MINIREVIEW

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Stem *vs* non-stem cell origin of colorectal cancer

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Colorectal cancer (CRC) is one of the most common cancers in the western world and is characterised by deregulation of the Wnt signalling pathway. Mutation of the adenomatous polyposis coli (*APC*) tumour suppressor gene, which encodes a protein that negatively regulates this pathway, occurs in almost 80% of CRC cases. The progression of this cancer from an early adenoma to carcinoma is accompanied by a well-characterised set of mutations including *KRAS*, *SMAD4* and *TP53*. Using elegant genetic models the current paradigm is that the intestinal stem cell is the origin of CRC. However, human histology and recent studies, showing marked plasticity within the intestinal epithelium, may point to other cells of origin. Here we will review these latest studies and place these in context to provide an up-to-date view of the cell of origin of CRC.

We first need to briefly introduce normal intestinal homeostasis and stem cells (for a recent in depth review see Vermeulen and Snippert, 2014).The intestinal epithelium has a remarkable capacity for self-renewal; every 4–5 days, the majority of the epithelial cells within the gut are replaced. The intestinal stem cells (ISCs), which are responsible for the epithelial renewal, reside at the bottom of the crypt (Figure 1). The immediate daughter cells of the stem cells proliferate for a finite number of times themselves before fully differentiating. These transit amplifying (TA) cells are the major producer of the epithelial cells and are situated directly above the stem cells (Figure 1A). In an intestinal crypt there are 5–16 ISCs per crypt and roughly 120–150 TA cells. Only few specialised cells (e.g., tuft cells, neuroendocrine cells and Paneth cells) live longer than the average 4–5 days. This rapid turnover of the intestinal epithelium led to the assumption that the long-lived ISCs are the most likely cell of origin for tumourigenesis.

PLASTICITY OF STEM CELL IN THE INTESTINE

The ISCs at the bottom of the crypt are defined by high Wnt activity, a characteristic they share with CRC tumour cells. In recent years, studies have shown that there is not just a single defined ISC pool in the intestine, but rather cells in the stem cell niche that can replace and compensate each other and therefore can also change their expression profile. There are at least two functional different ISCs, the crypt base columnar cells (CBCs) and the slow-proliferating label-retaining cells. With the discovery of the ISC marker LGR5, it was shown that these CBCs are the main pool of actively cycling ISCs (Barker *et al*, 2007).

Other ISC markers with a broader expression at the base of the crypt have been discovered that label more than the Lgr5 + ve cells, for example, *Lrig1* (Powell *et al*, 2012), *Hoxp1*, *Bmi1* and *mTert* (Montgomery *et al*, 2011). Although protein expression data have suggested these to be tightly located (i.e., at position +4), RNA expression data have placed *Hoxp1* and *Bmi1* relatively broadly at the base of the crypt (Sangiorgi and Capecchi, 2008; Muñoz *et al*, 2012).

Given that this set of markers are expressed in a wider range of cells at the base of the crypt helps to explain the studies that show that LGR5 + ve cells are dispensable for homeostasis of the intestine. Here LGR5 + ve cells were killed using diphtheria toxin, but the intestinal homeostasis was unperturbed (Tian *et al*, 2011). However, following radiation, the LGR5 + ve cell-depleted intestine was unable to recover (Metcalfe *et al*, 2014), showing that in certain circumstances the other cells in the stem cell niche are not able to compensate this loss.

As expression data have placed many ISC markers rather broadly at the base of the crypt, a recent study has taken a functional approach using *in vivo* imaging to define stem cell capacity at the base of the crypt. Ritsma *et al* (2014) showed there are about 16 LGR + ve cells in a crypt, some of which are located towards the centre of the crypt and others which are higher up, located at the border of the crypt base. Importantly, the probability of these centre and border stem cells to stay in the crypt over time and function as a stem cell was different. The 'central cells' were more likely to retain stem cell capacity compared with the 'border cells'.

