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A single dividing cell population with imbalanced fate drives oesophageal tumour growth

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Abstract

Understanding the cellular mechanisms of tumour growth is key for designing rational anti-cancer treatment. Here we used genetic lineage tracing to quantify cell behaviour during neoplastic transformation in a model of oesophageal carcinogenesis. We found that cell behaviour was convergent across premalignant tumours, which contained a single proliferating cell population. The rate of cell division was not significantly different in the lesions and the surrounding epithelium. However, dividing tumour cells had a uniform, small bias in cell fate so that, on average, slightly more dividing than non-dividing daughter cells were generated at each round of cell division. In invasive cancers induced by Kras^{G12D} expression, dividing cell fate became more strongly biased towards producing dividing over non-dividing cells in a subset of clones. These observations argue that agents that restore the balance of cell fate may prove effective in checking tumour growth, whereas those targeting proliferation may show little selectivity.

Introduction

Epithelial tumours form when the cellular homeostasis of normal tissue is locally disrupted so that cell production exceeds cell loss (Fig. 1a). This may result from the rate of tumour cell division being faster than that of normal cells. A second possibility is that in tumours such as squamous cell carcinomas (SCC) that consist of a mixture of dividing and non-dividing cells, the proliferating tumour cells produce a higher proportion of dividing than

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Author Contributions

JF designed and performed experiments, analysed data and wrote the manuscript, PG analysed data and wrote the manuscript, BDS analysed data and wrote the manuscript, PHJ initiated and supervised the project and wrote the manuscript.

non-dividing daughters¹. This bias in cell fate results in a progressive expansion in the proliferating cell population. Thirdly, the rate of cell loss may be decreased within the tumour relative to the rate of cell production. Here we set out to resolve which of these mechanisms contribute to squamous tumour growth in the oesophagus.

Further insights into the pathogenesis of oesophageal SCC, currently the 6th commonest cause of cancer death worldwide, are urgently needed as even with the most aggressive treatment the majority of patients will die from their disease^{2, 3}. Oesophageal SCC is strongly associated with tobacco exposure, and may be preceded by the development of non-invasive lesions called high-grade squamous dysplasias (HGD)^{4, 5}. Oesophageal carcinogenesis has been successfully recapitulated in rodents, either by exposing animals to the mutagenic DNA alkylating agent diethylnitrosamine (DEN), which is found in tobacco smoke, or by replicating some of the genomic alterations found in human SCC in transgenic mice^{6–12}.

Despite the availability of mouse models, quantifying the behaviour of proliferating cells within intact tumours remains challenging. One potential approach is lineage tracing, in which expression of a heritable genetic label is induced in individual proliferating cells (Supplementary Fig. 1a-c)^{13, 14}. As the progeny of the labelled cell proliferate and differentiate, they generate clusters of labelled cells, termed clones, as they derive from a single cell. Analysis and statistical modelling of the number of cells in cohorts of simultaneously labelled clones sampled over a prolonged time course has revealed normal cell behaviour and how it is altered by specific oncogenic driver mutations in several tissues, including the oesophageal epithelium^{15, 16}. However, the application of this approach to tumours has hitherto been frustrated by extensive inter- and intra-tumoural heterogeneity, which renders the inference of cell behaviour infeasible¹⁴.

Resolving tumour cell behaviour requires a protocol that generates highly similar lesions at a defined time point. Murine oesophageal epithelium is an ideal tissue in which to address this challenge. The normal tissue consists of layers of keratinocytes with proliferation confined to the basal layer¹³. Lineage tracing has shown that the oesophageal epithelium contains only one type of proliferating cell, termed a progenitor. The outcome of individual progenitor divisions is unpredictable, generating either two progenitors or two non-dividing, differentiating cells or one cell of each type (Fig. 1b). In homeostasis, the probabilities of each division outcome are balanced so that an equal number of progenitors and non-dividing cells are generated across the progenitor population. Wounding, however, drives the progenitors local to the injury to transiently produce an excess of proliferating daughters until the epithelium is repaired^{13, 17}. Mutations inactivating the Notch pathway also bias fate towards proliferation, suggesting the plasticity in progenitor cell behaviour that enables wound healing is a potential vulnerability during neoplastic transformation^{15, 18}.

Chemical carcinogens such as DEN induce oesophageal tumours but the number of lesions and the time they take to develop varies widely between animals⁶. We drew on the widely used and well-characterised two-stage carcinogenesis protocol in the skin, speculating that following mutagen treatment with a tumour promoting agent might drive the formation of a cohort of tumours sharing a similar phenotype¹⁹. We noted that side effects of the

multikinase inhibitor Sorafenib include induction of SCC of the skin and head and neck in patients treated for liver, kidney and thyroid cancers^{20–22}. This motivated us to test whether Sorafenib could promote oesophageal tumour formation in DEN treated mice.

