Mutation, clonal fitness and field change in epithelial carcinogenesis

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Abstract

Developments in lineage tracing in mouse models have revealed how stem cells maintain normal squamous and glandular epithelia. Here we review recent quantitative studies tracing the fate of individual mutant stem cells which have uncovered how common oncogenic mutations alter cell behaviour, creating clones with a growth advantage that may persist long term. In the intestine this occurs by a mutant clone colonizing an entire crypt, whilst in the squamous oesophagus blocking differentiation creates clones that expand to colonize large areas of epithelium, a phenomenon known as field change. We consider the implications of these findings for early cancer evolution and the cancer stem cell hypothesis, and the prospects of targeted cancer prevention by purging mutant clones from normal-appearing epithelia.

Keywords: epithelial carcinogenesis; field change; cancer

Introduction

Cancer is thought to evolve from genomic alterations in individual stem/progenitor cells that generate clones within a tissue as they proliferate [1]. If these clones persist, they may undergo additional genetic alterations to form a ‘field’ of mutant cells susceptible to further transformation, eventually resulting in the formation of dysplastic lesions and malignant tumours [2]. Whilst the clonal evolution hypothesis is widely assumed, until recently there have been few data on how oncogenic genomic alterations affect cell behaviour. The development of genetic lineage tracing in transgenic mice is now revealing both normal cell dynamics and how these are perturbed by common driver mutations. Here we review evidence revealing how specific mutations alter cell dynamics and enhance clonal fitness in cancer initiation and carcinogenesis.

Neutral competition: stem cells in homeostatic tissue

Tissues such as the intestinal epithelium and the squamous epithelia of the oesophagus and epidermis are continually turned over [3]. Cells are shed from the surface and must be replaced by proliferation in the underlying cell layers. In the intestine, the stem cells which maintain the epithelium reside in the base of the crypt, from which cells differentiate and populate the epithelium covering the finger-like intestinal villi (Figure 1) [4]. The squamous epithelia of the epidermis and oesophagus comprise sheets of keratinocytes. Proliferation is restricted to the deepest, basal layer (Figure 1). When basal cells differentiate, they first exit the cell cycle and subsequently migrate through the overlying cell layers, eventually reaching the tissue surface (Figure 1). In normal tissues, each cell division generates one differentiating and one proliferating daughter cell on average to ensure cellular homeostasis [5].

It was long assumed that epithelial maintenance was underpinned by stem or progenitor cells, each of which underwent asymmetric cell division to produce a stem cell and a differentiating cell. However, this view has been overturned by the results of lineage tracing studies in transgenic mice. By using inducible forms of Cre recombinase to express a conditional allele of a neutral reporter gene, such as a fluorescent protein, at clonal frequency, the fate of labelled progenitor cells and their daughters may be tracked within the normal cellular environment [6,7]. The combination of such genetic lineage tracing with three-dimensional imaging of intact sheets of epithelium allows large cohorts of clones to be analysed at single-cell resolution over time courses lasting a year or more [8]. An important insight has
been that clone size distributions at multiple time points can be used to generate simple quantitative models that capture the average behaviour of proliferating cells in homeostatic tissue [6,9]. This approach has since been applied in a series of studies in the epidermis, oesophagus, and intestine [10–15]. A recent development has been in vivo video imaging techniques, which complement the long-term lineage data with direct visualization of the behaviour of individual cells over a few rounds of cell division [16,17]. The conclusion of such studies has been that epithelia are maintained by populations of functionally equivalent cells that renew the tissue through ‘population asymmetry’, so that the balanced production of stem and differentiating cells is achieved across the population rather than at a single cell level [18]. This revised understanding has important implications for the fate of mutant clones in cancer initiation, which we will discuss below.