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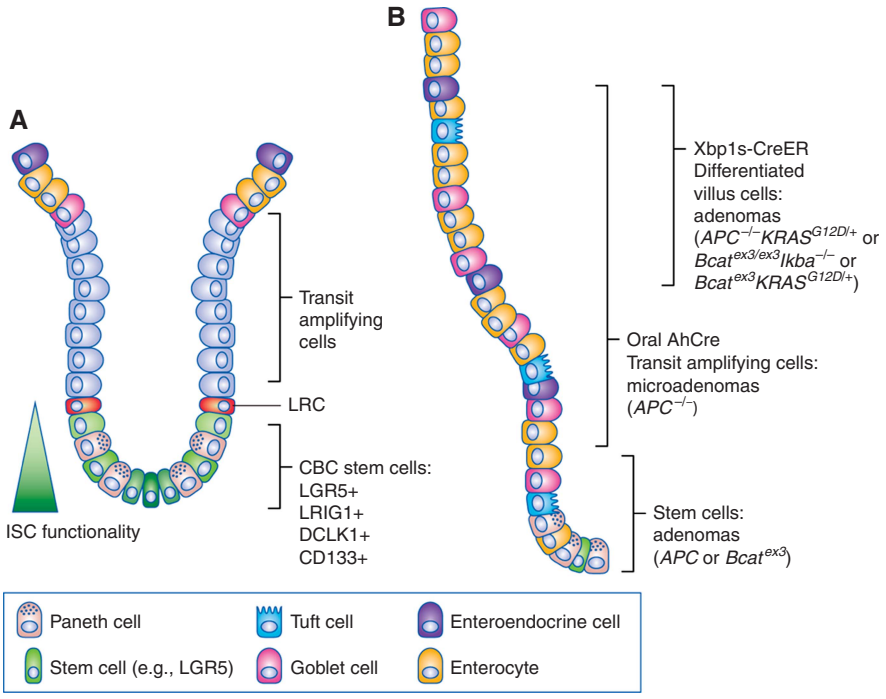

Figure 1. Methods to induce adenoma formation in stem cells and differentiated cells. (**A**) Overview of a crypt in the small intestine. The crypt base columnar (CBC) cells are located at the bottom of the crypt in between the Paneth cells. In addition to the expression of several stem cell markers, the functionality of the intestinal stem cell (ISC) is determined by its location, with the 'centre' ISC having the highest ISC functionality. (**B**) The expression of specific stem cell markers has been used to induce cre-driven recombination in ISCs, leading to adenoma formation, either by loss of *Apc* or $Bcat^{ex3}$ mutation. Oral administration of low-dose β-naphthoflavone has been used to induce *AhCre*-dependent *Apc* loss in cells above the crypt base, resulting in formation of microadenomas. Recombination in differentiated villus cells only resulted in adenomas when *Apc* loss or $Bcat^{ex3}$ mutation was combined with *Kras* mutation or NFKB activation (villus purification in $VilCre^{ER}$ mice *or* $Xbp1sCre^{ER}$).

This was not absolute as there is a constant transfer of cells between these two regions. Therefore the functionality of an ISC is defined by its position (as has been previously speculated (Sansom and Näthke, 2013)) rather than the expression of a specific protein marker.

Label-retaining studies using radioactive thymidine precursor (Potten *et al*, 1974) and recently by H2B-GFP/-YFP(Roth *et al*, 2012; Buczacki *et al*, 2013) lineage tracing have identified that there are a set of long-lived cells within the intestine that can act as stem cells as well and repopulate the intestine post damage. These cells express secretory markers and can be thought of as early progenitor cells, which fall back into the stem cell niche following damage and potentially dedifferentiate to develop full stem cell potential. In a complementary manner, cells that express Dll1 in the crypt mark secretory progenitors, which do not normally act as stem cells, but post damage can repopulate the crypt (Van Es *et al*, 2012).

Taken together these studies show that the normal intestine has great plasticity and non-stem cells could dedifferentiate to produce a stem cell-like phenotype, which is able to repopulate the intestine. This suggests that there may also be an increased repertoire of cells that could act as the cell of origin for colorectal cancer.

INITIATING MUTATIONS IN CRC

In 1990, it was postulated that CRC was caused by the accumulation of mutations that drive tumour initiation and then progression (Fearon and Vogelstein, 1990). Indeed, it was postulated that this process could take over 10 years (Beerenwinkel *et al*, 2007). Loss of the *APC* gene is the likely initiating mutation followed by additional mutations in *KRAS*, *TGFB*, *PI3 Kinase* and *TP53* signalling pathway components. It is interesting to note when these mutations are modelled in the murine intestinal epithelium, apart from *Apc* loss, the other mutations alone have very minor impact on intestinal homeostasis and only yield tumours at very long latencies (*Kras*, *Tp53*, *Pten*). However, loss of *Apc* leads to a rapid Wnt deregulation and acquisition of a crypt-progenitor cell phenotype in the small intestine and colon (Sansom *et al*, 2004).

