Defining the mode of tumour growth by clonal analysis

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Recent studies using the isolation of a subpopulation of tumour cells followed by their transplantation into immunodeficient mice provide evidence that certain tumours1-4, including squamous skin tumours5-8, contain cells with high clonogenic potential that have been referred to as cancer stem cells (CSCs). Until now, CSC properties have only been investigated by transplantation assays, and their existence in unperturbed tumour growth is unproven. Here we make use of clonal analysis of squamous skin tumours using genetic lineage tracing to unravel the mode of tumour growth in vivo in its native environment. To this end, we used a genetic labelling strategy that allows individual tumour cells to be marked and traced over time at different stages of tumour progression. Surprisingly, we found that the majority of labelled tumour cells in benign papilloma have only limited proliferative potential, whereas a fraction has the capacity to persist long term, giving rise to progeny that occupy a significant part of the tumour. As well as confirming the presence of two distinct proliferative cell compartments within the papilloma, mirroring the composition, hierarchy and fate behaviour of normal tissue, quantitative analysis of clonal fate data indicates that the more persistent population has stem-cell-like characteristics and cycles twice per day, whereas the second represents a slower cycling transient population that gives rise to terminally differentiated tumour cells. Such behaviour is shown to be consistent with double-labelling experiments and detailed clonal fate characteristics. By contrast, measurements of clone size and proliferative potential in invasive squamous cell carcinoma show a different pattern of behaviour, consistent with geometric expansion of a single CSC population with limited potential for terminal differentiation. This study presents the first experimental evidence for the existence of CSCs during unperturbed solid tumour growth.

Different models have been proposed to explain tumour heterogeneity and growth1-3,7-8. In the stochastic model, all tumour cells are considered equipotent and only a fraction (due to genetic and/or epigenetic changes) will contribute to tumour growth. By contrast, in the CSC model, only a fraction of tumour cells have high clonogenic potential, and give rise to a predictable hierarchical mode of tumour growth. These two models of tumour growth are not mutually exclusive and competition may still arise in the hierarchical model. Until now, CSC properties have been demonstrated by the ability of a subpopulation of tumour cells to reform a tumour identical to the parental tumour on transplantation into immunodeficient mice. This ‘gold standard’ assay has some caveats as defined many paradigms in cancer biology relevant to human pathology9-11,12. In this protocol, mice are first treated with the carcinogen DMBA (7,12-dimethylbenz[a]anthracene) followed by continuous administration of the mitogen TPA (12-O-tetradecanoylphorbol-13-acetate). During the course of TPA administration, benign tumours, known as papilloma, appear (initiation) and grow steadily (promotion), with a fraction eventually progressing into invasive tumours. Papilloma are characterized by a skin excrescence that expands slowly and presents a stereotypical architecture similar to normal epidermis with a basal layer of proliferative tumour cells expressing K5/K14 and suprabasal layers of non-proliferative cells expressing K1/K10, which finally enucleate and form a stratum corneum structure (Fig. 1a). In contrast with normal homeostasis, in which the rate of cell production matches the rate of loss, during tumour growth this balance is shifted towards proliferation, with the diameter of the papilloma doubling in size every month (Supplementary Fig. 1). Although these tumours are extremely useful in dissecting the different steps of tumour initiation, promotion and malignant progression, and has been instrumental in defining many paradigms in cancer biology relevant to human pathology9-11,12. In this protocol, mice are first treated with the carcinogen DMBA (7,12-dimethylbenz[a]anthracene) followed by continuous administration of the mitogen TPA (12-O-tetradecanoylphorbol-13-acetate). During the course of TPA administration, benign tumours, known as papilloma, appear (initiation) and grow steadily (promotion), with a fraction eventually progressing into invasive tumours. Papilloma are characterized by a skin excrescence that expands slowly and presents a stereotypical architecture similar to normal epidermis with a basal layer of proliferative tumour cells expressing K5/K14 and suprabasal layers of non-proliferative cells expressing K1/K10, which finally enucleate and form a stratum corneum structure (Fig. 1a). In contrast with normal homeostasis, in which the rate of cell production matches the rate of loss, during tumour growth this balance is shifted towards proliferation, with the diameter of the papilloma doubling in size every month (Supplementary Fig. 1). Although these tumours...
contain a mass of amorphous material arising from the terminal differentiation of tumour cells, the proportion of differentiated cells remains relatively constant over time (Supplementary Fig. 1), indicating that tumours grow mostly through basal epithelial cell expansion.

