Stem cell functionality is microenvironmentally defined during tumour expansion and therapy response in colon cancer

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Solid malignancies have been speculated to depend on cancer stem cells (CSCs) for expansion and relapse after therapy. Here we report on quantitative analyses of lineage tracing data from primary colon cancer xenograft tissue to assess CSC functionality in a human solid malignancy. The temporally obtained clone size distribution data support a model in which stem cell function in established cancers is not intrinsically, but is entirely spatiotemporally orchestrated. Functional stem cells that drive tumour expansion predominantly reside at the tumour edge, close to cancer-associated fibroblasts. Hence, stem cell properties change in time depending on the cell location. Furthermore, although chemotherapy enriches for cells with a CSC phenotype, in this context functional stem cell properties are also fully defined by the microenvironment. To conclude, we identified osteopontin as a key cancer-associated fibroblast-produced factor that drives in situ clonogenicity in colon cancer.

In recent years, in vivo lineage tracing experiments and quantitative models have resolved the dynamics of the intestinal stem cell (ISC) population1–4. It was found that in the homeostatic murine intestine each crypt contains five to seven functional stem cells1,2. However, the number of cells that express purported stem cell markers such as Lgr5 is much larger (n ~ 16 per crypt)4,5, and many of these cells indeed have stem cell potential in response to tissue damage or in clonogenic assays6. Therefore, which cells function as stem cells in the normal gut largely depends on their position within the niche, and stem cell functionality and identity are distinct properties1,7,8.

Previously, we elucidated how oncogenic mutations impact ISC dynamics and alter their behaviour during tumour initiation9, and it was established that early adenomatous outgrowths retain a hierarchy in which stem-like cells drive expansion10–12. In parallel, the presence of cancer stem cells (CSCs) in established human cancers has been assessed. In these studies, tumours are typically disrupted and single cell suspensions are injected in immune-compromised mice to determine the frequency of CSCs, and the markers that distinguish these cells13–15. Critically, such artificial assays test stem cell potential rather than the stem cell functionality that drives tumour expansion. It has also been reported that LGR5+ cells in colon cancer xenografts are actively clonogenic and able to function as CSCs16; however, it is unclear whether LGR5+ cells form a rare population, or if essentially all cancer cells can function as stem cells17. Recently it was established that cancer cells at the invasive front contribute most to tumour expansion, but how this relates to the CSC model remains largely unresolved18. An important caveat of the CSC hypothesis is that differentiated cancer cells are known to adopt stem cell properties following exposure to signals from the stroma19,20 and that ablation of Lgr5+ cells in tumours results in rapid repopulation by Lgr5− cells21. However, it is currently unknown if this is a rare phenomenon that only occurs in experimental settings, or whether this is also central to the biology of unperturbed colon cancer tissue. Answering this question is key to our understanding of colon cancer biology. In addition, the role of CSCs in driving resistance to chemotherapy has not been elucidated in established tumour tissues. Therefore, we set out to adapt the marker-free clonal tracing strategies that we have developed in the murine gut to define the properties of CSCs in human colon cancer in situ, and to determine to what extent these are regulated intrinsically or by the environment, both in unperturbed tumour growth and during treatment.

Results

Colon cancer growth dynamics. Primary human colon cancer cultures were established as described in refs 12,13. Subcutaneous injection of these lines (Co100, CC09) as well as a cell line cultured in the presence of serum (HCT-15) in immune-compromised mice resulted in tumours with a well-differentiated morphology reminiscent of human colon cancers (Supplementary Fig. 1).