Here we report that the combination of DEN followed by Sorafenib was effective at inducing cohorts of tumours that are sufficiently similar to permit the quantification of cell dynamics using transgenic cell proliferation and lineage tracing assays. We resolved cell behaviour in promoter treated epithelium, HGD and SCC. The data argues that tumours contain a single proliferating cell population whose behaviour is only subtly perturbed from that of normal progenitors.

Results

Sorafenib promotes the formation of high grade dysplasias

We set out to determine if Sorafenib could promote squamous carcinogenesis in the oesophagus, beginning by investigating the effect of treatment with Sorafenib alone. 10 days of dosing induced a marked dose-dependent increase in proliferation marker expression (Fig. 1c,d, Supplementary Fig. 2a-c). Treatment for 28 days at a 50mg/Kg dose resulted in decreased phosphorylation of ERK and AKT (Fig. 1e-g, Supplementary Fig. 2d-f). However, tissue integrity was preserved and no tumours developed (Supplementary Fig. 2g, Supplementary Table 1), even when the duration of treatment was extended to 56 days. Apoptosis was negligible (Supplementary Fig. 2h). Hence, Sorafenib on its own, increased proliferation but was not tumourigenic.

Next we studied the combination of Sorafenib and the mutagen DEN. Mice were administered Sorafenib or vehicle control for 28 days after 56 days of exposure to DEN (Fig. 2a). The combination of DEN and Sorafenib generated an average of 3 macroscopic tumours per animal with histological features of HGD (loss of normal differentiation, nuclear atypia and loss of cell polarity extending through the entire thickness of the epithelium) (Fig. 2b-e, Supplementary Table 1)⁴. In contrast, animals treated with DEN and vehicle developed small areas of focal hyperplastic epithelium at a frequency of less than one lesion per mouse (Fig. 2d,e, Supplementary Table 1).

We next characterised the DEN/Sorafenib induced HGD further. Consistent with the disruption of terminal differentiation seen histologically, we observed a loss of expression of the cornified envelope precursor proteins LOR and FLG in lesions by immunostaining (Fig. 2f, Supplementary Fig. 3a). Expression of KRT14, confined to the basal layer in normal epithelium, was widespread, and KRT4, normally detected in the first suprabasal layer was only seen in and above the 3rd and 4th suprabasal cell layers (Fig. 2f). We also observed increased expression of the keratinocyte stress induced proteins KRT6, induced in squamous tumours in humans and mice and KRT17, a regulator of tumour associated transcription (Fig. 2g, Supplementary Fig. 3b)^{23–25}. We detected apoptosis in only 0.08 +/- 0.05% (mean +/- s.e.m.) of cells (Supplementary Fig. 2h).

The dysplastic cells did not breach the basement membrane but protruded into the underlying submucosa (Fig. 2h). 3D confocal imaging revealed substantial stromal

remodelling beneath the lesions. There was marked angiogenesis and lymphangiogenesis with a leukocytic infiltrate including CD11b and CD3 positive cells closely associated with the deep margin of HGD, features also seen in murine and human squamous dysplasia and SCC (Fig. 2i-k, Supplementary Fig. 3c)26–30. We concluded that Sorafenib promotes tumour formation in DEN treated epithelium.

We next investigated whether the common histological phenotype of the lesions was reflected at a transcriptional level. Whereas the transcriptomes of HGDs and normal adjacent epithelium differed markedly, there was comparatively little variation between HGDs (Fig. 3a). Gene ontology categories that were significantly different between normal epithelium and HGD included keratinocyte differentiation and inflammation associated transcripts, consistent with our findings above (Supplementary Tables 2, 3). Transcripts of genes involved in RNA splicing, a process dysregulated in epidermal carcinogenesis, were also differentially expressed (Fig. 3b)31. We validated the relative expression of selected mRNAs in epithelium and HGDs by quantitative RT-PCR, observing marked upregulation of cytoskeletal associated transcripts (Fig. 3c).

Clonal origin of HGD lesions

Malignant transformation is thought to be a process in which lesions originate from the progeny of a single cell that progressively acquires multiple oncogenic genomic alterations32, 33. However, dysplastic lesions in several mouse and human tissues have been shown to be polyclonal34–39. We therefore investigated the clonal origin of the HGD lesions by genetic lineage tracing. We induced a heritable genetic marking with the confetti allele, which labels cells with one of four different fluorescent proteins (Supplementary Fig. 1a,b) 40. Reporter expression was induced in individual basal cells by transient expression of *cre* recombinase-mutant oestrogen receptor fusion protein (*cre*^{ERT}) from a xenobiotic inducible *Cyp1a1* (*Ah*) promoter following injection of low dose β -naphthoflavone and tamoxifen in Ahcre^{ERT}Rosa26^{flConfetti/wt} mice41. Animals were then treated with DEN and Sorafenib and epithelial wholemounts of HGD lesions imaged (Fig. 3d). If lesions arose from the clonal progeny of a single cell, they would be either unlabelled or completely labelled with a single colour. If, however, HGD derived from multiple cells, variegated labelling with multiple different coloured clones would be seen. We observed a mean of 2.7 clones (range 1-6) in 9 labelled lesions, which ranged widely in size (n=5 animals) (Fig. 3e-g). Similar results were obtained when the experiment was repeated with a single colour labelling system in Ahcre^{ERT}Rosa26^{flYFP/wt} mice, in which cells are labelled with a yellow fluorescent protein (YFP) reporter (Supplementary Fig. 1c, Supplementary Fig. 3d-g)13. We conclude that HGD have multiple cells of origin.

