and colleagues were the first to prospectively identify CSCs in a solid malignancy[134]. Using similar xenotransplantation assays, they identified a breast cancer cell population characterized by high CD44 and low CD24 expression that recapitulated the original tumor phenotype and developed from as few as 100 transplanted cells. Conversely, transplantation of tens of thousands of the alternate cellular phenotypes did not give rise to new tumors. Since that time, a multitude of studies have been published characterizing CSC populations across a wide variety of solid organ malignancies, including CNS, pancreatic, head and neck, and CRCs[82,230-232].

The CSC theory, aside from the contribution to our understanding of tumor biology, has potential far-reaching clinical implications. Like their normal tissue counterparts, CSCs have been shown to display increased chemoresistance and radioresistance[129,221,233-236]. Traditional cancer therapies typically target the rapidly dividing tumor cell population and, as increasing evidence suggests, may preferentially spare the CSC component of the tumor[129,234]. This may explain the often-encountered clinical scenario in which a tumor has apparent complete volumetric tumor reduction followed by subsequent local recurrence. As such, the CSC theory suggests that not only will our therapeutic targets need to be re-

envisioned with a focus on the CSC, but our methods for measuring therapeutic efficacy will need to be revised as well.

Stem cell hierarchy in colorectal tumors

Tumorigenesis within the colon follows an adenoma-carcinoma sequence first described in the early 1990s by Fearon and Vogelstein. The observation that colorectal tumors arise from a series of mutations that lead to the activation of oncogenes, inactivation of tumor suppressor genes and result in unregulated growth, has provided the framework for our understanding of tumor biology in the colon. While it is clear that mutations in multiple genes are required for malignant transformation, fewer changes are sufficient for benign tumor growth[237]. Additionally, the fact that stochastic acquisition of mutations within various combinations of signaling pathways can lead to cancer suggests that acquisition of CRC is an inevitable, temporally dependent event[218]. Incorporating this concept into the CSC model implicates these mutations to occur within the long-lived stem cell, leading to an accumulation of multiple mutations over time[216] (Figure A2.3). The mutated stem cell can, in turn, give rise to additional mutated stem and progenitor cells through symmetric and asymmetric division, seeding tumor growth with mutated, transformed and heterogeneous cells. In this fashion, the CSC is capable of nurturing its own microenvironmental niche, as the survival of its diverse population is selected by the surrounding tumor stromal cells. In support of this idea, Vermeulen and colleagues showed that establishment and

maintenance of the CRC stem cell niche is dependent on the Wnt signaling pathway orchestrated by myofibroblasts, suggesting that microenvironmental cues are as critical to the molecular diversity of tumors as are mutations[117].

Metastatic spread of disease is also consistent within the CSC theory. Independent subclonal populations within the tumor are endowed with different functional properties, but only selected clones have the potential to metastasize to distant organs[224]. In this model, the metastatic cells might originate from a monoclonal expansion of the original clonal cell population. But over time, development of additional genetic mutations enable responsiveness to environmental signals and acquisition of metastatic properties; namely the ability to invade the surrounding region, intravasate through vasculature, evade the immune system and extravasate at a distant site[224]. In support of this acquired diversity, metastatic tumors have the potential to significantly diverge morphologically from the primary tumor. Recent evidence from gene-expression microarrays support the CSC model for metastases in epithelial tumors, including colon cancer[216].

While direct evidence for the origin of CSCs in human cancer is lacking, elegant mouse experiments by Clevers and colleagues demonstrated that ablation of the Apc gene in the Lgr5-expressing progenitor cell population was sufficient to drive development of intestinal adenomas. In contrast, when Apc was deleted in the more differentiated TA cell compartment, macroadenomas did not develop. Experiments from this well-studied intestinal tumor model system

suggest that tumorigenesis is the result of malignant transformation specifically of a somatic tissue stem cell[238].

Identity of colorectal cancer stem cells

Certain barriers complicate the identification and isolation of CSCs within a tumor. Among these obstacles is the fact that stem cells are relatively scarce and lack a unique morphology that is easily distinguished from its progeny *in vivo*[239], and that CSCs are defined functionally by their ability to initiate a tumorigenesis and, as such, can only be truly identified *post hoc*. Despite these hurdles, multiple studies have demonstrated that small, isolatable populations of human tumor cells exist that are capable of recapitulating the phenotype of the parental tumor when transplanted and grown in immunodeficient mice. To date, these cell populations have been isolated based on expression of cell surface markers and have been shown to compromise approximately 1% of the total number of cells within the cancer (Table A2.1).