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We introduced a marker-free lineage tracing system, using a tamoxifen-inducible Strawberry expression vector (LV-indLS2)\(^2\), allowing random and permanent labelling of individual cells and their offspring independent of cell identity (Fig. 1a). Monoclonal cultures were established to limit genetic heterogeneity. In vitro, we confirmed that the induction of Strawberry expression was dose-dependent, random and a neutral event that does not impact on cellular fitness (Supplementary Fig. 2a-c). Dose dependency was confirmed in vivo (Supplementary Fig. 2d). For further studies we selected the dose that yielded sufficient clones for analysis, but showed no clone collision (Supplementary Fig. 2e-h). The resulting distributions of clone sizes showed no signs of scaling, confirming that we successfully avoided clone merging\(^2\) (Supplementary Note 1). Next, we induced clones in small tumours (~100 mm\(^3\)) and isolated tumours on at least five time points (4–42 days) (Fig. 1b–d and Supplementary Fig. 3a–d). To investigate the impact of the immune-compromised mouse strain employed, or the location of injection, we included the analysis of tumours grown in both NOD-scid gamma (NSG) and nude mice, as well as orthotopic tumours located in the caecal wall (Supplementary Figs. 1e,f and 3a,b). We analysed the induced clone sizes manually and by automated image analysis, which revealed excellent agreement (Supplementary Note 1). Two-dimensional (2D) analysis of tissue sections was sufficient to approximate clonal volumes, as demonstrated by 3D tissue analysis (Fig. 1f,g and Supplementary Note 1). Over time, the average clone size increased, as expected in expanding tumours (Fig. 1e, Supplementary Fig. 3b–d).
Fig. 2 | Stochastic modelling and data inference identify spatiotemporal regulation of stem cell function. a, Diagram explaining the stochastic model for tumour growth. With rate $\lambda$, clonogenic cells stochastically divide with probability $a$. Cells become non-clonogenic with probability $(1-a)(1-h)$, or all cells within a clone lose their clonogenicity with probability $(1-a)h$. $\lambda$ indicates effective proliferation rate (cell divisions per day), $a$ is mode of tumour growth ($a = 1 - \frac{h}{1 + \frac{h}{2}}$ for surface growth and $0.5 < a \leq 1$ for exponential growth), and $h$ is heterogeneity of growth between clones. See Supplementary Note 1 for details. b, Heatmaps, depicting goodness of fit (inverse and normalized least-squares distance) as a function of $\lambda$ and $h$ on expanding clones (clone size $> 1$ cell) in Co100, HCT-15 and CC09 xenografts. Dots indicate optimal fit. s.c., subcutaneous; ortho., orthotopic. Error bars represent s.d. of clone size distribution over time in Co100 xenografts: experimentally determined (black dots) and model-predicted (dashed line) clone size distributions using best fit and 95% confidence interval (CI, grey shade) as found in b. Red and blue dots represent experimental data obtained from subcutaneous and orthotopic xenografts in NSG mice, respectively. c, Average measured (solid lines) and predicted (dashed lines) clone size over time for all (black lines) or proliferating clones (red lines) in Co100 tumours. d, Measured (solid line) and predicted (dashed line) s.d. of clone size over time. In e-f, data are presented as mean ± s.e.m. f, Experimentally measured fraction of single-cell clones, one week after clone induction (black dots). The model prediction using optimal fit parameters for the indicated cell lines is shown as red lines. Data are presented as mean ± s.d. g, Inferred percentage of clonogenic cells in the tumour edge, depicted in circle diagrams for Co100, HCT-15 and CC09. Source data for b-f are provided in Supplementary Table 1.

and Supplementary Table 1). Of note, a wide range of clone sizes was detected, especially at later time points, indicating extensive variability in clonal outgrowth despite the monoclonal origin of these cells.

