Combined Changes in Wnt Signalling Response and Contact Inhibition Induce Altered Proliferation in Radiation Treated Intestinal Crypts

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Running Head
Modelling Proliferation in Irradiated Crypts

Highlight Summary
A systems-level analysis, utilising experiments and computational modelling, reveals how mechanisms that control cell dynamics in healthy crypts are altered to produce pre-cancerous changes and initiate tumours.

Competing Financial Interests
The authors declare no competing financial interests.

Abstract
Curative intervention is possible if colorectal cancer is identified early, underscoring the need to detect earliest stages of malignant transformation. A candidate biomarker is the expanded proliferative zone observed in crypts prior to adenoma formation, also found in irradiated crypts. However, the underlying mechanism is not known. Wnt signalling is a key regulator of proliferation and elevated Wnt signalling is implicated in cancer. Nonetheless, how cells differentiate Wnt signals of varying strengths is not understood. We use computational modelling to compare alternative hypotheses about how Wnt signalling and contact inhibition affect proliferation. Directly comparing simulations with published experimental data revealed that the model that best reproduces proliferation patterns in normal crypts stipulates that proliferative fate and cell cycle duration is set.
by the Wnt stimulus experienced at birth. The model also showed that the broadened proliferation zone induced by tumourigenic radiation can be attributed to cells responding to lower Wnt concentrations and divide at smaller volumes. Applying the model to data from irradiated crypts after an extended recovery period permitted deductions about the extent of the initial insult. Applying computational modelling to experimental data revealed how mechanisms that control cell dynamics are altered at the earliest stages of carcinogenesis.

Introduction

The intestinal crypts of Lieberkühn are closely-packed, test-tube shaped invaginations that cover the surface of the intestine. Crypts are lined with a monolayer of epithelial cells arranged in a proliferative hierarchy (Figure 1a), and house stem cells that are responsible for the rapid and constant renewal of the intestinal surface. The stem cell compartment is positioned at the crypt base, comprising slow-cycling stem cells interspersed between Paneth cells in the small intestine, and related secretory cells in the colon (Sato et al., 2011). Stem cells produce multipotent progenitor cells that migrate upwards and differentiate to produce both absorptive and secretory cells. In the large intestine, once cells reach the crypt collar they lose contact with the underlying basement membrane, extrude and undergo apoptosis (Watson et al., 2009; Eisenhoffer et al., 2012); in the small intestine, they continue to migrate towards the tip of the villus, a finger-like projection that is connected to at least six surrounding crypts, before they are extruded.

Several signalling pathways contribute to the normal regulation of these processes. A decreasing concentration gradient of Wnt signalling factors, produced by Paneth cells and the mesenchymal cells surrounding the stem cell compartment, regulates cell proliferation along the crypt-villus axis (Gaspar and Fodde, 2004). Stem cells are positioned closest to the source of Wnt ligands, which diffuse upwards, so that Wnt exposure decreases for transit-amplifying and differentiated cells, triggering growth arrest and differentiation. Another important signalling pathway involved in the
maintenance of proliferating cells is Notch (Fre et al., 2005). Notch proteins and their receptors are active at the crypt base in the stem cell niche (Crosnier et al., 2006). The combination of these and other signals coordinate maintenance of the stem and transit-amplifying cell populations and also direct binary cell fate decisions between secretory or absorptive lineages (Riccio et al., 2008).

The coordinated program of cell division, migration, differentiation and death/exfoliation ensures that the epithelial monolayer is completely renewed every few days. Furthermore, the regular clearance of cells from the epithelium ensures that cells carrying transforming mutations, which can occur frequently in this highly proliferative environment, do not remain sufficiently long enough to disrupt homeostasis. Nonetheless, colorectal cancer (CRC) is a common human disease. Tumours originate in crypts and are usually initiated by inactivation or mutation of the adenomatous polyposis coli (Apc) gene (Fodde and Brabletz, 2007; Humphries and Wright, 2008). Heterozygous germline mutation in Apc is responsible for the heritable condition Familial Adenomatous Polyposis (FAP), which causes patients to develop numerous benign polyps in their gut lumen. These polyps typically progress to CRC, and FAP patients present with CRC earlier than sporadic cases (Alberts et al., 2002; Boman et al., 2004). The mechanisms responsible for the cancerous changes induced by Apc mutations involve its role as a scaffold protein in the β-catenin destruction complex: loss of the wild type APC protein activates the canonical Wnt pathway by stabilising β-catenin. In addition, loss of APC also directly causes defects in cell migration and adhesion, due to the stabilising effects of APC on cytoskeletal proteins, including F-actin and microtubules (Näthke, 2006).

Curative intervention is possible if CRC is identified early, which makes biomarkers that permit the detection of early stages of tumour development an important tool for the community. One characteristic of adenomatous polyps is an expansion of the proliferative zone in crypts. In healthy crypts, the mitotic distribution peaks at positions 40-50% along the long crypt axis (Wright and Alison, 1984; Trani et al., 2014). However, in hyperplastic and adenomatous crypts, this distribution
broadens, such that mitotic cells are more evenly distributed along the entire crypt length (Figure 2, first and second columns) (Wiebecke et al., 1974; Wong et al., 2002; Fatehullah et al., 2015). Interestingly, the same effect has been observed in crypts following radiation treatment with both high-energy $^{56}$Fe ions and gamma rays, but before the formation of adenomas (Trani et al., 2014). Such radiation treatment initially leads to increased proliferation in surviving crypts – a regenerative response that replaces cells that were killed (Wright and Alison, 1984). Immediately following radiation, tissue is highly disorganised (François et al., 2013) making it difficult to investigate any change in cell size or dynamic behaviour until tissue structure is restored, within two weeks for non-lethal irradiation (Potten, 1990). However, as shown in Figure 2 (third column), the broadening of the mitotic zone persists and is still detectable 90 days after irradiation, suggesting that mutated cells generated as a result of radiation have persisted. This provides a useful biological model to determine how radiation causes changes in the distribution of mitotic cells along surviving crypts and how this leads to tumour formation. In our investigations, we concentrate on the period up to three months after the initial regenerative response, when tissue organisation is restored.

