CHAPTER TWO

Multitasking Paneth Cells in the Intestinal Stem Cell Niche

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Contents

1. PANETH CELLS: FROM SCHWALBE AND PANETH TO THE DISCOVERY OF THEIR SECRETORY ROLE

1.1 Early Studies Anticipate Paneth Cells' Multitasking Functional Nature

Granulated cells in the fundus of the crypts of Lieberkühn were first described in 1872, along with the other cells of the intestinal epithelium,

Advances in Stem Cells and their Niches, Volume 2
ISSN 2468-5097
https://doi.org/10.1016/bs.asn.2017.12.001

by Schwalbe, a German anatomist, histologist, and anthropologist (Schwalbe, 1872). Nevertheless, they take their name from Joseph Paneth, an Austrian histologist and physiologist who first performed their detailed morphological analysis in 1888 (Paneth, 1888). For many decades after their discovery, however, the role of Paneth cells (PCs) remained obscure due to mainly observational studies limited by the available methods and mostly focused on the morphological features of PCs and their granular payload.

In 1899 Möller reported on the presence of PCs in several animal species including mouse, guinea pig, hox, sheep, and horse (Möller, 1899). Given the broad spectrum of species found to host PCs and to feature acidophilic granules and mucus in the small intestinal epithelial lining, the question arose whether they represented independent zymogenic cells or were involved in mucus production (Bizzozero, 1892). Bizzozero claimed to have found cells with a dual Paneth/goblet cell fate, therefore raising the possibility of PCs representing goblet cells precursors (Bizzozero, 1892). Few years later, Klein brought evidence supporting the independent zymogenic function of PCs, distinct from goblet cells, based on staining analysis with mucus-specific dyes (Klein, 1906). Mols and Cowdry studied the effects of different diets in murine PCs and observed that their intracellular content changed depending on the specific diet fed (Cowdry, 1934; Georges, 1930), thus suggesting, already at these very early days, specific roles for this cell type in metabolism and nutrient sensing to be confirmed nearly a century later (Rodriguez-Colman et al., 2017; Yilmaz et al., 2012).

In 1937 Hertzog analyzed PCs in man and reported on their reduced abundancy in the duodenum. Also PCs multiplicity and distribution did not appear to be influenced by sex or age and, of note, they occasionally appeared in colon, stomach, and appendix in association with pathological conditions (Hertzog, 1937). The latter observation led to additional studies on the alleged function of PCs in disease (Creamer, 1967a, 1967b; Creamer & Pink, 1967; Lewin, 1969a; Trier, 1966, 1967). The most accurate description came from studies by Lewin who described and counted the appearance and number of PCs in the small and large intestine. Of note, PC multiplicity was shown to be affected upon inflammation depending on the specific type of pathology and the degree of the inflammatory damage. Decreased PC numbers were found in acute inflammation of the small intestine and in Crohn's disease (CD). Interestingly, PCs were also found to proliferate upon inflammation (Lewin, 1969a, 1969b, 1969c). Contrary to what observed in the small intestine, an increased number of PCs was found in the colon in association with ulcerative colitis (UC) and other inflammatory

diseases of the distal bowel. As of today, it is still unclear whether Paneth cell metaplasia (PCM), i.e., the appearance of PCs in the large intestine, plays any functional role in the tissue response to inflammatory insults and in the increased colon cancer risk associated with inflammatory bowel disease (IBD). The role of PCs in IBD will be discussed further in depth in a later section of this chapter.

PCs have also been observed within intestinal tumors: several studies have reported on the presence of lysozyme-positive neoplastic cells in adenomas and carcinomas of the colon, often with proliferative features. The number of PCs in colonic tumors was higher in proximal lesions when compared with distal neoplasias (Lewin, 1968). Notably, PCs have been observed in colonic tumors from patients with but also without a history of IBDs. As for the biogenesis of PCM in the colonic mucosa, early studies proposed that these Paneth-like cells arise through metaplastic changes brought about by the altered inflammatory and/or neoplastic milieu (Lewin, 1968). The term Paneth cell *metaplasia* (from the Greek *metaplasmos* = change in form) was therefore employed to indicate the conversion of a cell type into another. Metaplastic PCs have been found in a broad spectrum of pathological conditions ranging from IBDs to cancer (Paterson & Watson, 1961; Symonds, 1974).

The origin and fate of PCs and their context-dependent proliferative state were investigated by Cheng and Leblond who found that PCs exist in a variety of maturation stages in vivo as a measure of the size of their granules (23% with granules smaller than 2 μM and classified as "young" PCs; 63% with granules between 2 and 3 μM; and 14% "old" PCs with granules larger than 3 μM) (Cheng, 1974). These studies also showed that PCs were postmitotic in homeostatic conditions and persisted for long terms at the bottom of the crypt, unlike most intestinal epithelial cells that actively migrate upward along the crypt–villus axis to be then exfoliated by apoptosis within 5–6 days.

1.2 PCs: The Secretory Bodyguards of the Crypt

The large acidophilic granules apically secreted into the crypt lumen that earmark PCs have been the object of several electron microscopy studies. The secretory function of the PC is prominent throughout its different subcellular compartments featuring an extensive Golgi apparatus and very pronounced endoplasmic reticulum. However, the function of the secretory granules remained elusive until lysozyme was identified as one of their main components (Deckx, Vantrappen, & Parein, 1967). As lysozyme is a strong

antibacterial agent, a role was proposed for PCs in host defense mechanisms in response to bacterial flora.

Subsequently, two additional families of antimicrobial proteins, namely, secretory phospholipases (e.g., *Pla2g2a*) (Senegas-Balas et al., 1984), endowed with additional lipid modifying function, and α-defensins (also known as cryptdins for their location at the bottom of the crypt) (Ouellette & Cordell, 1988; Ouellette, Miller, Henschen, & Selsted, 1992) were found to be expressed by murine PCs and specifically localized in their secretory granules.

These studies sparked several additional efforts toward the elucidation of the mechanisms underlying PC secretion and the environmental stimuli that trigger it. To this aim, whole crypts were detached from the lamina propria and exposed to bacteria such as *Salmonella typhimurium*, *Escherichia coli*, or *Staphylococcus aureus*. A potent induction of granule secretion and consequent antibacterial activity was observed upon stimulation of these ex vivo isolated crypts with bacterial cells. More than 90% of the microorganisms to which the crypts were exposed were killed. Notably, most of the antimicrobial activity appeared to be elicited by cryptdins, as shown by the observed 70% reduction in bactericidal function of the granules upon exposure to antibodies directed against cryptdin and cryptdin-like enzymes. Exocytosis of PC granules was also observed upon exposure to bacterial surface products such as lipopolysaccharide (LPS) in a dose-dependent manner (Ayabe et al., 2000). Furthermore, granule secretion can be triggered by a broad range of stimuli including acetylcholinergic (Qu, Lloyd, Walsh, & Lehrer, 1996; Satoh, 1988) and toll-like receptor agonists (Rumio et al., 2004, 2012). More recent studies suggest that secretion of the granules by PCs is triggered by bacterial cells or antigens in an indirect way and requires interferon gamma (IFN-γ), mainly secreted by leukocytes coisolated with the crypts. To prove that stimulation of PC secretion is indirectly stimulated by bacterial antigens, crypts were cultured ex vivo as self-renewing organoids or "miniguts" (this technique is more accurately described further in this chapter) and the basal or luminal side of such structures exposed to LPS, flagellin, peptidoglycan (PGN), heat-inactivated *E. coli*, and live bacterial cells (Farin et al., 2014). No change in PC morphology or granule secretion was observed after 12 h. Furthermore, no changes in intracellular or secreted lysozyme expression were observed. PC expression of bacterial receptors like Cd14 and Toll-like receptors 2,3,7,8, and 9 remained unchanged in the cultured organoids when compared to PCs in vivo, the latter also indicating that these receptors are retained in ex vivo cultured crypts.

Consequently, several inflammatory molecules were tested for their capacity to induce discharge of PC granules. Among several molecules including interleukin 22 (IL22) and 6 (IL6), tumor necrosis factor, and IFN-γ, only the latter proved to be a potent inducer of granule secretion by PCs (Farin et al., 2014).

Additional in vivo experimental evidence for the role of IFN-γ as the main inflammatory molecule responsible for granule extrusion was provided by injecting wild-type and IFN-γ knockout mice with anti-CD3 antibody to trigger T cell activation and IFN-γ expression. When wild-type mice were administered the CD3 antibody, PCs degranulated as shown by decreased lysozyme content. Notably, no PC degranulation or any decrease in PC multiplicity was observed in in CD3-treated IFN-γ knockout mice (Farin et al., 2014).

