Cellular protection mechanisms that minimise accumulation of mutations in intestinal tissue

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Summary

The epithelial lining of the intestine is constantly exposed to a hostile environment containing a mixture of gastric acids, consumed harmful substances and microbes. It is widely accepted that the intestine has multiple mechanisms to protect itself against tissue damage. Here, we review three cellular protection mechanisms that protect intestinal tissue against accumulation of somatic mutations: the conveyor belt-like structure, stem cell competition and crypt fusion. We highlight the events that can perturb these cellular protection mechanisms, and their impact on accumulation of new (oncogenic) mutations. Lastly, we review the potential of in-vitro and intravital microscopy techniques to study the dynamics of these protection processes. These studies may identify new targets that can be used to manipulate cellular protection mechanisms in such a way that accumulation of new mutations can be reduced. Importantly, reducing mutation accumulation has the potential to delay aging, and the initiation and progression of diseases such as colorectal cancer.

Key words: colorectal cancer, aging, mutations, cellular protection mechanism, stem cells, dedifferentiation, stem cell competition, crypt fission, crypt fusion, regeneration, intravital microscopy

Introduction

The lumen of the intestine is a hostile environment and the intestinal epithelium is constantly challenged by intrinsic and extrinsic factors, such as gastric acids, consumed harmful substances and pathogenic microbes. These challenges can result in damage, such as disruption of the epithelial lining and loss of stem cells. Under homeostatic conditions the intestine has an impressive capacity to protect itself against tissue damage. The very dynamic nature of the intestinal epithelium enables a fast regenerative response that can maintain epithelial integrity. Here we review how this dynamic nature of the intestinal epithelium also results in three cellular protection mechanisms that minimise the accumulation of new mutations in the intestine, thereby protecting against aging and development of colorectal cancer. In addition, we focus on challenges that can perturb these protection mechanisms.

Cellular protection mechanism no. 1: conveyor belt-like organisation

The vast majority of intestinal epithelial cells are short-lived

One way the intestine protects itself against the accumulation of mutations is by imposing a short lifetime on the vast majority of intestinal cells. This is a result of the morphology of the intestinal epithelium, which is a repetitive sheet of crypt-villus units (fig. 1) [1]. Intestinal stem cells that reside at the bottom of so-called crypts of Lieberkühn – little invaginations into the intestinal epithelium – fuel the fast turnover of the intestinal epithelium [1]. These stem cells give rise to progenitor cells in the transit amplifying compartment, located a bit higher up the crypt-villus-axis, that subsequently differentiate into all specialised lineages while traveling upwards along the villus in a conveyor belt-like fashion (fig. 1) [1]. The differentiated cells in the villus fulfil the physiological functions of the intestine, including nutrient uptake by enterocytes, hormone production by enteroendocrine cells and mucus production for protection and lubrication by goblet cells. Upon arrival at the tip of the villus, ~5 days after birth of the cells, differentiated cells are shed into the lumen [1]. Only these short-lived differentiated villus cells are exposed to the hazardous environment of the intestinal lumen [2]. Since they get shed into the lumen within a week, any genomic damage that occurs in these cells cannot manifest or be propagated. Of note, the colonic epithelium does not contain villi, but does function as a conveyor belt as differentiated cells are shed at the surface of the colonic epithelium.

Long-lived intestinal stem cells can accumulate new mutations

The small pool of long-lived stem cells that maintain the epithelium are positioned in the intestinal crypts, away from the lumen, which minimises possible harm to these cells (fig. 1) [2]. The fact that these multipotent stem cells can accommodate the fast turnover of the intestinal epithelium has been known for decades [3]. However, their ex-
act identity remained uncertain for a long time. Already in the early 1970s, Cheng and Leblond identified the proliferative crypt base columnar (CBC) cells residing at the bottom of the crypt interspersed between Paneth cells [4]. However, their functional role as stem cells was only confirmed relatively recently, after leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) was found to mark these CBC stem cells [5]. Lineage tracing experiments in which these cells are labelled with markers that are inherited by daughter cells showed that LGR5+ CBC cells give rise to all differentiated cell types present in the intestine and that they can do so over prolonged periods of time [5]. Moreover, when single LGR5+ cells are isolated and placed in defined culture conditions, they can form mini-guts (organoids) that contain crypt-villus units and harbour all intestinal cell types [6]. Together, these findings show that LGR5+ CBC cells are multipotent and have the capacity to self-renew, indicating that these cells are bona fide stem cells in the intestine.