To elucidate the mode of growth of human colon cancer we developed a stochastic model of tumour growth (Fig. 2a and Supplementary Note 1). In this model, we distinguish clonogenic and non-clonogenic cells. At each time point, clonogenic cells either proliferate (probability $a$) or lose clonogenic capacity (probability $1-a$) with an effective rate $\lambda$, while non-clonogenic cells are inert. This dynamic is similar to models used to study clonal dynamics during homeostasis\(^1\), but differs in that $a \geq 0.5$ to capture the accumulation of malignant cells in growing tumours. We consider an exponential mode of growth (constant $a$) and a surface mode of growth ($a = \frac{h}{1 + \frac{h}{2}}$). Importantly, we also include how clonogenicity is lost in the stochastic model: either driven by the environment such that all cells in a clone lose clonogenicity simultaneously (probability $h$) or through an intrinsic process such that this loss is randomly distributed between cells and clones (probability $1-h$). Note that if loss of clonogenicity is fully defined by the environment $h = 1$, while for $h = 0$ loss of clonogenicity is completely intrinsic and the model is equivalent to a strictly hierarchical CSC model.
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**Fig. 3 | Mode of growth predicted by clonal dynamics confirmed at the macroscopic scale.** **a,** Ki67 staining of a subcutaneous grafted Co100 tumour. Bottom, magnification of the indicated area in the top panel. Scale bars, 1 mm. Image is representative of five tumours. **b,** Ki67 staining (yellow) of Co100 orthotopically grafted xenograft. Right, magnification of the indicated area in the left image. Scale bars, 500 μm (left) and 100 μm (right). Images are representative of five tumours. **c,** Average correlation ($R^2$) of tumour size measurements of tumour xenografts of the indicated cell lines with either the exponential volume (white bar) or surface growth model (black bar). Average $R^2$ of volume and surface growth were compared using two-tailed Student’s t-test. **d,** Average tumour volumes at 5-day intervals for Co100, HCT-15 and CC09 tumours. Best fit of volume growth ($V(t^\gamma) = V_e \beta^{\alpha t} + V_s \beta^{\beta t}$) is shown as a solid line, and the surface growth model ($V(t^\gamma) = V_e \beta^{\alpha t} + V_s \beta^{\beta t}$) is indicated by a dashed line. $V$, tumour volume (mm$^3$); $V_e$, initial tumour volume (mm$^3$); $V_s$, average clone size; $\gamma$, growth rate (1 per day) under exponential growth; $\alpha$, rate of volume under surface growth; $\beta$, initial tumour radius under surface growth; $\gamma$, growth rate (1 per day) under exponential growth. In **c** and **d**, data are presented as mean ± s.e.m. Sample sizes are as follows: Co100 ($n=29$ tumours), HCT-15 ($n=25$ tumours) and CC09 ($n=23$ tumours). Individual data points are shown in Supplementary Fig. 5a,b.

The model comprising the three parameters $\lambda$, $a$ and $h$ thus captures the rate and mode of tumour growth, as well as the process underlying the loss of clonogenicity. We numerically generated clone size distributions from the model for a wide range of parameters. By comparing the numerical distributions with the lineage tracing data using the Akaike information criterion, we found that the model with $a$ determined by surface growth best describes the expansion of solid cancers (Supplementary Note 1). Using the method of least-squares we next determined the goodness of fit for all combinations of parameter values of $\lambda$ and $h$ (Fig. 2b). Using the best-fit parameter values we accurately described the size distributions of expanding clones (Fig. 2c and Supplementary Fig. 3e-h), average clone size and the variance in the clone size distribution over time within the xenografts (Fig. 2d,e). We found that, for each of the xenografts, $\lambda$ ranged from 0.15 to 0.35 effective divisions per day. Critically, $h$ tended to approximate 1 for all cancers (Fig. 2b), suggesting that these tumours do not contain an intrinsic hierarchical organization. The lack of an intrinsic hierarchy was underlined by the ability of the model to estimate the proportion of single cell clones from the size distribution of larger clones (≥2 cells) (Fig. 2f), indicating that single cell clones and expanding clones are representations of the same dynamics. This rules out the possibility that two distinct populations—differentiated cells and CSCs—were initially labelled. These analyses imply that functional CSCs within established cancer tissue are defined by the environment, and mostly reside at the surface of the tumours. Indeed, the estimated fraction of CSCs on the tumour edge approaches 100% (Fig. 2g and Supplementary Note 1). Of note, analysis of subcutaneous tumours from nude mice and NSG mice resulted in similar inferred parameters, as did orthotopically grown tumours, suggesting that the composition of the remnant immune system or the location of tumour grafting does not impact these fundamental growth dynamics (Fig. 2b,c and Supplementary Fig. 3).

