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Chapter 6

SIGNALING PATHWAYS, GENE REGULATION AND DUODENAL NEOPLASIAS

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ABSTRACT

The *duodenum* is located at the crossroads of the three main secretions of the gastrointestinal (GI) tract -gastric, pancreatic and biliary. Despite the detrimental nature of these chemically dissimilar juices upon unprotected cells, the duodenal mucosa remains unharmed, keeping pace with cell renewal. Although anatomists consider the *duodenum* to be part of the small bowel, gastroenterologists regard the *duodenum* - together with the esophagus and the stomach- as part of the upper digestive tract. The latter view may lie on the fact that the *duodenum* has special biological attributes that are at variance with the rest of the small intestine. In this chapter we review signaling pathways affecting the duodenal mucosa, its putative stem cells and their offspring, TAP cells, and their differentiated cells (Paneth cells, goblet cells, enterocytes and neuroendocrine cells) as well as its villous fronds. Classical and more recently discovered stem cell candidate markers are tabulated. In celiac disease intolerance to gluten protein leads to villous atrophy. In contrast to the normal duodenal mucosa that is characterized by short crypts and long villous fronds, villous atrophy is typified by long crypts, high numbers of mitosis and absence of villous formations. We submit that the high numbers of mitosis in enlarged crypts mirrors increased mitotic activity in TAP cells but not in stem cells, as mitoses are not recorded in position +5 cells or in cells at the bottom of the duodenal crypts (the domain of stem cells).

There is an increasing awareness of the supportive role played by Paneth cells in maintaining CBC stem cell homeostasis. Paneth cells contain the potent antimicrobial enzyme lysozyme. Recent developments indicate that in celiac disease many Paneth cells are superseded by lysozyme-producing mucus glands. This metaplastic antimicrobial adaptation might mirror a reprogramming of stem cells, to signals generated by the

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pathogenic duodenal flora recently detected in celiac disease. All nonampullary duodenal adenomas are tubular. We submit that these adenomas are dysplastic aberrations of the crypts. The lack of villous fronds in duodenal adenomas as well as in celiac disease is puzzling. The possible signaling responsible for the abrogation of villous fronds in these two diseases of the duodenal crypts is discussed. Duodenal adenomas often concur with gastric duodenal metaplasia, a non-neoplastic mucosal change triggered by duodenal micro-environmental factors such as high pH and chronic inflammation, and celiac patients develop villous atrophy, but only if gluten protein is present in the duodenal micro-environment. The purported signaling of the duodenal micro-environment to stem cells in villous atrophy and cryptal adenomas, is receiving much attention at this laboratory.

1. INTODUCTION

Herophilos, a Greek physician (300 BC) found that the length of the first portion of the small intestine was equal to 12 finger diameters (approximately 25 cm).

He called this portion δωδεκαδάκτυλον (dodecadaktylon, in Latin, *duodenum*). In spite that anatomists regard the *duodenum* as a portion of the small bowel, gastroenterologists maintain that it is part of the upper digestive tract, together with the esophagus and the stomach. The reason for the latter view may lie on the fact that the *duodenum* has special biological attributes that are at variance with the rest of the small intestine:

i. The duodenum is located at the crossroads of the three main secretions of the gastrointestinal (GI) tract -gastric, pancreatic and biliary. Despite the detrimental nature of these chemically dissimilar juices upon unprotected cells, the duodenal mucosa remains unharmed, keeping pace with cell renewal.
ii. At histology, the duodenal mucosa show particular submucosal glands, the Brunner glands; their secretions buffers the disparate pHs in the duodenal microenvironment.
iii. The bottom of the duodenal crypts (where Paneth cells reside) show the highest expression of Peroxisome proliferator-activated receptor beta or delta (PPARβ/δ), a nuclear receptor encoded by the *PPARD* gene, than in the rest of the small intestine [1].
iv. The repertoire of Paneth cell antimicrobials differs between duodenum and ileum; cryptdin 4 and cryptdin-related sequences (CRS) 4C peptides are expressed at progressively increasing amounts (10^1- and 10^4-fold, respectively) comparing duodenum and ileum in conventionally reared mice [2].
v. The gene expression of the liver enriched transcription factor HNF6 is more abundant in the *duodenum* than in the jejunum [3].
vi. Lectines cause upregulation of the gene expression of the proinflammatoty cytokines interleukin (IL)-8, tumor necrosis factor in the *duodenum* but not in the ileum in mice [4].
vii. A duodenum-specific enhancer identified in the second intron of the human *ADA (*adenosine deaminase) gene is expressed at high levels only along the villi of the duodenal epithelium. Binding of PDX-1 and GATA-4 is absolutely essential for enhancer function. An additional enhancer binding sites for Cdx factors, for YY1, and for NFI family members has been identified [5].

viii. The *duodenum* is the most common site for extracolonic tumors in patients with familial adenomatous polyposis (FAP) [6].
ix. MUTYH is a mammalian DNA glycosylase that initiates base excision repair by excising adenine opposite 8-oxoguanine and 2-hydroxyadenine opposite guanine, thereby preventing G:C to T:A transversion caused by oxidative stress. In MUTYH-null mice treated with KBrO3, the mean number of small intestinal tumors dramatically increased to 61.88, whereas it was 0.85 in wild-type mice. The tumors developed predominantly in the *duodenum* [7].

In this chapter, pertinent literature regarding putative SCs, TAP cells and villous formations in the duodenal mucosa in health and disease, is presented. Possible differences in signaling and gene regulation between normal donors, patients with villous atrophy in celiac disease (an autoimmune mucosal ailment) and patients with duodenal neoplasias of the crypts, are discussed.

2. STEM CELLS: LOCATION IN THE CRYPTS AND SIGNALING PATHWAYS

Stem cells are multipotent, self-replicating, with capacity to generate daughter cells committed to terminal differentiation. The estimated number of stem cell population within the crypt, varies from 0.4% to 60% of the crypt [8] with variation from species to species [9, 10].

The position of the stem cells in intestinal crypts has been much debated. For some, stem cells are "placed" at position +4 relative to the crypt bottom [11].

For others, a stem cell zone consists of small, undifferentiated, cycling Crypt Base Columnar (CBC) cells, intercalated with Paneth cells [12]. It has been postulated that CBC cells represent a population of stem cells more ready to respond to stimulating signals generated from adjacent mesenchymal cells such as BMP antagonists like Noggin or Gremlin. There is, however, a mounting awareness that in rapidly renewing adult tissues, the 2 stem cell compartments coexist and work coordinately [13].