These observations raise several important questions. Why is the growth of the papilloma relatively slow? Classical theories of tumour development often involve unbridled replication of the tumour-maintaining cell population, leading to geometric expansion of the tumour mass. However, papilloma arise sporadically following DMBA/TPA treatment, after which they grow slowly, giving rise to a heterogeneous size distribution (Supplementary Fig. 1). Is the characteristic stem/progenitor cell hierarchy of normal tissue recapitulated in the tumour? Are proliferative tumour cells equipotent, or is the papilloma sustained by a distinct population of tumour cells with stem-cell-like properties? To address these questions, we used a quantitative lineage-tracing strategy that has proved successful in resolving cellular hierarchy and stem-cell fate in normal epithelial tissues.

To mark individual tumour cells, we made use of an inducible genetic lineage tracing system (Fig. 1b). Following the administration of tamoxifen (TAM) to K14CREER/Rosa-YFP mice bearing skin tumours induced by DMBA/TPA treatment, we were able to label cells at clonal density within the papilloma (Fig. 1c). Titrating down the dose of TAM to 0.35 mg generated yellow fluorescent protein (YFP) expression in around 1% of basal tumour epithelial cells (Fig. 1d and Supplementary Fig. 2), a density that allows the fate of individual labelled cells to be traced over time. In the weeks following TAM administration, some YFP-labelled basal cells actively divided (Fig. 1e), and formed clones containing several basal cells as well as suprabasal cells that reach the top of the cornified layer (Fig. 1f), demonstrating that labelled tumour cells are capable of generating all cell types that comprise the tumour. Interestingly, the initial shape of the marked tumour clones is reminiscent of the column of marked cells (sometimes termed epidermal proliferative units) observed in the interfollicular epidermis during normal homeostasis.

To gain further insight into the proliferative potential of labelled tumour cells, we first quantified the size and persistence of clones across a range of time points from 3 days up to 7 weeks post-labelling (Fig. 2a–c). Analysis of the junction between normal epidermis and papilloma excluded the possibility that tumour clones arise from cells coming from normal epidermis (Supplementary Fig. 3). Whereas the frequency of labelled tumour clones (number per sectional area) remained relatively constant during the first 2 weeks after TAM administration, this number had dropped by around 3 weeks, and by 7 weeks involved only 20% of the initial clones (Fig. 2b), indicating that only a fraction of proliferative tumour cells present long-term self-renewal potential. The near-absence of active caspase 3$^+$ cells in these tumours (0.28 ± 0.03% active caspase 3$^+$ tumour epithelial cells) indicates that clones were lost through differentiation rather than by apoptosis. (Supplementary Fig. 4). Of the clones that did survive at 7 weeks post-labelling, many were enormous, containing several hundreds to thousands of cells, and sometimes comprising an entire lobe of the tumour (Fig. 2a, c). These data indicate heterogeneity in the proliferative potential of tumour cells, and indicate that a fraction may conform to the CSC paradigm in vivo by massively contributing at the clonal level to tumour growth.

Although the stratified architecture is conserved in papilloma, its folded structure does not facilitate whole-mounting. Instead, clones can only be visualized through the acquisition of sectional data. As a result, total clone size could only be scored at single-cell resolution for individual clones by three-dimensional (3D) reconstruction using confocal analyses of serial (25 μm) sections (Supplementary Fig. 5). Following this procedure, we scored a limited number of clones over a 7-week time course (Fig. 2c), from which several qualitative features emerged. First, single-cell clones, which account for some 50% of the total YFP-labelled clones at 3 days post-labelling, showed a decline only around day 9, indicating that the loss rate of terminally differentiated cells is relatively slow in comparison to the cell division rate.