To validate the inferred model of tumour expansion we evaluated the distribution of proliferative cells within xenografts by Ki67 staining (Fig. 3a,b). This revealed a clear tendency for proliferation to take place in the edge regions of the tumour (the outer ~300 μm), similar to what we observed in human primary tumours (Supplementary Fig. 4a,b) and other reports [28,29]. Of note, xenografts did not show evidence for extensive cell death at the analysed time points (Supplementary Fig. 4d). The subcutaneous tumours were well-vascularized and although small hypoxic regions could be detected by immunohistochemistry for HIF-1α, these were not restricted to the tumour centres (Supplementary Fig. 4e-g). More importantly, we confirmed the prediction of the model that surface growth is the primary mode of tumour expansion in these cancers using macroscopic tumour volume measurements (Fig. 3c,d and Supplementary Fig. 5). We take the ability to predict macroscopic tumour growth characteristics from clone size distributions within the tissue, thereby bridging several scales of magnitude, as strong evidence for the validity of our model in providing a quantitative description of tumour growth dynamics.

**Spatiotemporal regulation of growth.** The most striking finding of our analyses is that CSC functionality is spatiotemporally organized in cancer tissue, at a spatial scale that is larger than the largest clones we detected; that is, the values inferred for $h$ are close to 1. This implies that individual clones that were labelled do not show evidence for hierarchical organization. However, within Strawberry-positive clones we do find heterogeneous expression of TCF optimal promoter-green fluorescent protein (TOP-GFP) (Fig. 4a), a read-out for Wnt signalling activity that was previously used to identify
CSCs as evidenced by their superior ability to initiate subcutaneous tumours\(^{29,30}\). In addition, differentiation markers displayed a heterogeneous expression pattern within individual clones (Fig. 4b). This confirms that a cell capable of both self-renewal as well as multi-lineage differentiation was indeed marked, conforming to the definition of a stem cell. To interrogate the relationship between CSC identity and functionality we took advantage of the fact that in situ clono-genic cells reside predominantly at the xenograft edges. Differential enzymatic digestion of the cells from the outside and inside regions of the xenografts followed by RNA sequencing revealed a significant enrichment of genes associated with proliferation in the tumour edge (Fig. 4c,d), in line with the Ki67 staining and surface growth kinetics. This enrichment was not observed for gene signatures associated with quiescent stem cells\(^6\), CSCs\(^{29}\) and ISCs\(^{30}\), or individual CSC-associated genes (Fig. 4e). More specifically, no differences in the proportion of LGR5\(^+\) cells (Fig. 4f) or TOP-GFP levels were detected between the edge and the centre of Co100.G7 xenografts. In contrast, images of edge and centre regions showing TOP-GFP (green) and nuclear stain, Hoechst (blue), representative of eight independent tumours. AC133 expression or TOP-GFP levels were detected between the edge and the centre of Co100.G7 (n = 8) xenografts. In the two-tailed paired Student’s t-test was used. NS, not significant. All data presented as mean ± s.d.
results indicate a discrepancy between cells positive for CSC markers, which reside homogeneously throughout the cancer tissue, and functional CSCs, which reside at the tumour edge.