It is well established that Wnt signalling is a key regulator of proliferation, but exactly how cells ‘interpret’ Wnt signals of different strengths to decide different fates is not well understood. In addition, although increased levels of Wnt signalling resulting from mutations in Apc or β-catenin is clearly implicated in CRC, details about the relationship between how much Wnt signal strength is required to affect specific cellular processes are not clear. Similarly, it is not known how subtle changes in Wnt signalling can contribute to early tumourigenesis. The broadening of the mitotic distribution, together with the known contribution of Wnt signalling to proliferation, is the focus of the investigations presented here. Specifically, using computational modelling we determine the cellular behaviours that account for the measurable changes in the distribution of mitotic cells in response to gamma radiation. We explore different hypotheses for the effect of Wnt signalling and altered contact inhibition on proliferation.
Using experimental data that shows the altered distribution of dividing cells in intestinal crypts following irradiation, prior to tumour development, we compare different hypotheses about factors that govern proliferation in this epithelial tissue. Each hypothesis is reflected in a unique model of division and is applied to a three-dimensional (3D) computational crypt model with a geometry constructed from 3D image data of small intestinal crypts (Appleton et al., 2009) (Figs. 1b, c and Figure S7). First, we use parameter fitting techniques to identify the model that most accurately produces the pattern of division in untreated and irradiated crypts post-recovery. Subsequently we compare these two cases to identify the cause for the shift in the distribution of mitotic cells following radiation treatment. Finally, we examine crypts after an extended recovery period following irradiation to determine how many 'radiation-damaged' cells and/or crypts, defined as 'mutant', remain.

We find that in the optimal model for cell division, cell-cycle duration is Wnt-dependent, and proliferative status – whether a cell stays in cycle or exits – is determined by the Wnt concentration it experiences at division. Moreover, we find that the broadening of the proliferative zone along the crypt axis following irradiation is due to a lowering of the threshold of Wnt required for cells to proliferate and/or a loss of contact inhibition, such that cells can withstand greater compression and divide at smaller sizes. Combining these effects gives the optimal fit. Thus, the effects of radiation on proliferation can be explained by changes in both cell cycle control and contact inhibition. After a three month recovery period, our simulations predict that a heterogeneous population of crypts will exist, consisting of individual monoclonal crypts that contain either healthy or mutated cells. Notably, in our models we exclude changes in mechanical properties, suggesting that changes induced by radiation can be explained solely by differences in the response of cells to Wnt and to compression.
Results

Experimental Data

We consider the experimental data generated and published by (Trani et al., 2014), reproduced here for convenience. These data were generated in mice heterozygous for a low penetrance Apc mutation (Apc$^{1638N/+}$). Unlike in animals with the more penetrant Apc$^{Min/+}$ genotype, the distribution of mitotic cells in crypts in control (untreated) Apc$^{1638N/+}$ mice is indistinguishable from that in wild type animals. At least four male mice were exposed at 6-8 weeks of age with 4Gy of whole-body gamma radiation. Their intestinal tissue was used to prepare 3D images that were then analysed. Twenty jejunal crypts per mouse were selected randomly in 3D images and the position of mitotic cells measured relative to total crypt length following a 48 hour and a 3 month recovery period. The raw data is shown as a bar histogram in Figure 2.

For comparison with computational simulations, we applied a nonparametric kernel-smoothing algorithm to these data. This generates a distribution (Figure 2, blue curve) that represents the pattern of mitotic events in a crypt, smoothing out the noise present in the original data, which is due to the relatively small number of observations. We used the Matlab routine ‘fitdist’ with a Gaussian kernel, using the default bandwidth (theoretically optimal for estimating densities for the normal distribution). The ‘smoothed data’ is used in the following for parameter fitting.

Computational Models

In silico experiments are conducted on a 3D lattice-free, agent-based crypt model (Materials and Methods). The geometry of the in silico crypt is defined by the dimensions of healthy small intestinal crypts from the jejunum of a 6 week old male WT mouse (Table S1, Figure S7). Cells are represented by deformable spheres constrained to lie on this surface. They exert forces on one another, calculated using a log-exponential law. Cell death occurs at the crypt collar, above a threshold height. Model parameters are summarised in Table S2.
Within this 3D framework, we seek to identify a model of cell proliferation that most accurately predicts the distribution of mitotic cells observed in control (un-irradiated) crypts. This model should also inform us of the changes responsible for the alterations observed in irradiated tissue post-recovery, preceding tumour formation (Figure 2, first and second columns). We compare six models that vary in the rules governing proliferative status and cell cycle duration. For comparison we include models previously applied to the study of crypt dynamics (Meineke et al., 2001; van Leeuwen et al., 2009; Osborne et al., 2010; Buske et al., 2011a; Dunn et al., 2012b).

We first consider a simple Pedigree model that assumes a generation-based approach to cell division: each proliferative cell undergoes a fixed integer number of symmetric divisions before terminally differentiating. In all cells, the length of the cell cycle is uniformly distributed, $\mathcal{U}[10,14]$ hours. From here on we refer to this as Model 1. The second model (Model 2) is an extension to the first and includes a longer cell cycle duration for stem cells, $\mathcal{U}[22,26]$, which are included as a distinct population from the transit amplifying cells. This model was implemented in the first cell-centre model of a 2D cylindrical crypt and we include it here to permit comparison (Meineke et al., 2001).

