A close-up of colon cancer

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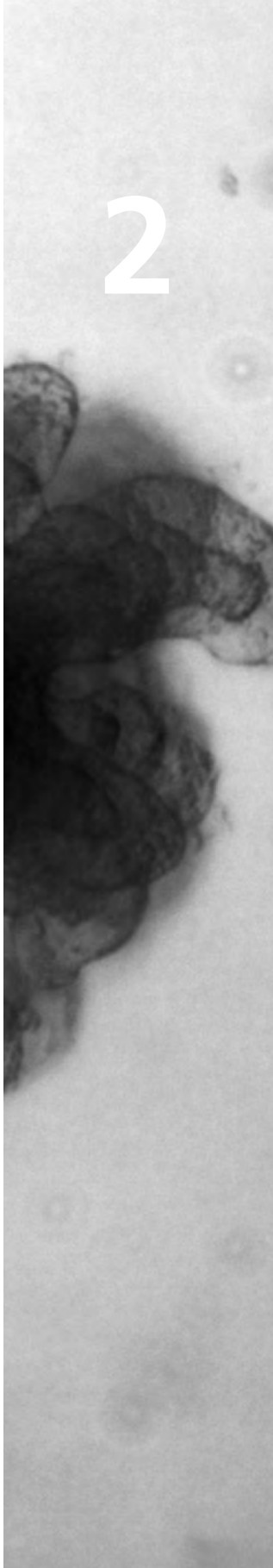
General introduction



Normal intestinal epithelial homeostasis

Small and large

In past decades, molecular and imaging techniques have fuelled our understanding of histology, pathology and molecular biology. This research is performed on human material as well as on experimental animals, most notably the mouse due to its predilection to be genetically manipulated. In addition, whereas nowadays in the field of cancer research the colon is of most interest, much of the current knowledge on the intestinal tract has been obtained from studies of the small intestine. This organ is longer, thus yields more tissue to work with and it is more neatly organized than the colon. Furthermore, when subjecting mice to those mutations that exclusively cause colonic neoplasms in men, mice tend to develop tumors primarily in the small intestine. Although no one will disagree that men are no mice, and that the small intestine is not the large intestine, others' and our studies have focused on those aspects that are alike in mice and men, in the small intestine and the colon.

The research discussed in this thesis is a built on foundations, laid down by others that have made important advances in the understanding of human colorectal cancer by studying the small intestine of mice. Therefore, when speaking of the intestine, this refers to the small intestine. When applicable, a difference between small and large will be noted in the text. Generally, the information on the biology of the intestine has been obtained from mouse studies.

The intestinal system

Although the intestine is localized inside the body, it is a continuum with the outside world. Compared to other contact areas such as the skin or oropharyncheal mucosa, it harbors the highest microbial load of all organs. The first line of defense consists of a cellular monolayer that protects us from these microbiota. The intestinal epithelium has a remarkable capacity to combine these defensive tasks with its role in the absorption of nutrients and water. Nutrients are primarily absorbed in the small intestine; the colon is the place of water uptake.

The intestinal epithelial monolayer is one of the most rapidly renewing tissues in the mammalian body. In order to achieve an absorptive capacity for digestion and uptake of nutrients from food, the intestine contain an enormous surface that is compressed in a tube, which in humans can stretch out to a length of five meters. The small intestinal epithelium is organized in crypt-villus units (Figure 1). In these crypts of *Lieberkühn*, the cells proliferate for a couple of rounds to generate sufficient progeny. After three proliferative cycles, cells exit the crypt and differentiate into a number of phenotypically distinct lineages and migrate up the villus, to perform digestive, absorptive and protective tasks. The large intestine (or colon) lacks these villi. There, crypts are larger and differentiated cells are localized in the upper half of the crypts. When cells reach the tip of the villus in the small intestine, or the upper part of crypts in the large intestine, they die off

and are shed in the lumen of the intestine. Every five days the total layer of intestinal epithelium is renewed.