Queries and comments

If +4 stem cells and CBC stem cells are two different cell systems, the pertinent question is: How cells, evolving from rapidly dividing CBC reach the TAP cell domain in the crypt? Rationally not by "jumping over" +4 cells, but, by either intercoursing signal information with +4 stem cells, or through by-passing the +4 stem cell niche in the crypt cuff via cellular predetermined corridors, one for CBC cells and one for +4 cells. If the latter is the alternative, then two cellular independent cell columns might exist: one for the off-springs of +4 stem cells and the other for off-springs of CBC stem cells. Other questions: Are TAP cells in these two columns proceeding at the same speed on their route to the villi? If so, do differentiated cells "mingle" in the 2 columns? In this context it should be remembered that six or more independent crypts surround a single villus, resulting in an equal number of parallel columns of epithelial cells running toward the villus tip.

2.1. Signaling Pathways

Several signaling pathways such as Wnt, bone morphogenic protein (BMP), PTEN-controlled PI3K/Akt kinase and Notch are involved in intestinal development and homeostasis, including stem cell regulation, lineage specification and maturation [14].

2.1.1. Wnt Transduction Signaling

In the canonical pathway, the Wnt ligand interacts with its cognate receptor, Frizzled, and a co-receptor from the lipoprotein receptor-related protein 5/6 (LRP 5/6) families [14, 15]. Wnt signalling, which is transduced through βcatenin/TCF4, maintains the undifferentiated state of intestinal crypt stem/progenitor cells via cell cycle control and inhibition of differentiation, controls migration and localization of epithelial cells along the crypt-villus axis, directs early secretory lineage development and terminal differentiation of Paneth cells [14, 16]. Paradoxically, the terminal differentiation of Paneth cells at the crypt bottom requires Wnt signals. In humans, the canonical Wnt signaling is also involved in the control of the migration/invasion behavior of mesenchymal stem cells (hMSC) [17, 18].

3. STEM CELL MARKERS

3.1. Detecting Stem Cells in Position+4

Several markers may identify stem cells at +4 position: CD34, cKit, or the Hoechst dye that define Side Population, Musashi-1 [19, 20, 21, 22, 23, 24, 25, 26].

3.2. Detecting CBC Stem Cells

Lgr5/Gpr49 gene, that encodes an orphan G protein-coupled receptor, characterized by a large leucine-rich extracellular domain, labels CBC cells. These cells invariably express the Ki67 cell cycle marker, providing a simple means of their identification amongst noncycling Paneth cells. The average cycling time of CBC cells is about 1 day, ruling out that they are quiescent, while transit-amplifying progenitors (TAP) cells cycle every 12 h [9].

$Lgr5^{hi}$ cells generate all cell types of the small intestine epithelium through life. Using the marker Bmi1 (a gene encoding a component of a Polycomb Repressing Complex 1) long term tracing is observed with kinetics that are surprisingly identical to that of $Lgr5^{hi}$ cells [27]. $Lgr5^{hi}$ and Bmi1 may mark overlapping, if not identical, cell population. Both Bmi-1 and CBC cells produce offspring within days [28].

Recently, PHLDA1 was found expressed in discrete crypt base and some position +4 cells in the human small intestine. Hence, PHLDA1 is a putative epithelial stem cell marker in the human small intestine [29].

Intestinal stem cells receive support from their own specialized progeny Paneth cell markers (lysozyme, defensin A1 and stem-cell markers (Lgr5, Olfm4, Tnfrsf19, Cdca) [30]. Paneth cells serve as multifunctional guardians of stem cells, by providing essential niche signaling support: EGF, Wnt3 and Notch.

Only the direct neighbors of Paneth cells, the Lgr5 stem cells, receive strong Wnt signaling, further increased by R-spondin.

Doublecortin- and Calmodulin Kinase-Like 1 (DCAMKL1), a microtubule-associated protein is expressed in the small intestinal crypt cells [26]. Mushashi-1, a gene that encodes an RNA-binding protein labels both ISCs and progenitor cells [8].

Prominin-1(Prom1)/CD133, a pentaspan trans-membrane glycoprotein identify intestinal CBC cells and TAP cells located above the Paneth cells. Hence, Prom1 marks a much larger stem cell/transit-amplifying progenitor compartment [28, 31]. A summary of the candidate markers of intestinal stem cells is shown in Table 1.

Table 1. Candidate markers of intestinal stem cells

+4 cells marker	CBC cells marker	+4 and CBC cells marker	Crypt Base marker	Crypt base (incl. Paneth cells) marker	Crypt-villous axis marker
Bmi 1 Sox9 mTert PHLDA1	Lgr5 Ascl2 Olfm4	Musashi 1	CD133/ Promin	CD24	Decamkl 1

4. CLONALITY

Human small bowel crypts are clonal, with all the differentiated lineages originating from a common stem cell, whereas the contribution of several crypts leads to villi being polyclonal. Patches of mutated crypts share the same mtDNA mutation and therefore one parent mutated crypt must have divided by fission, over time, to produce daughter crypts sharing the same mutation [32]. In mice, exposure to a slightly higher dose of radiation (> 1 Gy) yielded 6 additional so-called ‘clonogenic’ crypt cells with the capacity to regenerate entire lost crypts. On the other hand, as many as 30–40 clonogenic cells are identified per crypt, following higher doses of radiation (8–10 Gy) [33]. Under normal conditions, clonogenic cells are believed to represent early TAP cells. Many more short-lived progenitors rapidly divide before giving rise to a differentiated progeny.

5. STEM CELL NICHES

Stem cells are thought to reside in a ‘niche’ towards the base of the crypt and their activity is regulated by the paracrine secretion of growth factors and cytokines from surrounding mesenchymal cells. [12]. The niche includes all cellular and non-cellular components that interact in order to control the adult stem cell, to physical contact and diffusible factors [34].

Recent studies showed, however, that Lgr5+ cells can repopulate the crypt without a mesechymal niche. Asymmetry of crypt–villus organoids was established by the localized presence of Wnt-producing Paneth cells. [27].

5.1. Integrin Signaling

Integrin-linked kinase (ILK) plays a role in integrin signaling-mediated extracellular matrix (ECM)–cell interactions and also acts as a scaffold protein in functional focal adhesion points. ILK performs crucial roles in the control of human intestinal cell and crypt–villus axis homeostasis— especially with regard to basement membrane fibronectin deposition—as well as cell proliferation, spreading, and migration. The basement membrane directs ECM components, such as type IV collagen, laminins and fibronectin, key players in the establishment and maintenance of tissue morphology [35].