The third model is also based on previous work (Gregorieff and Clevers, 2005). It assumes a linear, decreasing gradient of Wnt along the crypt axis that is normalised to 1 at the crypt base and 0 at the crypt collar (Figure 1a) (Gregorieff and Clevers, 2005). A Wnt concentration threshold is defined, such that above this threshold Wnt stimulates a full Wnt response. In this model, a cell remains proliferative only if it resides in the region with concentrations of Wnt above the threshold. Similar to Models 1 and 2, we compare two scenarios (corresponding to Models 3 and 4 respectively): with uniform cell cycle durations ($\mathcal{U}[10,14]$ hours) for all proliferative cells; or Wnt-dependent cell cycle duration (so that the length of the cell cycle is proportional to the Wnt stimulus a cell receives).
the latter case, cells located towards the base of the crypt where stem cells reside have a longer cell cycle than those positioned further upwards. The cell cycle duration is $U[22, 36]$ hours for cells at the crypt base, and $U[10, 14]$ hours at the position where the Wnt threshold is reached. Cell cycle length decreases linearly between these two spatial limits.

A limitation of Models 3 and 4 is that loss of the Wnt stimulus (exposure to concentrations lower than the threshold) causes a cell to differentiate immediately. Such an abrupt exit from the cell cycle as a cell moves to a region of sub-threshold Wnt is biologically unrealistic, as it permits a cell to abandon the cell cycle at any point. Despite this limitation, these models are evaluated here to allow a direct comparison to previously described computational crypt models that implement this assumption (e.g. (van Leeuwen et al., 2009; Osborne et al., 2010; Buske et al., 2011b; Dunn et al., 2012b; Mirams et al., 2012)).

To overcome the limitations of Models 3 and 4 we define a new model, which stipulates that proliferative status be assigned at birth, depending on whether a newly born cell receives a sufficiently high Wnt signal to remain in cycle. Unlike Models 3 and 4, in this scenario proliferative cells will always complete the cell cycle, even if they move into a region of Wnt that is below the threshold. By coupling either uniform cell cycle duration (regardless of spatial location) or Wnt-dependent cell cycle duration (as in Models 3 and 4) to this Wnt response we define two new models, Models 5 and 6.

A summary of these six models is provided in Table 1. To evaluate and compare them, we sweep across the range of possible proliferation parameters and identify the optimal values for each model by comparing them to data acquired in tissue.
We also examine the effect of density-dependent inhibition of proliferation: in each model compression of a cell by its neighbours beyond a set amount will prevent it attaining a threshold volume and halt progression through the cell cycle (Nurse, 1985; Gao, 1997; Dietrich et al., 2002). The cell will undergo mitosis once it has attained the threshold volume (Küppers et al., 2010; Dunn et al., 2012b; Leontieva et al., 2014). We sweep across possible threshold volumes normalised to the maximum cell volume.

**Optimal Model of Wnt Response**

We seek to identify the model and parameters that most accurately reproduce the distribution of mitotic cells observed in crypts from control and irradiated mice (Figure 2, first and second column). We conduct a two-dimensional (2D) parameter sweep for each model, varying both the Wnt concentration threshold and the volume threshold for contact inhibition. For Models 1 and 2, we represent the response to a varying Wnt concentration threshold by sweeping over the average number of generations that mitotic cells are allocated before differentiating (Materials and Methods). For each parameter set, we grow an in silico crypt to a homeostatic steady state and then simulate for a further 1000 hours, with the position of each mitotic event in this 1000 hour window recorded.

We compare the histograms that describe the mitotic distribution for each of the models with the smoothed mitotic distribution derived from experimental measurements (Figure 2, second row). This permits comparison of the error in each of the 'bins' (using equal width for the simulated and experimental data), which corresponds to the difference between simulated and experimental results. Here we use the sum of squares of the differences in each bin to define the objective function for comparison.
An example of such a sweep for Model 6 is shown in Figure 3. The threshold volume for contact inhibition varies along the x-axis, while the Wnt concentration threshold varies along the y-axis. Note that we omit to show the case for a volume threshold or Wnt threshold of 1, as in either of these scenarios proliferation will not occur. The set of parameters that minimise the objective function when compared with the experimental data for the control mice (Figure 2, first column) are shown in blue, corresponding to a volume threshold of 0.9 and a Wnt concentration threshold of 0.6. Thus, in this model, cells that are smaller than 90% of the maximum volume will not undergo division, and only cells born in the lower 40% of the crypt will be proliferative.

Similarly, we identify the parameters for each division model that produce the best fit for the smoothed mitotic distribution observed in irradiated mice (Figure 2, second column), using the assumption that all proliferating cells will have been affected by irradiation. For Model 6, a volume threshold of 0.6 and a Wnt concentration threshold of 0.5 produce the closest match to the experimental data (Figure 3, red). Compared to the parameters identified for the control case, this means that a cell can divide at a smaller volume, and when experiencing a lower Wnt concentration. This produces a broadened proliferative zone, with cells dividing higher up the crypt.

Parameter sweeps were conducted for each of the six models (Figs. 3, S1-5). For both scenarios – control and irradiated tissue - Model 6 gave the smallest error between experimental and simulated data using the optimal parameters described above. The variation in error between the simulated and experimental data across the parameter domain is shown in Figure 4 for all six models evaluated (see also Table 2). To facilitate direct comparison of the best fitting parameters in each case, in each subplot, the blue circle identifies the parameter set that most closely matches the experimental data for untreated crypts, and the red circle for the irradiated crypts.
Based on these results, we can deduce the changes in contact inhibition and proliferation that are required to produce the altered mitotic distribution following irradiation. We observe that in all models, except Model 2, the threshold volume for contact inhibition decreases, and the Wnt concentration threshold decreases, or both. These results are also reflected by the increase in the mean and standard deviation of the simulated positions of mitotic cells in irradiated compared to control crypts (Table S3), consistent with the changes observed experimentally. The simulated distribution of mitotic cells in Model 2 does not follow this trend (i.e. the mean height decreases), suggesting that it is not an appropriate model to describe changes in proliferation in crypts.

Our result that cells in irradiated crypts have a lower threshold for contact inhibition predicts that cells will divide at smaller volumes. Consistently, for each model the average cell volume in simulated crypts is smaller for irradiated crypts compared to controls by 1-10% (Table S4).