ACQUISITION OF MUTATIONS

How do cells acquire mutations and what are the direct consequences? It is generally believed that the main source for mutations in the intestine is due to DNA replication errors and carcinogenic exposure. Given the high proliferation of the TA cells and the more restricted zone of proliferation of the ISCs, the TA cells are more prone to acquire a mutation. However, as long as the cell migration is not perturbed, these cells would be shed from the intestine within 3–4 days. Given the relatively mild phenotypes arising from mutation of most of the major tumour suppressors and oncogenes, a TA cell with a single mutation would be lost within a couple of days. The short lifespan of these proliferating cells reduces the risk of tumour initiation.

An additional mechanism to prevent accumulation of mutated cells in the crypt is the neutral drift of the ISCs. Until recently it was believed that ISCs divide by asymmetric cell division, which means an ISC gives rise to one TA cell and one ISC. Instead, the ISC division follows a principle of random replacement of ISCs, a process called 'neutral drift' (Lopez-Garcia *et al*, 2010; Snippert *et al*, 2010). This means that a single ISC in a crypt can be replaced by any of the other ISCs in the crypt. In a scenario with 5 functional ISCs, a marked wild-type stem cell has a 1/5 (20%) chance to populate the whole crypt and replace all the other ISCs.

In an elegant study by Vermeulen *et al* (2013) the consequences of oncogenic mutations on ISC fitness has been studied. If one ISC acquires a neutral mutation it has a high risk of being replaced by a normal stem cell within the crypt. The probability for an *Apc* mutation, for example, to become fixed, that is, to populate the whole crypt is 42% ($P_R = 0.62$, $N = 5$ stem cells), which means in the majority of cases the mutated cell will be replaced by one of its wild-type stem cell neighbours and will be consequently lost. The advantage of a *Kras* mutation ($Kras^{G12D}$) to populate the whole crypt is even higher (about 72%), which was confirmed in another study (Snippert *et al*, 2014). Although these studies have not taken into account the different positions of the tracked stem cell at start of the observation, it demonstrates the mutation itself has a major impact on the stem cell fitness.

Furthermore it shows that even if a stem cell acquires a mutation, there is a high chance that the cell will be lost, even if it has an advantage on the stem cell fitness. This might explain why CRC takes years to develop, even in patients with a genetic predisposition (germline $APC^{mut/+}$) to familiar adenomatous polyposis (FAP) disease.

STEM CELLS AS CELL OF ORIGIN

The discovery of expression markers for ISCs has enabled functional approaches to be used to test whether they can act as a cell of origin for intestinal tumourigenesis. Several studies (see Table 1) have shown that *Apc* deletion specifically in *Lgr5*+ (Barker *et al*, 2009), *Lrig*+ (Powell *et al*, 2012, 2014), *CD133/Prominin1* (Zhu *et al*, 2009) and other cells can provoke rapid adenoma formation. Bmi1+ve cells were also able to form small intestinal adenomas when an activating *β*-catenin mutation was targeted to these cells (Sangiorgi and Capecchi, 2008). Thus it appears that Wnt activation in the stem cells is sufficient for adenoma formation in the mouse. One important caveat is that most of these approaches have been limited to the generation of intestinal adenomas (most of which form in the small intestine).

NON-STEM CELL ROUTES

In contrast to this plethora of studies showing adenomas originating from stem cells, there is a more limited literature on tumours developing via non-stem cells routes in the mouse. This is in part owing to the rapid turnover of these cells (one would predict from Cre induction to gene and protein turnover about 48 h).