Kras^{G12D} expression in combination with DEN/Sorafenib treatment results in invasive squamous cell carcinomas

We speculated that altering the protocol might generate invasive SCC rather than HGD. We noted that mutations, amplifications or overexpression of growth factor receptor tyrosine kinases and genes in their downstream signalling pathways, including KRAS, are present in the majority of human oesophageal SCCs42–44. Furthermore, oncogenic KRAS mutations are frequent in cutaneous SCCs in patients treated with Sorafenib45. These observations led

us to investigate the effects of inducing the oncogenic *Kras*^{G12D} mutant in oesophageal epithelium after DEN exposure and prior to treatment with Sorafenib (Supplementary Fig. 1d)46. This protocol resulted in lethal, invasive SCCs in 11/14 animals (Fig. 4a-d, Supplementary Table 1). We observed increased phospho-Erk levels in the carcinomas, consistent with *Kras* activation in the lesions (Fig. 4e). Consistent with our findings in dysplasias, KRT14 stained positive throughout the tumours, while expression of terminal differentiation marker LOR was abrogated (Supplementary Fig. 4a,b). The carcinomas shared a similar transcriptome (Fig. 4f). Among the differences in transcription between HGD and SCC were significant induction of the inflammation associated mRNAs *Ifng*2 and *Fcer1g* and changes consistent with *Kras* activation in SCC (Fig. 4g,h, Supplementary Fig. 4c). A limitation of inducing SCC with this protocol is that we were unable to use lineage tracing to determine whether the tumours were monoclonal or polyclonal in origin.

Proliferating cell dynamics in Sorafenib treated epithelium

The data above show that the combination of DEN and Sorafenib was effective in inducing HGD and SCC that shared similar features. We therefore set out to quantify how proliferating cell behaviour changed in promoter treated epithelium, HGD and SCC.

We began by analysing the effect of Sorafenib treatment by using the dilution of a transiently expressed transgenic histone-green fluorescent protein (HGFP) to infer the mean rate of epithelial cell division13, 47, 48. Transgenic animals were treated with doxycycline (dox) to induce HGFP transcription from a synthetic dox-regulated promoter, resulting in high level expression of HGFP throughout the epithelium (Supplementary Fig. 1e)49, 50. Following withdrawal of dox, HGFP levels were measured by confocal imaging of epithelial wholemounts. Histone mRNA is short lived, so HGFP message is unlikely to persist following dox withdrawal and the cessation of transcription51, 52. Assuming that the rate of HGFP protein degradation is small compared with the cell cycle time, the rate of HGFP decrease will reflect the rate at which it is diluted by cell division (Fig. 5a-c, Supplementary Table 4). Analysis of HGFP levels indicated the mean cell division rate rose from once every 3.5 days in controls to a rate of once a day during Sorafenib treatment (Supplementary Fig. 5a,b, Supplementary Note 2.1).

To address how the fate of individual progenitor cells was perturbed by treatment with Sorafenib, we used lineage tracing, inducing sparse genetic labelling with YFP to yield well-separated clones in *Ahcre*^{ERT}*Rosa26*^{flYFP/wt} mice during a course of Sorafenib treatment (Fig. 5d)13. 12 days after clonal induction, animals were sacrificed, and multicellular YFP expressing clones (233 from 3 animals) imaged (Fig. 5d-f, Supplementary Fig. 5c-f, Supplementary Table 4). To assess whether the clone size data could be accommodated within the cell fate paradigm of normal OE (Fig. 5g), we implemented cell fate rules in a stochastic model, which was simulated for a wide range of parameters (Supplementary Note 1.2.2). Taking the cell cycle times determined by HGFP dilution as a prior, we found the best match of model and data using Bayesian inference (Fig. 5f). This confirmed that the data complies with the same paradigm as normal epithelium, but with a marked increase in both the proportion of divisions leading to asymmetric fate and the rate of cell stratification, in line with the increased cell division rate (Fig. 5f,g, Supplementary Table 5, Supplementary

Note 2.2). We subsequently repeated the experiment analysing clones in Sorafenib treated epithelium 21 days after induction. The observed clone size distribution at 21 days accurately matched the predicted clone size distribution based on the parameter estimates from the 12 day experiment, confirming our cell fate model (Supplementary Fig. 5g-j, Supplementary Table 4). We further tested the predictions of the lineage tracing analysis by independent assays, which were consistent with model predictions (**Methods**, Supplementary Fig. 5k-q, Supplementary Note 2.2). We concluded that Sorafenib established a new ‘steady-state’ with a faster rate of cell turnover than control tissue, sustained by the same single dividing cell population that maintains the normal epithelium.