Overall, results from our simulations show that broadening of the distribution of mitotic cells in pre-cancerous conditions – in this case modelled by observations from crypts exposed to gamma irradiation – can be caused by a lowered threshold for proliferating cells to respond to Wnt signals, or the ability of such cells to divide at smaller volumes, or a combination of both. We refer to these cells collectively as ‘mutant’ cells. Thus far, we have assumed a scenario in which all proliferating cells are altered by irradiation. In reality, this is unlikely, and therefore to reproduce the shift in mitotic distribution, the characteristics we have identified for mutant cells would be more dominant, i.e. more exaggerated, in the more realistically occurring, smaller number of mutant cells. Nonetheless, we can extrapolate from these results to predict that cells colonising the epithelium after irradiation carry mutations in genes contributing to Wnt responses and cell size.
Mutant Colonisation of the Crypt

Crypts usually are clonal (Ponder et al., 1985; Bjerknes and Cheng, 1999). This means we can ask whether the altered cell properties of mutant cells we have identified are sufficient to ensure dominance of a mutant population over healthy epithelial cells. Using Model 6, which most accurately reproduces experimental data, we determined the probability of a crypt with an initially heterogeneous population of control and mutant epithelial cells to become colonised solely by mutant cells. We compare the scenario in which mutant cells adopt the parameters identified for the 48hr gamma irradiated data (Figure 3, red) with the control case, in which mutant cells are identical to untreated, healthy cells (Figure 3, blue). For each initial proportion of mutant cells we performed 500 simulations, and tracked the probability that mutant cells colonise the entire crypt in each case (Figure 5a). Note that we assume that the mechanical properties of mutant and healthy cells remain identical.

The blue curve in Figure 5a shows that, if assigned the same proliferative properties as control cells, the probability that mutant cells colonise a crypt is equal to the initial proportion of mutant cells, as expected. Comparing the blue and red curves shows that when assigned the properties identified from the 48hr gamma irradiated data (Figure 3, red), mutant cells are more effective at colonising the crypt than control cells, despite identical mechanical properties. Moreover, if the radiation insult affected 40% or more of proliferating cells, this is sufficient to guarantee complete conversion of a crypt to mutant cells. Under these conditions, 499 out of the 500 simulations were colonised by mutant cells, and therefore the 95% confidence interval for the probability of a crypt being taken over by non-mutant cells (when 40% of the initial cells were mutant) is (-0.0019, 0.0059). It follows that there is less than a 1% chance (at the 95% significance level) of a crypt initially comprised of 40% mutant cells to become colonised by non-mutant cells. If a lower percentage of cells were affected by radiation, either the mutant or healthy cells could take over. Figure 6b shows an example
of a simulated crypt that initially contained 10% mutant cells, which became colonised entirely by mutants after 400 hours.

Figure 5c shows the average time taken for either mutant or healthy cells to colonise a crypt, which decreases as the proportion of the corresponding cell-type increases. The longest time required to reach clonality is approximately 800 hours (33 days), and the mean time was 586 hours (24 days). That means in the tissue used by (Trani et al., 2014) to investigate the mitotic distribution in crypts three months post-irradiation, all crypts in the samples consisted of either healthy or mutant cells. Based on our simulation results, we predict that all crypts in the 3-month ‘recovered’ tissue were dominated solely by mutant cells if at least 40% of the proliferating cells were affected by irradiation. In contrast, we expect a mixed population of mutant and healthy crypts if the radiation affected no more than 40% of proliferating cells, as either cell type could 'win out'. That the tumour burden in the irradiated animals is ten times higher than in untreated controls (Trani et al., 2014) suggests that the number of mutant crypts was indeed high. However, since only macroscopically detectable tumours were scored it is impossible to know how many crypts were transformed.

Recovery Post Irradiation

Immediately after irradiation many cells in crypts die, and there is a rapid proliferative response to repair this injury that restores normal architecture by 2 weeks for the most severe non-lethal dose (Wright and Alison, 1984; Maj et al., 2003). The data showing an altered mitotic distribution even three months later is consistent with the idea that mutations persisted in some of the cells during the recovery period. Our models show that by this time, individual crypts are populated entirely by either such mutant or healthy cells (Figures 5a, c). Next, we used Model 6 to predict the composition of recovered tissue and asked, what is the proportion of crypts that are dominated entirely by mutant cells that most closely fits the experimental data?
We calculated the mitotic distribution for a mixed population of crypts using the individual distributions identified for single control and irradiated crypts (Figure 3, blue and red histograms). To calculate the distribution for a heterogeneous crypt population (e.g., 10% mutant crypts and 90% healthy crypts) we combined the distributions in the correct ratio (e.g., 0.1 times the mutant distribution with 0.9 times the healthy distribution). In this way we constructed a mixed population of crypts for all ratios of healthy to mutant, and compared the resulting mitotic distributions with the recovered data three months post-irradiation. Figure 6b shows that the error between the simulated data and the recovered experimental data is minimum for a population consisting of 64% mutant crypts and 36% control crypts. The histogram corresponding to this ratio is shown in Figure 6c.

Crucially, the error is one order of magnitude smaller than if we assumed a homogeneous population of crypts containing cells with unique proliferation parameters (i.e. if we derived new parameters following the approach used in Figure 3). This suggests that a mixed population of homogeneous crypts, individually containing either all control or all mutant cells, explains the experimental data much better than a homogeneous population of crypts. Further, we can infer that the initial burst of radiation produced mutations in no more than 40% of proliferative cells in each crypt.

Discussion

Cells within tissues receive instructive cues to regulate proliferation and differentiation. Changes in how this information is processed lie at the core of many diseases — particularly cancer — and can act as biomarkers for detection and therapy response. Concurrently, preceding full transformation, tissue aberrations are already detectable, such as the altered distribution of proliferating cells in pre-adenomatous intestinal crypts exposed to Gamma radiation (Trani et al., 2014). In addition to
providing potential biomarkers, such changes can reveal how mechanisms that control cell behaviour in healthy crypts are altered to initiate tumours.