Second, from the size distribution (Fig. 2c), there is further evidence of proliferative heterogeneity. Although the spectrum of clone sizes is broad and continuous at 3 days post-labelling (with clones of ten cells or more coexisting with clones of only two cells), by 9 days the distribution becomes bimodal (Fig. 2c). Of the clones involving multiple cells (and therefore derived from proliferative cells), a minority continue to grow and expand rapidly over the time course, whereas others generate clones with fewer than ten cells that become progressively lost. At 7 weeks post-labelling, clones still present significant heterogeneity in size ranging from dozens of cells to several thousands of cells (Fig. 2c).

Although data from the 3D reconstruction provides qualitative insight into the proliferative cell dynamics, to further resolve the properties of these populations, we turned to a quantitative analysis. Because acquisition of a large statistical ensemble of clone sizes from serial reconstruction is impractical, we used data from independent sections, which could be obtained in larger numbers. Fortunately, analysis of data from the 3D reconstruction revealed a robust quadratic relationship between sectional clone size, $n_{3D}$, and total clone size, $n_{3D}$, which allows the statistical distribution of the latter to be reliably reconstructed from the former (Supplementary Fig. 5 and Supplementary Information). This quadratic dependence, $n_{3D} = n_{2D}^2/3$, follows from the architecture of the tissue that constrains clones to grow laterally as cohesive ‘pancake-like’ structures once the clone has spanned the suprabasal cell layers.

Data from the serial reconstruction indicate that papilloma are sustained by a cellular hierarchy in which a minority population of
tumour cells with stem-cell-like properties gives rise to a more transient progenitor cell pool. Motivated by lineage-tracing studies of normal epidermis and stem cells, we propose that the progenitor cell population follows a stochastic pattern of fate in which cell division can lead to all three fate outcomes: two dividing cells, two non-dividing, and one dividing and one non-dividing (Fig. 2d). Further, we propose that the stem-cell-like population divides rapidly, leading to a similar pattern of stochastic fate involving self-renewal and the generation of progenitor cell progeny (Fig. 2d). With this model, we made use of the sectional clone size data (Fig. 2e) to fit the experimental parameters (for further details of the modelling scheme and fitting procedure, see Supplementary Information). This procedure is simplified considerably by the divergence in size of the progenitor- and stem-cell-derived clones, with the former dominating the distribution for smaller clone sizes and early time points, and the latter contributing substantially to the larger clone sizes and later time points. From the predicted dynamics, we obtain an excellent fit to the clone size distributions over the wide time course (Fig. 2f) if we suppose that the stem-cell-like population accounts for just 20% of the labelled tumour proliferative cells, and take both the progenitor and stem cell fates to be approximately balanced with some 80% of divisions leading to asymmetric fate outcome while the remaining 20% lead to either symmetrical duplication or differentiation, with equal probability (Fig. 2d). Moreover, the inferred stem-cell-like tumour cell division rate of twice per day is approximately four times larger than the progenitor cell division rate in the tumour, and an order of magnitude larger than the progenitor cell division rate in normal tissue. Finally, as expected from the analysis of the serial reconstruction, the loss rate of terminally differentiated cells is slow, at around once per week.

To test the predictions of the model, we performed a double-labelling nucleotide pulse-chase assay to estimate the proportion of cells that re-enter the cell cycle after 12 h. To this end, we first administered a pulse of 5-ethyl-2'-deoxyuridine (EdU) to mice bearing papilloma, and administered 5-bromo-2-deoxyuridine (BrdU) 12 h later, and analysed double-labelled basal cells 4 h after that (Fig. 3a). Significantly, whereas virtually no double-positive cells can be found in normal epidermis on the edge of the tumour (Supplementary Fig. 6), the incidence of small clones was significantly diminished. Interestingly, all of the largest clones contained cells that contact the stroma and endothelial cells as well as cells that have lost their cohesion with the rest of the clone, and present signs of epithelial to mesenchymal transition with a fibroblastic-like morphology (Fig. 4d, e, h). Most cells within these clones present no sign of terminal differentiation despite having undergone many rounds of division (Fig. 4e), indicating that these clones are comprised of proliferative cells with a very low probability of terminal differentiation.