There are important differences between stem cell behaviour in the intestine and squamous epithelium. In the intestine, it is the competition for space among the 5–10 stem cells in the intestinal stem cell niche at the crypt base that balances proliferation and differentiation [12,14,19]. The migration of a differentiating cell out of the crypt base is linked to the division of a neighbouring stem cell, which always produces two stem cell daughters, in a ‘differentiation/replacement’ event (Figure 1). The stem cells adjacent to the differentiating cell have equal odds of dividing to replace it. Thus, a single stem cell expressing a reporter gene generates cohesive clones that expand and contract over time in a process of neutral competition, depending on whether a labelled cell or an unmarked neighbour replaces a given differentiating cell. The fate of an individual clone is thus unpredictable, but eventually it will either be lost by differentiation or come to populate the entire crypt through ‘neutral drift’. Once a crypt has been occupied, the marked clone, or indeed any clone carrying a ‘neutral’ mutation, will persist [12,14]. This phenomenon was originally described by Schmidt et al., who termed this conversion to crypt monoclonality ‘crypt purification’ [20].

In contrast to the intestine, in epidermis and oesophageal epithelium, there is no spatially restricted niche. Both tissues are maintained by single populations of functionally equivalent progenitor cells [9–11,13,15]. As in the intestine, progenitor cell division is linked with the migration of a nearby differentiating cell out of the basal layer. However, progenitor divisions may have one of three possible outcomes, generating two progenitor cells, two differentiating cells or one cell of each type [9,10] (Figure 1). The outcome of an individual cell division is unpredictable, but the probabilities of each outcome are balanced. Thus, across the population, equal proportions of progenitor and differentiating cells are generated. As in the intestine, the fate of a labelled

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**Figure 1.** Neutral competition between stem cells in intestinal and squamous epithelia. (A) Structure of intestinal epithelium. Tissue is organized into finger-like processes (villi) alternating with pits (crypts). Stem cells (light blue) lie between Paneth cells (pink) at the base of the crypt. Stem cells differentiate, leaving the crypt base to generate cycling progenitors (grey), which give rise to the four post-mitotic cell types [enterocytes (dark blue), enteroendocrine cells (purple), goblet cells (orange), and Paneth cells], which migrate onto the villus, from which they are shed. Figure adapted from [62]. Figure modified from Zecchini V, Domaschenz R, Winton D, Jones P (2005) Notch signaling regulates the differentiation of post-mitotic intestinal epithelial cells. *Genes and Development* 19: 1686–1691 with permission from Cold Spring Harbor Laboratory Press. (B) Top-down view of crypt base. Stem cell 1 differentiates and leaves the crypt base (arrow), to be replaced by division of an adjacent stem cell (2 or 3), each of which has an equal probability of dividing. (C) Clonal progeny of a stem cell with a neutral mutation (e.g., a reporter gene). Division of the cell generates a clone which may expand and contract, eventually either being lost through differentiation or populating the entire crypt, so the mutation is fixed in the tissue. (D) Mouse interfollicular epidermis and oesophageal epithelium consist of layers of keratinocytes. Proliferation is confined to the basal cell layer, which contains a single population of progenitor cells (blue) that generate post-mitotic keratinocytes (red), which subsequently leave the basal layer and migrate to the surface, from which they are shed. When a differentiated cell exits the basal layer (1), a nearby progenitor divides, generating either two progenitor cells, two differentiating cells, or one cell of each type with the probabilities shown. (E) Two possible fates of progeny of a single progenitor cell over three rounds of division. Most clones are eventually lost by differentiation (left), but by chance a few expand to a size where differentiation of all progenitors is unlikely. There is no limit on clonal expansion within the basal layer.
clone is a matter of chance. Many clones are lost by differentiation within a few rounds of division. However, a few will expand to such a size where the probability of all their constituent progenitors dividing to generate two differentiating daughters is very low, so the clone persists long term [9,10]. Unlike the intestine, there is seemingly no limit on clonal expansion within the basal layer in epidermis and oesophageal epithelium.

It is worth highlighting that proliferating epithelial cells are highly adaptable. For example, following injury, epidermal and oesophageal cells adjacent to the wound transiently switch their behaviour to produce an excess of proliferating cells until the epithelium is repaired, when they revert to homeostasis [10,15,21]. In the intestine, differentiating cells have the potential to revert to a stem cell phenotype and repopulate the crypt base if stem cells are ablated [22–24]. Such injury responses may contribute to the tumour-promoting effect of wounding in epithelial tissues [25,26].