Cell dynamics in dysplasia

Next we investigated proliferating cell behaviour within HGD lesions. To map the location of proliferating cells, animals were injected with EdU (Fig. 6a)53. After 24 hours, the vast majority of EdU+ cells were found at the edge of lesions (Fig. 6b). However, 5 days later, residual EdU+ cells were found in the centre of the lesion, arguing non-dividing cells migrate from the proliferative rim to the core of the lesion where they accumulate (Fig. 6c). This was confirmed by HGFP dilution assay, which showed that cells retaining detectable levels of HGFP remained in the centre of the lesions for at least 38 days after dox withdrawal, but were absent elsewhere in the tissue (Fig. 6d,e). We concluded that HGD were characterised by a centripetal migration of cells from the proliferative margin to the non-dividing centre, a phenomenon also reported in human squamous tumours¹. A possible explanation for the retention of non-dividing cells is that the disrupted expression of proteins required for late terminal differentiation reduces the rate of shedding of dysplastic cells from the surface of the lesion in comparison with normally differentiated cells in the adjacent epithelium (Fig. 2f, Supplementary Fig. 3a).

Next we estimated the rate of cell division in HGD and the adjacent epithelium (Fig. 6f-i, Supplementary Fig. 6a-d, Supplementary Table 4). Analysis of HGFP dilution showed the mean cell division rate was once per day both in the lesion and the surrounding epithelium, assuming the stability of HGFP is not altered by neoplastic transformation (Fig. 6h,i, Supplementary Fig. 6d, Supplementary Table 5, Supplementary Note 2.1). To track the fate of individual cells in HGD, we performed lineage tracing in YFP reporter mice, imaging clones within wholemounts at 13 and 22 days post-induction (Fig. 7a-c, Supplementary Fig. 6e,f, Supplementary Table 4). The clone size distribution remained unimodal, consistent with dividing cells belonging to a single progenitor population following a defined stochastic fate. We therefore sought the simplest modification of normal cell dynamics that could accommodate the observed clone sizes, in the form of a constant statistical bias in cell fate towards the production of proliferating cells. We thus simulated the original model relaxing the previous restriction on equal cell fate ratios, and tested this model on the clone size distributions through Bayesian inference (Fig. 1b, Supplementary Note 2.3). This showed an excellent agreement between experiment and theory and gave a bias of around 4% towards divisions producing two cycling over two non-dividing cells (Fig. 7c,d, Supplementary Table 5, Supplementary Note 2.3). The rate of cell loss was also reduced (Fig. 7d, Supplementary Table 5, Supplementary Note 2.3). This is consistent with both the observed disruption of the differentiation process that occurs prior to cell shedding from the

epithelial surface and the accumulation of non-dividing cells in the lesion core (Fig. 2f, Supplementary Fig. 3a, Fig. 6c,e). Analysis of the clone size distribution in the surrounding epithelium indicated the cell dynamics were not significantly different from those of epithelium in mice treated with Sorafenib alone, suggesting that the effects of the combination of DEN and Sorafenib treatment on cell behaviour are confined to the HGD lesions (Supplementary Fig. 6g). To further validate the analysis, we repeated the experiment in mice carrying the confetti reporter allele, which allows labelling with four different colours, thereby reducing the probability that clones with the same colour merge to create a misleadingly large apparent 'clone' (Fig. 7e-g, Supplementary Note 2.3). We obtained similar results to YFP for all parameters but the loss rate, which was increased in the confetti experiment. This discrepancy may be attributed to the higher sensitivity of detection of labelled cells in the single colour assay, in which immunostaining was used to visualise YFP expression (see **Methods**). We concluded that a *single* proliferating cell population with a small constant cell fate bias towards the production of dividing cells is responsible for the growth of the dysplastic lesions (Fig. 7d).

This analysis made the counterintuitive prediction that the proportion of dividing cells amongst the total cell population should be *decreased* within HGD compared with the surrounding epithelium, as the decreased rate of cell loss in HGD would result in the accumulation of non-dividing cells. The proportion of cells expressing the proliferation associated antigen Ki67 was indeed reduced within HGD (Fig. 7h).