Table A2.1 – Colorectal Cancer Stem Cell Markers

Marker Name	Function(s)	References
CD44	Hyaluronic Acid Receptor; Cell Adhesion (Osteopontin, collagens and MMPs)	[82, 240, 241]
CD133/Prominin1	Self-renewal	[233, 242-244]
CD166/ALCAM	Cell Adhesion (Heterotypic/Homotypic)	[82, 241]
ALDH1	Enzyme - Alcohol Metabolism	[148]
Lgr5	Wnt-target gene, function unknown	[241]
EpCAM/ESA	Homotypic Cell Adhesion	[82]

An early study conducted by O'Brien et al. focused on validating CD133 as a colorectal CSC marker. In these experiments, $CD133^+$ and $CD133^-$ cells were isolated from both primary and metastatic human CRCs, and injected under the renal capsule of NOD/SCID mice. $CD133^+$ cells gave rise to tumors while explanted $CD133^-$ cells did not support tumor growth. Further, the regenerated $CD133^+$ tumor cells could be serially transplanted and still retain the parental tumor morphology[136]. This observation has been recapitulated by other groups[245]. Furthermore, the $CD133^+$ tumor cells showed exponential *in vitro* growth as tumor spheres, while maintaining the ability to generate new tumors when injected into immunodeficient mice. Upon withdrawal of growth factors, the cells within the tumor spheres gradually differentiated, resulting in loss of CD133 expression, and subsequent loss of their tumorigenic potential. Clarke's group used similar xenograft techniques to show that $CD44^+/CD166^+/EpCAM^{HIGH}$ cells isolated from human CRC could also establish a phenocopied tumor while no growth was observed with $CD44^-/CD166^-/EpCAM^{LOW}$ cells. In addition to CD133, CD166, CD44 and EpCAM, a potential colon cancer stem cell marker is proposed to be the somatic intestinal stem/progenitor cell marker Lgr5[246]. The importance of any one specific CSC marker identifying a "true" CRC stem cell population remain in flux, and several recent studies have questioned whether the CSC population remains static (e.g. expresses one specific marker, such as CD133, continuously throughout the course of disease), or whether this expression is variable and potentially cyclic[82,247-250]. A recent and elegant examination of CD133 surface expression in glioblastoma multiforme highlighted

this point by illustrating that the underlying PTEN signaling status represented a better correlation with CSC function than CD133 cell surface expression[249].

The question of whether these cell surface markers have functional relevance to the CSC population or whether they act simply as surrogate markers for CSCs remains unclear. Many of these proteins, such as CD133, have unknown function. Others, such as CD44 (hyaluronic acid receptor) and CD26 (dipeptidyl peptidase IV), have known functions; however, their functional relevance to tumorigenesis is uncertain and it is quite likely that these proteins have additional, currently unknown roles which may be relevant to cancer initiation or progression. As an example, CD166 is a member of the immunoglobulin super-family and is known to form homo-dimeric complexes as well as hetero-dimeric complexes with CD6 on lymphocytes to facilitate cell-cell interactions. Recent work in our laboratory has shown that CD166 marks the stem cell niche in the intestinal crypt in both mice and humans[153]. This suggests that CD166-expressing cells are important for the establishment and maintenance of the endogenous intestinal stem cell niche and, by extension, the CSC niche. Additionally, CD166 and other CSC marker proteins possessing cell-cell interactions may function to establish a pre-metastatic niche in target organs such as the liver, preparing a site to which migrating CSCs can home and establish metastatic deposits[153,251-254].