Tilting the odds towards proliferation: oncogenic mutations

Cancer initiation occurs when an oncogenic mutation arises in an individual stem/progenitor cell. A recent study used lineage tracing to resolve mutant stem cell dynamics in the intestine [27]. A stem cell carrying a reporter allele has a 50 : 50 chance of replacing an adjacent wild-type cell that leaves the crypt. In contrast, clones null for Apc, inactivated in the majority of human colorectal adenomas and carcinomas, have an 80% probability of replacing a differentiating neighbour, with the consequence that Apc mutant clones have a substantially increased likelihood of taking over the entire crypt base and persisting long term (Figure 2). Clones lacking one copy of Apc also have an advantage (60% probability of replacement), and hence increased odds of crypt replacement, and the clone persisting for long enough to lose the other Apc allele. This said, the advantage of Apc−/− cells over those with one copy of the gene is small, so several null clones may be lost before a heterozygous crypt is colonized. With a KrasG12D mutation, the odds of replacement are also substantially increased at 80% [27]. In contrast, a Tp53R172H mutation confers no significant benefit, unless the intestine is injured in a model of colitis, when the odds of replacement rise to 60% (Figure 2). This observation may explain the increased prevalence of TP53 mutation in human colorectal cancer associated with colitis compared with sporadic cases [27,28]. It is important to stress that mutant cell fate remains stochastic, so even with their competitive advantage half of all Apc−/− and a quarter of KrasG12D clones will not succeed in taking over their crypt. Nevertheless, as crypt takeover allows persistence of cells carrying an oncogenic mutation and acquisition of further mutations, even a small advantage over wild-type cells may have the potential to increase cancer risk.

For some mutations, the crypt architecture may restrict the expansion of the mutant cell population. However, in mouse intestine, KrasG12D mutant cells may escape from this constraint by triggering a process termed ‘crypt fission’, where the crypt splits to form two adjacent crypts carrying the mutation [29]. Crypt fission occurs infrequently in wild-type mice, but at a 30-fold higher rate in crypts replaced by KrasG12D mutant cells, creating clusters of several mutant crypts. Crypt fission also occurs in humans and may contribute to the expansion of mutant clones in human colorectal epithelium, discussed below [28,30].

A similar imbalance of fate towards proliferation occurs in the Tp53 mutant clones found in sun-exposed human epidermis. Deep sequencing of human sun-exposed epidermis reveals 14% of basal cells carrying non-synonymous TP53 mutations [31]. Consistent with earlier studies, most of the mutations observed were cytosine (C)-to-thymidine (T) base pair changes and CC-to-TT double base pair changes, signatures of ultraviolet (UV) light [32–34]. The majority of TP53 mutations stabilize the protein, enabling the mutant cells to be detected by immunostaining, which does not detect the far lower levels of wild-type p53 [35–38]. Staining of sun-exposed human epidermis reveals frequent (30 per cm2) TP53 immunoreactive clones that range widely in size from 30 to 3000 cells [35,39]. Repeated exposure of mice to low doses of UV light, below the level which causes sunburn, generates similar immunoreactive Tp53 mutant clones in the epidermis [39,40]. A reanalysis of the size distribution of clones in both mouse and human epidermis argues that UV light drives the exponential expansion of TP53 copy of TP53 and the clone persisting for long enough to lose the other KrasG12D mutation, the odds of replacement are also substantially increased at 80% [27]. In contrast, a Tp53R172H mutation confers no significant benefit, unless the intestine is injured in a model of colitis, when the odds of replacement rise to 60% (Figure 2). This observation may explain the increased prevalence of TP53 mutation in human colorectal cancer associated with colitis compared with sporadic cases [27,28]. It is important to stress that mutant cell fate remains stochastic, so even with their competitive advantage half of all Apc−/− and a quarter of KrasG12D clones will not succeed in taking over their crypt. Nevertheless, as crypt takeover allows persistence of cells carrying an oncogenic mutation and acquisition of further mutations, even a small advantage over wild-type cells may have the potential to increase cancer risk.