Computational modelling is a powerful tool for testing hypotheses derived from experimental data. We describe a 3D computational model, geometrically-constrained according to the size, shape and composition of small intestinal crypts. Within this framework we compare six alternative hypotheses about the mechanisms that control cell division, which differ in how cells interpret Wnt signals to set their proliferative status and the duration of the cell cycle. We directly compare model simulations with experimental measurements to identify parameters that most accurately reproduce the situation in tissue. We focus on Wnt signalling as the major signalling pathway that regulates proliferation and differentiation in many tissues, particularly in intestinal crypts, where it is absolutely required. Changes in key proteins that regulate Wnt signalling are known to be key drivers of cancer in this tissue (Anastas and Moon, 2012; Polakis, 2012). Concurrently, we examine the role of density-dependent inhibition of mitosis to account for cell size. By sweeping over two parameters – a Wnt, or pedigree, threshold that defines when a cell is no longer proliferative, and a volume threshold that determines when the cell cycle pauses – we identify both the model and parameter set that most closely matches experimental data.

Our simulations reveal that the concentration of Wnt a cell experiences when it is generated by a division (i.e. when it is born) dictates its proliferative status. Furthermore, simulations suggest that cell cycle duration is proportional to Wnt stimulus, with cell cycle times decreasing linearly along the crypt axis. We predict the Wnt concentration threshold required to maintain cells in cycle such that cells residing in the lower 40% of the crypt receive sufficient Wnt to proliferate. We also find that, normally, the cell cycle will pause due to contact inhibition if cell volumes are less than 90% of equilibrium. Crucially, the same optimal model most closely recapitulates the mitotic distribution of gamma-irradiated crypts 48 hours post irradiation. In this precancerous situation, both the Wnt
concentration threshold and the threshold volume for division are lower than in the control. This means that cells proliferate at lower Wnt concentrations, and can divide despite not having reached normal size, which could happen under increased compression or because a checkpoint that links cell growth to mitotic entry is defective.

Mutations that stimulate Wnt signalling are common to almost all human tumours in intestinal tissue (Schneikert and Behrens, 2007; Polakis, 2012). Therefore, the finding that pre-cancerous cells are more sensitive to Wnt may appear inconsistent with elevated Wnt signalling in tumours (Anastas and Moon, 2012). However, an alternative way to interpret our results is that the cells act as if they perceive higher Wnt concentrations than they actually receive. Our models assume that Wnt ligands in the environment are unchanged, and parameter sweeps compare cellular response to external Wnt concentrations that vary spatially. Therefore in our simulations, a mutation that produces increased Wnt signalling in a cell is equivalent to a lowered Wnt stimulus threshold, making it appear as if a cell maintains its proliferative state at a lower Wnt concentration. Importantly, our modelling work supports the idea that mutations that cause or mimic increased Wnt signalling in addition to decreasing sensitivity to compression are sufficient to produce observable changes in proliferation patterns in pre-cancerous crypts, suggesting that even before overt tissue changes are in place, Wnt signalling is upregulated so that lower external Wnt concentrations can stimulate proliferation.

Similar considerations apply to the data and models for irradiated tissue. Radiation damage requires tissue repair, which involves up-regulation of Wnt signalling. Intestinal tissue damage causes local upregulation of Wnt5a to support tissue repair (Miyoshi et al., 2012). Further support for the idea that Wnt activation is involved in recovering from radiation-induced damage in crypts relates to the finding that lack of Mtg16 causes improved recovery of intestinal crypts and organoids from radiation damage (Poindexter et al., 2015). Mtg16 competes with beta-catenin for binding to (and thus activating) Tcf4 (Moore et al., 2008). In the absence of Mtg16 beta-catenin can activate Tcf4
more effectively (akin to increased Wnt signalling) and recovery from radiation is improved. In addition, radiation damage produces free oxygen radicals, which causes stabilisation of Hypoxia inducible factor alpha (HIF1a). Hif1a represses APC (Newton et al., 2010), which in turn activates Wnt target genes. Our finding that mutant cells respond to lower Wnt concentrations creates the same situation, and cells proliferate when they normally would not. However, in our model it is the concentration of Wnt required to stimulate proliferation that is lowered; in response to injury an increase in the locally available Wnt could have the same effect.

A novel distinction that our models can make is whether the proliferative state of a cell is decided when it is first produced by cell division, or whether it depends critically on (and thus varies with) its spatial position. Cells in the crypt move rapidly and can cover up to 50µM in 12 hours (Nelson et al., 2012). This means that the Wnt concentration cells experience could vary significantly between divisions. We find that the Wnt concentration a cell experiences is set when it emerges from a division, which means that the position where a cell is born is crucial for its fate. This is consistent with the emerging idea that differentiation signals are most effective in the G1 stage of the cell cycle, i.e. following mitosis (Dalton, 2015).

This behaviour is particularly relevant for stem cells, which reside at the crypt base. In the optimal model we identify, the high Wnt concentration at the crypt base induces a long cell cycle time (22-24 hours), such that stem cells can theoretically move a significant distance between birth and committing to the next cell cycle. However, stem cells may not move significantly unless they are positioned close to the stem cell niche boundary. This was recently suggested by lineage tracing experiments, which showed that the probability of a stem cell, or its progeny, to exit the stem cell zone is highest when positioned near the boundary between the stem cell niche and the transit amplifying compartment (Ritsma et al., 2014). Consistent with these data, our model shows that where a cell is born predicts whether it or its daughters will re-enter the cell cycle or differentiate.
Thus our models provide key insights into how signalling events in one compartment affect cellular behavior in another. This in turn explains the hierarchical organisation of the intestinal crypt and also the relationship between cell position and fate.