The role of CSCs in the establishment and maintenance of metastatic disease has been evaluated in several recent studies. Odoux and colleagues identified $CD133^{+}$ and $CD44^{+}/CD166^{+}/EpCam^{High}$ cells in samples of metastatic

CRC which maintained their CSC marker and histologic phenotypes in a limiting-dilution *in vitro* culture system as well as in *ex vivo* xenograft tumor models[255]. These results show that metastatic colorectal tumors possess similar CSC phenotypes and functionality as primary CRC tumors do. Further, $CD133^+$ cells from the CRC cell line SW480 have enhanced migratory ability *in vitro*[250]. Analysis of metastatic CRC samples from peritoneal washings and comparison to the CRC tumor cell line HCT116 by Botchkina et al., identified similar $CD133^+$ and $CD44^+/CD166^+/EpCam^{High}$ cell populations with tumorigenic potential similar to prior studies[256,257]. These studies suggest that the biological basis of metastatic establishment is similar to that of the establishment of primary colorectal tumors. New data from Clarke and colleagues in *ex vivo* models of human breast cancer stem cells suggests that the CSCs responsible for metastatic formation are the same CSCs as those that develop primary tumors. Additionally, these CSCs escape the primary site of tumor implantation in a xenograft model before there is an obvious histologically invasive phenotype at the primary tumor site. This provides experimental evidence that CSCs possess an invasive and migratory phenotype separate from that of the bulk tumor and that CSCs are largely responsible for the presence of disseminated malignancy[257].

Clinical implications of cancer stem cells in colorectal cancer

While it is clear from available evidence that CSCs play an important role in CRC development and metastasis, the prognostic impact of tumor CSC content in any particular tumor remains unsettled. The extent to which CSC marker expression patterns can be used to predict survival or response to therapy is also unclear. While individual CSC markers (and combinations thereof) such as CD44, CD166 and CD133[258-260] have been used to identify colorectal CSCs in specific patient populations, the prognostic and predictive utility of CSC markers remains uncertain, particularly in completely resected or widely metastatic disease[144,261-263]. Because the expression of these markers can be determined on virtually any type of tumor tissue (freshly isolated single-tumor cells, fresh-frozen tissue, archived formalin-fixed paraffin-embedded (FFPE) tissue, fine needle aspirates, or tumor cells isolated from peritoneal fluid or pleural effusions) using widely available technologies including flow cytometry, bright-field immunohistochemistry and multi-label immunofluorescence, the use of CSC markers and phenotype to predict clinical behavior such as metastatic potential and susceptibility to chemotherapy and radiation is an area of significant clinical importance. The ability to predict these behaviors will allow for more personalized and directed therapies based on tumor CSC phenotype.

Although surgical resection of metastatic disease is an option for some patients, the vast majority of cases of metastatic CRC are not amenable to curative surgical or radiation therapy, leaving chemotherapy and biologic therapy as the mainstays of treatment. While these treatments extend survival, they are

not curative. The stem cell theory of tumorigenesis and metastasis states that a primary mechanism of treatment resistance in metastatic disease is the resistance of CSCs to traditional chemotherapy. As standard cytotoxic therapies target rapidly dividing tumor cells with the goal of maximum cytoreduction, the underlying tumor-maintaining cells (CSCs) divide less frequently and express drug efflux pumps similar to somatic stem cells, rendering them less susceptible to chemotherapy. The clinical manifestation of this biologic phenomenon is that, although many tumors initially respond well to chemotherapy resulting in radiographic complete remission of disease, more often the CSC remains at the site of disease, undamaged by chemotherapy, and able to initiate disease recurrence. Chemotherapy resistance of CSCs has been described in a variety of epithelial malignancies including breast, lung, head and neck, and pancreatic cancer[234,236,264,265]. Recent studies have shown similar data for CRC as well. Using $EpCAM^{+}/CD44^{+}$ colon cancer xenografts, Dylla and colleagues showed that this was the only tumor-initiating cell population remaining following treatment with the cytotoxic drugs irinotecan and cyclophosphamide, and that these cells express high levels of ALDH1, a gene implicated in chemoresistance and a marker of CSCs[129,266-268]. Chemotherapy resistant CRC cell lines HT-29/5FU-R and HT-29/OxR are enriched in $CD44^{+}/CD133^{+}$ CSC phenotypic cells[269]. Together, these studies suggest the importance of targeting both the bulk cancer cells and the tumor-initiating cell if any systemic anti-tumor therapy is to ultimately be successful.