PC granule secretion was also shown to be mediated by calcium fluxes. By tracking Ca^{2+} dynamics in ex vivo crypts during granules secretion, a biphasic increase in cytosolic Ca^{2+} was revealed with the first phase mainly arising from intracellular storage, whereas the second depends on extracellular calcium uptake (Satoh, Habara, Ono, & Kanno, 1995). The second rise in cytosolic Ca^{2+} can be reduced by selectively blocking the calcium-activated potassium channel *IKCa1* (by means of clotrimazole or charybdotoxin), and by measuring the levels of cryptdin secretion. The observed 50% reduction in secretion also corresponded to a similar reduction in antibacterial activity of crypts exposed to *S. typhimurium* (Ayabe et al., 2000, 2002; Satoh et al., 1995).

The antimicrobial peptides (AMPs) secreted by PCs have been proposed to encompass two main functions: (i) protect the host from enteric pathogens and (ii) keep in balance and shape the composition of the microbiome (Vaishnava, Behrendt, Ismail, Eckmann, & Hooper, 2008). These two deeply intertwined functions are of great relevance since the composition and number of PCs might unfavorably alter the composition of microbiota, a condition known as dysbiosis, thus rendering the host more susceptible to a variety of infectious and noninfectious diseases (Candela et al., 2011; Dumas et al., 2006; Karlsson et al., 2012; Koren et al., 2011; Salzman & Bevins, 2013; Turnbaugh et al., 2006).

The analysis and study of AMPs like defensins in the mouse are made more difficult by the complex genomic structure encompassing several gene paralogs within a single locus, and by the strain-dependent composition of AMPs among inbred mice (Amid et al., 2009; Gulati et al., 2012; Shanahan, Tanabe, & Ouellette, 2011).

As previously stated, PCs are found in a wide range of species (Möller, 1899). Likewise, AMPs have also been conserved throughout evolution (Ouellette, 2011). One notable difference is represented by the presence of multiple defensins expressed by PCs in a broad spectrum of mammalian species, whereas their human counterparts only encode for two, i.e., defensins 5 and 6 (HD5, HD6) (Jones & Bevins, 1992, 1993). The function, activity, and structure of defensins have been discussed in depth in several literature reviews (Bevins, 2013; Clevers & Bevins, 2013; Lehrer & Lu, 2012; Ouellette, 2011).

2. THE INTESTINAL CRYPT STEM CELL NICHE

2.1 The Discovery of the Intestinal Stem Cell

The crypts of Lieberkühn were for long time suspected to harbor the elusive intestinal stem cells responsible to preserve homeostasis in one of the most dynamic and regenerative epithelial tissues. Indeed, seminal studies by Cheng and Leblond identified cycling cells located at the very bottom of the intestinal crypt, the so-called crypt base columnar cells or CBC's, and proposed them as the stem cells of the gut epithelium. Experimental evidence for the identity of CBCs as stem cells of the adult gut was provided by Barker in the laboratory led by Clevers, based on the identification of the transmembrane receptor *Lgr5* (leucine-rich repeat-containing G protein-coupled receptor 5) as a specific marker of cycling CBCs. Lineage tracing experiments were performed with a knock-in mouse model expressing both a green fluorescent protein (GFP) and a tamoxifen inducible C-recombinase under the control of the endogenous *Lgr5* gene promoter (*Lgr5*-eGFP-IRES-CreER^{t2}) (Barker et al., 2007). These mice, when bred with Rosa26-LacZ reporter animals and induced by tamoxifen, allow the excision of the roadblock (i.e., the stop sequences flanked by LoxP sites located upstream of the LacZ gene) and transcription of the reporter gene by Cre protein expression only in *Lgr5*$^{+}$ cells. In this way, LacZ expression, easily detectable by X-gal staining, will mark *Lgr5*$^{+}$ cells and their cellular progeny. Upon tamoxifen treatment, *Lgr5*-eGFP-IRES-CreER^{t2} mice gave rise to long ribbons of blue-labeled cells starting from the bottom of the crypt to the villus tip. These blue ribbons persisted in the intestine of the mice for more than 300 days and encompassed all the intestinal cell lineages, thus showing that *Lgr5*$^{+}$ have long-term self-renewal and multipotent differentiation capacity both in the small and large intestine.

Although stem cells have long been thought to divide infrequently, the intestinal epithelium almost entirely renews its cellular composition every 5 days suggestive of an actively proliferating stem or precursor cell. By using the above *Lgr5* knock-in mice, CBCs have been shown to divide approx. every 16 h (Snippert et al., 2010). Another common view on stem cells is relative to their mitotic division modality, i.e., in symmetric vs asymmetric fashion giving rise to two daughter cells with equal (two stem or two committed cells) or distinct (one stem cell and one committed progenitor) cell fates, respectively. By employing *Lgr5*-eGFP-IRES-CreER^{t2} mice bred with the Rosa26-confetti reporter, it was shown that *Lgr5*$^{+}$ stem cells divide symmetrically and that they follow a neutral drift dynamic (Snippert et al., 2010).

Several studies suggested that stem cells in epithelial tissues come in two flavors: rapidly and slowly cycling (Fuchs, 2009). *Lgr5*$^{+}$ cells divide every 16 h and as such represent frequently cycling stem cells thought to underlie intestinal homeostasis under physiological conditions. A more quiescent or slowly cycling stem cell type located at position +4 from the crypt base was first identified by Potten in 1974 based on its label-retaining properties (Potten, Kovacs, & Hamilton, 1974). Only several years later the first gene marker was identified to earmark the +4 label-retaining cells, namely, the *Bmi1* (B cell-specific Moloney murine leukemia virus integration site 1) gene. By following a lineage tracing approach analogous to that employed for the *Lgr5*$^{+}$ CBCs, *Bmi1*$^{+}$ cells at the +4 position were shown to give rise to all the differentiated lineages of the small intestine (Sangiorgi & Capecchi, 2008). Additional genes have also been proposed to specifically mark a subset of slowly dividing stem cells such as *mTert*, *Hopx*, and *Lrig1* (Carlone & Breault, 2011; Powell et al., 2012; Takeda et al., 2011). Although lineage tracing provides valuable information on specific cell types and their progeny, it mainly relies on the specificity of the gene promoter under which the Cre recombinase is driven. In situ hybridization analysis on single RNA molecules indicated that none of the aforementioned (+4) markers is uniquely expressed at a single position along the crypt–villus axis (Munoz et al., 2012). However, this is likely to be true for the vast majority of the genes/markers expressed by stem and progenitor cells in the lower intestinal crypt. Most recently, the laboratory led by Eduard Batlle identified a specific RNA-binding protein called Mex3a that, in combination with Lgr5, marks a specific population of quiescent stem cells resident in the lower crypt also located at around position +3/+4 (Barriga et al., 2017). Single cell RNAseq analysis of individual *Lgr5*$^{+}$ stem cells revealed the presence two

populations differing by their cycling rates with *Mex3a* expression earmarking the slowly cycling *Lgr5*$^+$ subpopulation. Lineage tracing analysis using *Mex3a* knock-in mice bred with the Rosa26 reporter line conformed that *Mex3a*$^+$/*Lgr5*$^+$ cells represent bona fide infrequently dividing stem cells in vivo (Barriga et al., 2017).

The presence of quiescent stem cells is of functional relevance as they have been proposed to underlie the regenerative response by compensating for the loss of rapid-cycling *Lgr5*$^+$ CBCs upon tissue insults (Yan et al., 2012). In the small intestine, tissue regeneration upon injury conditions has been shown to rely on the dedifferentiation of terminally committed cells. Terminally differentiated cells have long been thought to be unable to rewire their cell fate and reenter the cell cycle and acquire pluripotency. Recently, however, distinct differentiated lineages of the small intestine, i.e., PCs and *Alpi*$^+$ enterocytes, have been shown to act as stem/progenitor-like cells upon tissue injury (Roth et al., 2012; Tetteh et al., 2016). The issue of plasticity as an intrinsic property of specific intestinal cell lineages raises several questions on the underlying pathways and molecular cues which are of relevance for our understanding of analogous mechanisms in pathological conditions such as tumor plasticity and resistance to therapy.