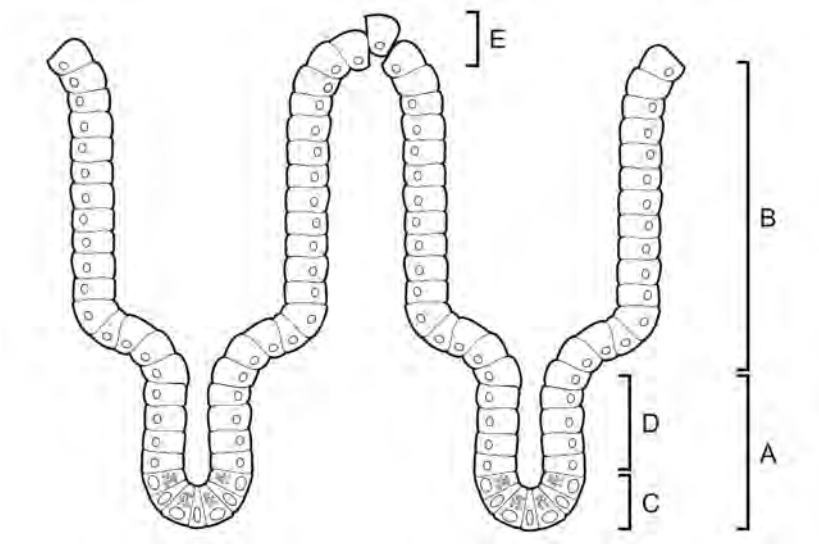


Figure 1. Schematic representation of the small intestinal epithelium.
The small intestinal epithelium consists of crypts (**A**) and villi (**B**). In crypts reside stem cells at the crypt base (**C**), which divide and form transit amplifying cells (**D**). These cells in turn proliferate for a number of cycles to move up the villus once differentiated. After a number of days, villus cells reach the villus tip (**E**), where they go into apoptosis and are removed from the villus by a process termed shedding.

When this process of cellular renewal goes awry, disease may occur. On the one hand, in case of defects that impede proliferation, enteritis develops. This is seen during infection with a number of micro-organisms, upon irradiation or upon treatment with noxious agents such as a number of chemotherapeutic agents and in inflammatory bowel disease, in which the immune system causes a damaging inflammatory response. These states in turn cause reduction of resorptive capacity, which may result in nutritional deficits, usually accompanied by rapid passage of undigested food: diarrhea. On the other hand, in the case of increased proliferation, DNA mistakes may accumulate and neoplasms can develop.

Development of intestinal epithelium

By embryonic day 9 (E9) in the mouse the first structure that bears the intestinal identity is formed. The embryo contains a tubular gut that is also referred to as the primitive gut, and the endodermal epithelial lining is morphologically similar from end to end[1-3]. The primitive gut can be divided into three pieces that develop into different structures. The foregut forms the oesophagus, the stomach and a small fragment of the small intestine, the duodenum. The midgut

forms the larger part of the small intestine and contributes up to the first half of the colon where the second half of the colon is derived from the hindgut. On E14.5, caused by mesenchymal signals that are not completely understood, the pseudostratified epithelium in a larger part of the midgut is remodeled to form intestinal villi, lined with a single layer of columnar epithelium[3]. Together, these events mark the birth of the small intestine.

To accustom newborn mammals to a diet of breast milk, which is rich in fat and contains components that are crucial for the first phase of life, such as immunoglobulins or the sugar lactose, the embryonic and newborn intestine are distinct from adult intestine on a number of aspects. One of the most important differences is in the digestive enzymes expressed on the epithelial brush border. In adults, sucrase isomaltase (*Si*) is the predominant enzyme to digest simple sugars[4]. This enzyme is absent in the neonatal intestine. Instead, to accommodate the digestion of the milk-sugar lactose, neonates express the hydrolase lactase (*Lph*)[5, 6]. To maximize the uptake of dietary lactose, an additional β-galactosidase is expressed similar in function to lactase, although it functions in the cellular cytosol instead of the brush border[7]. Whereas in adults all nutrients are degraded in the intestinal lumen to be absorbed by enterocytes, neonates possess an additional strategy for uptake of nutrients. In neonatal intestine, microvilli that cover enterocytes are shorter and leave space for orifices by which nutrient containing fluids can flow into cells through a system of microchannels. These fluids are collected in supranuclear vacuoles[6, 8]. Additionally, the cells have loose contacts with neighbouring cells, allowing a surplus amount of milk to pass the epithelial barrier into the lymph, where it can be directed towards the liver without time and energy consuming digestion by absorptive enterocytes. Together these measures are necessary to digest nutrients that are in the diet of the first period of life. There are however nutrients that are absent in milk but abundantly present in the adult diet of solid food. One of these is arginine[9]. Arginine is a *conditionally* essential amino acid, which is mainly essential during periods of growth and repair and therefore critical for the proper growth of the neonate. To produce necessary levels of arginine, a number of neonatal enzymes are expressed that facilitate its production from other amino acids. For example, the enzyme argininosuccinate synthetase 1 is highly expressed in neonates, whereas enzymes that catabolize arginine such as arginase are only found in the adult intestinal epithelium.