The clinical implications of CSCs in metastatic CRC are manifold and quite significant. First, while the bulk of tumor cells will succumb to cytotoxic and biological therapy, remnant treatment resistant CSCs will remain, leading to disease recurrence most likely with decreased susceptibility to chemotherapy (Figure A2.4). Second, CSCs likely possess dysregulated signaling pathways such as the p53, WNT and Notch pathways, which are not targeted by current therapeutic agents. Targeting of the p53 pathway has failed to be fruitful under *in vivo* conditions and targeting of the WNT signaling pathway has thus far proven toxic. Finally, in order to completely eradicate a tumor and all of the CSCs which contribute to its survival, they must be targeted in a directed and specific manner. All of the markers currently used to identify CSCs *in vivo* are expressed on a variety of normal somatic cells, including somatic stem cells. Therapies targeted at any single CSC marker, such as monoclonal antibodies conjugated to cytotoxic compounds, is likely to also damage the normal tissue stem cell compartment, potentially leading to unacceptable toxicity. Because of the difficulty in prospectively identifying and maintaining tumor-initiating cells *in vitro*, identification of CSC-specific compounds has been slow and complicated. The use of breast cancer cells induced into an epithelial-mesenchymal transition (EMT) and enriched for CSCs in a high-throughput compound screen identified salinomycin as a CSC-targeting agent[270] and induces apoptosis in a variety of human hematologic cancer cell lines[271]. However, its efficacy against purified CSCs or other solid tumors has not been evaluated either *in vivo* or *in vitro*. A novel, immunotherapy approach to targeting tumor-initiating cells has been

recently described by Herrmann and colleagues. Using MT110, a bi-specific antibody to EpCAM and human CD3[272], this group was able to eliminate primary human colorectal tumors in a xenograft model, as well as xenografts generated from the HT29 CRC cell line by inducing tumor-specific T-cell cytotoxicity while avoiding apparent toxicity to the host animal[273]. Interestingly, this method eradicated both the CSC component of the tumor as well as the bulk tumor cell population. It is uncertain, however, whether the anti-EpCAM portion of the antibody would bind to normal EpCAM-expressing intestinal epithelium, inducing a similar cytotoxic response in normal colon epithelium and colonic stem cells. Clearly, while important discoveries are being made in the identification of CSC-targeting compounds, much work, particularly in Phase 0 and Phase I human studies, remains to be carried out.

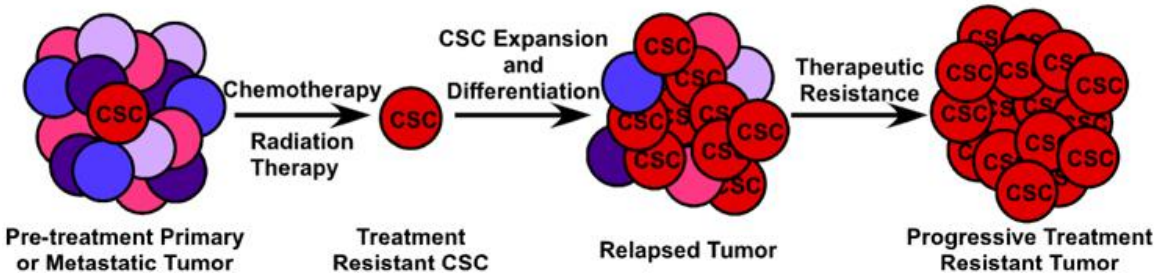

Figure A2.4. Clinical implications of cancer stem cells.

Clinical implications of the CSC model. Systemic chemotherapy and locoregional radiation therapy affect the more differentiated tumor cells but not the CSC. Following therapy, the treatment-resistant CSC remains and is able to re-populate the tumor and give rise to additional treatment-resistant CSC progeny as well as chemotherapy-sensitive differentiated cells. Clinically, this is seen as disease relapse. Further treatment with standard cytotoxic and biologic therapies will result in increasing numbers of CSCs, which presents clinically as progressive, completely treatment-resistant disease.