Our simulation results show that if radiation induces a conversion to a mutant state in at least 40% of proliferating cells, these mutants will have a sufficient growth advantage and can colonise an entire crypt, even without a change in mechanical properties. For lower proportions of mutant cells, non-mutant cells can also ‘win out’. Previous modelling work found that mutant cells needed to exhibit stronger adhesion to the substrate than healthy cells to colonise the crypt, however these investigations were constructed in a simpler, 2D geometry (Mirams et al., 2012). We predict the time for conversion to be short in either case, on average requiring fewer than 800 hours, consistent with idea that crypts are clonal (Ritsma et al., 2014). This leads to a situation with some crypts fully mutant and some fully non-mutant after a recovery period, with the ratio between these two types of crypts dependent on how many mutant cells were created by the initial insult.

We examined this situation in the context of such ‘recovered’ tissue that had been exposed to radiation and then was allowed to recover for three months before mitotic events were recorded. In this scenario, the mitotic distribution in the crypts remained broadened, suggesting that the tissue had permanently changed. We predict that the mitotic distribution in recovered tissue reflects division events in monoclonal crypts. Our analysis confirmed existence of two types of crypts: one comprised solely of mutant, the other solely of healthy cells, with a relative abundance of 64% and 36% respectively. Based on these numbers, we predict that the initial insult affected fewer than 40% of proliferating cells in each crypt.

Our result is consistent with the long time it takes for the development of fully transformed tissue. The fact that the altered mitotic distribution preceded tumour development is also consistent with
the idea that mutant crypts can expand and produce adenomas, and with the fact that additional mutations are required to develop the necessary growth advantage to fully transform and generate tumours (Fearon and Vogelstein, 1990). Overall the ability for radiation to induce initial tumorigenic changes is consistent with the delayed onset of colon cancer in patients receiving radiation therapy, and also professionals exposed to elevated radiation, such as astronauts (Chancellor et al., 2014).

Another important finding is that the response to contact inhibition is reduced after tumorigenic insult and that cells are smaller when they divide (Table 2). The complex relationship between cell cycle duration and cell size is affected by many different signalling pathways (Ginzberg et al., 2015), including Wnt, and our understanding of what governs mechanical properties of cell is just beginning to be understood. The limited data available suggest that cancer cells in situ are indeed softer, and more readily compressed than healthy cells consistent with our finding that mutant cells are smaller. However, to date, high resolution direct measurements of mechanical properties of cancer and healthy cells in situ are available only in the context of breast tissue (Plodinec et al., 2012). Further elucidating the relationships between different signalling pathways, mechanical properties and cell size requires the ability to measure cell size accurately, which is non-trivial in whole tissue. This is further complicated by the fact that the maximum volume change we predict is only 10% (Table S4). Using tissue sections to measure cell size only allows 2D measurement. If we assume that size changes result from a decrease in diameter rather than height, the maximum measurable change would be a 5% reduction in cell diameter. The curvature of crypts together with the natural variability of cell packing makes it unlikely that such a small difference can be measured reliably. An alternative, more sensitive approach is flow activated cell sorting (FACS). However, we predict size changes in situ where compression by neighbouring cells affects cell size. Thus, interpreting results generated from isolated cells requires first testing the assumption that mechanical properties of mutant and non-mutant cells are identical. Tissue organoids may be a useful experimental system to
explore this, as they are amenable to experimental manipulation and contain only epithelial cells. Indeed, when epithelial cells with homozygous mutations in Apc form organoids, they appear smaller when examined in cross section (Fatehullah et al., 2013). However, how these organoids relate to the situation in irradiated crypts needs to be established before valid conclusions can be drawn.

Importantly, our results help to identify mechanisms that are disrupted at the earliest stages of tumour development, thus providing potential biomarkers for CRC. Here, we only consider one signalling pathway, and one of its outputs: proliferation. However, the close fit between experimental and in silico results suggests that how cells interpret Wnt is sufficient to explain their proliferative behaviour confirming that it is at the core of the regulation of intestinal tissue. On the other hand, it is well established that other signalling pathways including Notch/Delta, BMP, and EGF also affect proliferation in the intestinal crypt and cross talk between them is likely to fine-tune behaviour of cells. Our results suggest that it may be the ability of these pathways to modulate Wnt signalling that is key. Including additional parameters in our model could help to identify their contribution. For example, it is possible that when the Wnt concentration threshold is low, Notch signalling, which normally inhibits differentiation into the secretory lineage, becomes irrelevant, but that if the Wnt threshold is high and cells require a significant Wnt stimulus to remain proliferative, Notch signals may have a more potent effect to maintain cells de-differentiated and closer to cycling.

**Materials and Methods**

**The 3D Crypt Model**

We construct a 3D computational crypt model using a discrete agent-based approach (Osborne et al., 2010; Dunn et al., 2012a, 2012b, 2013). Cells are modelled as deformable spheres, free to move on a 3D surface defined according to experimentally-measured crypt dimensions described below.
Cell-cell connectivity is governed by an interaction radius (here taken to be 1.5 cell diameters), such that all cells whose centres are closer than this radius are considered to be connected. This connectivity is used to implement attractive and repulsive forces between neighbouring cells.

We define four distinct cell types / proliferative states as shown in Figure 1c: stem (light blue), Paneth (black), transit amplifying (yellow) and differentiated (pink). Simulations are initiated with 15 Paneth cells, which are not proliferative and are restricted to sit positioned between stem cells at the base of the crypt by applying a retainer force, and 60 proliferating cells (these are stem cells in Models 1 and 2 and are transit amplifying cells in Models 3-6). Paneth cells are included to ensure that we do not overestimate the number of proliferative cells, and therefore mitotic events, at the crypt base. A linearly decreasing gradient of Wnt stimulus is defined along the long crypt axis (Figure 1), from 1 at the crypt base to 0 at the crypt collar. This is used to identify the Wnt stimulus received by any given cell according to its location along the z-axis, and thus to determine its proliferative status.

Stem cells are explicitly modelled only in Models 1 and 2, which are based on the Pedigree assumption. However, in the remaining models, cells located towards the base of the crypt may have more 'stem-like' properties and they may have a longer cell-cycle duration that is dependent on the strength of the Wnt stimulus. Sloughing occurs at the crypt collar, where cells are immediately removed from the simulation.