2.2 PCs and Crypt Development

Both the small and large intestinal epithelium develop by tubulogenesis from the posterior endoderm following extensive folding (Noah, Donahue, & Shroyer, 2011; Zorn & Wells, 2009). The embryonic gut tube is lined by a simple epithelium that condenses to form a pseudostratified epithelium where all the cells are attached to the basement membrane. Around e14, the gut epithelium assumes a columnar form, while it forms protruding structures called villi. Lineage specification is initiated at around e17 in proliferating cells confined in the intervillus region from where they subsequently invaginate to form intestinal crypts. Notably, whereas in the mouse crypt development occurs at early postnatal stages and is completed by the time of weaning, in man it occurs in utero (Darmoul, Brown, Selsted, & Ouellette, 1997).

Notwithstanding the absence of fully mature and granulated PCs in the mouse intestinal epithelium during the first 2 postnatal weeks, expression of defensins 1 and 6 was found in scattered fashion throughout the newborn intestinal lining thus raising the possibility of an innate immune role for these peptides at early perinatal stages (Darmoul et al., 1997).

De novo crypt formation occurs through a process called "crypt fission," a rate-limiting event in intestinal expansion and growth that consists in its essence of the division of a crypt into two daughter crypts (Clarke, 1972; St Clair & Osborne, 1985). Crypt fission represents a form of epithelial tube branching through which the newly formed crypts of Lieberkühn divide and multiply thus extending and widening the intestinal tract during postnatal development. The lumen of the resulting two daughter crypts can be of equal or unequal length as the result of symmetric and asymmetric crypt fission, respectively. However, the cellular and molecular mechanisms underlying crypt fission are still poorly understood.

Recently, Inke Nathke and collaborators observed that the number and relative position of PCs and *Lgr5*$^{+}$ CBCs at the bottom of the crypt are critical for the initiation of crypt fission (Langlands et al., 2016). Analysis of crypts undergoing fission revealed that the process can be divided into an early and a late phase (Langlands et al., 2016). During early fission, a change in cell patterning occurs with PCs absent from the middle of the crypt bottom due to their migration to either side of the site of bifurcation/branching. Simultaneously to this side clustering of PCs, *Lgr5*$^{+}$ CBCs form a distinct cluster in the middle of the crypt base thus marking the bifurcation site where, during the late fission phase, the epithelial lining is expanded upward until the duplication of the two daughter crypts is completed (Figure 1A).

The specific cell patterning observed during crypt fission reflects the intrinsic cell properties of *Lgr5*$^{+}$ CBCs and PCs, the latter showing high stiffness and increased adhesion to the basement membrane. High β4 integrin expression allows PCs to firmly anchor to the basement membrane when compared with other cell types.

In confirmation of previous studies showing that inhibition of ephrin (Eph) signaling causes misplacement of PCs from the crypt base (Batlle et al., 2002), inhibitory Eph protein fragments have been found to induce their mislocalization in ex vivo organoids though without altering the rate of crypt fission. However, altering PC localization skewed the balance between symmetric and asymmetric fission in favor of the latter, likely due to the formation of new branches further away from the crypts. Cell adhesion and integrin levels appear to play a central role in this process as shown by the observed 50% reduction in crypt fission in the presence of a β4 integrin antibody. It is therefore plausible to postulate that the enhanced adhesion, a feature of PCs when compared to their neighboring cells, plays a key functional role in the early phase of crypt fission (Langlands et al., 2016).

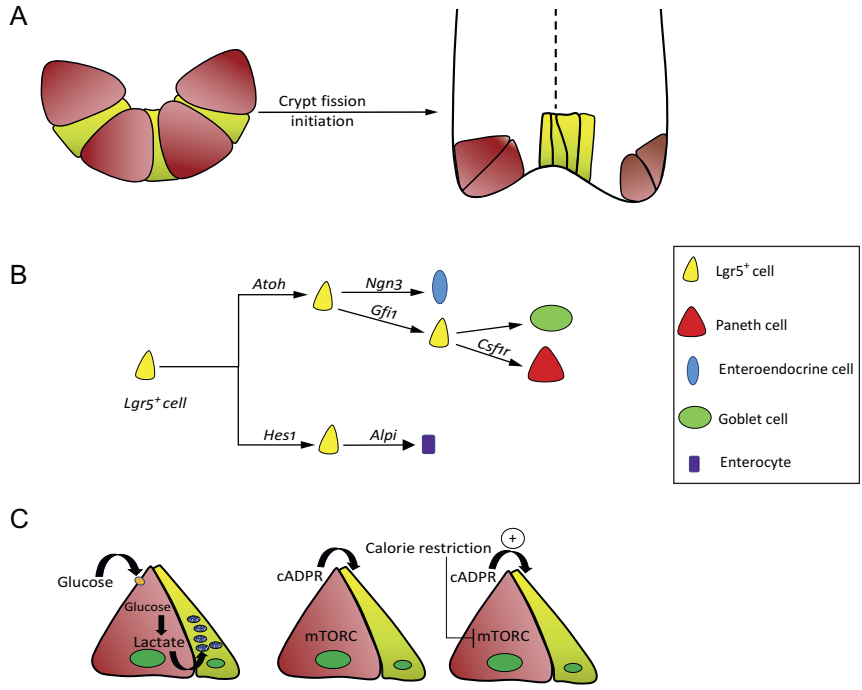

Fig. 1 (A) At the bottom of the crypt of Lieberkühn, Paneth cells (*brown*) and *Lgr5*$^+$ stem cells (*yellow*) cluster away upon crypt fission initiation (Langlands et al., 2016). The increased stiffness of Paneth cells forms harder regions on the sides of the branching crypt, in contrast with the "softer" Lgr5$^+$ stem cells positioned in the center. (B) Schematic diagram of the differentiation fates of the *Lgr5*$^+$ stem cell and the genes that underlie the specific commitment steps. (C) *Left*: Paneth cells (*brown*) sustain the metabolism of *Lgr5*$^+$ cells by using glucose as a substrate to secrete lactate that, during homeostasis, fuels oxidative phosphorylation in *Lgr5*$^+$ stem cells. *Center* and *right*: mTORC1 inhibition in Paneth cells leads to the secretion of cyclic ADP ribose (cADPR). During caloric restriction, mTORC1 is inhibited in Paneth cells leading the enhanced cADPR secretion and increased *Lgr5*$^+$ stem cell self-renewal and multiplicity.

2.3 Paneth-Specific Signaling Pathways: Wnt and Notch

Experimental evidence links de novo crypt formation to canonical Wnt signaling in view of the involvement of two of its downstream target genes (*EphB* and *cMyc*) in the migration and compartmentalization of Paneth and *Lgr5*$^+$ cells within the lower crypt. Cell migration within the crypt occurs in bidirectional fashion: whereas enteroendocrine cells, goblet cells, and enterocytes migrate upward along the crypt–villus axis, PCs descend in

opposite direction toward the bottom of the crypts. In the mouse prenatal intestine, the EphB2 and EphB3 receptors and their ligand Ephrin-B1 are expressed in complementary domains: both receptors and ligand are coexpressed in cells located at the periphery of the intervillus pockets, whereas expression of the EphB2 and EphB3 earmarks the cells present at the very bottom of the pockets (Batlle et al., 2002). In the adult small intestine, the expression pattern of the two ephrin receptors appears to be more complex with EphB3 being restricted to PCs in the crypt bottom, whereas *EphB2* expression earmarks the entire crypt bottom until position +4.

EphB3 receptor knockout mice are characterized by misplaced PCs throughout the crypt–villus axis, a phenotype not observed in $EphB2^{-/-}$ mice. Therefore, expression of the EphB3 receptor in PCs appears necessary for their correct positioning at the bottom crypt (Batlle et al., 2002). Furthermore, the nuclear β-catenin accumulation characteristic of PCs at the bottom of the crypt was lost in their misplaced equivalents in $EphB3^{-/-}$ mice thus indicating a noncell autonomous and localization-dependent regulation of Wnt signaling in PCs (Batlle et al., 2002). As previously mentioned, Wnt signaling plays a pivotal role in the intestinal epithelium with both $Lgr5^{+}$ CBCs and PCs are characterized by the accumulation of β-catenin in the nucleus where it binds with members of the family of Tcf transcription factors thus driving the expression of Wnt target genes (van de Wetering et al., 2002).