Architecturally, the neonatal intestine lacks fully developed crypts and instead has small buds surrounding the villi that are termed intervillus pockets[10] (Figure 2). Epithelial proliferation is restricted to these intervillus pockets and the proliferative rate is reduced compared to adults. Additionally, intervillus pockets are characterized by a lack of Paneth cells, the cell type that secretes antimicrobial peptides, found in adult crypt bases[11]. In the adult intestine, development of Paneth cells requires transcription factor Sox9 and expression thereof is driven by the Wnt-signaling pathway[12-14]. Taken together with the reduced proliferation in intervillus pockets, the neonatal to adult transition may mark an increase in Wnt-activity. A surprising report by Kim *et*

al. showed that during intestinal development the Wnt-pathway is active on the villus and not in the crypt and signaling utilizes transcription factor Tcf-3, a homolog of the major intestinal Wnt mediator, transcription factor Tcf4[15]. However, these data contradict the expression of Wnt-target genes Cd44, EphB2 and EphB3 that are localized in the intervillus pockets[16, 17]. Additionally, germ line knockout of Tcf-4 causes a loss of proliferation in intervillus pockets whereas villi are unaffected[17, 18]. In the first week after birth, intervillus pockets deepen to form crypts, while the number of crypts steadily increases by crypt fission[19].

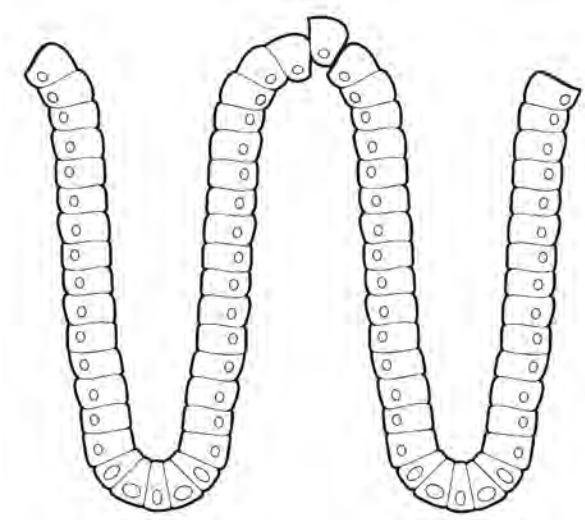


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Figure 2. Schematic representation of the neonatal small intestine.
The neonatal intestine is a scaffold of intestinal epithelium. Note that in contrast to adult intestine, no crypts are formed. Instead, intervillus pockets surround the villus.

Although the neonatal to adult epithelial transition has not been carefully studied in humans, many aspects are conserved between mice and humans. In terms of the expression of digestive enzymes, human neonates are born with a more mature intestine then mice and there is a larger overlap in the expression of enzymes required for a diet of milk and the adult diet of solid food. Thus, in the last phase of embryonic development, the intestinal epithelium develops into a specialized tissue that accommodates ingestion of those nutrients most important for the first stage of life. Later, it progresses to a tissue that is adapted to the adult diet. During this developmental switch a number of changes in the intestinal epithelium are simultaneously orchestrated. These include changes in the expression of digestive enzymes, changes to the cellular architecture and morphological changes to the tissue.

Adult intestinal crypts

At approximately 21 days after birth, the intestine achieves its final, adult form, which is maintained throughout further life. The coarsest division to functionally categorize intestinal epithelial cells is between proliferative and differentiated cells. Differentiation can occur into one of six distinct cell types but the epithelium is mainly made up of absorptive enterocytes. Goblet cells lie interspersed between the enterocytes and generate mucins that cover the intestine with a layer that is protective against microbial components. Defects in the synthesis of this mucus layer have been shown to cause a greater tendency towards mucosal inflmammation[20, 21]. Enteroendocrine cells are rare cells that produce hormones such as somatostatin, motilin and vasoactive intestinal peptide. A fourth cell type, the Paneth cell, resides at the base of the crypt. These cells are mostly known for the production of antimicrobial peptides[22]. Microfold cells, or M-cells have a role in antigen presentation. The function of the sixth cell type, Tuft cells, remains more elusive. Likely, these cells are a secretory cell type as well, and produce local opioids[23].

A first step of differentiation occurs when pluripotent intestinal epithelial stem cells leave their niche and initiate a differentiation program. These cells then acquire features of differentiation, while still progressing through the cell cycle a limited number of times and are therefore termed *transit amplifying* cells (TA cells). On the crypt villus junction, cells exit the cell cycle. Differentiated cells remain for a number of days on the villus and slowly migrate upward, until they are shed into the lumen of the intestine. Paneth cells remain in the crypt base where they are interspersed with crypt base columnar cells that were identified as the intestinal stem cells[24]. In the homeostatic intestine, a complex multitude of signaling pathways converges. Among these pathways are morphogenic pathways, intercellular contact pathways and pathways that signal in a cell-autonomous fashion (for a short conceptual explanation, see box 1).

Box 1

A concise explanation of signaling pathways.