Crypt Geometry

The measurements that define the surface of the crypt model are based on the dimensions of a healthy small intestinal crypt taken from the jejunum in a 6 week old male wild type mouse (Table S1, Figure S7a). The tissue was prepared for imaging as previously described (Appleton et al., 2009).
The sample was imaged on a Zeiss 710 multi-photon microscope and morphological measurements of the 3D dataset made using Volocity (Perkin Elmer) software. 3D models of single crypts were made from 3D datasets using Imaris (Bitplane) software and the dimensions presented here are based on averages of >500 crypts.

We use the cross-sectional area of experimentally measured crypts at 10μm intervals up the long axis to infer the crypt radius at these points, starting 10μm from the crypt base (Table S1). In order to specify the dimensions of the crypt for all heights we fit a fifth degree polynomial function to these radii (another fit could be used, however the chosen fit approximates the data points well and is not over specified):

\[ r(z) = r_1z^5 + r_2z^4 + r_3z^3 + r_4z^2 + r_5z + r_6, \]

where

\[ r_1 = 8.365 \times 10^{-7}, \]

\[ r_2 = 1.613 \times 10^{-4}, \]

\[ r_3 = 1.70 \times 10^{-2}, \]

\[ r_4 = -3.013 \times 10^{-1}, \]

\[ r_5 = 5.051, \]

\[ r_6 = -14.979. \]

This function is used to calculate the radius of the crypt, \( r \), at a given distance along the long axis, \( z \) (Figure S7b). We define the radius of the crypt base according to the surface of an ellipse, with a
minor axis of 15.3973, and a major axis of 16.6968 µm. These axes are determined according to the radius of the crypt 10µm from the base.

Typically the crypts of the large intestine are longer. While the dimensions used in the 3D computational model allow for accurate comparison with the radiation data, the overall behaviour we observe in simulations does not change if we instead studied larger intestinal crypts.

Cell-Cell Mechanics

Interactive cell forces, which mimic cell-cell adhesion and limited compressibility between neighbouring cells, are modelled using a force law (Figure S8) that acts along the lines between centres of neighbouring cells (Pathmanathan et al., 2009; Dunn et al., 2013). Mechanically, all cells behave the same due to adhesion and compressibility. Let \( \mathbf{r}_{ij} \) be the position of the centre of cell \( i \).

The force acting on this cell due to a neighbouring cell \( j \) is calculated as

\[
F_{ij}(t) = \begin{cases} 
  k_{ij} s_{ij} \log \left( 1 + \frac{|\mathbf{r}_{ij}| - s_{ij}}{s_{ij}} \right), & |\mathbf{r}_{ij}| < s_{ij}, \\
  k_{ij} s_{ij} \exp \left( -\alpha \frac{|\mathbf{r}_{ij}| - s_{ij}}{s_{ij}} \right), & |\mathbf{r}_{ij}| \geq s_{ij},
\end{cases}
\]

where \( \mathbf{r}_{ij} \) is the vector from cell centre \( i \) to cell centre \( j \) at time \( t \), \( \mathbf{r}_{ij} \) is the corresponding unit vector, \( s_{ij} \) is the natural length of the spring connecting cell centres \( i \) and \( j \), which increases from 0.5 to 1 over the first hour of the cell cycle (van Leeuwen et al., 2009), \( k_{ij} \) is the spring constant, and \( \alpha \) is a constant that represents the level of volume exclusion, here set to 4. The constant \( \alpha \) is a multiplication factor set to 0 if \( i \) and \( j \) are both Paneth cells, and 1 otherwise. This implements differential adhesion between Paneth cells and other cells similar to Sulsky et al. (1984). In addition to nearest-neighbour forces, a retainer force acts only on Paneth and stem cells, is included:

\[
F_i^r = \gamma_i \mathbf{e}_z.
\]
where \( \boldsymbol{z} = (0, 0, 1) \) and \( y_z = 75 \) if the cell is a paneth or stem cell, or \( y_z = 0 \) otherwise. These parameters are chosen to keep stem and paneth cells restricted to the crypt base, without inducing excessive cell compression.

By neglecting inertial terms relative to dissipative terms, the velocity of cell centre \( \mathbf{r}_i \) is given by

\[
\eta_i \frac{d\mathbf{r}_i}{dt} = F_{	ext{df}}(\mathbf{r}_i) + F_{	ext{fr}}
\]

where \( \eta_i \) is the drag coefficient for the motion of cell centre \( \mathbf{r}_i \). As we consider cells to be uniform, \( \eta_i = \eta \) for all \( i \). By iterating in small time intervals, cell positions are updated at each time step using the Forward Euler method, with the timestep chosen so that reducing it further results in indistinguishable simulations. Parameters used are given in Table S2.

Parameter Sweeps

For Models 1 and 2, the equivalent of sweeping over the Wnt threshold height is to sweep over the number of possible generations each dividing cell can have (Figs. S1, 2). The number of generations that can be assigned to dividing cells is bounded: any more than 6 generations will effectively prevent differentiated cells from being present in the crypt. We sweep over the average number of generations assigned to the dividing population so that when a non-integer number of generations is assigned, the fractional part of the number is used to assign the percentage of the dividing population to have the pedigree found by rounding up, and the remaining percentage will have the pedigree found by rounding down. For example, if the average number of generations is 2.7, then we assign 70% of the population to have pedigree 3, and 30% to have pedigree 2.

Density-Dependent Inhibition of Mitosis

Density-dependent inhibition of mitosis prevents cells from progressing through the cell cycle if they do not have a sufficiently large volume. Instead, such cells ‘pause’ the cell cycle, and remain in G1.
phase until their volume increases beyond the threshold. The volume of a cell is determined by the balance of all cellular forces, and thus serves as a proxy to how crowded the cells are in a specific region, which is believed to affect cell division (Alberts et al., 2002; Dietrich et al., 2002; Küppers et al., 2010).