6. STEM CELL PLASTICITY

ISEMF's are an integral part of the epithelial stem cell niche, and regulate epithelial stem cell function by paracrine secretion. In mice with experimental colitis the number of bone marrow derived ISEMF's may be as high as 70%. Donor-derived cells have been observed forming cellular columns in a crypt to- apical fashion which is morphologically similar to indigenous ISEMF's [35].

7. PROGENITOR CELLS SIGNALING

3 H-thymidine and BrdU, or antibodies against markers such as Ki67, indicate that the lower two thirds of crypt cells are synthetizing DNA. They are called transit amplifying population (TAP). The asymmetric division of the intestinal stem cells maintains the continuous supply of crypt cells [35, 36]. In mice crypt TA progenitors divide every 12–16 h, generating some 300 cells per crypt every day [38]. Newly formed TAP cells reside within crypts for 48–72 h undergoing up to six rounds of cell division as they migrate apically towards the villous [37]. During this journey up the crypt, TAP cells become committed to specific cell fates, start to differentiate and exit into the villous epithelium as mature absorptive enterocytes, mucus-secreting goblet cells, or hormone-producing enteroendocrine cells [30]. Progenitor cells retain the ability to divide until they terminally differentiate [12]. Short-lived multipotent "Mix" progenitors presumably derive directly from multipotent stem cells and generate different epithelial lineages [39].

7.1. Notch Signaling

Stem cells and TAP cells express Notch receptors (N1 and N2) and Hes1, a Notch target gene implicated in intestinal cell differentiation.

Secretory cells express Notch ligands (Dll1, Dll4, Jag1 and Jag2) [38].

7.2 EphB Signaling

EphB signaling promotes cell-cycle reentry of progenitor cells and accounts for approximately 50% of the mitogenic activity in the adult mouse small intestine. Ephrin ligands and their Eph tyrosine kinase receptors are membrane bound proteins that regulate cell migration [39, 40, 41, 42]. EphB receptors are key coordinators of migration and proliferation in the intestinal stem cell niche [43]**.** Eph/ephrin signaling regulates cell-to-matrix adhesion EphB2 and EphB3, establishing a boundary between proliferative and differentiated cells [44].

7.3. Cdx2, Math 1(Atho1), GATA, ADA Signaling

The transcription factor Cdx2 is related to the differentiation of intestinal epithelial cells. Math1-positive epithelial cells co-expressing Cdx2 are found in normal intestinal mucosa from humans and mice. The basic helix–loop–helix transcription factor Math1, is instrumental in the cell fate decision of enteroendocrine, goblet, and Paneth cells in the intestine [45, 46].

A *duodenum*-specific enhancer is located in intron 2 of the human Adenosine deaminase (ADA) gene. This enhancer has been shown to rely on PDX-1, GATA factors, and Cdx factors for its function. *Duodenal* nuclear extracts contain five distinct DNase I footprints within the enhancer [47, 48, 49].

8. DIFFERENTIATED CELL SIGNALING

8.1. Paneth Cells

Paneth cells are terminally differentiated cells characterized by large eosinophilic, refractile granules that contain several anti-microbial compounds, important in immunity and host-defense. These cells are made in pairs and die at the base of the crypts. Paneth cells are the longest-lived differentiated cells within a crypt (in mice estimated to be around 23 days). By extrapolating from the lifespan of a mouse (approximately 2 years) to that of a human (approximately 70 years), the estimated turnover time of human Paneth cells reported is 2.2 years [8].

8.1.1. Wnt Signaling

Paneth cells home towards the source of Wnt signals, that is, the CBC cells domain. Conditional deletion of the Wnt receptor Frizzled-5 abrogates expression of these genes in Paneth cells in the adult intestine. Thus, Wnt signals in the crypt can separately drive a stem-cell/progenitor gene programme and a Paneth-cell maturation programme. In intestinal cancer, both gene programmes are activated simultaneously [50]. Ablation of one of the Wnt receptors, *Fz5*, with a *K19Cre* transgene, caused mislocalization of Paneth cells in the villi [51].

8.1.2. β-Catenin/TCF Signaling

β̃catenin/TCF signalling is essential for maintaining the proliferative/undifferentiated state of intestinal epithelial cells and determining cell positioning along the crypt axis by controlling the expression of EphB and ephrinB genes. β̃catenin and TCF inversely control the expression of the EphB2/EphB3 receptors and their ligand ephrin-B1 along the crypt-villus axis. In EphB2/EphB3 null mice, Paneth cells do not follow their downward migratory path, but scatter along crypt and villus. [52, 53, 54]

8.1.3. Musashi-1 Signaling

Musashi-1 (Msi-1), a RNA-binding protein, known as a putative marker of intestinal stem cells suppresses expression of Paneth cell specific genes, presumably through a pathway independent from Notch or Wnt. Msi-1 is a negative regulator of Paneth cell differentiation, and may contribute to maintain the undifferentiated phenotype of intestinal stem cells [55].

8.1.4. PPARβ/δ Signaling

The peroxisome proliferator–activated receptor (PPAR*β/δ* is a nuclear hormone receptor expressed at the bottom of the crypt of the small intestine. PPARs belong to the superfamily of nuclear receptors and their ligands, most of them fatty acids or derivatives (prostaglandins and leukotrienes). PPAR*β/δ* acts on Paneth cell homeostasis by down-regulating the expression of the *Indian hedgehog (Ihh*) signaling pathway, a signal sent by mature Paneth cells to their precursors, negatively regulating their differentiation. The expression of PPAR*β/δ* is highest in the *duodenal* mucosa [1].

8.1.5. EphB Signaling

Ephrin-B1 and Ephrin-B2 are expressed on differentiated cells along the villus. As cells migrate upwards, along the crypt and farther from the Wnt source, present at the crypt base, EphB expression decreases and Ephrin-B ligand expression increases, thereby preventing downward migration [16]. Paneth cells migrate downward into the crypt base from their site of origin in an EphB3-dependent manner [9]. Because Paneth cells do not express Ephrin-B ligands and only express EphB3, their upward migration is prohibited, and, subsequently, they are forced to remain at the crypt base. Deletion of EphB3 results in aberrantly localized Paneth cells. EphB2, not present on Paneth cells is found to be expressed on CBCs and decreases in expression from just above the Paneth cells toward the crypt top. EphB3 expression is restricted to CBCs and Paneth cells at the crypt base [56].