A Morphogenic signaling pathway is activated by binding of a soluble signals to a receptor. These signals, often small soluble proteins, were found to alter tissue morphology and were therefore termed morphogens. Conceptually however, there is little difference between morphogenic signaling, cytokine signaling and many forms of hormonal signaling. To explain morphogen signaling, an analogy was made to an ecological model, the *source-sink* model . In morphogenesis, the source is usually a cell that produces morphogen. The sink cell harbors the receptor for this morphogen. Between source and sink, a gradient of this particular morphogen exists as long as the molecules can diffuse freely. The sink cell can react differently depending on the amount of signal it perceives. Therefore, the phenotype of the sink cell is determined by the amount of morphogen protein available

for the sink cell. This, in turn, is determined by the position of the sink cell in the gradient of a morphogen. Thus patterning of tissues is regulated by the formation of morphogens and the coupling of a cells position in these gradients to their phenotype. Paradigmatically, signaling can be autocrine (where similar cell types in the same tissue are both source and sink) or paracrine (different cell types signal to one another), although this distinction is not exact since signaling between two cells within the same tissue may well be regarded as paracrine. Considering that multiple morphogenic pathways are involved in the intestine, a complex network of morphogenic gradients can be detected. Therefore, the combination of morphogens that a cell receives is intimately involved in localization of cells[25]. These aspects are all instrumental for governing cellular homeostasis and cell fate. In cellular contact signaling, the cell that produces the activating ligand is physically connected to the cell receiving this signal in order to initiate pathway activity. Since no gradients can arise from one cell to another, there is no conceptual rationale to term these cells source and sink cells. In cell-autonomous pathways, a stimulus from within a cell directs signaling. Obviously, these stimuli such as alterations in oxygen, nutrients or cell fate are largely derived from outside the cell or from morphogen- or cellular contact pathways, although a distinction may be made based on a signal that is intentionally generated by other cells to direct cell fate versus a signal that is a result of homeostatic changes (these may include oxygen deprivation, physical damage irradiation, initiation of a transcriptional profile).

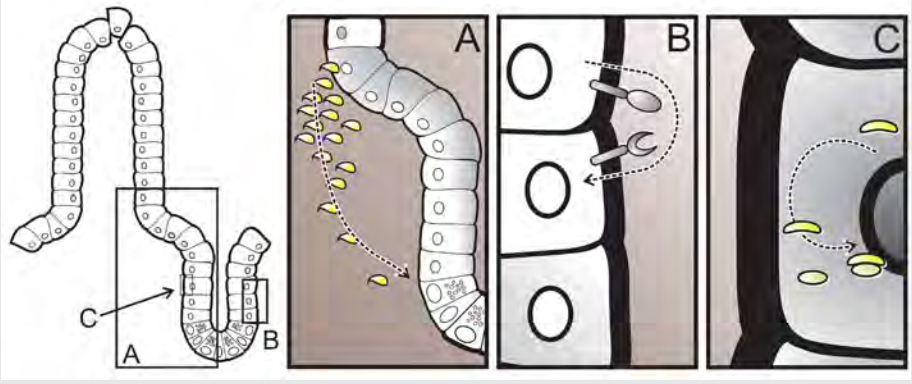

Figure 3. schematic representation of three kinds of molecular signaling.
The left panel depicts a crypt-villus unit in which three areas are indicated where different kinds of signaling may occur. (**A**) In morphogenic signaling source cells secrete a morphoge that forms a gradient towards sink cells, signaling may be toward distant cells. (**B**) In cellular contact signaling a cell receives the signal from an adjacent cell. (**C**) In cell autonomous signaling the signal is derived from within the cell.

A principal difference between the three categories of pathways is the distance that can be bridged by the signal. Whereas morphogens can cross distances of one or multiple

cell diameters or even tissues, intercellular contact signaling can be no further than between adjacent cells. Cell autonomous signaling has little direct effects on adjacent cells. Where morphogenic pathways may direct cell fate and thereby alter activity of cell autonomous pathways, these signaling cascades may, in turn, affect production of morphogens. Therefore, these pathways are profoundly interrelated during tissue homeostasis (Figure 3).
In this thesis, references are made to a number of pathways which are reviewed in depth in the following references[26-31].

Intestinal stem cells

In past years, research on intestinal stem cells has skyrocketed. An important step in understanding the biology of these stem cell has been the identification of several genes specific to stem cells. These discoveries fuelled research identifying and investigating these cells.
According to the current stem cell paradigm, tissue specific stem-cells harbor a combination of two unique properties: *multi lineage differentiation* capacity and *self-renewal* capacity. The extent to which cells harbor these properties is termed *stemness*. Multi lineage differentiation capacity is the property of one cell to become all different cell types that reside in one tissue. In the case of the intestinal epithelium, these are enterocytes, goblet cells, enteroendocrine cells, Paneth cells, M-cells and Tuft cells. The capacity for self-renewal is the property of one stem cell to form new stem cells. In case of a tissue in which the cells are constantly renewed such as in gastrointestinal epithelia this translates into the property of a cell to populate the tissue with its daughter cells in the long term.