Consistent with the notion that most cells in invasive SCCs are proliferative, about 70% of cells in SCC are Ki67+ and continuous administration of BrdU for 5 days labelled about 90% of undifferentiated cells in invasive SCCs (Fig. 4i–k and Supplementary Fig. 8). Although heterogeneity of clone size could reflect tumour cells with a range of proliferative potential, the exponential form of the cumulative clone size distribution of individual tumours (Supplementary Fig. 9) is equally consistent with a single equipotent proliferative cell population, biased towards symmetric self-renewal, with a broad distribution of cell cycle times (Supplementary Information). Within this paradigm, we estimate an average cell division time that varies between 1 and 2 days (according to the tumour). This figure is consistent with the results of a BrdU–EdU double-labelling assay, which revealed that, whereas 23% of SCC cells acquire BrdU or EdU expression following a short pulse, the frequency of double-labelled cells after 16 h chase was below 2% (Fig. 4l–n and Supplementary Information).

In summary, our data present the first attempt to dissect the mode of tumour growth using clonal analysis by lineage tracing in mice, and demonstrate for the first time the existence of tumour cells with stem-cell-like properties in an unperturbed solid tumour, consistent with the CSC paradigm. The neutral competition of tumour cells uncovered here in skin papilloma is reminiscent of the dynamics found in normal tissue and indicates that this hierarchical mode of tumour growth is mediated at the cellular level by stochastic cell fate decisions. This mode of tumour growth, involving the chance expansion and extinction of...
neighbouring clones, may explain the clonal heterogeneity found in many different tumours13. In future studies, it will be important to define the mechanisms regulating the balance between proliferation and differentiation during tumour progression, the influence of the external environment and other physical constraints on tumour expansion, and whether this stochastic mode of tumour progression also operates in other types of cancer.

METHODS SUMMARY

Squamous skin tumours were induced by treating K14CREER/Rosa-YFP mice with the two-step DMBA/TPA chemical carcinogenesis protocol25. Clonal YFP expression in skin tumours was performed by administering 0.35 mg or 1 mg TAM to K14CREER/Rosa-YFP mice bearing benign or malignant skin tumours. Immunostainings were performed as described24. Quantification of the total clone size was determined by counting the number of YFP-positive cells in 3D reconstruction of the whole clone using serial thick sections analysed by confocal microscopy. Two-dimensional clone size was determined by counting the number of YFP cells in a single tumour thin section and its relation to the total clone size was inferred using the algorithm defined in the main text and Supplementary Information. BrdU–EdU double-labelling approach was performed as described25. For further details see Supplementary Methods.

Full Methods and any associated references are available in the online version of the paper.

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METHODS

Skin tumour induction and clonal YFP expression. K14CREER and Rosa-YFP mice were obtained from JAX. Mouse colonies were maintained in a certified animal facility in accordance with European guidelines. Mice were treated with DMBA and TPA as previously described. Briefly, mice were treated with DMBA at postnatal day 23, 25 and 27 with DMBA (9,10-dimethyl-1,2-benzanthracene) and then treated weekly for several weeks with TPA (12-0-tetradecanoylphorbol-13-acetate). 7-week-old mice were treated again twice with DMBA. 0.35 mg Tamoxifen (TAM) was administered intraperitoneally to K14CREER/Rosa-YFP mice bearing papilloma and 1 mg to mice bearing SCC. The tumours were surgically excised at different time points after TAM injection.

Measurement of tumour growth. Skin tumours were measured using a precision calliper allowing to discriminate size modifications >0.1 mm. Tumour surface was measured every week with the formula $A = \pi(dD/2)$, where $d$ is the minor tumour axis and $D$ is the major tumour axis.