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Figure 2. Oncogenic mutations bias cell fate. (A) Mutation of intestinal stem cells. The mutations shown increase the probability of the mutant stem cell (red) replacing an adjacent wild-type differentiating cell, and increase the chance that mutant clones will replace wild-type stem cells in the crypt. (B) Mutation of squamous progenitor cells. In the oesophagus, a Notch inhibiting mutation (DNMaml1) blocks progenitor cell divisions resulting in symmetric differentiation, so clones expand exponentially and are no longer lost by differentiation. In epidermis exposed to UV light, Tp53 stabilizing mutations imbalance the fate of epidermal progenitor cells in favour of proliferation. Indicates the increase in probability of divisions producing two progenitor daughter cells in UV-exposed Tp53 mutant progenitors; the probabilities of the other two division outcomes decrease as shown. When UV exposure ceases, mutant cells revert to normal behaviour.

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mutant clones by increasing the probability of symmetric divisions generating two progenitor cells [41,42]. On cessation of UV exposure, the clones return to homeostatic behaviour, with some clones being lost through differentiation but the persisting clones expanding, so that the proportion of mutant cells is maintained. These findings imply that most of the TP53 mutant cells in human epidermis arise by UV-driven proliferation of pre-existing mutant cells rather than new mutations. The mutant cell population will self-maintain over the winter, when there is little UV exposure, and expand during the summer, perhaps explaining the supralinear relationship between non-melanoma skin cancer incidence and life-time UV dose, as cancer risk is dependent on the proportion of TP53 mutant cells [43,44]. Use of sunblock should thus be lifelong, as the proportionate reduction in risk will increase with age [41]. A more dramatic change in squamous progenitor fate results from Notch inhibiting mutations. Inactivating mutations of the Notch pathway are very common in squamous carcinomas [45–48]. Lineage tracing of Notch inhibited clones has been modelled by expressing a dominant negative mutant of the Maml1 gene (DNMaml1) fused to a fluorescent protein to allow lineage tracing in transgenic mice [49]. Expression of DNMaml1 in scattered oesophageal progenitors both accelerates their proliferation and abolishes cell divisions that result in two differentiated cells. As well as imbalancing cell production in favour of proliferating cells, this also blocks clone loss by differentiation, rendering the DNMaml1 clones functionally ‘immortal’ [49]. In addition, the mutant cells actively drive out their wild-type neighbours by increasing the probability of differentiation of wild-type cells bordering the clone. In combination, these alterations in cell behaviour result in the epithelium being completely replaced by DNMaml1-expressing cells within a year. Surprisingly, the Maml1 mutant mice do not develop tumours and oesophageal integrity is preserved. This can be attributed to the fact that once the mutant cells have colonized the epithelium, the cells become crowded and the symmetric differentiation division outcome is restored [49]. The ability of the epithelium to establish a new ‘steady state’ by normalizing the probability of differentiation suggests a robust mechanism to defend the tissue against aggressive mutations. This may operate once cells become crowded, as restricting the contact area of keratinocytes with the underlying basement membrane promotes their differentiation. Similar cell crowding linked with slowing clonal expansion is seen in Tp53 mutant clones in human sun-exposed epidermis once they reach more than a thousand cells in size [39,41].

Colonization and ethnic cleansing: field change

A striking phenomenon in intestinal, squamous, and other epithelia is field change, the formation of large clonal regions of preneoplastic epithelium which have an increased risk of transformation [2,28,30,50–52]. The ability of Ras mutant cells to trigger crypt fission and the aggressive expansion of DNMaml1 clones in oesophageal epithelia suggest that mutation-driven changes in cell dynamics underpin the development of field change [29,49]. It seems likely that as well as interacting at the intracellular level, mutations may also interact via alterations in cell dynamics. Clonal frequency induction of DNMaml1 in the oesophageal epithelium of mice already carrying Tp53 mutant clones creates rare double mutant clones. These expand more rapidly than clones carrying Tp53 alone, but at the same rate as those which just express DNMaml1 [49]. Over time, the double mutant clones colonize large areas of the epithelium, illustrating how a mutation which confers clonal dominance can expand and immortalize pre-existing mutations with a much weaker growth advantage.