Box 2

Experimental procedures to assess multilineage differentiation and self-renewal capacity.

In order to assess *multi lineage differentiation capacity* a technique termed *lineage tracing* is most frequently used (Figure 4A,B). Using this technique, a genetic trait is applied to a progenitor cell. This cell will maintain this trait throughout life and progeny cells inherit this trait. Most frequently, an inducible form of *Cre* recombinase is expressed in mice under the endogenous promoter of a gene that putatively marks a certain progenitor cell population. This mouse is then crossed into an inducible reporter allele that allows imaging of the inheritable genetic trait upon Cre-mediated recombination. In this fashion, cells are marked in the *Cre*-bearing cell population exclusively. Often, visible genetic markers are

used, such as LacZ or eGFP. The presence of all cell lineages within the marked cells is proof of the multi lineage differentiation capacity of this cell[32].

Self-renewal capacity can be measured *in vivo* and *in vitro*. In the context of the intestine, it suffices to show long-term maintenance of progeny by a cell. In case of dividing cells this shows that these cells are capable of generating offspring without differentiating themselves, suggesting self renewal of stem cells. *In vitro* assessment of *self-renewal capacity* of intestinal cells can be shown by generation of epithelial organoids[33]. Intestinal organoids are cultures of primary intestinal epithelial tissue, that harbor all cell types characteristic for the adult intestine. The capacity of single cells to reconstitute an organ that can be long-term maintained is proof of self-renewal capacity. In this fashion, different populations can be compared to identify those markers that harbor the two stem cell capacities to the highest extent.

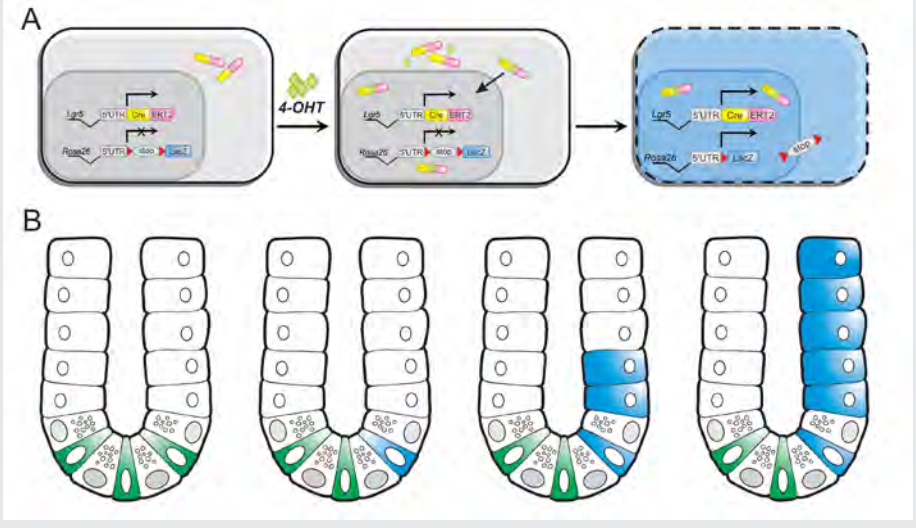

Figure 4. Strategy for lineage tracing.
(**A**) Cells harbor both a reporter allele capable of imposing a visible trade and a knockin allele in which CreERT2 is expressed under the promoter of a specific gene of interest, in the case of this example under the promoter of the stem cell marker Lgr5. when these cells are treated with 4 hydroxy tamoxifen (4OHT), CreERT2 translocates to the nucleus and activates the reporter allele. In this fasion cells are marked and remain marked even after expression of the specific gene of interest resides. (**B**) schematic representation of lineage tracing over time. Cells that express the gene of interest become blue immediately. After a number of days, if these cells possess multi lineage differentiation capacity, different cell lineages that descend from the firstly marked cells are marked with blue.