Cytosolic levels of β-catenin are tightly controlled by a multiprotein complex encompassing the tumor suppressors Apc (adenomatous polyposis coli) and Axin, the Ser/Thr kinases GSK3β (glycogen synthase kinase 3-beta) and CK1 (casein kinase 1), protein phosphatase 2A (PP2A), and the E3-ubiquitin ligase β-TrCP. In the absence of Wnt ligands, this "destruction complex" phosphorylates β-catenin promoting its ubiquitinilation and degradation by the proteasome (Stamos & Weis, 2013). Of note, canonical Wnt/β-catenin signaling has been shown to induce maturation of PCs in the mouse small intestine: expression of the Paneth-specific gene program is lost in the intestine of $Tcf4^{-/-}$ embryos (van Es et al., 2005). In particular, failure to express defensins and cryptdins caused PCs maturation defects. Also, the frizzled (Fzd) family of receptors encompasses integral membrane proteins featuring seven -transmembrane-spanning domains function in canonical Wnt signaling. Remarkably, *Fzd5* knockout mice exhibit an identical phenotype to that of $EphB3^{-/-}$ animals with misplaced PCs with no β-catenin nuclear accumulation, in confirmation of the positional cues provided by Wnt signals along

the crypt–villus axis (Batlle et al., 2002; van Es et al., 2005). The Wnt-driven maturation of PCs and their pronounced nuclear β-catenin accumulation is intriguing in view of the essential role played by canonical Wnt signaling in the regulation of stemness in the intestinal crypt. Hence, the same pathway controls the onset and maintenance of both multipotent and fully differentiated (and postmitotic) lineages.

Studies aiming at the identification of the Wnt signals required for PC maturation and maintenance have been performed by first establishing the expression patterns of Wnt ligands in the intestinal epithelium (i.e., Wnt3; uniquely expressed in PCs) and in the surrounding mesenchyme (Wnt2b, Wnt4, and Wnt5a). Notably, conditional ablation of Wnt3 throughout the intestinal epithelium ($Wnt3^{fl/fl}/Vil^{CreERT2}$) does not affect PCs, indicative of functional redundancy between mesenchymal and epithelial Wnt ligands in vivo (Farin, Van Es, & Clevers, 2012). The latter was confirmed (i) by the impaired growth of ex vivo cultured crypt organoids derived from the $Wnt3^{fl/fl}/Vil^{CreERT2}$ mice upon removal of the floxed *Wnt3* allele and (ii) by the ability of exogenously added Wnt3 to rescue the growth impairment (Farin et al., 2012).

The orphan G protein-coupled receptor Lgr4 is expressed in small intestinal crypts above the PC zone (i.e., in the TA region), and in CBCs and in a subset of PCs (Mustata et al., 2011). $Lgr4^{-/-}$ mice show reduced epithelial proliferation without any significant defect in the differentiation of absorptive, enteroendocrine, and goblet cell lineages. However, the absence of specific PC markers such as lysozyme and cryptdin 4 was also noticed in $Lgr4^{-/-}$ mice, likely indicative of a PC maturation defect. Accordingly, ex vivo cultured crypts from the *Lgr4* knockout mice failed to form fully mature organoids and showed decreased expression levels of several stem cell-specific markers and Wnt targets such as *Axin2*, *Sox9*, and *Lgr5*. Accordingly, reactivation of Wnt signaling by addition of lithium chloride (LiCl) to the organoid culture medium partially rescued their growth impairment (Mustata et al., 2011).

Among the Wnt targets observed to play a key role in PC onset, *Sox9* is expressed before the appearance of PCs in the embryonal gut and its expression overlaps with that of cryptdins in the intervillus pockets. Inducible genetic ablation of *Sox9* results in the disappearance of PCs and a decrease of goblet cells (Bastide et al., 2007; Mori-Akiyama et al., 2007). As a consequence of the altered tissue morphology and absence of PCs, the crypts of $Sox9^{fl/fl}$ mice present an enlarged proliferative compartment extended to the whole crypt bottom (Bastide et al., 2007; Mori-Akiyama et al., 2007).

Constitutive *Sox9* expression as a consequence of loss of *Apc* function has also been shown to induce formation of ectopic PCs in the mouse colon (Feng et al., 2013). By employing a colon-specific promotor (*Cdx2*) to delete the *Apc* tumor suppressor gene, it was shown that adenoma formation is accompanied by the appearance of ectopic lysozyme-expressing Paneth-like cells. These metaplastic PCs were observed in regions outside of the crypt base, often at new crypt budding sites, coincident with high *Sox9* expression as the result of the induced *Apc* deletion. The latter is not surprising in view the role of *Sox9* as Wnt target, but it is noteworthy that *Sox9* upregulation in the colon of these mice preceded the changes in β-catenin subcellular localization possibly due its easier detection, when compared to β-catenin stabilization, at early time points after *Apc* inactivation in the colon epithelium (Feng et al., 2013).

Alongside canonical Wnt, also Notch signaling plays an important role in PC differentiation and maturation, mainly through one of its downstream target genes, namely *Math1*, encoding for a basic helix–loop–helix transcription factor. Studies performed on *Math1* knockout mice revealed the ablation of all secretory lineages including Paneth and goblet cells, and enteroendocrine cells (Yang, Bermingham, Finegold, & Zoghbi, 2001). Thus, *Math1* is required for progenitor cells to commit toward a secretory fate. Notably, studies performed on mosaic *Math1*-null crypts showed that commitment toward a secretory fate improves regeneration upon small bowel resection (SBR). *Math1*-deficient crypts showed decreased regenerative capacity after SBR when compared to proficient crypts, suggesting that secretory lineages may play a (niche) role in the response to tissue injury (Shroyer et al., 2007).

Constitutive expression of an active form of the Notch 1 receptor in the intestinal epithelium causes signs of malnourishment at postnatal day 1 (P1) and death at P3 as a consequence of the inhibition of all secretory lineages, a phenotype opposite to that observed in *Hes1* knockout mice characterized by an excess of secretory cells at the expenses of enterocytes (Fre et al., 2005). Hence, constitutive Notch 1 expression upregulates *Hes1* expression while downregulating *Math1*, a key gene in secretory lineage development.

Notch signaling inhibition has also been studied: mice treated with dibenzazepine showed an increment in PCs numbers, with abnormal lysozyme staining, i.e., cytoplasmic and diffused rather than in the characteristic granular pattern, indicative of alterations in PCs formation upon Notch signaling inhibition (VanDussen et al., 2012). Upon further analysis, both the

number and size of PC granules were shown to be decreased in addition to the expression of goblet-specific markers, suggesting the establishment of an intermediate phenotype in between the Paneth and goblet lineages upon Notch signaling inhibition. These findings highlight the importance of Notch signaling in terminal differentiation of PCs (VanDussen et al., 2012). Screens performed on *Math1*-proficient and -deficient mice led to the identification of the target genes of this transcription factor critical for the establishment of the secretory cell fate (Shroyer, Wallis, Venken, Bellen, & Zoghbi, 2005). The *Gfi1* gene was found to be significantly downregulated upon *Math1* ablation. Likewise, *Gfi1* knockout mice were deprived of mature PCs together with a significant reduction of goblet cells and an increase in enteroendocrine cells. In these mice, secretory progenitors although already committed, are unable to differentiate into mature PCs (Figure 1B) (Shroyer et al., 2005).

Another Paneth-specific gene known to affect Notch signaling in the crypt bottom encodes for the receptor of colony stimulation factor 1 (*Csf1r*), whose *Csf1* ligand is expressed by cells in close proximity to PCs. Csf1 receptor-deficient mice ($Csf1r^{-/-}$) are characterized by a marked reduction in fully mature PCs (Huynh et al., 2009, 2013). Goblet cell multiplicity was also highly increased as a consequence of the *Csf1r* genetic ablation indicative of faulty cell fate determination in the common goblet and PC precursor. These results were also confirmed in ex vivo cultured crypts, where $Csf1r^{-/-}$ organoids showed decreased clonogenicity and size, and reduced number of budding events. These experiments also suggested a role in PC maintenance for the Csf1 receptor: conditional *Csf1r* deletion by villin-Cre in organoids resulted in PCs loss (Figure 1B) (Akcora et al., 2013).

In view of the main secretory function of PCs, it is not surprising that proteins involved in vesicle trafficking and secretion play significant roles in PC maturation. In particular, two classes of vesicle proteins have been investigated in gut epithelial homeostasis, namely, Rab8A and αSNAP (soluble *N*-ethylmaleimide-sensitive factor attachment protein alpha). The Rab family of small GTPases regulates both the sorting of transported molecules to the correct vesicles and vesicle delivery to target membranes. Of note, Rab8A has been linked to trafficking and secretion of Wnt ligands as it mediates anterograde transport of Gpr177 (wntless), a Wnt-specific transmembrane transporter.