Histology, immunostaining and imaging. Tissues were pre-fixed for 2–8 h in 4% paraformaldehyde (PAF) and washed in sucrose 30% solution to remove the excess of PAF before being embedded in OCT. Samples were sectioned serially in thin 6–7 μm or thick 25–30 μm sections using a CM3050S Leica cryostat (Leica Microsystems GmbH). The following primary antibodies were used: anti-K5 (rabbit, 1:1,000, Covance), anti-β4 (rat, 1:200, BD), anti-K14 (chicken, 1:1,000, Covance), anti-GFP (rabbit, 1:1,000, Molecular Probes), anti-K10 (rabbit, 1:2,000, Covance), anti-active caspase 3 (rabbit, 1:600, R&D Systems), FITC (fluorescein isothiocyanate)-coupled anti-BrdU (mouse, 1:100, BD). EdU staining was performed following the manufacturer’s instructions (Invitrogen). Sections were incubated in blocking buffer (PBS, normal donkey serum (NDS) 5%, BSA 1%, Triton 0.2% or 0.5% for thin and thick sections, respectively) for 1 h at room temperature. Primary antibodies were incubated in blocking buffer overnight at 4 °C. Sections were rinsed three times in PBS and incubated with secondary antibodies diluted at 1:400 for 1 h at room temperature for thin sections or overnight at 4 °C for thick sections. The following secondary antibodies were used: anti-rabbit, anti-rat, anti-chicken conjugated to AlexaFluor488 (Molecular Probes), to rhodamine Red-X (Jackson ImmunoResearch) or to Cy5 (Jackson ImmunoResearch). Nuclei were stained in Hoechst solution (1:1,000) and slides were mounted in DAKO mounting medium supplemented with 2.5% Dabco (Sigma). In all immunofluorescences, Hoechst nuclear staining is represented in blue.

Confocal pictures were acquired using Zeiss LSM 510 or Zeiss LSM 780 confocal microscopes using Zen software. Images were median-filtered (3 to 3) and are represented as orthogonal projections of 20–65 optical sections in 0.3–1 μm increments rendered using Zen software.

Analysis of clone size. Total clone size was determined by counting the number of YFP-positive cells in 3D reconstruction of confocal analysis of the whole clone using serial thick sections.

2D clone size was determined by imaging 30 to 1,330 clones from at least 8 different tumours from different mice at each time point. 6–7 μm sections were analysed to number the cells and the total clone size was inferred after correction as described in the Supplementary Information. The density of basal clones was determined by quantifying the number of basal clones per area of tumour and corrected for the increase in the total surface during tumour growth.

Proliferation assays. To measure the kinetics of tumour cell proliferation, mice were treated with EdU (pulse) and chased for 12 h followed by BrdU administration and another chase of 4 h. Tissues were first stained for K5. Sections were then washed in PBS and fixed again in PAF 4%. After washing in PBS, tissues were incubated for 20 min in HCl 1 N at 37 °C, washed three times and incubated overnight with FITC-coupled anti-BrdU antibody (mouse, 1:100, BD). The tissues were washed in PBS the next day and the EdU staining was performed following the manufacturer’s instructions (Invitrogen). Slides were mounted in DAKO mounting medium supplemented with 2.5% Dabco (Sigma).

FACS quantification of YFP basal tumour cells. 3 days after TAM administration to the K14CREER/Rosa-YFP mice bearing skin tumours, tumours were digested in collagenase I (Sigma) for 2 h at 37 °C on a rocking plate. Collagenase I activity was blocked by addition of EDTA (5 mM). After tumour digestion, cells were first incubated in PBS complemented with 30% FCS to block Fc receptors for 15 min at room temperature. Immunostaining was performed using phycoerythrin-conjugated anti-α6-integrin (clone GoH3; BD Pharmingen), allophycocyanin-Cy7-conjugated anti-Epcam (clone G8.8; Biolegend). YFP-positive tumour cells were quantified within the Epcam^−/α6-integrin^ population after forward scatter, side scatter selection and Hoechst dye exclusion. Fluorescence-activated cell sorting analysis was performed using BD LSR Fortessa and FACSDivia software (BD Biosciences).

Theory. See Supplementary Information.