In theory only one functional stem cell population should suffice to maintain tissue homeostasis. To date however, multiple genes that mark distinct progenitor cell populations are reported in the small intestinal epithelium of which the following three are representative: *Lgr5*$^{+ve}$ stem cells, *Bmi1*$^{+ve}$ stem cells and *mTert*$^{+ve}$ stem cells[24, 34-36]. Although *mTert*$^{+ve}$ cells and *Lgr5*$^{+ve}$ cells seem to belong to distinct populations, *Bmi1* mRNA was found in both *Lgr5*$^{+ve}$ and *mTert*$^{+ve}$ populations[35, 37]. The three populations differ in cellular kinetics and localization within crypts. The position at the ultimate base of the crypt is referred to as +0, and higher cells are counted according to location from the crypt base. *Lgr5*$^{+ve}$ cells are the cells located at the crypt base between Paneth cells (also referred to as crypt base columnar or CBC cells). These cells cycle daily and are located at the +0 position in the crypt, whereas *Bmi1*$^{+ve}$ cells cycle somewhat slower and are located primarily at the +4 position in the crypt. Although cells exist in both populations that do not express markers of the other population, these two populations reportedly give rise to one another[36, 38]. *mTert*$^{+ve}$ stem cells are located in only a proportion of crypts[35, 39]. Under normal circumstances these cells are localized primarily at the +5 position and they are largely non-cycling. Upon damaging insults, such as irradiation, CBC and +4 cells die off[40]. As a result, *mTert* cells enter the cell cycle and give rise to more rapid cycling stem cells such as *Lgr5*$^{+ve}$ CBC stem cells[19]. In other systems, such as the bone marrow and the skin, indeed two populations of stem cells exist of which one is fast cycling and a second one is slow cycling or quiescent[41-43]. Since cycling cells are affected by a number of damaging insults such as irradiation, a quiescent reserve population may be a helpful backup for regeneration upon these insults. Additionally, limiting the number of divisions of a stem cell may protect from accumulating mutations throughout life. Arguing against a backup population that limits maximal divisions during normal homeostasis, rapid cycling stem cells were shown to be capable of populating the epithelium for the lifetime of mice[44]. Whether this is also the case in humans, remains to be investigated.
During homeostasis, there is an overlap between CBC stem cells and +4 stem cells and between *mTert* stem cells and +4 stem cells[35, 37]. Thus, the +4 stem cell population seems to consist of an inhomogeneous cell-population. Furthermore, for other populations as well limited knowledge exists on the homogeneity within the population. Since in homeostatic conditions the crypt harbors multiple populations that all have certain levels of stemness (e.g. CBC and +4), it is likely that a hierarchy exists between these populations. Respective self-renewal capacity and multipotency of these populations as could be measured by the capacity to form organoids may shed light on the position of these cells in the stem cell hierarchy. Additionally, for all three populations, limited knowledge exists on the homogeneity within the population.

Currently, many challenges still exist in stem cell research. Two main fields of interest revolve around stem cell differentiation and regeneration: (1) identification of differentiating signals and (2) the possibility to form stem cells from more differentiated cells (de-differentiation).

Upon massive loss of stem cells, for example as a result of gamma irradiation, both CBC and +4 cells are lost[40] and repopulating epithelium may be descendent from a reserve population of stem cells that is quiescent (possibly *mTert*$^{+ve}$ cells) or possibly from differentiated cells that give rise to stem cells in the process of de-differentiation. It was recently shown that cells that have a more differentiated phenotype are capable of reverting to a stem cell phenotype upon stressful events such as gamma irradiation[45]. Additionally, activation of both Wnt-signaling and NFκB signaling in cells is capable of oncogenic transformation of differentiated cells into tumors, whereas under normal circumstances, tumors are solely derived from stem cells[46]. It is currently unknown what the contribution of dedifferentiation is to tissue regeneration and tumorigenesis in the absence of these mutations.

It has been postulated that increasing the distance between stem cells and the Wnt-source may be the single explanation for removal of cells from the stem cell pool and thus differentiation[17, 47]. Potentially however, signaling pathways that remove stem cells actively from the stem cell pool balance Wnt-signaling to maintain a constant stem cell number. An additional potential role for these pathways may be to protect the stem cell pool from damaged cells that could jeopardize long-term homeostasis by their decreased fitness. Identification of these pathways fuels our understanding of normal homeostasis and derailment during oncogenesis. Potentially, utilizing these pathways to differentiate cells may aid in therapy directed against cancer.

Translational control in the intestine

Stem cells are increasingly recognized as critical players during tissue regeneration and tumorigenesis. Whereas these processes are intricately regulated by many different cell types and molecular signals, the contribution of pathways that regulate the control over protein synthesis is increasingly acknowledged. Proteins constitute the largest proportion of cellular contents. One of the critical steps in protein synthesis is in the rate of translation of mRNA to protein. Regulation of translation is complex and relevant for both cellular homeostasis and disease.