The effect of DNMaml1 in creating ‘supercompetitor’ clones is a mammalian example of a concept formulated more than 30 years ago in studies of Drosophila. It was observed that slow-growing cells in imaginal discs were replaced by faster-growing neighbouring cells [53]. It turned out that the slow-growing cells were heterozygous for the deletion of a Minute gene which resulted in their being outcompeted by neighbouring wild-type cells [54]. Subsequent work identified other genes linked to cell competition, the most prominent being dm, the Drosophila homologue of MYC [55]. Clones overexpressing dm expanded until they reached the compartment boundaries not only by proliferating faster than their wild-type neighbours, but also by actively eliminating these cells through apoptosis. dm was thus termed a ‘supercompetitor’.

Whilst tissues are able to tolerate regions of field change without losing epithelial integrity, they confer an increased risk of malignant transformation. For example, carcinogen-exposed oesophageal epithelium expressing mutant Maml1 develops several-fold more tumours than wild-type epithelium [49]. These results suggest that cancer may arise from the transformation of cells within a patchwork of supercompetitor clones that have colonized fields of normal epithelium, carrying any pre-existing oncogenic mutations with them.

Perspective

In conclusion, the evolution of cancer is dictated by a complex interplay between mutation and clonal dynamics. Oncogenic mutations alter the balance of cell fate to create clones with a competitive advantage, which may both persist and colonize a tissue. This results in an increased risk of cancer development upon the acquisition of further mutations. These findings have important implications for both cancer biology and translational research on cancer prevention.

It has been argued for some years that in addition to microenvironmental and genetic heterogeneity, epithelial tumours may contain a discrete subpopulation of
‘cancer stem cells’ (CSCs), which have the potential to regenerate the tumor after therapy [56]. Testing this hypothesis has proved challenging [57]. Lineage tracing studies to quantify tumor cell dynamics in mice have thus far only proved feasible in benign lesions, and analysis of human tumors has relied on xenotransplantation of sorted candidate CSCs, grossly perturbing the cellular microenvironment [6,19,58,59]. The studies highlighted here show that single oncogenic mutations tilt stem cell behaviour towards the production of proliferating cells, but do not fundamentally change cell dynamics [27,41,49]. It remains to be seen whether the multiple mutations and genomic rearrangements in human solid tumors merely adjust the division rates and outcomes of normal stem cells or fundamentally change the paradigm of cell behaviour. However, given that different oncogenic mutations have divergent effects on stem cell fate, it seems probable that cells of different subclones within the same tumor may have different cell dynamics depending on the mutations they harbour [60,61]. Currently, the CSC hypothesis is neither proven nor excluded in epithelial tumours, but must be considered alongside other potential causes of proliferative heterogeneity such as genetic and microenvironmental variation [56].

An improved understanding of how mutation alters clonal behaviour in early carcinogenesis is essential if we are to design rational interventions to reduce cancer risk. Thus far, genomic sequencing has been focused on advanced tumors, revealing their complex genetic architecture [60,61]. As sequencing methods improve, they may permit analysis of mutant clones within normal-appearing human tissues, allowing personalized stratification of cancer risk and targeting of screening in carcinogen-exposed subjects. Such analysis may ultimately enable the identification of candidate oncogenes for targeted chemo-prevention by ‘clonal purging’, the elimination of specific mutant clones associated with high risk of transformation. Thus, the future will see a convergence of our knowledge of driver mutations, their effect on cell fate, and insights into how we may use this information to inform patient care.

**Author contribution statement**

PHJ wrote the manuscript with input from JF and DJA.

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