$Rab8a^{-/-}$ mice lack fully mature PCs and are instead characterized by immature cells with decreased numbers of granules (Das et al., 2015). Expression analysis of the intestinal epithelium of $Rab8a^{-/-}$ mice showed

downregulation of several Wnt targets such as *Axin2* and *Ascl2* together with decreased nuclear β-catenin levels and reduced lysozyme and cryptdin 5 expression. When cultured ex vivo, crypts from *Rab8a*$^{-/-}$ mice showed less budding events and decreased clonogenicity. Accordingly, the negative effect brought about by *Rab8A* ablation can be rescued by adding Wnt3 in the organoid medium. The observed effect on cell maturation seems to be specific for PCs and Wnt secretion, as goblet cell numbers, mucin trafficking, packaging, and secretion were unaltered (Das et al., 2015).

Trafficking and delivery to target membranes is followed by vesicle fusion that requires the SNARE complex, the postfusion disassembling of which is regulated by the aforementioned factor αSNAP. A pronounced reduction in PC differentiation was reported in ex vivo cultured crypts derived from mice carrying a single amino acid substitution in the *αSNAP* gene leading to its decreased expression in the intestine (Lechuga, Naydenov, Feygin, Jimenez, & Ivanov, 2017). These results point to a selective inhibition of terminal differentiation of PCs due to the *αSNAP* gene defect and to a novel functional role for vesicle trafficking and fusion in the regulation of secretory cell fate.

2.4 PCs Constitute the Main Epithelial Intestinal Stem Cell Niche

The physical association of PCs and *Lgr5*$^{+}$ CBCs at the bottom of the crypt is suggestive of an additional function for this multitasking secretory lineage, namely, as niche cells capable of regulating the activity of stem cells in homeostasis and during regeneration upon tissue insults. Sato and collaborators in the Clevers' laboratory noticed that *Lgr5*$^{+}$ stem cells were extremely inefficient when employed to establish ex vivo organoids unless cocultured with PCs, which increased organoid multiplicity by at least 20-fold (Sato et al., 2011). Based on this seminal study, PCs were proposed to represent the main epithelial niche for *Lgr5*$^{+}$ stem cells.

As discussed earlier, Wnt and Notch signaling play rate-limiting roles in regulating both stemness and in establishing secretory lineage fate; likewise, a tightly controlled balance between the two pathways is crucial to regulate intestinal homeostasis (Tian et al., 2015). Specific Wnt and Notch factors encoded by PCs, namely, Wnt3, Dll1, and Dll4 (delta-like ligands 1 and 4), underlie their niche function (Sato et al., 2011). The organoid assay (further discussed later) and the possibility of culturing these two cell types ex vivo have

been instrumental for the identification of these and other key regulatory niche cues (Sato et al., 2009).

Due to their close physical association with *Lgr5*$^{+}$ cells, PCs exert their niche function both through secreted short-range factors and by membrane-bound signaling ligands. Moreover, apart from their signaling modalities, PCs provide additional support to *Lgr5*$^{+}$ stem cells in a contact-dependent manner (Gracz et al., 2015). Although these requirements are well established for rapidly cycling *Lgr5*$^{+}$ CBCs, little is known about the niche requirements of other stem cell types, e.g., the more quiescent stem cells located at position +4.

Additional stem cell niche factors are thought to be secreted by the surrounding stroma. In particular myofibroblasts have been shown to secrete Wnt ligands (Vermeulen et al., 2010). Studies on Porcupine (*Porcn*), a gene encoding for an *O*-acetyltransferase essential for Wnt ligands secretion, showed that its genetic ablation in epithelial cells does not impair intestinal homeostasis and regeneration upon tissue insults. However, concomitant genetic *Porcn* ablation and administration of a specific porcupine inhibitor (C90) leading to impaired Wnt secretion in both the epithelial and stromal compartment significantly reduced *Lgr5*$^{+}$ CBCs multiplicity in homeostasis and stalled proliferation and recovery after tissue injury (Kabiri et al., 2014). These studies highlight the relative stromal vs epithelial contribution in the secretion of Wnt and other ligands known to regulate intestinal stemness and must be taken into consideration when addressing the in vivo niche role of PCs. Accordingly, in vivo depletion of PCs, as observed in *Sox9* knockout mice, does not results in any major differentiation defect among the different intestinal epithelial cell lineages (Mori-Akiyama et al., 2007). Likewise, deletion of the *Atoh1/Math1* gene encoding for a transcription factor necessary for the commitment of secretory lineages results in a loss of PCs without affecting epithelial morphology and homeostasis (Durand et al., 2012). Similar results were obtained by knocking out other genes (e.g., *Gfi1,* Shroyer et al., 2005*; Spdef,* Gregorieff et al., 2009). However, it is unclear whether the apparent absence of PCs in these mouse models is limited to fully mature PCs or is extended to its immature progenitors which could still exert some of the relevant niche functions. Also, as reported later, PCs are likely to play key roles (both as niche and stem-like cells) in the regeneration of the intestinal epithelium upon inflammation and other forms of tissue injury. As such, mouse models depleted of PCs should be challenged by inflammatory insults to fully evaluate the consequences of their partial or complete loss.

PCs have mainly been studied in inbred C57BL/6J (B6) mice. This is of relevance as expression of PC-specific genes, and pathways may differ among distinct inbred strains and as such differentially affect their secretory, niche, and other functions as illustrated by the case of the secretory phospholipase A2 (*Pla2g2a*). The *Pla2g2a* gene, specifically expressed in PCs, represents a major genetic modifier of intestinal tumor multiplicity in the Apc^{Min} mouse model (Dietrich et al., 1993; MacPhee et al., 1995). While the C57BL/6J strain carries a *null* secretory phospholipase A2 allele ($Pla2g2a^{-/-}$) due to a stop codon mutation, other inbred strains (e.g., FVB, AKR, BALB/C) are *Pla2g2a* proficient ($Pla2g2a^{+/+}$). When bred into the $Apc^{Min/+}$ background, B6 animals show a strikingly high multiplicity of upper GI adenomas (approx. 90). In contrast, $Apc^{Min/+}/Pla2g2a^{+/+}$ mice are characterized by a pronounced reduction in intestinal tumor numbers. In a recent study, our laboratory has shown that the intracellular pool of phospholipase A2 downregulates Wnt signaling during homeostasis by modifying the subcellular localization of the yes-associated protein 1 (Yap1) protein (Schewe et al., 2016). Upon inflammation, Pla2g2a is secreted into the lumen from where it activates a cascade of downstream signaling events culminating in the synthesis of prostaglandins and Wnt activation, thus supporting the regenerative response to the inflammatory insults (Schewe et al., 2016).

Overall, it appears that PCs exert their niche function throughout a complex and diverse network of secretory auto- and paracrine pathways and by their physical association with stem cells.

2.5 PCs and Nutrient Sensing: A Matter of Metabolism?

As mentioned earlier in this chapter, already the first studies on PCs observed a change in their content upon feeding mice with different diets (Cowdry, 1934; Georges, 1930) thus suggesting a functional connection with metabolism.

Although a set of defined factors including Notch and Wnt stimuli have been proposed to constitute the stem cell niche (Sato et al., 2011), very little is known about the metabolic requirements of $Lgr5^{+}$ CBCs and their niche. More recently, analysis of Paneth and $Lgr5^{+}$ cells revealed a striking metabolic dichotomy: while PCs are earmarked by high glycolytic activity, $Lgr5^{+}$ CBCs rely on mitochondrial oxidative phosphorylation for their metabolic needs (Rodriguez-Colman et al., 2017). Notably, PCs, characterized by a high glycolytic activity, secrete lactate which is taken up by the $Lgr5^{+}$ stem cells to fuel oxidative phosphorylation. As such, PCs provide a metabolic niche to $Lgr5^{+}$ stem cells.