To test if the loss of clonogenicity of clones in the centre is reversible, we re-transplanted the tumour centre and detected a rapid recapitulation of the surface growth mode, indicating that clonogenicity is instilled by an appropriate environment (Fig. 5a). Furthermore, we compared the clonogenicity of tumour cells derived from the edge and centre of xenografted tumours using the limiting dilution assay. In vitro clonogenicity of cells from the xenograft edge and the centre was identical (Fig. 5b). The lower clonogenicity found in vitro (~1/300) compared to that obtained using our marker-free lineage tracing in vivo (~1/1) supports the notion that cell-extrinsic factors determine cell fate. We found that expression of TOP-GFP does correlate with the observed clonogenicity in both limiting dilution and tumour initiation assays (Fig. 5c,d), but does not reflect the spatial regulation of clonogenicity as found in established tumours. More generally, although an inverse relationship exists between the clonogenicity observed in vitro and the time it takes until a small tumour has formed (Fig. 5e), no relationship between this clonogenicity and the time it takes until a small tumour has formed in vivo (<0.001) was detected (Fig. 5f). This points to a marked discordance between the cells that drive tumour expansion and the cells that show tumour initiation ability in transplantation assays.
Confirming the role of the environment, RNA profiling revealed that capacity, and that in fact all malignant cells can function as CSCs.  

Again, the absence of intrinsic differences between cells from clonogenic and non-clonogenic parts of the tumour suggests that the environment rather than the intrinsic characteristics of a cell determines its clonogenic capacity, and that in fact all malignant cells can function as CSCs.
Fig. 7 | Chemotherapy does not fundamentally alter the growth dynamics of cancer. a, Co100 tumours were treated with a combination of oxaliplatin (3 mg per kg, 1× per week) and 5-FU (15 mg per kg, 2× per week) and lineage tracing was performed. Viable tumour cell volume over time is shown, together with surface model fits (dashed lines). Control data are from Fig. 3d. N = 29 (control) and 26 (Oxa-5-FU) tumours; data are presented as mean ± s.e.m. b, c, Representative immunofluorescence images (b) and quantification (c) of TOP-GFP (green) expression in control or treated Co100 (n = 8 and 9) tumours. d, e, Representative images (d) and quantification (e) of in situ hybridization of LGR5 mRNA in control and treated Co100 tumours; n = 4 (control) or 5 (Oxa-5-FU) tumours per group. In c and e, data are presented as mean ± s.d. f, Example images of clones detected in the presence of therapy. Scale bars, 100 μm (b, d, f). g, Relative clone frequency (heatmap colours) per binned size (in columns) and time (rows) for treated Co100 tumours. Numbers of clones and tumours (in parentheses) are indicated to the right of each time point. h, Inference of the temporally changing clone size distributions with the stochastic model. Dots indicate optimal fit (black, Oxa-5-FU; red, control) for the heatmap shown in Fig. 2. Data are presented as mean ± s.d.; source data for f–h are provided in Supplementary Table 1. i, Ki67+ cells (yellow) are in the proximity of stromal cells (αSMA, green). Scale bars, 100 μm. j, Average distance of either all or proliferating (Ki67+) cells to the nearest αSMA+ fibroblast in treated Co100 tumours (n = 20,000 cells from seven tumours). Error bars represent s.e.m. k, Image of a treated Co100 tumour, showing mStrawberry clones and αSMA+ cells (green). Scale bar, 500 μm; image is representative of four tumours. l, Mean distance of either large clones (clone > 10 cells) or small clones to the nearest αSMA+ fibroblast in Co100 tumours treated with oxaliplatin-5-FU (n = 100 clones from four tumours; error bars represent s.e.m.). In c, e, j, l, groups were compared using paired two-tailed Student’s t-test.
(Fig. 5g). Immunofluorescent staining for activated fibroblasts, characterized by αSMA expression, revealed a strong enrichment in the tumour edge (Fig. 5h). Importantly, we could also demonstrate a clear co-localization between Ki67-positive proliferating tumour cells and activated stromal cells (Fig. 5i,j and Supplementary Fig. 4c,h,i). Larger clones were located significantly closer to stromal cells (Fig. 5k,l).