Since the LGR5+ stem cells are long-lived, whereas the differentiated cells in the intestine only have a short lifetime, the LGR5+ stem cells are vulnerable to accumulation of mutations in, for example, cancer driver genes. In 2009, Barker et al. showed that LGR5+ stem cells can indeed function as cells-of-origin for intestinal adenoma [7, 8]. Mice developed intestinal adenomas only when an Apc mutation was introduced into the long-lived LGR5+ stem cells, but not when the same mutation was introduced into the short-lived differentiated cells. Thus, the fast turnover of the intestine and its conveyor-belt-like structure can protect against the accumulation of mutations in the vast majority of intestinal epithelial cells that are exposed to the hazardous environment of the lumen. Only a small pool of long-lived stem cells located at the bottom of shielded crypts can accumulate damage and mutations, and therefore have the potential to act as cells-of-origin for intestinal cancer.

The stem cell pool can be replenished upon tissue damage

Under homeostatic conditions, the chance to accumulate mutations depends on the lifetime of a cell, and this therefore happens, as discussed above, predominately in LGR5+ stem cells. However, a large body of work suggests the existence of a pool of “reserve” stem cells that are in a relative quiescent state and characterised by DNA label-retention [9] and the expression of Lrig1, Bmi1, mTert, Hopx and Mex3a [10–15]. Since many of these proposed mark-

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**Figure 1:** The cellular protection mechanisms against the accumulation of mutations. Cartoon of the crypt-villus units of the small intestine. Shown are the three protection mechanisms: 1. conveyor belt-like structure, 2. stem cell competition and 3. crypt fusion.
ers are enriched at the border of the stem cell niche, four cell diameters from the crypt bottom (+4 position), these cells are often referred to as +4 cells (fig. 1). However, the identity and even the very existence of quiescent “reserve” stem cells have been controversial and heavily debated [16]. It has been suggested that, instead of being dedicated reserve stem cells, +4 cells are progenitors that can repopulate the LGR5+ stem cell pool through dedifferentiation (for a review see [17]). This notion is supported by lineage tracing experiments that showed that damage can induce the reversion of committed progenitors to LGR5+ stem cells, including secretory progenitors [18, 19], enteroocyte precursors [20], Paneth cells [21] and a population of goblet cells [22, 23]. This dedifferentiation is, at least in part, mediated by chromatin remodelling, which can make genomic regions important for stemness more accessible [22]. The thought that there is no dedicated “reserve” stem cell pool is further strengthened by two independent studies that showed that a key marker of the reserve stem cell pool (Bmi1) actually marks mature enteroendocrine cells and that these cells can be recalled into the stem cell compartment upon damage [22, 23]. Other studies showed that the +4 markers do not mark a specific population of cells, but are expressed in cells throughout the crypt, including in LGR5+ stem cells [11, 13, 16, 24, 25]. These data indicate that, similar to mammary tissue [26], the identity, behaviour and fate of cells cannot always be linked to a single molecular profile or specific marker. Since cells are highly plastic and can gain or lose stem cell traits, it may be better to define a stem cell by its function than through markers.

**Tissue damage drives progenitor dedifferentiation and affects accumulation of mutations**

Regardless of the identity or state of stem cells, the current literature agrees on the existence of a pool of cells that has the potential to replenish LGR5+ stem cells upon tissue damage. Consequently, mutations that are acquired in cells just above the stem cell zone can persist when these cells revert back to a stem cell state, which prevents cells from being transported to and lost at the tip of the villus. The activation of NFκB signalling seems to be required for this dedifferentiation [27, 28]. An activating mutation in the proto-oncogene β- catenin in non-stem cells leads to adenoma formation only when NFκB signalling is simultaneously enhanced [27]. In addition, Apc mutations in Delta1+ tuft cells do not induce tumourigenesis under homeostatic conditions [29]. However, when NFκB signalling is enhanced by dextran sulphate sodium-induced colitis, APC loss in tuft cells does lead to intestinal tumours [28]. Thus, enhanced NFκB signalling may result in dedifferentiation of committed progenitors, thereby unmasking oncogenic events that can potentially lead to tumour initiation.