Little is known about the role of translational control in the intestinal epithelium, but the connection between translation, proliferation and tumorigenesis, established in other cell types may be of equal interest in intestinal homeostasis. In the following section a number of components that govern translation and influence cellular proliferation or tumorigenesis are highlighted. One of the critical regulators of protein synthesis is mammalian target of rapamycin (mTOR). This protein kinase functions as a master regulator of translation, cell cycle progression, motility and transcription[48, 49]. The control over translation and cell cycle progression converge in the downstream activation of eIF4E[50]. This eukaryotic translation initiation factor is involved in directing ribosomes to the 5'cap structure on mRNAs. Inhibition of mTOR causes translation attenuation and cell cycle arrest[50]. However in the absence of 4E-BP proteins that normally inhibit eIF4E function, blocking mTOR does not effect either cell cycle progression or translation. Additionally, overexpression of eIF4E causes cell cycle progression and development of cancer,

implicating the importance of control over cap-dependent translation in both processes[51]. To achieve these effects, eIF4E co-operates with oncogenes of the MYC family of transcription factors[51].

In the intestinal epithelium, C-MYC is known for its effector role to cause proliferation downstream of the Wnt-signaling pathway[17, 52]. Interestingly, C-MYC deficient enterocytes have reduced translation and overexpression of C-MYC in B-cells causes increased translation[52, 53]. Translational effects, proliferation and oncogenic effects of C-MYC in B-cells could be rescued by haploinsufficiency of the ribosomal protein L24, thus linking MYC to proliferation and cancer through upregulation of translation[53]. The pro-proliferative and tumorigenic role of C-MYC may be the result of its capacity to directly increase components of the translational machinery[54]. But conclusive data linking the proliferative and oncogenic role of MYC in the intestine to its function in the regulation of translation has yet to be found.

The regulation of activity of translation initiation factor eIF2α introduces another level of complexity to translational regulation. eIF2α is part of a ternary complex directing met-tRNA to the 5'cap of mRNAs and phosphorylation of this subunit by one of four homologous kinases causes translation attenuation. One mode of eIF2α phosphorylation is by the PKR like, endoplasmic reticulum (ER) localized kinase (PERK)[55]. This kinase is a component of the unfolded protein response (UPR), a pathway that is activated upon accumulation of misfolded proteins in the ER. UPR activation results from accumulation of unfolded proteins inside the ER, which may be the result of various stimuli such as differentiation, inflammation, physical damage or DNA mutation[56-60]. The ER may therefore be regarded as a sensory organelle that detects cellular impairment, which in turn activates a signaling cascade. Activation of the UPR aims at restoring a balance inside the ER, but cell fate changes may occur along the way. For example, overexpression of the UPR transcription factor XBP1(s) was found to be a model for multiple myeloma[61], knockout of XBP1 in the intestinal epithelium causes hyperresponsiveness of enterocytes to bacterial antigens[57] and Perk-eIF2α signaling causes cell-cycle arrest[62].

Taken together, protein translation capacity likely plays a critical role in proliferation and tumorigenesis. It is tempting to speculate on the existence of two categories of mechanisms that influence protein translation: 1) factors that influence translational capacity, and 2) factors that limit translational output. Translational capacity of a cell is likely linked to structural and metabolical alterations, such as the size of the ER, the amount of nutrients and the number of ribosomes, factors that are all rate limiting. Examples of factors that can increase translational capacity are transcription factors C-MYC and XBP1, as well as the amount of eIF4E protein and possibly other eIFs. Factors that limit translational output may do this regardless of translational capacity available and interference may be rapid. They thus function more or less as an on-off switch that is superimposed on the translational capacity. Examples of such limiting factors may be phosphorylation of eIF2α and 4EBP factors.

Intestinal neoplasms

Development of colorectal cancer (CRC) is the third most prevalent cancer among men and women, to come only after lung cancer, prostate and breast cancer. Yearly, CRC is diagnosed in approximately 12.000 patients in the Netherlands of which almost half die as a consequence of this disease. Stratification of patients with CRC has generated a number of stronger and weaker risk factors for disease, fuelling clinical and experimental research on CRC. Two high risk populations have been identified. These include patients with inheritable CRC syndromes and patients that suffer from inflammatory bowel disease. CRC syndromes include a number of syndromes in which patients have germline mutations in genes that regulate differentiation (Smad4, Lkb1, Pten), proliferation (Apc) or the rate of acquiring additional mutations (Msh2, Mlh1 etc.). Patients with inflammatory bowel disease have an increased risk of CRC that correlates with colonic localization, severity, duration and extent of inflammation[63, 64]. The largest group of patients develops CRC spontaneously. In these patients a benign adenoma develops over the course of what may be well over ten years into an invading carcinoma[65]. These patients have been stratified and a number of risk factors were found of which male gender invariably exceeds others[66, 67].