To ascertain a direct effect of cancer-associated fibroblasts (CAFs) on clonogenicity, we established co-cultures of cancer cells with primary murine and human fibroblasts, and performed medium transfer experiments. Tumour cells expanded more effectively in co-culture, or when exposed to fibroblast-conditioned medium compared to control conditions (Fig. 6a,b and Supplementary Fig. 6a-c), suggesting a mechanistic role for CAF-secreted factors in driving clonogenic outgrowth. Osteopontin (OPN, gene name Spp1) was the most abundantly expressed secreted factor by CAFs in our xenograft models, and we found that this protein was able to induce tumour cell proliferation in vitro (Supplementary Fig. 6d,e). To confirm that CAF-secreted factors modulate clonogenicity in vivo, Co100 cultures harbouring the LV-indLS2 vector were transduced with a human OPN overexpression vector (Co100. OPN) (Supplementary Fig. 6f,g). Following subcutaneous injection of Co100.OPN cells in nude mice we witnessed accelerated tumour growth compared to control xenografts (Fig. 6c,d). OPN was ubiquitously expressed in Co100.OPN tumours as compared to control xenografts, where it was restricted to CAF-rich regions (Fig. 6e). We predicted that the homogenous presence of OPN would drive clonogenic outgrowth throughout the tumours independently of CAFs, thereby uncoupling CAF presence and clonogenicity within the tissue. To assess this, we performed clonal tracing in Co100. OPN tumours as described before, quantified clone size distributions at various time points, and subsequently analysed these with our inference strategy (Fig. 6f and Supplementary Fig. 6h,i). We also confirmed that in this setting no intrinsic CSCs are present; that is, \( h \approx 1 \). More importantly, we found that the clone size distribution was altered, and that the variation in clone sizes was significantly reduced in Co100.OPN xenografts (Fig. 6g,h). In combination with the accelerated growth rate, this shows that a larger proportion of cells contributes to tumour expansion, and that clonogenicity is more homogeneously distributed throughout the Co100.OPN cancers. Indeed, when we performed a spatial analysis of clone sizes we detected that the initially observed increased clone sizes at the tumour edges, where the CAFs reside, was now absent (Fig. 6i-k).

This was further supported by increased proliferation in the central regions of Co100.OPN tumours, while no significant difference in the abundance of CAFs was observed there (Supplementary Fig. 6j-l). Together, these data indicate that, by overexpression of key CAF-secreted factors, we can uncouple the clonogenic potential of cancer cells from the stroma, and link it to individual secreted factors. This confirms the robustness of our model predictions, and provides a direct mechanistic link between CAF-secreted factors and cancer cell clonogenicity in situ.

**Clonogenic dynamics under treatment.** CSCs are assumed to be resistant to chemotherapeutics, and to drive relapse of the disease \(^{13,20}\). Critically, this notion is also based on analysis of CSC marker expression and transplantation assays. We have employed our tracing system to elucidate the clonogenic dynamics within tissues exposed to therapy. We established small xenografts (~200 mm\(^3\)) and initiated treatment with the clinically relevant combination fluorouracil (5-FU) and oxaliplatin. We titrated the dose to a level that significantly reduced tumour expansion (Fig. 7a), but did not kill all cells, to allow assessment of the putative differential sensitivity of various populations. Analysis of the CSC markers TOP-GFP and LGR5 in this setting corroborated previous reports that the percentage of cells expressing these markers was increased (Fig. 7b-c)\(^{16,22}\). Next, we performed lineage tracing within the treated xenografts and inferred the parameters that optimally describe tumour expansion under these conditions (Fig. 7d-h). \( \lambda \) was slightly decreased, reflecting the slower growth when therapy is applied, but we did not detect an impact on the \( h \) parameter, which still approximated 1, indicating that clonogenicity was dictated by the environment. Thus, although tumour growth was effectively delayed and cells expressing CSC markers were enriched, no differences in the fundamental expansion dynamics were observed, and this expansion is probably governed by the microenvironment. Indeed, similar to untreated cancers, we detected an increase in Ki67 at the tumour edges and in close proximity to CAFs (Fig. 7i,j), and in treated tumours clones in the vicinity of CAFs were also larger (Fig. 7k,l).