Prospectus

The CSC hypothesis is revolutionizing the understanding of tumor initiation and progression, however much remains to be elucidated regarding the role of these specialized cells in metastasis and response to therapy, arguably the most clinically important aspects of tumor biology. While a number of studies have shown a correlation between the expression of CSC markers such as CD133, CD44, CD166[91], Lgr5[274] and Bmi1[275] and survival, much less is known about the correlation of the expression of these markers and the CSC phenotype in metastatic disease. More importantly, almost nothing is known about the functional relevance of these markers for tumor behavior. In a case-controlled study, Horst et al. examined CD133 expression in colonic tumors from patients with or without synchronous liver metastases and found increased expression of CD133 in the metastatic tumors compared to the localized tumors, but found no effect on proliferation, migration, or invasion when it was knocked down in cancer cell lines[91,276]. They concluded that while CD133 was highly prognostic for development of metastases, it had no functional relevance to the tumors. An alternative view holds that, although CD133 expression *per se* is not relevant to the metastatic phenotype, a pathway involving CD133 likely is important. As so little is known about the function of CD133, or many of the other CSC markers currently used, a better understanding of the functions and interactions of these proteins in cancer and normal somatic stem cells will be critical in furthering our understanding of the function and therapeutic targeting of the CSC.

Additional work is also needed to determine either a reliable surface identity of the CRC stem cell population, or more likely, given the phenotypic and genetic variability between different tumors and over time in a single tumor a panel of markers that precisely identifies the CSC. To date, most studies have evaluated one to at most four CSC markers in identifying a CSC population. This is largely due to technical limitations in the use of fluorescently labeled antibodies and the spectral limits of detection and fluorophore separation of most flow cytometers and microscopes. The use of new technologies such as quantum dot-antibody conjugates will allow for the simultaneous detection of increasing numbers of CSC markers and more precise CSC identification[277].

Currently, screening assays for the effectiveness of novel chemotherapeutic compounds largely rely on their *in vitro* cytotoxicity. The CSC model therefore has important implications and provides exciting new tools with respect to the design of new assays to test anticancer therapies. Three-dimensional tumorsphere culture systems can be generated from cancer cell lines or primary tumor cells enriched for CSC marker expression (and proven to be tumor-initiating cells in xenograft models) and used in high-throughput compound screens similar to current assays. Secondary screens can then be performed on promising compounds using orthotopic and heterotopic xenograft models of the sorted cell lines and tumors. One issue that must be addressed, however, is the need for a standardized methodology for identification and culturing of CSCs in order to allow clinically meaningful comparisons between different experimental compounds.

Because of the related features and functions of CSCs and normal somatic stem cells, it is clear that a significant limitation to designing compounds which target the CSC will be limiting their effects on the normal somatic stem cell. If somatic intestinal stem cells are damaged by drugs targeted to colorectal CSCs, it is likely that gastrointestinal toxicity would be unacceptably high and, unlike current chemotherapies which spare the stem cell population, may be fatal. Thus, while the use of normal intestinal stem cells to understand colorectal CSC biology is important, the identification of novel and unique CSC targets distinct from somatic stem cells is critical[278].

Conclusions

Increasing evidence supports the presence of a CSC or tumor initiating cell as the cause of tumor establishment, progression, and relapse and metastasis. Identification of the origin of the CSC remains elusive in human CRC, however progress is being made in mouse models of intestinal cancer. The precise role of the CSC in these tumorigenic steps of CRC also remains unclear. Additionally, the interaction of colorectal CSCs with the cellular microenvironment, both at the site of tumor initiation and at sites of metastatic deposit, must be further investigated. This is particularly needed given the importance of the microenvironmental niche in the function and maintenance of somatic stem cells. Finally, in order to specifically target CSCs while sparing somatic intestinal stem cells, it will be critical to identify unique molecules and dysregulated pathways in the CSC population when compared to the somatic stem cell population. A better understanding of these aspects of somatic and CSC biology will be necessary in order to effectively target CSCs and ultimately developing cures for advanced and metastatic CRC.

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Figure Contributions

Unless noted here or in the figure legend all images and data were generated by Trevor Levin. Figure contributions below denote collection of raw data. All final image layout and design performed by Trevor Levin and Melissa Wong unless noted.

Figure 2.3 (B) Paige Davies
Figure 2.5 (B, C) Anne Powell, Adria Dismuke
Figure 2.8 (C, D) Paige Davies, (E) Alian Silk
Figure 2.9 (A, B) Eric Anderson

Figure 3.1 (D) Nick Smith
Figure 3.2 (C) Nick Smith
Figure 3.3 (A) Nick Smith (B) Quantification by both N.S. and T.L.
Figure 3.4 (E, F) Nick Smith

Figure A1.3 (A, B) James Tanyi
Figure A1.8 (A-H) Patsy O'Brien

Appendix 2, all figures conception and design by Eric Anderson

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