The route of cancer initiation via NFκB-induced dedifferentiation of committed progenitors may not be surprising. In colorectal cancers, and also pancreatic and gastric carcinomas, this pathway is often activated by inflammation or through mutations. NFκB signalling can, for instance, be enhanced by activating mutations in Kras, which occur in ~40% of all human colorectal cancers [30]. In mice, simultaneous induction of β-catenin and Kras mutations within differentiated villus cells induces the re-expression of stem cell markers, and leads to dedifferentiation and stem cell potential [27]. Moreover, in these mouse lesions are often formed at the villus regions, suggesting that WNT and KRAS-mediated dedifferentiation enables cells to function as cells-of-origin [27]. This phenomenon may explain the so-called “top-down” adenomas that are observed in the clinic, where adenomas on the surface of the colorectal lumen form on top of "normal" looking crypts [31]. However, it should be noted that these studies are predominantly performed in mouse models, in which whole populations of cells are transformed by oncogenic mutations. Future studies are required to indicate whether the route of dedifferentiation also occurs in human colorectal cancers, where adenomas arise from an oncogenic event in a single cell.

Taken together, the short life-time of the vast majority of cells is a strong cellular protection mechanism in intestinal tissue, since it results in loss of most newly acquired mutations. Therefore, mutations can only manifest in long-lived stem cells or in more differentiated cells that dedifferentiate into cells with stem cell characteristics that live long enough to induce a tumour.

**Cellular protection mechanism no. 2: stem cell competition**

**Most stem cells are lost over time as a result of stem cell competition**

Although the geometry of the crypt-villus unit protects long-lived stem cells better than short-lived differentiated cells against harmful substances present in the intestine [2], new mutations can be introduced in the stem cells upon proliferation. Fortunately, not all mutations that arise in stem cells are propagated as a result of a second protection mechanism present in the crypt: stem cell competition [32, 33]. The crypt contains ~14–16 LGR5+ stem cells interspersed with Paneth cells, which, together with the stroma, function as a niche for stem cell maintenance [34]. On average, LGR5+ stem cells proliferate symmetrically every 21.5 hours [35], and as soon as stem cells lose touch with a Paneth cell, they are primed for differentiation and move up along the crypt-villus axis (fig. 1). This process was illustrated by lineage-tracing experiments in LGR5-multicolour “confetti-mice” [32]. In these mice, injection of tamoxifen resulted in recombination of a confetti-construct, which stochastically induced expression of one of four confetti colours specifically in LGR5+ stem cells, and this colour was inherited by all daughter cells [32]. Despite induction of different confetti colours in different individual LGR5+ stem cells within the same crypt, most crypts contain stem cells of a single confetti colour after 1 to 6 months (fig. 1). These data led to the neutral drift stem cell competition model: upon each stem cell division, the number of stem cells exceeds the available positions within the niche, which is counterbalanced by loss of a stem cell from the niche. This constant stem cell division and stem cell loss leads to the expansion or extinction of stem cell clones and ultimately to clonality of all stem cells in the niche (fig. 1). By use of multi-day intravital microscopy, we directly visualised this process and showed that stem cells can be passively displaced from the stem cell niche after the division of proximate cells, implying that stem cell fate can be uncoupled from division [36]. Moreover, our experiments showed that all LGR5+ stem cells (~14–16) are able to contribute to the stem cell competition. However, cells at the border are more suscep-
Stem cell competition can eradicate mutant cells
The model of stem cell competition predicts that a mutation can remain in a crypt long term only when it is present in the one stem cell that wins the competition; mutations in the other stem cells will get lost. To test this, two independent studies were done using sporadic induction of oncogenic mutations often found in colorectal cancer (Apc, Kras or TP53) in combination with lineage tracing; they demonstrated that oncogenic clones can indeed get lost from the stem cell niche [39, 40]. However, the stem cell competition is not always completely neutral. For example, instead of having a 50% chance of displacing a neighbouring stem cell in neutral competition, Apc<sup>hm</sup> and Kras<sup>G12D</sup> mutations lead to a 62% and 78% chance, respectively, to outcompete a neighbour [39, 40]. Interestingly, a TP53 mutation does not affect stem cell competition under homeostatic conditions, but it gives a competitive advantage (58%) when colitis is induced [39]. Mutations can potentially also give a disadvantage in the competition. With use of time-lapse microscopy of organoids, it has recently been shown that Ras<sup>V12</sup>-transformed cells have an altered metabolism that promotes active extrusion of these cells from nontransformed epithelial tissues [41]. Obviously, if this also holds true for Ras<sup>V12</sup>-transformed intestinal stem cells, this mechanism decreases the strength of these cells in stem cell competition and therefore the ability of the Ras<sup>V12</sup> mutation to be maintained in intestinal epithelial tissues.