Queries and Comments

The general consensus is that Paneth cells migrate to the base of the crypts whereas all other differentiated cells migrate upwards towards the tip of the villi. This notion would imply that only +4 cells are able to generate Paneth cells while CBC cells are unable to do so. It appears reasonably to assume, however, that if CBC cells also produce Paneth cells, then at least some Paneth cells would migrate sidewise in the crypts and not downwards.

8.1.6. BMP/Hedgehog Signaling

Members of the BMP and hedgehog (*HH*) families direct the positioning of the crypts in the niche through and interplay between epithelial and subepithelial cells [43, 56, 57, 58, 59, 60, 61].

8.1.7. SOX9 Signaling

SOX9 is a transcription factor found in stem/progenitor cells that maintains the multipotent and proliferative capacity of stem/progenitor populations. Sox-9 expression precedes Fz-5-dependent Paneth cell maturation. Since the *Sox9* conditional knockout mice display a complete absence of Paneth cells, *Sox9* is identified as a crucial Wnt-dependent gene in Paneth cell maturation. Sox9 mutant mice display an increase in crypt cell proliferation as well as an increased number of cells expressing c-MYC and CyclinD1, indicating that Sox9 may play a role in negative feedback of the Wnt pathway. Recently, it was found that CD24 is expressed in multipotent IESCs (Sox9EGFPlo) population and marks IESCs that form organoids in culture. Thus, single IESCs generate crypt-like units in the small intestine without a detectable mesenchymal cell component [62]. Flow cytometry of jejunal epithelial preparations revealed a CD24(+) CD45(-) fraction comprising ~1% of the cells. Flow cytometry with anti-lysozyme antibodies demonstrated that Paneth cells comprise ~30% of the CD24 (lo) subfraction [63].

8.1.8. Other Signaling Pathways

Paneth cells express EGF, TGF-a, Wnt3, the Notch ligand Dll4and CD24+; they are essential signals for stem-cell maintenance in culture. Co-culturing of sorted stem cells with Paneth cells markedly improves organoid formation. Genetic removal of Paneth cells in vivo results in the concomitant loss of Lgr5 stem cells. Lgr5 stem cells compete for essential niche signals provided by a specialized daughter cell, the Paneth cell [64].

8.1.9. Paneth Cells Are Involved in IgA-Mediated Acquired Immunity

It is generally accepted that Paneth cells do not participate in the synthesis of polymeric immunoglobulin receptor (pIgR) or the secretion of immunoglobulin A (IgA) in the small intestine. Polymeric Immunoglobulin Receptor mRNA (pIgR) and IgA are colocalized in the secretory granules of human Paneth cells. These findings suggest that, in addition to their well-recognized role in innate immunity, Paneth cells are involved in IgA-mediated acquired immunity in the intestinal tract [65, 66, 67].

8.2. Goblet Cells

In situ hybridization has been used to study mRNA expression of mucin genes in *duodenum.* The pattern of mucin gene expression in fetal duodenum reiterated the patterns observed during intestinal ontogenesis, with MUC2 and MUC3 expression in the surface epithelium and MUC6 expression associated with the development of Brünner's glands . A regulatory role for mucin gene products in gastroduodenal epithelial cell differentiation was suggested [68]. Atoh1 (Math1 for mouse, Hath1 for human), is a basic loop-helix transcription factor and downstream component of the Notch signaling pathway; it is

negatively regulated via Notch signaling and expressed in secretory progenitor and mature secretory cells. Musashi 1 is required for differentiation of the secretory lineage (goblet, enteroendocrine and Paneth cells) [69].

8.3. Enterocytes

8.3.1. Notch Signaling

In concert with the Notch pathway, β-catenin maintains intestinal stem cells and controls their differentiation [70, 71, 72, 73]. Deletion of Math1 causes depletion of the goblet, Paneth, and enteroendocrine cell lineages in the small intestine, suggesting that Math1 is essential for progenitor cell commitment to one of three epithelial adult cell types, and that Math1-negative progenitors become enterocytes [55]. Intestinal stem cells can be distinguished by their enriched expression of the Notch ligand, Delta (Dl), through which ISCs control Notch activity of their differentiating daughters. Notch induces absorptive enterocyte differentiation [15]. High levels of Delta result in enterocytic differentiation. HNF-1α cooperates with Cdx2 and GATA-4 in the modulation of proliferation, acquisition of morphological features, and the initiation of the enterocyte differentiation program [74]. In the *duodenum* GATA factors are necessary for crypt cell proliferation, cell differentiation including absorptive enterocyte gene expression. [75].

8.3.2. Epidermal Growth Factor (EGF) Signaling

Signaling by epidermal growth factor (EGF) is associated with intestinal proliferation. Organoids derived from single stem cells were cultured for more than 8 months. Villin1 mature brush borders and apical alkaline phosphatase demonstrated fully polarized enterocytes [76].

8.3.3. Reelin/ Disabled-1 (Dab1) Signaling

Reelin receptors [apolipoprotein E receptor 2 (ApoER2) and very low density lipoprotein receptor (VldlR)], Disabled-1 (Dab1) protein and integrins alpha3 and beta1 are found in enterocytes. The Disabled-1 and VldlR protein signals are detected in the crypt and villus cells, being particularly abundant in the terminal web domain of the enterocyte. The ApoER2 protein signal is found in the upper half of the villi but not in the crypts [77].

8.4. Enteroendocrine Cells

8.4.1. Neuroendocrine Cell Signaling

Enteroendocrine cell differentiation is controlled positively by the transcription factor Ngn3 [78]. Additional transcription factors such as Pdx1, and Neurod1 are required for terminal differentiation of enteroendocrine cells [79]. Reg IV (RELP), a regenerating protein family member, is constitutively expressed in neuroendocrine cells of the intestinal mucosa. The human helix-loop-helix transcription factor Hath1 regulates the embryonic differentiation of neural and other intestinal secretory lineage cells.

GFI1, a transcriptional repressor that functions downstream of *Math1* to select Paneth/goblet versus entero-endocrine cell fates among intestinal secretory progenitors, is expressed in enteroendocrine cells, but not in goblet or Paneth cells. *Gfi1* is *Gfi1−/−* mice lack Paneth cells, have fewer goblet cells, and supernumerary entero-endocrine cells. Low levels of Delta support the enteroendocrine lineage [78, 79].