Other studies involving dietary modulation suggested that PCs play a major role in sensing the organism's nutritional status. In particular, Yilmaz et al. showed that, upon longevity-promoting calorie restriction (CR), PCs elicit *Lgr5*$^{+}$ stem cell function by downregulating mTORC1 (mechanistic target of rapamycin complex 1) signaling (Yilmaz et al., 2012). Of note, CR seems to primarily affect PCs but not CBCs.

mTORC1 inhibition in PCs leads to the activation of the ectoenzyme bone stromal antigen 1 (Bst1) which in turn triggers the secretion of cyclic ADP ribose (cADPR). During caloric restriction, this paracrine factor is secreted by PCs and favors stem cell self-renewal eventually resulting in an increase of *Lgr5*$^{+}$ CBCs' multiplicity. This effect could be mimicked in vivo by administration of rapamycin, the main mTORC1 pharmacological inhibitor. However, in a subsequent study, Igarashi and Guarente reported a more detailed analysis of the effects of CR with mTORC1 signaling being inhibited in PCs but upregulated in intestinal stem cells (Igarashi & Guarente, 2016). In this scenario, mTORC1 activation is mediated by SIRT1 in *Lgr5*$^{+}$ cells leading to an increase in protein synthesis and an increase in CBC multiplicity. Notably, in this study the in vivo administration of rapamycin was shown to abolish CBC expansion rather than mimicking CR effects (Igarashi & Guarente, 2016). Although a model was proposed where PC signaling could override any direct nutrient sensing in *Lgr5*$^{+}$ CBCs, further studies will be required to elucidate how drugs that modulate CR pathways may exert opposing effects on different cell types (Figure 1C).

2.6 Culturing Intestinal Crypts ex vivo as Organoids

Seminal work by the laboratories led by Clevers and Kuo's led to the establishment of novel 3D culture methods for the ex vivo culture of mouse intestinal crypts as organoids or "miniguts," i.e., long-lived and self-renewing structures recapitulating the crypt–villus organization of the intestine and encompassing both *Lgr5*$^{+}$ and PCs as the main units of the stem cell niche, in addition to other differentiated lineages (Ootani et al., 2009; Sato et al., 2009). Both methods successfully established long-term epithelial cultures from the small and large intestine by employing matrigel and collagen, respectively. Both studies identified R-spondin, the main ligand of the Lgr5 receptor and a potent canonical Wnt signaling amplifier, as the key growth factor for the successful organoid culture. Other important factors include the epithelial growth factor and the Tgf-β inhibitor Noggin.

Organoid cultures are also partly dependent on the Wnt3A ligand, normally present in the basolateral membrane of PCs (Farin et al., 2016). Recently the combination of Wnt3A, R-spondin, and Noggin (WRN) has been found to be sufficient to grow ex vivo intestinal organoids from most companion and large animals (Powell & Behnke, 2017).

The availability of an ex vivo culture system that, although in the absence of a mesenchymal/stromal niche support, recapitulates the complex in vivo structure of the intestine, stimulated and improved additional studies through the genetic and biochemical modifications of the whole crypt or its specific cell lineage components (Andersson-Rolf et al., 2016, 2017; Koo et al., 2011; Rodriguez-Colman et al., 2017; Schewe et al., 2016).

As organoids can also be established from patient-derived tumors, biobanks of human 3D cultures are currently being established from tumor material and matched healthy tissues for several purposes including the preclinical assessment of (chemo) therapeutic approaches, and studies of stem cell tracing and plasticity within neoplastic lesions, tumor heterogeneity, and metabolism. The establishment and in vitro maintenance of organoids from patient-derived colon cancers require different and specific factors depending on the tumor subtype, also in reflection of the heterogeneity of the spectrum of malignancies affecting the large bowel (Fujii et al., 2016).

As mentioned earlier, the main limitation of the currently available organoid culture methods is the lack of an intact stromal microenvironment. Also, since in its original formulation the protocol employs whole intestinal crypts as the biological substrate to establish organoids, this precludes functional studies of specific cell lineages within the stem cell niche, and in particular its two main components, namely, the *Lgr5*$^{+}$ CBCs and PCs.

In order to include and preserve the stromal and extracellular matrix microenvironment where intestinal crypts normally reside, colonic stroma has been de- and recellularized with myofibroblasts, endothelial cells, and epithelial cells (whole organoids or sorted cells) (Chen et al., 2016). This approach promises to provide additional insights into the normal gut physiology, not currently feasible with the canonical organoid culture protocol.

As for the second limitation, *Lgr5*$^{+}$ stem cells and PCs tend to physically interact when coincubated to then give rise to organoids (Sato et al., 2011). This key feature is the basis for the "organoid reconstitution assay" (ORA) further developed and implemented by us and others (Igarashi & Guarente, 2016; Schewe et al., 2016). Sorting by FACS of CBCs and PCs allows their genetic and/or biochemical modification (or their isolation from genetically modified or treated mice) prior to their coincubation and reconstitution into

organoids, thus providing a handy functional study tool. A recent example of the usefulness of ORA is represented by the demonstration of the striking metabolic dichotomy between PCs (glycolytic) and *Lgr5*$^{+}$ stem cells (oxidative phosphorylation) and the functional relevance of these metabolic needs for the maintenance of homeostasis (Rodriguez-Colman et al., 2017).

3. PCs, DYSBIOSIS, AND IBD

The intestinal microbiome hosts a diverse and abundant group of microorganisms known to be essential for gut homeostasis and development, as shown by studies on germ-free mice. Several environmental factors affect the composition of the microbiome such as diet (Claesson et al., 2012; Wu et al., 2011), oxygen levels, and pH (Bevins & Salzman, 2011). In the laboratory mouse, inbred strain-specific variations in the composition of the microbiome have been shown (Gulati et al., 2012), analogous to the observed variations in PCs' multiplicity, distribution, and secretory function (Salzman & Bevins, 2013). Dysbiosis, i.e., the unfavorably altered composition of the microbiota, is central to a broad spectrum of human pathologies, among other IBD.

The term IBD is comprehensive of two main chronic and relapsing pathologies of the intestinal tract, namely, UC and CD (Kaser, Zeissig, & Blumberg, 2010). Like many other pathological conditions such as diabetes, atherosclerosis, obesity, and cancer, IBD is nowadays thought to result from inappropriate inflammatory responses to dysbiosis in genetically susceptible individuals (Figure 2A) (Salzman & Bevins, 2013). One of the main differences between UD and CD is the affected region of the gastrointestinal tract. UC is characterized by chronic inflammation of the colon, initiating in the rectal region, and subsequently spreading proximally, with tissue damage usually limited to the mucosa or submucosa featuring local crypt inflammation (*cryptitis*) and abscesses with neutrophilic exudate in the lumen. CD instead affects both the large bowel and the distal part of the small intestinal tract, namely, the ileum where PCs are more abundant. At the microscopic level, CD's distinctive features include thickening of the submucosa, transmural inflammation, fissuring ulceration, and granulomas (Kaser et al., 2010).

As a consequence of the primary role played by PCs in the secretion of AMPs and in the control of the gut microbiota during homeostasis, their dysfunction (either due to genetic defects or to environmental "stressors" such as infections, obesity, and graft vs host disease) has been shown to be

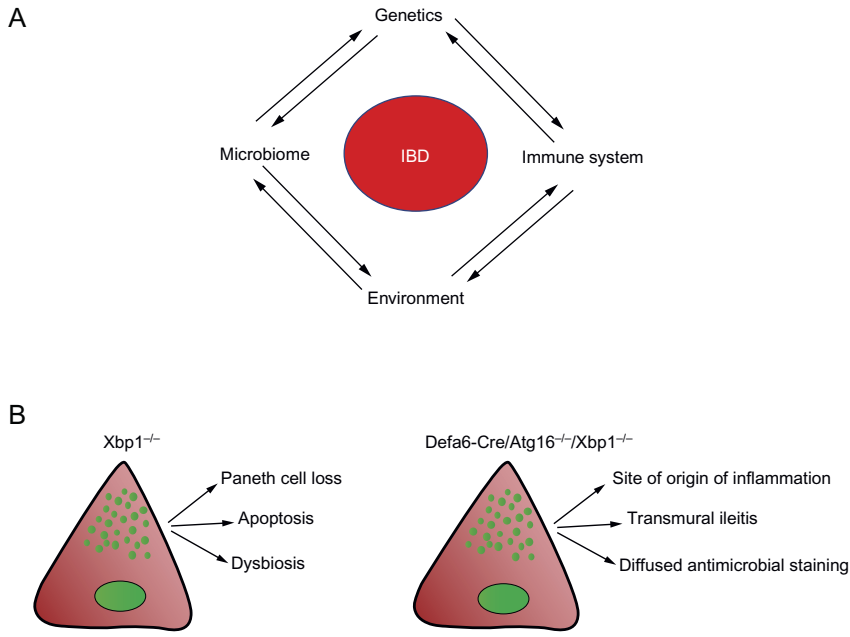

Fig. 2 (A) Inflammatory bowel disease (IBD) is a complex multifactorial disease dominated by four major cross-interacting components, namely the genetic background of the host, the gut microbiome, the host's immune system, and the environment. (B) Genetic depletion of the *Xbp1* gene encoding for a key ER stress response protein causes Paneth cell loss, increased apoptosis, and dysbiosis. Combined genetic loss of the *Xbp1* and *Atg16* genes in Paneth cells results in diffused lysozyme staining reminiscent of IBD and transmural ileitis, suggesting that PCs represent the site of origin of intestinal inflammation (Adolph et al., 2013).

causative in a broad spectrum of dysbiosis-associated pathologies, including IBD (Salzman & Bevins, 2013). The complex multifactorial nature of IBD can be in fact reduced to the interaction of a quartet of host-derived and environmental factors: the genetic predisposition and susceptibility of the host, the intestinal microbiota, the immune system, and the above-mentioned environmental stressors (Clevers & Bevins, 2013).