Even in a non-neutral competition, a stem cell that acquires a mutation is likely to be outcompeted by one of the ~15 wild-type stem cells and, as a consequence, this mutant cell will be expelled from the niche, and transported to and lost at the villus tip [39, 40]. Thus, acquisition of an oncogenic mutation may influence the fitness of a cell in the stem cell competition, but is not deterministic, and can be eradicated owing to stem cell competition.

Perturbation of niche factors alters the stem cell niche and stem cell competition
Stem cell competition can be quite accurately described by a relatively simple one-dimensional stochastic model based on only two parameters: the number of stem cells per crypt and the rate at which they are replaced by a neighbour and get lost [32, 33, 36, 37] (for review see Vermeulen and Snippert [17]). The number of stem cells determines the chance of an individual stem cell to win the competition, whereas both parameters determine the speed of the competition. These parameters are tightly controlled by the stem cell niche. The niche provides cues in order to accurately balance stem cell proliferation and differentiation, controlling the number of stem cells and therefore also the protection potential of stem cell competition [42]. The niche factors that control stem cell numbers are produced by Paneth and mesenchymal cells, and include Wnt ligands (e.g., Wnt3a), Notch ligands (Dll1, Dll4), bone morphogenetic protein (BMP) antagonists (e.g., Noggin and Gremlin) and epidermal growth factor (EGF) [34, 43–48]. Importantly, the stem cell zone, and therefore stem cell competition, is altered when these signals are perturbed. For example, blocking Delta-Notch signalling between stem and Paneth cells results in quick differentiation of stem and progenitor cells into postmitotic goblet cells [44, 49]. Moreover, inhibition of BMP signalling by Gremlin or Noggin leads to hyperproliferative crypts and the formation of ectopic crypts at the villus compartment [50, 51]. In addition, when Wnt signalling is reduced by manipulating Wnt proteins directly or by manipulating a regulator of Wnt signalling R-spondin, the number of stem cells is decreased [52]. As predicted by the one-dimensional stochastic model for stem cell competition described above, lineage tracing experiments showed that reducing the number of LGR5<sup>+</sup> stem cells results in faster stem cell competition, observed as accelerated drift of stem cells toward monoclonality [52]. Together these studies show that niche factors tightly control the number of stem cells and the composition of the crypt, thereby controlling stem cell competition and its ability to minimise the accumulation of new mutations.

Cellular protection mechanism no. 3: crypt fusion
Crypt fusion and fusion can influence stem cell dynamics
As a result of stem cell competition, stem cells within crypts become monoclonal over time. However, this is not a static situation: crypts can undergo fission and fusion (fig. 1). During crypt fission one crypt divides into two crypts [53], and this process mostly takes place during postnatal intestinal elongation and during regenerative responses [54–57]. In adulthood, crypt fission still occurs during homeostasis, although at lower levels [58–60]. Using intravitral microscopy, we recently uncovered crypt fusion, which seems to be an almost exact reverse phenomenon of crypt fission where two crypts fuse into one daughter crypt [61] (fig. 1). Under homeostatic conditions, crypt fusion and fission occur at near similar frequencies and on average a crypt should at least undergo a fission or fusion event every 3 months [58, 61]. Crypt fission has the potential to spread monoclonal mutant crypts over the epithelium, and has been shown to be a mechanism through which mutant cells can expand beyond crypt borders [62]. This spread creates fields of genetically altered crypts that can predispose a tissue for cancer development (field carcinisation) [63, 64]. In the human intestine, fields of KRAS-mutated crypts have been observed surrounding colorectal cancers, indicating that this can be an initiating event in cancer development [65, 66]. In addition, fields of APC-deficient crypts have been found, which may play an important role in adenoma formation and expansion [67–69]. Thus, crypt fusion can induce the spread of mutated cells over the epithelium, which may enhance tumour initiation.