9. PATIENTS LACKING PANETH CELLS, GOBLET CELLS AND ENTEROENDOCRINE CELLS

Patients characterized by absence of Paneth, goblet, and enteroendocrine cells in the small bowel have a developmental block in differentiation for secretor cell lineages. [67]**.** Factors responsible for this process are: anomalies in Wnt/β-catenin signaling; Notch signaling, transcription factors Math1, and Hes1; homeobox transcription factors Cdx1 and Cdx2; Kruppel-like factors KLF4 and KLF5; transcription factor Elf3; platelet-derived growth factor (PDGF) A and its receptor, PDGF-Ra; the winged helix transcription factor Fkh6; the homeodomain transcription factor Nkx2–3; and *Hox* and *ParaHox* cluster genes, *Sonic hedgehog*, and bone morphogenetic proteins. The *duodenum* shows absence of goblet cells in both villi and crypts (confirmed by electron microscopy), with preservation of enterocytes. Mucus cell antigens MUC2, MUC5AC, TFF1, and TFF3; enteroendocrine products CMG, gastrin, and serotonin; or the early Paneth cell marker human defensin 5 (HD5) are all negative [67].

10. VILLOUS FORMATION SIGNALING

Villous development depends on the proliferation of mesenchymal cells, which support the villous lining. To transport absorbed nutrients, blood and lymph vessels should be formed within the villi.

Villous development does not rely on the proliferation of intestinal epithelial cells alone and requires a cytokine network including epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and bone morphogenetic protein (BMP) 2 and 4 [80].

10.1. Hedgehog Signaling

Sonic hedgehog and *Indian hedgehog* genes encode members of the Hedgehog family of cell signals. Both are expressed in gut endoderm, Because Hedgehog inhibition compromises villus formation, this signal is thought to work as a morphogen within the intestine. [3]. *Sonic hedgehog* mutants display among other somatic anomalies, *duodenal* stenosis. *Indian hedgehog* mutants show reduced epithelial stem cell proliferation and differentiation. Hence, Hedgehog signals are essential for organogenesis in the intestinal tract [59, 80, 81, 82].

11. VILLOUS ATROPHY (CELIAC DISEASE)

Celiac disease is a common immune-mediated condition in the proximal small intestine often generated by a permanent intolerance to cereal gluten. In diagnostic biopsies the *duodenal* mucosa may display total villous atrophy (Marsh type IIIC), with enlarged crypts showing increased number of mitosis (TAP cells) and absence of villi. Gluten-free diet restores the normal configuration of the *duodenal* mucosa with short crypts and long villi. In later years much attention has been focused on the abnormal microbiota present in the *duodenum* in patients with celiac disease: *bifido bacterium adolescentis/bifido bacterium animalis lactis, Bacteroides vulgatus, Escherichia coli* and rod-shape bacteria [83, 84, 85, 86].

Comments

Lysozyme is as an innate enzyme with potent antibacterial properties, which is strongly expressed in Paneth cells in normal duodenal crypts. There might exist a critical limit for the number of Paneth cells (intercalated with CBC cells) that can be housed at the base of single crypts. Recently, it was found at the bottom of enlarged crypts in celiac disease that Paneth cells could be absent [87]. Instead, lysozyme-rich mucus glands were found to replace the areas of Paneth cell domain (Figure 1) Apparently, duodenal crypts undergo mucus metaplastic transformation, possibly to increase the demands in antibacterial lysozyme required by the recently detected hostile bacterial flora. The possibility that in celiac disease, stem cells might have been re-programmed, from lysozyme-rich Paneth cells to lysozyme-rich mucus glandular metaplasia, was entertained. The molecular signals behind the abrogation of Paneth cells in duodenal crypts in celiac disease and its substitution by lysozyme-rich mucus metaplastic glands deserves further investigation.

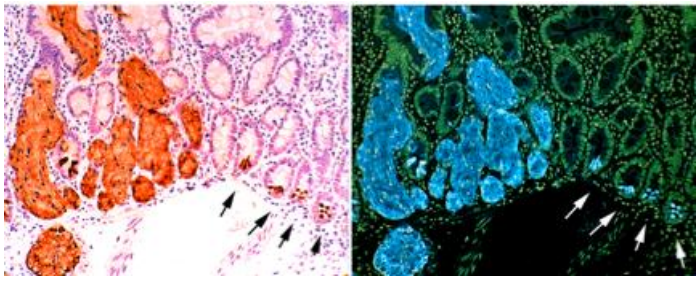

Figure 1. Duodenal mucosa with villous atrophy, antilysozyme immunostain. Left panel: Note normal Paneth cells (at arrows) in the right side of the picture. In the left part, lysozyme-rich mucus metaplasia of the crypts has replaced Paneth cells at the base of the crypts (antilysozyme immunostain x10). Right panel: Same picture using the INVERT function of a Photoshop program (Ps Adobe Photoshop CS3 extended) to highlight the phenomenon.

11.1. Mitosis

Increased number of mitotic figures is a dominant parameter of cell proliferation in patients showing villous atrophy in the duodenal mucosa [91]. The molecular signals that control mitosis in duodenal crypts in villous atrophy remain poorly understood [88].

Comments

The term proliferating cells (from Latin *proles*, offsprings*)* is here applied for cells in mitosis (M phase) that is, capable to produce daughter cells through cell division and not for cycling cells showing DNA-labeled synthesizing nuclei (S phase). This distinction is crucial, considering that in tumors, a proportion of the DNA synthesizing cells remain arrested in S phase or die, unable to generate daughter tumor cells. In this respect a human nuclear gene (named MTGM) that encodes a novel, small, integral mitochondrial inner-membrane protein is highly expressed in human tumor tissues. Over-expression of MTGM results in inhibition of "cell proliferation", stalling tumor cells in S phase. Hence, a proportion of the DNA synthesizing tumor cells is sterile. Accordingly, these tumor cells will not *proliferate*.

Figures 2, 3 and 4 depict sections from duodenal biopsies in patients with villous atrophy. Sectionion were challenged with the mitosis marker Anti-phospho Histone H3 (Ser 10). Figure 2 reveals enlarged crypts, high numbers of mitotic figures and absence of villous fronds. But the most significant finding is the absence of mitosis in position +5 cells and in cells at the bottom of the crypts (the stem cell domain) (Figures 3 and 4). This observation strongly suggests that proliferation affects exclusively TAP cells and that stem cells are not proliferating at the time of observation. The possibility that stem cells might also proliferate at the time of observation in sections from other patients with villous atrophy cannot be totally rejected. Nevertheless, mitotic cells in position +5 and at the bottom of the crypts were not found in a cohort of 56 biopsy-patients with villous atrophy (Rubio, in preparation).