As implemented in our earlier work (Dunn et al., 2013), cell volumes are approximated by averaging the interaction distance between each spherical epithelial cell and its overlapping neighbours, with adjustments for when the cell has fewer neighbours than expected, and using this distance as the effective radius of the epithelial cell of interest, \( r_e \). The volume is then calculated as that of a sphere of radius, \( r_e \). The equilibrium cell volume is calculated as the volume of an un-deformed sphere (1 cell diameter), at approximately 524 \( \mu m^3 \).

Simulations

Model simulations are conducted within the Chaste framework (Mirams et al., 2013), which can be accessed from http://www.cs.ox.ac.uk/chaste. Chaste is an open source software library, written in object-oriented C++ and constructed using agile programming techniques. The code that is used to run the simulations presented here is available to download from the Chaste website, where we have provided a tutorial specific to this publication:


(Note that the page currently requires a login with the username: reviewer and password: paper. On publication this page will be made public.)
References


tissue is a potential biomarker for detection with microultrasound. Submitted.


Figure 1: The structure of intestinal crypts. (a) A cartoon image of a single crypt, illustrating the decreasing concentration gradient of Wnt along the long crypt axis and highlighting the stem cell compartment, which consists of the Stem and Paneth cells. Nuclei displaced to the apical surface represent mitotic cells. (b) A 3D reconstruction of a single crypt, with the red surface corresponding to the lumen and the blue surface the basal surface of a crypt outlining its shape. This is used to define the dimensions of the computational crypt model (Materials and Methods). (c) The 3D computational crypt model. Stem cells are coloured blue, transit cells are coloured yellow, differentiated cells are coloured pink, and Paneth cells are coloured black.
Figure 2: Experimental data illustrating the change in the distribution of mitotic cells that occurs in response to tumour-inducing radiation. Shown are the mitotic distributions from (Trani et al., 2014) in crypts from the middle of the small intestine (jejunum) of male control mice (column 1), male mice irradiated with 4Gy of gamma radiation after a 48 hour recovery period (column 2) and mice irradiated and allowed to recover for 3 months (column 3). In the first row, the raw data is plotted as a bar histogram together with a smoothed data distribution (blue curve), which is the data fitted to a Nonparametric kernel-smoothing distribution (with normal distribution and a bandwidth of 10). The second row shows a sample from the smoothed distribution to illustrate the 'smoothed data' that is subsequently used for parameter fitting.
Figure 3: An example of a two-dimensional parameter sweep for Model 6. This shows the effect of increasing the volume threshold for contact inhibition (x-axis) and decreasing the Wnt concentration threshold (y-axis) on the distribution of mitotic cells. The optimal parameter set to fit to the control data is highlighted in blue (0.9, 0.6), the 48hr gamma-irradiated crypts in red (0.6, 0.5), and the 3 month recovered crypts in green (0.8, 0.5). The shaded regions have an error that is within 25% of the optimal parameters. These results illustrate that the effect of irradiation within Model 6 is both to decrease the Wnt threshold concentration and lower the volume threshold for contact inhibition: cells can divide at much lower volumes, and under a lower Wnt stimulus to cause widening of the mitotic distribution.
Figure 4: The log of the error between the simulated and experimental data varies with the parameters implemented in each model. Model numbers indicated on each plot. The red and blue circles mark the minimum error in each case for the control and irradiated cases respectively. The first contour is within 25% of the minimum, the second contour within 200% and the third within 400%, etc. In all models, save Model 2, the parameter sets that produce the minimum error in each case reveal that the proliferation (Wnt concentration) threshold and the volume threshold decrease from the control to the gamma irradiated case.
Figure 5: Colonisation of a crypt by mutant cells. (a) The probability that a population of mutant cells will colonise an entire crypt for different starting sizes of mutant populations as indicated. We show results for mutants with parameters that mimic control cells (blue), and for mutant cells that adopt the parameters identified for cells in the 48hr gamma irradiated data (red). The shaded region represents one standard deviation. (b) Simulation snapshots of a crypt with an initial heterogeneous population of 10% mutant and 90% healthy epithelial cells (blue and red, respectively; black shows Paneth cells). After 400 hours the mutant cells have colonised the crypt. (c) The average time taken (hours) for either mutant or control cells to colonise the crypt with increasing initial proportion of mutants. Shaded red region represents one standard deviation.
Figure 6: Identifying the initial proportion of mutant cells that explains the mitotic distribution in recovered crypts. (a) The experimental data for the distribution of mitotic cells in irradiated crypts after a recovery period of 3 months (smoothed data has been fit to a nonparametric kernel-smoothing distribution). (b) The log error between simulated and experimental data, for the indicated percentage of mutant crypts (x-axis). The lowest error between experimental and simulated data occurs when 64% of crypts are homogeneously mutated (black circle). (c) The mitotic distribution derived for a heterogeneous population of crypts, with 64% mutant crypts and 36% control crypts (error between simulated and experimental data indicated). (d) The best fit histogram of mitotic events in the simulated crypt to the recovered data, assuming a homogeneous population of cells (error between simulated and experimental data indicated).
Table 1: The six alternative models we consider. Each is constructed from five separate components, or rules. The first three rules (columns) determine the proliferative status of each cell, and the final two rules determine cell cycle duration. The results shown in Figs. 3, 6 and 7 correspond to Model 6.

<table>
<thead>
<tr>
<th>Model</th>
<th>Pedigree</th>
<th>Spatially-dependent on Wnt concentration</th>
<th>Spatially-dependent on Wnt concentration at birth</th>
<th>Uniform Cell Cycle Duration</th>
<th>Wnt-dependent Cell Cycle Duration</th>
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<tr>
<td>1</td>
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<td>-</td>
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<td>-</td>
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</table>

Table 2: The parameter sets and minimum error identified for each model when compared with experimental data.

<table>
<thead>
<tr>
<th>Model</th>
<th>Control</th>
<th>Irradiated (48hrs)</th>
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<td></td>
<td>C.I.</td>
<td>Proliferation parameter</td>
</tr>
<tr>
<td></td>
<td>C.I.</td>
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<tr>
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