Cell dynamics in squamous cell carcinoma

Finally, we investigated proliferating cell behaviour in SCC. We performed lineage tracing in triply transgenic Ahcre^{ERT}Rosa26^{flYFP/wt}Kras^{flG12D/wt} animals, induced after DEN exposure and prior to Sorafenib treatment (Fig. 8a-h, Supplementary Fig. 7a-d, Supplementary Table 4). The YFP allele was less efficiently recombined than the Kras^{G12D} allele, resulting in sparse labelling in tumours. In these experiments, the clones reflect the fate of labelled cells and their progeny from when they were labelled in normal appearing epithelium at the end of DEN treatment until they are sampled within SCC. We observed that 26/31 SCC contained an average of 5 (range 1-20) YFP positive clones (Fig. 8e). Two thirds of the clones had a size distribution quantitatively consistent with the cell dynamics seen in HGD (Fig. 8b,f,g, Supplementary Fig. 7c, Supplementary Note 2.4). The sizes of the vast majority of the remaining clones could be captured within the same model paradigm, but with a larger bias towards proliferating cell fate (Fig. 8c,h, Supplementary Fig. 7d, Supplementary Note 2.4). Only 2% of clones expanded significantly faster than the model prediction (Fig. 8d,f), suggesting that the same paradigm of progenitor dynamics applies to almost all clones in SCC, but with a variable bias in cell fate (Fig. 8i). Strikingly, as with HGD, the proportion of Ki67 positive cells was lower in SCC than in the surrounding normal epithelium (Fig. 8j, Supplementary Fig. 7e).

Conclusion

In summary, the DEN/Sorafenib carcinogenesis protocol generates multiple, synchronous and phenotypically similar oesophageal HGDs. Additional expression of an oncogenic Ras

allele generates invasive SCC. These tumours are sufficiently similar to allow the quantitative resolution of cell dynamics and offer a model of carcinogenesis that can be combined with transgenic tools to explore many other aspects of tumour biology.

Lineage tracing reveals that cells in dysplasias share a common dynamics despite their polyclonal origin. It remains to be resolved whether a single clone recruits surrounding cells or whether multiple clones collaborate in establishing a lesion.

Within oesophageal tumours, we show that dividing cells are a single population with a bias towards the production of dividing over non dividing daughter cells and no significant change in the rate of cell division (Supplementary Fig. 7f). In dysplasia cell behaviour is remarkably uniform with a small constant fate bias. In contrast, early carcinoma is characterised by the emergence of a sub-set of clones with an increased bias towards proliferation that compete for dominance within the tumour^{33, 54}.

Our results also argue that the ‘cancer stem cell’ hypothesis, which proposes that tumour expansion depends on a hierarchy of proliferating cells, does not apply to squamous neoplasia in mice^{55, 56}. We conclude that targeting imbalanced cell fate may provide an attractive strategy to arrest tumour progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Cell dynamics in oesophageal squamous carcinogenesis.

(a) Normal oesophageal epithelium is maintained by a single population of progenitor cells that divide to generate dividing (pink) and post mitotic cells (white), which exit the basal layer. In homeostatic epithelium cell production (green arrow) balances cell loss (red arrow) as proliferating cells generate equal proportions of dividing and non-dividing cells on average. In tumours, an excess of cells is generated locally through one or more of: faster cell division, indicated by the clock, an imbalance in cell fate with a bias towards producing proliferating over non-dividing progeny, or a decrease in the rate of cell loss relative to

the rate of cell production. **(b)** The outcome of individual progenitor divisions is unpredictable, generating two dividing progenitors or two non-dividing, differentiating cells in symmetric divisions or one cell of each type with the probabilities shown; r is the probability of a symmetric division outcome. In homeostasis, on average equal proportions of dividing and non-dividing cells are generated. During wound healing, local progenitor cells transiently generate an excess of dividing cells until the epithelium is repaired. The probability of generating two dividing cells is increased by β , a measure of cell fate bias towards producing proliferating over non-dividing progeny. **(c,d)** Proliferation in Sorafenib treated oesophageal epithelium. **(c)** Protocol. Animals were given Sorafenib or vehicle only (Control) for 10 days and injected with EdU (purple arrow) 1 hour before being culled. **(d)** Confocal z stacks showing 'top down' views of typical epithelial wholemounts, representative of 3 animals per group; stained for Ki67 (green), EdU (magenta), 40,6-diamidino-2-phenylindole (DAPI, blue). Scale bar, 50 μm . **(e-g)** Effect of Sorafenib on ERK phosphorylation. **(e)** Protocol. **(f)** Representative confocal images of epithelial cryosections stained for P-ERK (Thr202/Tyr204, green), basal marker ITGA6 (white) and DAPI (blue). Scale bar, 50 μm . Arrow, cells positive for P-ERK. Image is representative of sections from 3 animals/group. **(g)** Mean percentage of basal cells staining positive for P-ERK, (* $p=0.026$ by t test, $n=3$ animals/group). See Supplementary Table 4 for source data for **g**.