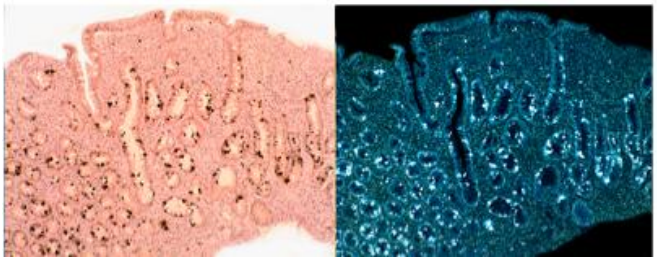

Figure 2. Duodenal mucosa with villous atrophy immunostained with Mitosis marker anti-phospho Histone H3 (Ser 10) (low power view MIT immunostain, x4). Left panel: Note numerous mitotic figures in TAP cells in elongated crypts. Right panel: Same picture using the INVERT function to highlight the phenomenon.

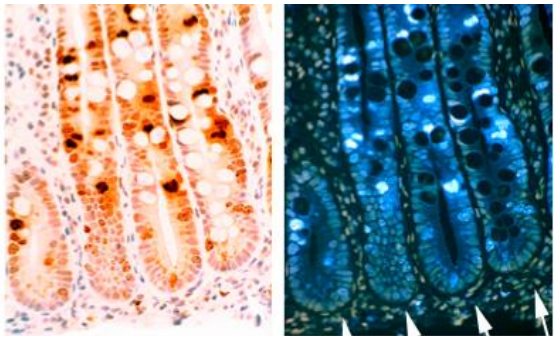

Figure 3. Duodenal mucosa with villous atrophy, immunostained with Mitosis marker anti-phospho Histone H3 (Ser 10) (x20). Left panel: Numerous mitotic figures in TAP cells. Note absence of mitotic figures in the stem cell domain, at basal aspect of the crypts. Right panel: Same picture using the INVERT function to highlight the phenomenon.

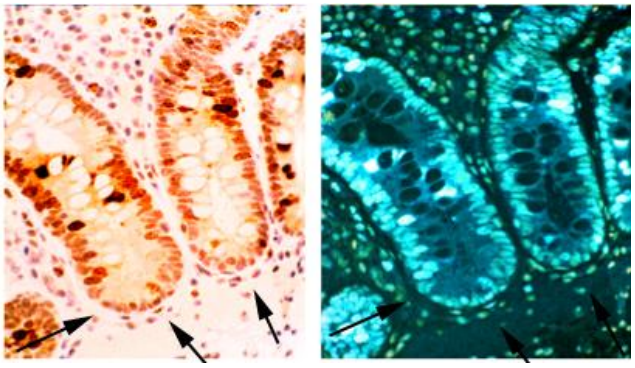

Figure 4. Closer view of duodenal mucosa with villous atrophy, immunostained with Mitosis marker anti-phospho Histone H3 (Ser 10) (x40). Left panel: Mitotic figures are seen in TAP cells. Note absence of mitotic figures in the stem cell domain, at basal aspect of the crypts (arrows). Right panel: Same picture using the INVERT function to highlight the phenomenon.

11.1.2. SUMOylation

One of the key characteristics of stem cells is their capacity to divide for long periods of time in an environment where most of the cells are quiescent. The miRNA pathway might be part of a mechanism that makes stem cells insensitive to environmental signals that normally stop the cell cycle at the G1/S transition [92]. Small ubiquitin-like modifiers (SUMOs) are attached to other proteins to regulate their function (sumoylation). SUMOylation is a downstream target of regulation through Ran, a small GTPase with important functions in both interphase nuclear trafficking and mitotic spindle assembly. Thus, SUMOylation plays a critical role(s) in mitosis [89].

Decreased sumoylation in inducible knockout mice of the E2 enzyme Ubc9 (*Ubc9fl/-/ROSA26-CreERT2* mice) leads to the depletion of the intestinal proliferative compartment and to the rapid disappearance of stem cells. Sumoylation maintains intestinal stem cells and the architecture, mechanical stability. Sox9 in the mutant epithelium at early stage revealed mislocalized Paneth cells throughout the crypt indicating that sumoylation controls allocation of proliferative and differentiated cell populations within the crypt [90]

11.1.3. Feed-back Grow Signaling

The geometric shape of the epithelial tissue in the *duodenum* can be understood as the steady state of a highly dynamic process. Many tissues have an inherent property of growing to a particular size. Self-renewing mucosas seem to ‘‘remember’’ their appropriate sizes, as they accurately regenerate to their original sizes following substantial erosions. Migration of intestinal cells is believed to result from the generation of cells in the crypts that transmits a pressure along the crypt-villus axis. Gaps left by the extruded mature cells at the top of the villi are immediately reoccupied. As a result, epithelial cells attached to each other by cell-to-cell contacts are forced to migrate upward.

The rate of cell renewal might be regulated by a negative feedback control. It has been submitted that size regulation is mediated by secreted factors called *chalones*. *Chalones* are secreted negative feedback factors that control the output of multistage cell lineages. As part of a negative feedback loop, *chalones* repress the proliferation of the cells that secrete the

chalones, so that when there is a high number or density of the cells, the corresponding high concentration of the *chalone* slows proliferation.

Contrariwise, the exfoliation of *chalone*-rich cells is a signal to stem cells to produce more cells, in order to restore mucosal homeostasis. There is a mounting interest in the mechanisms underlying the execution and regulation of the feedback signaling of intercellular communication. [91, 92, 93]

Comments

In patients with villous atrophy, the duodenal crypts are enlarged (up to 6 times) and the number of mitotic figures, dramatically increased. One conceivable explanation for the latter phenomenon might be that the *chalone*-producing differentiated cells in the villi -that normally refrain stem cells and TAP cells from overproducing mitotic cells- are absent in villous atrophy.

12. DUODENAL ADENOMAS AND CARCINOMAS

12.1. Genetic and Clinical Aspects

Duodenal adenomatosis is found in most patients with familial adenomatous polyposis. Familial adenomatous polyposis (FAP) results from inherited mutation of one APC allele. A 'second hit' mutation of the other allele renders the APC protein inactive and the inability of the destruction complex to degrade cytosolic β-catenin results in the over-transcription of target [12).

About 40% of the duodenal adenomas are sporadic and the remaining 60% from FAP patients [93]. The cumulative incidence of duodenal adenomatosis at age 70 years is 90% [6] and the risk of duodenal cancer in FAP is estimated to be between 100 and 330 times that for the general population, and the lifetime risk of developing duodenal cancer is approximately 4% [94, 95].