Deregulation of pathways in adenomas and sporadic cancer

The development and maintenance of cancer is a process of leviathan complexity in which a high number of distinct cell types and signaling pathways participate. In a simplified scheme, CRC develops from colonic crypts following a chain of events that is known as the adenoma to carcinoma sequence[65]. Many signals influence this process either locally or from a distance. Colon tumors have many resemblances to the normal intestinal epithelium and the mutational landscape of CRC gives an image of important control mechanisms in normal homeostasis. Those pathways that are important for proliferation (Wnt-signaling, Tyrosine kinase signaling) are often mutated in such as fashion that they signal constitutively[68]. Those pathways involved in differentiation (Tgfβ, Bmp) are lost.

In the epithelium itself, a first step in the development of sporadic carcinomas is malignant transformation. At first, crypts that have acquired mutations become hyperproliferative and a so called aberrant crypt focus (ACF) may appear. Over time, a proportion of these ACFs may develop into adenomatous polyps[69, 70]. These are premalignant lesions that can occur through hyperactivation of the Wnt-signaling pathway. Mutations cause loss of proliferation regulation and normal differentiation is disturbed. A niche is created where subsequent mutations may occur. Additional mutations include activation mutations in K-RAS, inactivation of transforming growth factor signaling by loss of transcription factor Smad4 and eventually loss of tumor suppressor P53. All these mutations guide the transition from normal intestine, via ACF to early and late adenomas and eventually to cancer. Interestingly, many Wnt-target genes upregulated in

adenomas compared to healthy colon are downregulated when adenomas progress into CRC[71]. Thus whereas the adenoma stage is indispensable for subsequent development of CRC, signaling that is necessary for its development needs to be lost in order to form a less differentiated cancer. In the development of sporadic CRC stromal cells play a key role. The immune system participates either through other cell types or directly in malignant tissue itself. The adaptive immune system modulates cancer development as it seems to function as a surveillance system that recognizes and kills malignantly transformed cells. In contrast, the Innate immune system promotes tumor development. It may increase proliferation, promote vascularization or facilitate infiltration[72]. In the innate immune system, transcription factor NF-κB plays a critical role during tumorigenesis[73, 74]. Activation of NF-κB can occur trough a number of receptors of which the Toll like receptor family has received much attention. Toll like receptor signaling links pathogen- and damage associated molecular patterns (PAMPs and DAMPs) to downstream signaling by activation of NF-κB and mitogen activated protein (MAP) kinases[75]. Mice that have a truncating mutation in the *Apc* gene (termed $Apc^{Min/+}$ mice) develop multiple small intestinal adenomas[76]. This is dramatically reduced when crossed into animals that lack the TLR adaptor protein MyD88. Although TLR signaling is important in both immune cells and directly on the epithelium, the pro-tumorigenic effect seems to depend primarily on epithelial MyD88 signaling[77]. Although it has been demonstrated that signaling of PAMPs and DAMPs through MyD88 plays a critical role in development of sporadic intestinal tumors, the contribution of individual receptors that transduce these signals has received little attention.

Gender disparity in CRC

Differences between men and women in prevalence of CRC were described historically[78]. Whereas hormones were implicated to mediate these differences, a large number of confounding risk factors were proposed as well. These included factors of environmental origin (toxins at work, night shifts) and factors of habitual origin (smoking, drinking alcoholic beverages, eating red meat, etc.)[79-82]. A study initiated by the Women's Health Initiative (WHI) collaboration was the first to establish a possible role for female hormones in the protection from CRC[83]. This large randomized placebo controlled trial was set up to investigate risk and benefits of postmenopausal hormonal replacement therapy (HRT). Large groups of postmenopausal women were treated with placebo or with a combination of conjugated estrogen and the progestin medroxyprogesterone acetate (MPA) and these women were followed up for an average duration of 5.2 years. A surprising finding was the protective effect of estrogen and progestin combination therapy on the development of CRC which amounted to almost 40% compared to placebo after 5 years of follow up[84]. Further analysis pointed out that although the prevalence of CRC was lower in the HRT treated group, CRC-related mortality was equal in both groups since CRC was more advanced in HRT treated women. This last notion renders the protective effects of HRT difficult to interpret. Later, the WHI performed a large follow up study evaluating the use of conjugated

estrogen monotherapy in women in which the uterus had been removed. In this study, no protective effect on development of CRC was found[85]. These results argue a protective role for progestins in general or MPA in specific. This protective effect is conferred by the steroid alone or in combination with conjugated estradiol. Whereas estradiol was shown have pleiotropic effects in the development many cancers[86], the role of MPA or other progestins is less well understood. Allthough clearly, steroid hormones have profound effects on CRC development, the differences in CRC prevalence observed between men and women may still rely on habitual or environmental factors. Thus, whereas the protective effect of HRT seems to be beyond doubt, whether it is explanatory for the gender bias found in CRC development remains to be investigated.

Taken together, development of CRC is a complex process in which different cell types play a role. Next to oncogenic mutations in epithelial cells, innate and adaptive immune signaling and hormonal signals may each contribute to development of this disease.

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