Table 1. Recent Studies

Targeted cells	Cre	Mutation	Tumourigenesis	Reference
Stem cells	Lgr5	$Apc^{-/-}$	Yes	(Barker *et al*, 2009)
	CD133 (Prom1)	$Ctnnb1^{ex3/+}$	Yes	(Zhu *et al*, 2009)
	Bmi1	$Ctnnb1^{ex3/+}$	Yes	(Sangiorgi and Capecchi, 2008)
	Dclk1	$Apc^{-/-}$	Only with inflammation	(Westphalen *et al*, 2014)
	Lrig1	$Apc^{+/-}$	Yes	(Powell *et al*, 2012)
Transit amplifying cells	AhCre (low oral dose)	$Apc^{-/-}$	Yes, but mainly microadenomas	(Barker *et al*, 2009)
	Xbp1	$Ctnnb1^{ex3/+}$	No	(Schwitalla *et al*, 2013)
Differentiated villus cells (via *in vitro* purification of villi cells)	$VilCre^{ER}$	$Apc^{-/-}$	No	(Schwitalla *et al*, 2013)
		$Apc^{-/-}$ $Kras^{G12D/+}$	Yes	

Our initial studies to delete *Apc* in the non-stem cell compartment using an oral dose of Cre inducer to spare the crypt stem cells led to the production of a number of small lesions that were retained within the intestinal epithelium. These however did not form tumours rapidly and even at 200 days post induction, many predominantly small lesions remained, though rarely one would progress to an adenoma. This was the first study to make a qualitative comparison between stem cells and TA cells as the cell of origin. Although the Lgr5+ve cells were much more efficient in adenoma formation, it is important to note that the mutated TA cells were not lost and the microscopic lesions found after 280 days were high in nuclear *β*-catenin (Barker *et al*, 2009) (Figure 1B).

Deregulation of Wnt signalling by loss of *Apc* in Dclk1+ tuft cells (that do not have stem cell characteristics (Nakanishi *et al*, 2012)) was not able to induce tumourigenesis. However, when these APC-deficient tuft cells where challenged with dextran sulphate sodium to induce colitis, even 3 months after induction, they formed colonic tumours (Westphalen *et al*, 2014). This suggests that Apc loss in a non-stem cell population could initiate tumourigenesis but would need extra events (e.g., inflammation) to progress to an adenoma.

These studies suggested that if cells acquire mutations and persist, additional events (e.g., mutations or inflammation) could then lead to adenoma progression.

The ability to 'dedifferentiate' and the link with pathways involved in inflammation was tested in more depth by Greten and colleagues. Activation of the NFKB pathway could provoke crypt-like structures in the villus and there was strong cooperation of Wnt pathway and NFKB pathway activation for tumour initiation. To test whether one of the reasons for increased tumour initiation was due to an expanded cell of origin population they used the Xbps1-CreER to target differentiated cells in the intestine. Xbps1 is an ER stress protein that is not expressed in stem cells of the intestine and thus allows recombination in more differentiated cells of the epithelium. Using this Cre, an activating *β*-catenin mutation was unable to transform the intestinal epithelium. However, when a *β*-catenin mutation was combined with increased NFKB signalling, tumours could be formed (Schwitalla *et al*, 2013).

Importantly, the same study showed that one of the consequences of a $Kras^{G12D}$ mutation in CRC was to activate NFKB signalling and hence *Kras* could also initiate tumourigenesis in differentiated cells when combined with Wnt pathway activation owing to loss of *Apc*.

Recently, it was shown that also changes in the intestinal microenvironment can initiate tumour formation in non-stem cells (Davis *et al*, 2014). Hereditary mixed polyposis syndrome (HMPS) is caused by aberrant expression of the bone morphogenetic protein (BMP) antagonist 'GREM1'. This BMP antagonist is usually expressed by mesenchymal cells, but owing to a gene duplication, in these patients, it is expressed by intestinal epithelial cells. The authors generated a mouse model with aberrant expression of GREM1 in the intestinal epithelium, which resulted in the formation of crypt structures in the differentiated villus compartment, similar to human HMPS. These crypt structures proliferated and were able to acquire additional mutations, which led to intestinal neoplasia.

Taken together, these studies showed that targeting Wnt pathway activation alone in non-stem cells was not sufficient to drive adenoma formation. However, additional mutations, activation of inflammatory pathways or changes in the microenvironment were able to increase the pool of cell of origin to non-stem cells.