Mut Y human homologue-associated polyposis is a recently described colorectal adenomatous polyposis with an autosomal recessive mode of inheritance. Patients affected by Mut Y human homologue-associated polyposis (MAP) have a high prevalence of duodenal polyposis and are at increased risk to develop adenocarcinoma of the duodenum [96].

12.2. Histopathology

Duodenal adenomas in FAP originate in monocryptal adenomas [97]. Early microadenomas show evidence of cellular differentiation; defects in the positioning of Paneth cells suggest disruption of the EphB2:EphB3 receptor system. FAP-associated duodenal epithelium has a significant increase in the number of Paneth cells and endocrine cells per crypt in comparison with controls [97]. Duodenal adenomas spread in a 'bottom-up' fashion with dysplastic cells migrating onto normal villi.

Sporadic nonampullary adenomas exhibit a tubular architecture in all cases [98, 99].

Comments

While reviewing a cohort of 308 duodenal adenomas from patients with familial adenomatous polyposis (FAP), or sporadic, we found in 40.6% (n=125), gastric duodenal metaplasia covering part of the adenomas [100].

Intestinal metaplasia of the esophagus (Barrett´s esophagus), intestinal metaplasia of the stomach, and metaplastic polyposis of the colon are lesions that may antedate neoplastic transformation. It is therefore conceivable that gastric metaplasia in the duodenum might have the same neoplastic proclivity as other metaplastic lesions in the gastrointestinal tract. The known carcinogenic effect of high concentrations of bile acids and pancreatic juices bathing the duodenal mucosa with gastric metaplasia might have set aflame adenomatous neoplastic transformation, either spontaneously (sporadic cases) or reinforcing genetic mutations in patients with FAP. The molecular events telescoping from gastric duodenal metaplasia to dysplastic crypts in duodenal adenomas remains mute. Gastrointestinal pathologists agree that all nonampullar duodenal adenomas are tubular lesions. This is remarkable considering that these tubular neoplasias evolve in a mucosa characterized by villous ornaments. Accordingly, nonampullar duodenal tubular adenomas are dysplastic lesions of the duodenal crypts (Figure 5). The molecular signals responsible for the abrogation of the natural villous formations in tubular adenomas of the *duodenum*, remains elusive.

It should be understood, however, that villous development requires a cytokine network including epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and bone morphogenetic protein (BMP) 2 and 4. Whether the lack of villous architecture in tubular adenomas of the *duodenum* is due to a switch-off of EGF, FGF, PDGF and/or BMP signaling during neoplastic transformation, remains to be elucidated.

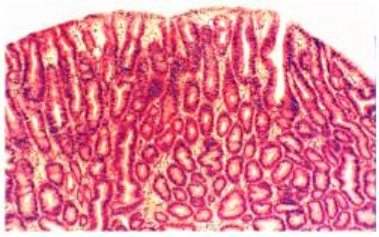

Figure 5. Tubular (cryptal) duodenal adenoma (H and E x 4).

12.3. Duodenal Neoplasia: Mice Models

Duodenal adenomas are also very common in the Min mouse model. Polyps in the small intestine of Min mice develop from cells located around the crypt-villus junction that proliferate inside the mucosa as a disorganized mass that give rise to a tumor. This abnormal migratory behaviour is likely the outcome of the β-catenin/Tcf target gene program autonomously activated in APC mutant cells and results in a further compartmentalization of EphB and ephrin-B expressing cells in the polyp areas. The initial founding polyp cells express high levels of EphB receptors and migrate abnormally inside the villus to avoid the area of high ephrin-B expression at the top positions of the crypts [52]. The morphological similarity of adenomas in the Min mouse and duodenal adenomas in humans suggest the Min mouse is a good model to study carcinogenesis in the proximal small intestine in FAP patients [97].

MUTYH is a mammalian DNA glycosylase that initiates base excision repair by excising adenine opposite 8-oxoguanine and 2-hydroxyadenine opposite guanine, thereby preventing G:C to T:A transversion caused by oxidative stress. MUTYH-null mice are susceptible to spontaneous and oxidative stress–induced intestinal tumorigenesis [7]. In MUTYH-null mice treated with KBrO3, the mean number of small intestinal tumors dramatically increased to 61.88 tumors, (0.85 in wild-type mice). The tumors developed predominantly in the *duodenum***.** MUTYH suppresses spontaneous tumorigenesis in mammals, thus providing experimental evidence for the association between biallelic germ-line MUTYH mutations and a recessive form of human hereditary colorectal adenoma and carcinoma [7].

12.4. Paneth Cell Translocation in Duodenal Adenomas

Duodenal adenomas from individuals with FAP show Paneth cell differentiation in 92% [30]. In small intestinal adenomas, loss of the wild-type *APC* allele leads to the nuclear localization of *β*-catenin and to the induction of *β*-catenin/tcf-mediated gene transcription. This reduces the expression of Ephrin ligands, leading to Paneth cells losing positional information, being found throughout the adenomatous epithelium. The disruption of Paneth cell positioning tend to be more pronounced in the larger lesions, and this is likely to be associated with the increased disruption of the *β*-catenin–tcf pathways [101].

Queries and Comments

Lysozyme immunostain detects not only differentiated Paneth cells but also Paneth cells precursors. In duodenal adenomas, lysozyme-expressing precursors (undetected in conventionally stained sections (HandE)) are found haphazardly distributed in adenomatous glands, even in the most superficial cell layers [102]. The latter finding suggests that if the innate code that orchestrates the direction of cell migration for Paneth cells is also valid for duodenal adenomas, then the stem cells that normally overly Paneth cells would have been exfoliated into the lumen of the organ [103, 104]. The alternative could be that human duodenal adenomas lack EphB2/EphB3 signaling, as EphB2/EphB3 null mice show misplaced Paneth cells, scattered along crypts and villi. Other explanations could be the ablation of one of the Wnt receptors, *Fz5*, with a *K19Cre* transgene, that causes mislocalization of Paneth cells in the villi or Sox9 mutation that induce mislocalized Paneth cells throughout the crypt.