All these data suggest a direct role of the tumour stroma in driving clonogenic outgrowth during therapy. Most critically, during treatment, no intrinsic functionally distinct population was detected, and similar to unperturbed tumour growth this reveals a direct mismatch between CSC phenotype and functionality within a tissue.

**Discussion**

Here, we have presented a marker-free and quantitative analysis of colon cancer growth dynamics and response to therapy. We found that cells with CSC functionality are not necessarily the same cells that express CSC markers. We conclude that the environment is dominant over cell-autonomous features in defining stem cell functionality. Furthermore, although chemotherapy enriches for a CSC phenotype, no functionally distinct population is selected and in situ clonogenicity remains defined by the environment. Although previous murine lineage tracing studies in intestinal tumours provided evidence for functional stem-like cells, these experiments focused on early adenomatous lesions containing few genetic aberrations, and within these lesions the crypt niche remains largely conserved\(^{9,11,32}\). Recent studies in carcinoma demonstrated a function for LGR5\(^+\) cells as CSCs, but also suggested a high plasticity of tumour cells following the ablation of specific populations\(^{19,21}\). We now demonstrate that switches in cell functionality defined by the microenvironment are the rule rather than the exception, and that this also applies to unperturbed tumours. Our findings are in agreement with a recent study that demonstrated clonogenic outgrowth in colon cancer predominantly takes place in the outer tumour regions, suggesting that tumour cell position may define clonogeneity\(^{18}\). Using the quantitative approach proposed here we have shown that the complete variability in clone sizes derives from spatiotemporal regulation and we thus conclude that a stem cell hierarchy is absent in colon cancer. Our work also provides mechanistic insight into the signals that dictate spatiotemporal growth dynamics. We identified one CAF-secreted factor, OPN, as a key regulator of in vivo clonogenicity. Although CAFs have previously been implicated in the dedifferentiation of tumour cells\(^{19,21}\) and tumours with high stromal content have been associated with poor prognosis and therapy resistance\(^{13,14}\), we now show that CAFs are principal actors in shaping tumour biology. Perhaps even more relevant, during treatment no functional enrichment for CSCs was detected, despite an increase in CSC marker expressing cells. Hence, therapies specifically targeting the CSC fraction are likely to fail, because non-clonogenic or differentiated cells display tremendous plasticity and will become clonogenic when they gain access to the right niche. We believe that strategies that either block activating signals from the stromal compartment, or that directly target the cell-intrinsic pathways that drive clonogenicity irrespective of differentiation state, are key to improving anticancer therapies. To conclude, our study provides a detailed account of the spatiotemporal expansion dynamics of colon cancers, and as such describes the neutral clonal dynamics within this tissue. We envision that our experimental system and analytical framework can be used to quantify clonal advantages that specific mutations provide, both in the absence and
presence of (targeted) therapeutic agents. This could enhance the value of xenograft models in the study of acquired resistance, and facilitate the development of novel approaches to circumvent this.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41556-018-0179-z.

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Author contributions

K.J.L., S.C.L., X.B., L.E.N., F.C.L., M.C.L., M. van d.H., A.v.O., N.L., F.d.S.E.M., J.O., P.V., G.H. and L.K. performed experiments. D.M.M., E.M. and L.V. developed the quantitative models. K.J.L., D.M.M., S.C.L., X.R.R., L.E.N., F.C.L., M.C.L., M.v.d.H., S.M.v.N., A.v.O., N.L., F.d.S.E.M., J.O. and P.V. advised on the work and commented critically on the manuscript. K.J.L., D.M.M., F.M.B. and L.V. conceived and designed research. D.J.W. and J.P.M. advised on the work and commented critically on the manuscript. All authors approved the content of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Methods

Primary human tumour material. Primary human colorectal tumour material was obtained from the AMC-AJCIC2-90 cohort, 90 AJCC stage II colorectal cancer patients who underwent surgical resection at the Amsterdam Medical Center (AMC). The Netherlands Cancer Institute, 1997–2006 (AMC-AJCIC90). The study is compliant with all relevant ethical regulations involving human participants, and was approved by the medical ethical committee of the AMC. Informed consent was obtained from all subjects.