Figure 2. Characterisation of dysplastic lesions.

(a) Protocol. Animals were treated with DEN for 8 weeks followed by vehicle control or Sorafenib for 4 weeks. (b) Representative epithelial wholemount with lesions (arrows) following DEN and Sorafenib treatment. Scale bar, 1 mm. (c) Haematoxylin & eosin stained section of a typical high grade dysplastic lesion, arrow indicates parakeratosis. Scale bar, 100 μ m. Image representative of 8 lesions. (d,e) Number of lesions per animal (d) and lesion area (e) in DEN and vehicle (DEN+V) and DEN and Sorafenib (DEN+S) treated animals. ** $p=0.003$ by t-test, $n=12$ per group. *** $p=0.0001$ by Mann-Whitney test, $n=28$ lesions for DEN+S and 15 lesions for DEN+V group. (f) Differentiation within lesions. Confocal image of cyrosection from lesion stained for a terminal differentiation marker, the cornified

envelope precursor protein LOR (green), KRT4 (magenta) and basal cell marker KRT14 (white). Scale bar, 200 μ m. **(g)** Expression of keratinocyte stress induced protein KRT6. Confocal image of cryosection stained for KRT6 (green) and basal cell marker ITGA6 (white). Scale bar, 200 μ m. Images **f,g** are typical examples of 3 lesions. **(h-k)** Stromal changes beneath HGD lesions. **(h)** Brightfield image of submucosa paired with wholemount shown in **b**. Arrows indicate lesions. Scale bar, 1 mm. Image representative of 8 wholemounts. **(i-k)** Projected confocal z stacks of stroma underlying lesions (arrowed) stained for **i**, capillary endothelial marker CD31 (green), lymphatic endothelial vessel marker LYVE-1 (magenta) and KRT14 (white); scale bar, 200 μ m. **(j,k)** Immune infiltrate in stromal wholemount stained for myeloid marker CD11b (green) and KRT14 (magenta) **(j)** and lymphoid marker CD3 (green) and KRT14 (magenta) **(k)**. Scale bar, 100 μ m. Each image is representative of at least 3 tumours. See Supplementary Table 4 for source data for **d** and **e**.

Figure 3. Transcription and origin of dysplasias.

(a) Hierarchical clustering of normalised expression values using complete linkage of transcripts from tumours and adjacent normal appearing epithelium. Heat map indicates Z scores. Arrow indicates sample of apparently normal epithelium intermediate between normal and tumour groups. (b) Interaction map of proteins implicated in RNA splicing encoded by transcripts downregulated in tumours. (c) Quantitative PCR with reverse transcription of selected transcripts in HGD compared with adjacent control epithelium, relative to *Hprt* messenger RNA. *Krt6b*, *Krt17* and *Sprr2f* represent differentiation-

associated transcripts, *Ccnd2* proliferation-associated and *Fcer1g* inflammation-associated mRNAs; *Raf1* is implicated in oncogenic signalling pathway. Values are means of $n=9$ independent biological replicates, normalised to control ($=1$). Error bars are s.e.m. * $P<0.05$ ** $P<0.01$; *** $P<0.001$ by Mann Whitney test. **(d-g)** Origin of HGD lesions. **(d)** Protocol. Single cell labelling was induced in $Ahcre^{ERT}Rosa26^{fl}Confetti/wt$ mice and animals subsequently treated with DEN and Sorafenib to induce lesions. Possible outcomes are shown. Clonal lesions, derived from a single cell would be either unlabelled or completely labelled with a single colour, while the presence of multiple coloured and/or unlabelled areas would indicate multiple cells of origin. **(e)** Oblique view of 3D rendered confocal Z stack of typical lesion, dotted white line indicates lesion edge, GFP green, YFP yellow, RFP red, and *Itga6* white. Scale bar, 200 μm . Image representative of 9 lesions. **(f)** Clones per lesion ($n=9$ lesions from 5 animals). Line indicates mean. **(g)** Clone area ($n=13$ clones, from 3 lesions in 3 animals). Line indicates mean. See Supplementary Table 4 for source data for **c**, **f** and **g**.

Figure 4. Expression of mutant Kras^{G12D} induces SCC.

(a) Protocol. *Ahcre^{ERT}Rosa26^{wt/flyFP}Kras^{wt/G12D}* transgenic mice were administered DEN prior to induction of the Kras^{G12D} mutant, followed by Sorafenib treatment for 42 or 56 days. (b) Haematoxylin & eosin staining of representative section of typical SCC, image typical of 26 tumours. Scale bar, 250 μ m. (c) Close up of area of invasion in b. Scale bar, 50 μ m, T, tumour, M, muscle. (d) Survival of induced *Ahcre^{ERT}Rosa26^{wt/flyFP}Kras^{wt/G12D}* (Kras^{G12D}) and *Ahcre^{ERT}Rosa26^{wt/flyFP}Kras^{wt/wt}* (WT) animals (P=0.0001 by Log Rank test, n=11 per group). Arrows indicate time points of sampling for clonal analysis (see Fig.