13. DUODENAL NEOPLASIA SIGNALING

13.1. Wnt-β-Catenin Signaling

In genetically engineered mice, aberrant Wnt signaling causes tumors predominantly in the small intestine [97, 98]**.** In adenomas nuclear β-catenin is found in DCAMKL-1-positive cells. Thus, nuclear translocation of β-catenin distinguishes normal and adenoma stem cells within the intestinal tract of mice. Duodenal adenomas are thought to progress to duodenal cancer in a stepwise manner, with the accumulation of genetic mutations (such as *APC*, *k-RAS*, *p53*).

13.2. TGF-β Signaling

Sporadic and FAP-related adenomas show APC and KRAS mutations. BRAF mutations, p53 alterations, and DNA mismatch repair abnormalities are rare. [96, 99]. Regardless of their anatomic location and whether they were sporadic or FAP-related about 75% of the duodenal adenomas show Wnt signaling pathway abnormalities. In fact loss of control of Wnt signaling by the tumour suppressor protein APC is an early event in adenoma formation. Crossing the Lgr5-EGFP mice with APCmin mice allow to examine Lgr5 expression within the premalignant adenomas, which spontaneously arise in their intestine as a result of chronic activation of the Wnt signaling pathway. Lgr5-GFP expression is restricted to a small number of cells within large adenomas. Lgr5 marks the limited population of tumor-initiating/ propagating cells thought to exist within cancer, the so-called cancer stem cells [105].

13.3. Eph Signaling

Mice with adenomatous polyposis (APCMin/+) developed invasive adenocarcinoma in the small intestine when EphB signaling is inhibited, thus highlighting the significance of EphB receptors as tumor suppressors. EphB signaling regulates the size of the proliferative compartment without an apparent effect on the number of stem or progenitor cells [43, 106].

13.4. PTEN/PI3K Signaling

The PI3K signaling plays an important role in cell survival, proliferation, growth, and tumorigenesis. Central to this pathway is PI3K, a lipid kinase, composed of both a regulatory subunit, p85, and a catalytic subunit, p110 [107].

13.5. Oncogene Signaling

Disruption of cell polarity is a hallmark of cancer. Basic mechanisms of cell polarity, often targeted by oncogenic signaling pathways, induce deregulation of asymmetric cell divisions of stem or progenitor cells. Cell-polarity mechanisms are important for the diversification of cell shapes and regulation of the asymmetric cell divisions of stem cells. Cell-polarity proteins are known proto-oncogenes or tumor suppressors. [108].

13.6. Mutation Signaling

While mutations in the *p53* and microsatellite instability, occur at approximately similar frequencies in small and large intestinal adenocarcinoma, an inactivation of the adenomatous polyposis coli (*APC*) gene, the gatekeeper mutation in colorectal cancer, is only infrequently seen in small bowel carcinomas [106]. Mutations in *BMPR1*a, *SMAD4* and *PTEN* (phosphatase and tensin homolog -mutated in multiple advanced cancers 1-, give rise to

intestinal polyposis syndromes, suggesting a role for BMP and PTEN signaling in the intestinal crypt. BMP signaling is a negative regulator of ISC proliferation [10, 109, 110, 111, 112].

13.7. Methylation

Hypermethylation seems to be a general feature of both FAP-related duodenal carcinomas as well as sporadic duodenal carcinomas with the exception of the PAX6 gene, which is methylated only in FAP-related carcinomas [113].

13.8. Cancer Stem Cells vs. Clonal Evolution

The cancer stem model is highly hierarchical with unique self-renewing cell type at the apex, whereas the clonal evolution model attributes much of the intratumorsal variation to subclonal difference in the mutational profile, and all -except the terminally differentiated cells- may have some self-renewal capacity. Accordingly, in the cancer stem cell model, a phenotypically distinct and generally rare cell type maintains the tumor´s growth, whereas in the clonal evolution model, the dominant subclone(s), sustains it [114].

13.9. Cancer Stem Cell Niche

The cancer stem cell (CSCs) relies on a similar niche, dubbed the "CSC niche," which controls their self-renewal and differentiation [115].

13.10. Cancer in Celiac Disease

Small bowel adenocarcinomas have sporadically been reported in patients with celiac disease [116].

14. NET TUMORS

A loss of heterozygosity (LOH) of the MEN1 gene and/or the centromere 11 is demonstrated in approximately 50% of MEN1-associated duodenal NETs. Allelic deletion of the MEN1 gene in microadenomas may reflect a pivotal genetic event in the development of multifocal gastrin and somatostatin cell neoplasms in the duodenum of MEN1 patients [117]. Predominant or exclusive expression of somatostatin was found in 26% of duodenal neuroendocrine tumors (NETs). None of the patients fulfilled the criteria of a somatostatinoma syndrome [118].

15. "SIDE POPULATION" CELLS

The cancer stem cell (CSC) hypothesis suggests that neoplastic clones are maintained exclusively by a small subpopulation of cells that give rise to phenotypically diverse cancer cells [119].

16. NEOPLASTIC CELL MARKERS

Prominin-1 (CD133). CD133 expression is not restricted to the stem cell zone of the crypt, but expressed all over the intestinal epithelium [28, 31, 103] Direct genetic labeling of adult stem cells in the mouse small intestine, reveal that adenomas arise directly from adult stem cells. Recently, CD 166 was found 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 [120]. Prom11/C-L mice containing a Cre-dependent mutant allele of β-catenin show disruption of crypt architecture and a disproportionate expansion of Prom11 cells at the crypt base. The progeny of these cells replace the mucosa of the entire small intestine with neoplastic tissue, characterized by focal high-grade intraepithelial neoplasia and crypt adenoma formation. Although all neoplastic cells arise from Prom11 cells in these mice, only 7% of tumour cells retain Prom1 expression, strongly suggesting that cance, is generated by a rare population of cancer stem cells [121]. *Lgr5*-GFP expression is restricted to a small number of cells within large adenomas, suggesting that *Lgr5* marks not only the normal intestinal stem cells, but also the rare population of tumor-initiating/propagating cell, the so-called cancer stem cells [122].

PHLDA1 [29] is expressed in discrete crypt base and some position +4 cells in the human small (and large) intestine. In small adenomas, PHLDA1 is expressed in a subset of undifferentiated and predominantly Ki-67-negative neoplastic cells, suggesting that a basic hierarchy of differentiation is retained in early tumorigenesis. In large adenomas, carcinomas, and metastases PHLDA1 expression became widespread, with increased expression and nuclear localization at invasive margins. The integrins ITGA2 and ITGA6 were downregulated in response to PHLDA1 suppression, and accordingly cell adhesion to laminin and collagen was significantly reduced. PHLDA1 is a putative epithelial stem cell marker in the human small (and large) intestine [29].

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