In contrast to the spread of mutations via crypt fission, crypt fusion has the potential to eradicate mutations from the epithelium, since it enables stem cell competition to eradicate mutant cells even in the situation where all stem cells in a crypt contain a particular mutation. When a crypt...
containing mutant stem cells fuses with a wild-type crypt, the stem cell competition “restarts” and the mutant cells can be outcompeted by the wild-type cells (fig. 1). Therefore, crypt fusion may be a third important cellular protection mechanism against accumulation of mutations, and has the potential to counteract the spread of mutations by crypt fission. Since crypt fission and fusion significantly influence stem cell competition, it will be important to investigate the stem cell dynamics during crypt fission and fusion. Moreover, both processes should be incorporated into models describing stem cell competition in the intestine.

Tissue damage alters crypt dynamics
As mentioned before, crypt fission and fusion can influence stem cell competition. Because of its recent discovery, the molecular mechanisms underlying crypt fission are yet unknown, whereas more is known about crypt fission. For example, it has been found that crypt fission occurs more frequently in response to damage, including intestinal resection, irradiation and chemotherapy treatment [54, 55, 70, 71]. This response may (partly) function through the transforming growth factor-beta (TGFβ) signalling pathway, since loss of the receptor TGFβR2 significantly reduces crypt fission events [72]. Interestingly, increased crypt fission is also observed in diseased colonic epithelia from patients with Crohn’s disease and ulcerative colitis [73], which both induce an inflammatory response and lead to an increased risk of colorectal cancer. Thus, damage and inflammation induce crypt fission, which may be linked to tumour initiation. In addition to damage and inflammation, crypt fission may also be induced by genetic mutations. For example, in the mouse small intestine, the number of crypts monoclonal for the KRASG12D mutation can expand by crypt fission with an increased rate compared to wild type crypts (>30-fold), and this creates fields of KRASG12D mutated crypts [40]. As mentioned before, activating KRAS mutations can enhance NFκB signalling, which is associated with inflammation, again suggesting a link between inflammation and colorectal tumour initiation.

Future perspectives
In this review we have given an overview of the current knowledge about the cellular mechanisms present in the intestinal epithelium that minimise accumulation of new mutations, including the conveying belt-like structure, stem cell competition and crypt fission. Future research is required to reveal the exact dynamics of these processes and how each of them contributes to the protection against the accumulation of mutations. Interestingly, once we understand these processes in more detail, one could think about manipulating them to optimise their protective capacity. For example, inhibition of crypt fission might lead to decreased spread of new mutations over the epithelium. On the other hand, induction of crypt fission might result in the depletion of mutant crypts and a reduced spread of oncogenic mutations. In addition, expanding the number of stem cells per crypt might increase the chance that a mutant stem cell will be expelled from the niche and be depleted from the tissue.

Live imaging of the intestinal epithelium, such as organoid imaging and intravital microscopy, will greatly help in understanding the cellular protection mechanisms and in finding ways to manipulate them. In contrast to techniques that draw a static picture of the dynamic nature of intestinal tissues, live microscopy can be used to visualise intestinal tissues, cells and processes over time. Organoid imaging [6] will be instrumental in monitoring intestinal dynamics at subcellular resolution (e.g., [41, 74, 75]). However, it is important to realise that organoids – as any other 3D culture model – lack the in-vivo microenvironment, such as the surrounding stroma and immune cells. Recent advantages in high resolution intravital microscopy, and the development of a variety of imaging windows [76], enable the visualisation of the fate and behaviour of cells and lineages in living mice for several days [77–79]. We recently developed the abdominal imaging window [80, 81], which was used to study intestinal tissue homeostasis [36, 61] and intestinal tumour progression [82]. In addition, it enabled us to uncover new aspects of the intestinal cellular protection mechanisms, such as crypt fusion, which on static images cannot be discriminated from crypt fission [61]. In the future, live imaging technologies will be instrumental in understanding whether and how manipulating cellular protection mechanism affects tissue homeostasis and how this affects the fate of cells that have acquired mutations in for example cancer driver genes. We believe that manipulation of the cellular protection mechanism gives us the ability to reduce the accumulation of new mutations, which provides great potential to influence aging and the induction and progression of diseases such as cancer. With new microscopy techniques, we expect to make big steps in this direction in the near future.

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