8). **(e)** Typical cryosection of SCC in induced $Kras^{G12D}$ animal treated with Sorafenib, stained for Phospho-ERK (P-ERK, red) and ITGA6 (white). Scale bar, 200 μ m, 100 μ m, inset. **(f)** Gene expression array analysis was performed on 12 SCCs from 4 $Ahcre^{ERT}Rosa26^{flYFP/wt}Kras^{flG12D/wt}$ animals, induced after DEN exposure and prior to Sorafenib treatment. Hierarchical clustering of normalised expression values using complete linkage of transcripts from SCCs. Heat map indicates Z scores. **(g,h)** Quantitative PCR with reverse transcription of selected transcripts in SCC compared with control epithelium **(g)** or HGD **(h)**. Transcript levels normalised relative to *Hprt* messenger RNA. *Krt6b*, *Krt17* and *Spr2f* are differentiation-associated, *Cnd2* and *Ctnnb1* proliferation-associated and *Myd88*, *Fcer1g* and *Ifngr2* inflammation-associated transcripts. *Raf1*, *Pten* and *Igf2* are implicated in oncogenic signalling. Values are means of n = 4 independent biological replicates, normalised to control (=1). Error bars are s.e.m. * $P < 0.05$ ** $P < 0.01$; *** $P < 0.001$ by Mann Whitney test. See Supplementary Table 4 for source data and exact P values for **d**, **g** and **h**.

Figure 5. Sorafenib accelerates tissue turnover.

(a) Protocol. Transgenic R26^{M2rtTA}/TetO-HGFP mice were treated with Sorafenib and doxycycline (dox) to induce expression of a Histone2BJ-GFP fusion protein (HGFP). After withdrawal of dox, HGFP is diluted by cell division. HGFP levels were quantified at the time points shown. (b) Confocal images show ‘top down’ views of representative unstained epithelial wholemounts at indicated times. Scale bar, 20 μ m. (c) HGFP dilution. Box plots of HGFP fluorescence intensity in at least 300 cells per animal ($n=3$ animals per time point, 4 animals at 72h) following Dox withdrawal. Box indicates 25th and 75th percentiles, centre

line is median, whiskers indicate range. **(d-f)** Lineage tracing in Sorafenib treated $Ahcre^{ERT}Rosa26^{-/fl}YFP$ transgenic mice. **(d)** Protocol. Clonal density labelling was induced (green arrow) after 28 days of Sorafenib treatment, which continued for a further 12 days. Animals were injected with EdU (purple arrow) 1 hour before being culled. **(e)** Rendered confocal z stacks of wholemounts showing representative lateral views of clones; green is YFP and white is basement membrane marker ITGA6. Scale bar, 20 μ m. Image is typical example of 233 clones. **(f)** Clone size distribution (frequency of clones containing a given number of cells) in Sorafenib-treated animals, 12 days post labelling. Points are experimental data (n=233 clones from 3 animals), lines model predictions for the best fit parameters and the blue area error margins (standard deviation), see Supplementary Note 1 and 2.2. **(g)** Progenitor cell behaviour inferred from clone size distributions in Sorafenib and control treated animals, showing a marked increase in the cell division and stratification rates and in the proportion of divisions leading to asymmetric fates, i.e. generating one dividing and one non-dividing cell. See Supplementary Table 4 for source data for **c** and **f** and Supplementary Table 5 for fitting error margins in **g**.

Figure 6. Proliferation, migration and cell retention in HGD lesions.

(a-c) EdU lineage tracing. (a) Protocol. Lesions were induced and animals injected with EdU (purple arrow) 1 or 5 days before culling. (b,c) ‘Landscape’ views of rendered confocal Z stacks of wholemounts 1 day (b) or 5 days (c) after labelling; white dashed line indicates lesion margin, EdU (red), basal marker ITGA6 (white). Scale bar, 100 μ m. Images are typical of 3 tumours per time point. (d,e) Label retention in HGD lesions. (d) Protocol. Lesions were generated in R26^{M2rtTA}/TetO-HGFP animals, followed by dox to induce HGFP expression. After dox withdrawal, HGFP diluted for 38 days. (e) Rendered confocal Z stacks showing ‘landscape’ view of wholemount. Dashed white line is lesion margin. HGFP (green) and ITGA6 (white). Scale bar, 50 μ m. Image is representative of 3 lesions. (f-i) Measuring the cell division rate in HGD and adjacent epithelium. (f) Protocol. Lesions were generated in R26^{M2rtTA}/TetO-HGFP mice. dox was then given for 4 weeks, withdrawn and