Genetic susceptibility predisposes the individual to IBD and, likewise, family history does represent a risk factor as shown by genome-wide association studies (GWAS). A multitude (>150) of IBD-associated single nucleotide polymorphisms (SNPs) have been established as risk loci, 28 of which common to both CD and UC. Of note, the very first genes to be identified are not only highly and specifically expressed in PCs but also exert essential roles in PC's secretory function (Cho, 2008; VanDussen et al., 2014) including

host-bacterial interaction and response (e.g., *NOD2*) (Hugot et al., 2001; Ogura et al., 2001), ER stress and unfolded protein response (*XBP1*) (Kaser et al., 2008), and autophagy (*ATGL16L1*) (Cadwell et al., 2008; Cadwell, Patel, Komatsu, Virgin, & Stappenbeck, 2009).

3.1 Nod2 in IBD and PCs

Although its physiological function in the intestine remains elusive, the *Nod2* gene, encoding for a member of the nucleotide-binding oligomerization domain–leucine-rich repeat (NOD-LRR) family of proteins, is specifically expressed in PCs and is most pronounced in the ileum of both healthy controls and IBD patients (Ogura et al., 2003). A role in bacterial sensing through innate and adaptive immunity was initially hypothesized and later confirmed by the laboratory of Richard Flavell (Kobayashi et al., 2005). Strikingly, PCs from *Nod2*-deficient mice were not able to sense specific microbial antigens such as muramyl dipeptide (MDP), a conserved bacterial PGN. Nevertheless, $Nod2^{-/-}$ mice did not display any overt symptoms of intestinal inflammation and were not significantly susceptible to DSS-induced colitis. However, they were resistant to lipopolysaccharide (i.e., Toll-like receptor agonists) challenge with MDP priming, in confirmation of the role of *Nod2* in sensing and responding to bacterial antigens by activating the adaptive immune system either directly or by enhancing the production of α-defensins (Kobayashi et al., 2005). As such, Nod2 is essential to protect the host from intestinal bacterial infection. However, it still remains to be established whether the PC defect in MDP sensing is the only and even main mechanism through which *Nod2* mutations are related to CD development. Rather than representing the main initiating factor for the disease, *Nod2* gene defects might alter the physiological response to pathogenic bacteria and predispose the individual to CD.

Additional studies in germ-free mice revealed that lack of lysozyme expression also characterizes PCs from $Nod2^{-/-}$ mice due to specific lysosomal degradation (Zhang et al., 2015). When AMPs are synthetized in PCs, they are sorted into specialized secretory granules called dense core vesicles (DCV). Accordingly, Nod2 is also recruited to AMP-containing DCVs that, in turn, is required for the DCV localization of the multiprotein kinase Lrkk2 and the GTPase Rab2a (Zhang et al., 2015). Defects in the Nod2–Lrkk2–Rab2a axis result in the lysosomal degradation of lysozyme (Zhang et al., 2015).

3.2 ER Stress and Unfolded Protein Response in IBD and PCs

As part of their secretory function, PCs are characterized by a pronounced endoplasmic reticulum and Golgi apparatus, and by large secretory granules. As such, they act as protein factories responsible for the synthesis of many proteins with antimicrobial and essential for intestinal homeostasis. In secretory cells, nascent proteins enter the ER as unfolded polypeptide chains to complete folding and maturation. The folding capacity of the ER of secretory cells is targeted on the rate of nascent proteins entering the ER. Specific sensors monitor the ER lumen and signal to other cellular components. Whenever the rate of unfolded nascent proteins exceeds the folding capacity of the ER, a condition known as ER stress, the unfolded protein response (UPR) is triggered (Ron & Walter, 2007). In this process, the molecular sensor and ER chaperone immunoglobulin-binding protein B (BIP, HSPA5, or GRP78) play a central role. During homeostatic conditions BIP is bound to the luminal side of three master regulators of the UPR response, namely, transmembrane proteins inositol requiring 1 (IRE1), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6). Upon ER stress activation, BIP dissociates from these sensors thus triggering their activation. The downstream pathways involve, among others, the Paneth-specific transcription factor X-box-binding protein-1 (XBP1) whose mRNA is converted upon ER stress to an active form by the IRE1 nuclease. This leads to the modulation of protein synthesis to adapt the ER folding capacity to the specific requirements of the cell. Whenever ER stress is not solved, apoptosis is triggered (Ron & Walter, 2007; Schroder & Kaufman, 2005).

PCs, as many secretory cells, are characterized by a basal ER stress level. Of note, the small intestine is the only tissue reported to date to express an isoform (IRE1β) of the proximal sensor IRE1 (Walter & Ron, 2011). Remarkably, in *Xbp1* knockout mice ER stress was detected in the intestinal epithelium through GRP78 upregulation in association with an inflammatory phenotype reminiscent of human IBD with crypt abscesses, ulcerations, and leukocyte infiltration (Kaser et al., 2008). Furthermore, PC with normal granules was barely detectable together with a substantially decreased expression of cryptdins 1, 4, and 5. Also, PC apoptosis, most likely triggered by the failure of solving ER stress, was a feature of *Xbp1* genetic ablation. Last, *Xbp1* knockout mice were more susceptible to infection by gram-positive bacteria and to DSS-induced colitis (Figure 2B).

3.3 Autophagy in IBD and PCs

The term autophagy refers to the natural process through which cellular components, like organelles or large protein complexes which cannot be removed by the proteasome, are degraded and recycled in orderly and regulated fashion through the lysosome. Although the original function of autophagy is likely to have arisen as an adaptation to starvation or nutrient deprivation, it has evolved as a quality control process for organelles and proteins and to regulate energy homeostasis (He & Klionsky, 2009). Moreover, it is also employed by the cell to degrade microorganisms (also referred to as xenophagy), as shown by the increased susceptibility to infections caused by intracellular pathogens upon mutation of autophagy genes (Glick, Barth, & Macleod, 2010). Autophagy can also be selective when it triggers the degradation of specific damaged organelles, such as mitochondria or peroxisomes.

The formation of autophagic vesicles earmarks autophagy (Mizushima, Yoshimori, & Ohsumi, 2011) and is dependent on the translocation of the mTOR substrate complex from the cytosol to the ER. Through recruitment of PI3 kinases to the ER, vesicle formation and elongation are started. The final stage of this process is mediated by a number of autophagy (Atg)-related proteins, namely, Atg12, Atg5, and Atg16, which form a complex with Atg8 and phospholipid phosphoethanolamine, that catalyzes further elongation and maturation of the autophagic vesicles. Once mature, they are transported to lysosomes where they undergo degradation.

The autophagy machinery interfaces with cellular stress and response pathways, including those controlling immune responses and, in turn, inflammation. Large autophagic vesicles were observed to be prominent in PCs after irradiation (Gorbunov & Kiang, 2009). Several mice carrying hypomorphic variants of different *Atg* genes (i.e., *Atg16*, *Atg5*, and *Atg7*) were characterized by apparently mature PCs though with secretory granules decreased in size and numbers (Cadwell et al., 2009). Defects in granules exocytosis leading to decreased AMP secretion into the lumen and differences in microbiome composition were also reported in these mice (Cadwell et al., 2009).