'TOP-DOWN *VS* BOTTOM-UP' DEBATE

Thus, far this review has been restricted to discussion of small intestinal adenoma formation in mouse models, so what of the human CRC? Obviously the cre-lox lineage tracing experiments performed in mice cannot be done in humans, but naturally occurring methylation or mitochondrial mutations can be used to study the fate of stem cells in human samples. Already in 2001 Shibata and coworkers (Yatabe *et al*, 2001) indicated a model in which several stem cells per crypt are present and are constantly replaced. More recently Baker *et al* (2014) were able to study clonal advantage of stem cells in humans by tracking stem cells with unique somatic mitochondrial mutation. Here the authors confirm the neutral drift theory in humans and show that the number of functional stem cells in a human colonic crypt is similar to the number in mice (5–6).

However, the histology of human CRC and early lesions in patients with FAP had triggered a debate over the cell of origin of CRC. This was owing to the observation that dysplastic cells are mainly found at the luminal surface of the colon with normal crypt cells underneath. When the lab of Bert Vogelstein microdissected several spontaneous adenomas they found that only cells at the top of the crypt had mutations in *APC*, whereas the underlying crypts with their respective stem cells revealed no such mutations. This led to the so called 'top-down' model, where tumour initiation starts at the top of the crypt and then spreads laterally and consequently also downwards towards the normal crypt (Shih *et al*, 2001).

In contrast, it has been observed in patients with familial predispositions in the *APC* gene, the dysplastic cells often occupy entire single crypts (monocryptal adenoma), which can also be found, but rarely, in spontaneous colorectal adenomas. This 'bottom-up' model would predict that the stem cell at the bottom of the crypt is the cell of tumour initiation and populates the entire crypt (Preston *et al*, 2003).

UNANSWERED POINTS/CONSIDERATION

After the lessons from the induced pluripotent stem cells, it is perhaps unsurprising that differentiated cells in the intestine could form a tumour if given enough oncogenic events. The key question is, could it happen in patients? From the discussion of the rapid turnover of the intestinal epithelium, this already suggests a hierarchy of cells that would be able to be transformed. The likelihood of acquiring a mutation and giving rise to a phenotype in a cell that is differentiated is unlikely due to its stop in proliferation and short lifespan. However, a TA or a progenitor cell, which is about to undergo a number of cell divisions, would dilute out any remaining protein after mutation of the gene. If the mutation conferred a selective advantage that allowed a cell and its daughter cells to persist, then this could result in a potential cell of origin for cancer.

However, for a cell to gain three mutations, for example, biallelic loss of *APC* and a *KRAS* mutation would be highly unlikely. Instead one could imagine perhaps a model where an intestinal stem cell has a novel *APC* mutation which then due to drift (and an selective advantage) makes an entire crypt $APC^{mut/+}$ throughout. One could then envisage a second mutation in a daughter cell that persists to form a microadenoma. Further mutations such as *KRAS* mutation could then drive *bona fide* tumour formation.

Another possibility is that certain mutations (e.g., KRAS or BRAF) would select for mutations in an inflammatory environment via a non-APC route of tumourigenesis (e.g., traditional serrated adenomas). These mutations could occur in long-lived differentiated cells and give rise to adenomas owing to a changed microenvironment.

One question still unclear is, what would be the clinical implications of a stem cell *vs* a non-stem cell route for carcinogenesis? The mouse studies would suggest that a stem cell would need fewer mutations to form an adenoma and then would grow more rapidly. This might suggest that these tumours may acquire fewer mutations than ones in non-stem cells and therefore they may be more sensitive to treatment. It is interesting to note that the Hanahan lab identified signatures from human CRCs that could be compared with stem cells, TA cells and more differentiated cells, suggestive that tumours might keep the features of the cells from which they arose (Sadanandam *et al*, 2013). Although only a low number of patients were used there was some suggestion of potential prognostic and predictive potential of these signatures.

CONCLUSION

Overall, we show that the intestinal stem cells are defined by great plasticity. Wnt activation in the ISCs shows that they are very potent in initiating adenoma formation. The TA cells are also able to form adenomas, but not as potent as the stem cells. In addition, it became evident that even differentiated cells are able to initiate tumourigenesis but require additional events (i.e., mutations, inflammation or changes in the microenvironment). Therefore, we would conclude that the ISCs are the most potent cells for transformation, but certainly not the only possible cells of origin for colorectal cancer.

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