samples collected over 72 hours. **(g)** Confocal image, representative of 6 lesions, from edge of dysplasia showing HGFP fluorescence (green), basal marker ITGA6 (white) and tumour associated cytokeratin KRT6 (magenta). Scale bars, 50 μm and 20 μm , respectively. Arrows indicate the border of dysplasia (HGD) and adjacent epithelium (A). **(h)** Ratio of HGFP intensities in adjacent epithelium (A) to HGD after 72 hours ($n=6$ lesions). **(i)** Mean cell generations (number of completed cell divisions since 24 hours post dox withdrawal) in HGD (black; $n=4$ animals (24h and 48h); 6 animals at 72h) and adjacent epithelium (purple; $n=3$ animals per time point) (see **Methods**, Supplementary Note 2.1, Supplementary Fig. 6b,c). Error bars are of the logarithmic HGFP fluorescence levels, due to mouse-to-mouse variation (see discussion in Supplementary Note 2.1). The dashed line represents a slope of 1/day. A least-squares linear regression yields a slope of 0.99 ± 0.01 (s.e.m.)/day in HGD and 0.97 ± 0.06 in adjacent epithelium, which estimates the cell division rate (see Supplementary Note 2.1). See Supplementary Table 4 for source data for **h** and **i**.

Figure 7. Cell dynamics in HGD lesions.

(a-d) Lineage tracing in dysplastic lesions. (a) Protocol. After generating dysplasias in *Ahcre^{ERT}Rosa26^{fl/YFP}* mice, YFP was induced at clonal density and samples imaged 13 days later. (b) Rendered confocal z stacks showing typical 'landscape' view, representative of 19 lesions, containing YFP+ clones (green, magenta is EdU and white is ITGA6). Scale bar, 100 μ m. (c) Clone size distributions 13 days after induction. Points are experimental data (n=100 clones), lines model fits and blue area indicates standard deviation. Note that sizes > 40 may be attributed to clone fusions (see Supplementary Note 2.3). (d) Model: dynamics of proliferating cells (pink) in dysplasias, adjacent epithelium is shaded purple,

see Supplementary Table 5 for error margins. **(e-g)** Cell dynamics in HGD in Ahcre^{ERT}Rosa26^{flConfetti/wt} mice. **(e)** Protocol. Dysplasias were generated prior to induction of the confetti reporter at clonal density. Samples were imaged 10 days or 21 days later. **(f)** Rendered confocal z stacks showing 'landscape' view of a representative example of 21 lesions containing one or more confetti clones (yellow is YFP, red RFP, magenta is DAPI). Scale bar, 100 μ m. **(g)** Clone size distribution 10 days after induction. Points are experimental data (100 clones from 3 animals), line model fit and blue area indicates standard deviation (see Supplementary Note 2.3). **(h)** Mean Ki67+ cells as a percentage of total nucleated cells in HGD and adjacent epithelium (A) (**p<0.0072 by t-test, n = 5 tumours, from 4 mice). See Supplementary Table 4 for source data for **c**, **g** and **h**.

Figure 8. Cell dynamics in Ras-driven SCCs.

(a) Protocol. $Ahcre^{ERT}Rosa26^{wt/flyFP}Kras^{wt/G12D}$ transgenic mice were administered DEN prior to simultaneous induction of the $Kras^{G12D}$ mutant and YFP, followed by Sorafenib treatment for 42 or 56 days. (b-d) Examples of clones. Reconstructed 3D rendered confocal z stacks of representative clones in vibratome sections of SCCs stained for YFP (green) and ITGA6 (white). Scale bar, 100 μ m. Images typical of 147 clones. (e) Clones per tumour (n=31 tumours, from 16 animals). Line indicates mean. (f) Clone sizes at 42 and 56 days after induction. (g, h) Clone size distributions 42 days after induction. Points are

experimental data, lines model predictions and blue area indicates standard deviation. Data are binned in intervals of 10 cells per clone (**g**) and 100 cells per clone (**h**), respectively. (**g**) Clones < 150 cells (n=65 clones) overlying predictions of dysplasia model (Fig. 7d). (**h**) Clones > 150 cells (n=33 clones), with theoretical prediction (exponential function, see Supplementary Note 2.4). Clone size is rescaled (size relative to mean clone size). (**i**) Proliferating cell fate in oesophageal squamous cell carcinoma. indicates cell fate bias towards producing proliferating progeny, low in smaller SCC clones (blue), higher in larger SCC clones (green). (**j**) Mean percentage Ki67+ cells/total nucleated cells in SCC and adjacent epithelium (A), (*p=0.022 by t-test, n=4 tumours). See Supplementary Table 4 for source data for **f-h** and **j**.