Several defects in autophagy, autophagy-related genes, and PC morphology have been observed in IBD patients (Stappenbeck & McGovern, 2017). As previously mentioned, SNPs in the *ATG* and related genes were found by GWAS to predispose to IBD (Cadwell et al., 2008, 2009; Liu et al., 2017). More recently, a seminal study by Adolph et al. has functionally linked ER stress and autophagy in a specific cell type, the PC (Adolph et al., 2013).

Here, mouse models were developed that were defective in the *Xbp1* gene in the small intestine. Upon induction of ER stress in the intestinal epithelium, autophagy was observed mostly at the bottom of the crypts where PCs reside. To assess whether autophagy was induced to ameliorate ER stress, mice defective in both Xbp1 and Atg16L1 (or Atg7) in intestinal epithelium-specific fashion were generated (Adolph et al., 2013). These mice lacked UPR-induced autophagy resulting in constitutive ER stress in the absence of autophagy. Notably, the intestines of the double-mutant mice featured a strong inflammatory reaction with tissue damage reminiscent of CD. In view of the potent autophagy induction in *Xbp1* defective mice at the bottom of the crypts, PCs are likely to be the culprit of the CD-like phenotype of these mice. Indeed, Paneth-specific ablation of *Xbp1* and *Atg16L1* (or *Atg7*) recapitulated the phenotype of the above mice where the same genes were inhibited in the whole intestinal epithelium, thus providing additional experimental evidence for PCs as the cellular site where ER stress and autophagy are functionally linked and as the origin of intestinal inflammation in IBD cases linked to ATG and ER stress loci (Adolph et al., 2013). As for the molecular mechanisms underlying the autophagy-driven amelioration of ER stress, upregulation of the NF-κB pathway was observed in the double-mutant mice. Accordingly, use of an NF-κB inhibitor led to a decrease in the overall number of cells undergoing apoptosis and a partial reversion of the inflammatory phenotype caused by the combined *Xbp1* and *Atg16L1* genetic deletion. Notably, NF-κB activation upon concomitant loss of *Xbp1* and *Atg16L1* function was not observed in germ-free mice again highlighting the relevance of the microbiome in these experimental settings and providing a suggestive functional connection between ER stress, autophagy, and the microbiome in IBD (Adolph et al., 2013).

The above findings open novel therapeutic scenarios for IBD patients, as illustrated by the use of NF-κB inhibitors to ameliorate and possibly resolve inflammation. Moreover, patients without SNPs in *Atg*-related genes, autophagy could be stimulated by treatment with rapamycin and mimicking caloric restriction (Yilmaz et al., 2012). Also, it is well established that IBD patients carry an increased risk to colon cancer. From this perspective, the notion that PCs are the site of origin of inflammation may also have implications for IBD-associated colon cancers in view of previous observations pointing at the capacity of PCs and their progenitors to dedifferentiate and take active part in the regenerative response to tissue damage (Roth et al., 2012). Further studies will be required to elucidate whether PCs might also represent the cell of origin of IBD-associated colorectal cancer.

3.4 The Role of Immunity and Environmental Factors

The immune system plays important roles in IBD in response to bacterial antigens, proteins, and RNA. Innate or in-born immunity is a prerequisite for the activation of the adaptive immunity which in turn seems to be causative for the tissue damage in IBD. Animal models of IBD, along with a more detailed understanding of the immune response in the digestive tract, have led to an unifying hypothesis relative to the role of the immune system in IBD. An inappropriate mucosal immune response to normal intestinal components leads to a local imbalance in cytokines resulting in a neutrophil and monocyte influx with subsequent secretion of oxygen radicals and enzymes, leading to tissue damage (Brown & Mayer, 2007).

The incomplete penetrance of IBD animal models and the heterogeneity of the disease can be accounted for by additional environmental factors. Several epidemiological studies have shown increased IBD risk in migrants from countries at low- to high-risk countries (Segal, 2016). Furthermore, since the 19th century the incidence of IBD has steadily increased in westernized countries (Segal, 2016). Diet and vitamin D intake are supposedly additional modifying risk factors to IBD. Low dietary fiber intake is associated with increased IBD risk as fibers are metabolized by bacteria into short-chain fatty acids that inhibit transcription of proinflammatory mediators (Ananthakrishnan et al., 2013, 2014). Furthermore, low zinc intake might impair autophagy and modulate immune functions (Ananthakrishnan et al., 2015; Liuzzi, Guo, Yoo, & Stewart, 2014). Additional factors correlated with higher risk of developing IBD are lack of exercise and sleep, although more experimental evidence is needed to link the latter functionally to IBD (Blumberg, 2016).

3.5 PCs as IBD-Specific Disease Markers

The broad range of processes coming together in IBD is indicative of the complexity and heterogeneity of the disease (Figure 2A). In view of their striking multifunctionality ranging from the secretory and bactericidal function to innate gut immunity, PCs seem to be endowed with the capacity to integrate cues from dietary nutrients and the microbiota and translate them in to stem cell niche regulatory signals in homeostasis and tissue regeneration. Accordingly, changes in PCs numbers and/or morphology or mutations in PC-specific genes underlie the etiology of at least a subset of IBD cases (Stappenbeck & McGovern, 2017). Stappenbeck and McGovern

have proposed a classification of CD subtypes based on the detailed characterization of PC morphology (granules size and numbers) and IHC staining of AMPs like lysozyme (Lyz1) and defensins (Stappenbeck & McGovern, 2017). In this fashion PCs can be studied retrospectively in archival resection specimens or from biopsies collected via endoscopy.

3.6 PCs as Stem Cell Niche in Inflammation

The stem cell niche role of PCs has been mostly studied in homeostasis, and little is known on their function upon inflammation. Several reports suggest a decrease in mature PCs' multiplicity in inflammation. One of the genes responsible for the incomplete maturation of PCs in inflammation is protein kinase C iota or (*Prcki*). *Prcki* ablation in the intestinal epithelium did not affect PC number and granules. However, the PC-specific marker lysozyme was shown to colocalize with alcian blue-positive cells characteristic of the goblet lineage. Hence, inflammation triggers PC dedifferentiation to acquire an intermediate secretory precursor of both Paneth and goblet cells (Nakanishi et al., 2016). Expression analysis of PRCKI in IBD patients revealed an inverse correlation with disease progression (Nakanishi et al., 2016).

The secretory phospholipase A2 group 2a (*Pla2g2a*) gene was shown to be able to modify and improve the PC niche function upon inflammation (Schewe et al., 2016). Contrary to the role of *Pla2g2a* in homeostasis where the protein downregulates Wnt signaling in PCs, gene- and protein-based assays showed highly increased *Pla2g2a* expression and secretion upon inflammation leading to an increase in canonical Wnt in PCs. When secreted, Pla2g2a, next to exerting its antibacterial function, binds to the M-type phospholipase A2 receptor 1 (Pla2R1). This interaction triggers an autocrine cascade of downstream events culminating in increased Wnt signaling and improved regenerative response to the inflammatory tissue damage (Schewe et al., 2016). These observations open new therapeutic options by stimulating PC functions in IBD to improve tissue regeneration (Schewe et al., 2016).

4. CONCLUSIVE REMARKS

4.1 Multitasking PCs in Health and Disease

The strikingly broad spectrum of functions covered by PCs is reflected by the plethora of molecular and cellular mechanisms through which these

secretory cells have been linked to several human pathological conditions related to dysbiosis, from obesity to graft vs host disease, inflammation, and cancer. The more recently elucidated niche role of PCs in supporting and modulating intestinal stem cell function possibly represents the integration hub of their multiple tasks. By sensing environmental factors like dietary changes and/or inflammatory stimuli, PCs seem to be capable of translating them into modifications of stem cell numbers, activity, and regenerative capacity. This is achieved either directly or indirectly by coordinated stress sensors such as UPR, ER stress, autophagy, and possibly many others. And yet, we are possibly only scratching the surface of the true functional identity of PCs. Many fascinating aspects remain unsolved: what is, if any, the functional role of PCM in colonic inflammation and cancer? Can mature PCs and their secretory precursors, next to their niche function, directly contribute to the regenerative response to tissue insults by dedifferentiating and acquiring stem-like properties? Analogous to their regulatory role in response to caloric restrictions, can Paneth-like cells located in the colon alter their metabolic needs and niche functions upon Western-style nutrients known to increase colon cancer risk? Ongoing and future studies will contribute to the elucidation of these and other questions and possibly lead to PC-tailored therapeutic strategies for IBD and GI-tract cancer patients (Figs. 1 and 2).

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