In vivo experiments. The study is compliant with all relevant ethical regulations regarding animal research. All in vivo experiments in this study were approved by the Animal Experimentation Committee at the Academic Medical Center in Amsterdam (DEC103141) and performed according to national guidelines. Female nude (HsdAthymic Nude-Foxn1nu) mice (6–12 weeks old) were obtained from Envigo. NOD-scid IL2renull mice (NSG; NOD.Cg-Pkdckid Il2rgtm1Wjl/SorJ) were bred in our facility. Animals were randomly assigned to experimental groups, and no blinding was performed during the experiments. Animals were only excluded from analyses when no tumours appeared. Animal sample sizes were estimated on the basis of previous work.

Cell culture and constructs. Human primary colon cancer cultures were established as described previously and cultured as spheroids in polyHEMA (poly(2-hydroxyethyl methacrylate, Sigma) coated flasks (Corning). Spheroid cultures expressing a T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) driven GFP reporter (TOP-GFP) for Wnt signalling have been described previously. Primary colon cancer medium contains advanced DMEM/F12 (Life Technologies), supplemented with N2 (Life Technologies), 1-glutamine, glucose, HEPES, heparin, insulin, epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), as described previously. Primary fibroblast cultures were established by mechanical disruption and digestion of fetal intestinal tissue using Liberase (Sigma), and cells were filtered through a 70 µm filter and cultured on tissue culture plates (Life Technologies). Co-cultures of primary colon cancer cells and fibroblasts were performed in primary cell culture medium without EGF and FGF HCT-15 (ATCC) and HT29 (Sanger) cells were cultured in DMEM (Life Technologies) supplemented with 10% FCS (Life Technologies). Spheroid cultures were lentivirally transduced with LV-indLS2 or LVPLX-D-OPN and single-cell clones were selected on 96-well plates with an SH800 cell sorter (Sony). For in vitro chemotherapy, cells were treated with either oxaliplatin (Sigma) or 5-FU (Sigma) at the indicated concentrations. Cells lines were authenticated by STR profiling and mutation analysis and were regularly tested for mycoplasma. pWXPXL-D-OPN was generated by cloning of human OPN-full length (OPN-FL) from pDEST-OPN-A (Addgene) into the pWPXL lentiviral expression vector (Addgene) using the Pmel restriction site.

Xenograft studies. Subcutaneous tumour growth and label induction. To generate in vivo tumours, 50,000 human primary colon cancer cells in medium were mixed at a 1:1 ratio with matrigel (Corning) and injected subcutaneously into both flanks of nude mice. Mouse expression was induced when the tumour reached a size of ~100 mm³, by a single intraperitoneal injection with 0.05 mg mouse 4-OH-tamoxifen (Sigma) dissolved in sunflower oil (Sigma). Tumour growth was monitored twice a week using calipers, using the formula: 4/3π×(length × width × height). Chemotherapeutic treatment of subcutaneous tumours. For in vivo chemotherapeutic treatment of Co100 xenografts, small subcutaneous tumours were first grown as described above. At a tumour size of 200–300 mm³, treatment was started. Mice received a combination of oxaliplatin (5 mg/kg, 1x per week) and 5-FU (15 mg/kg, 1x per week) for 4 days after the start of treatment with a single 4-OH-tamoxifen dose was given to induce clone labelling. Treatment was continued until the tumours were collected.

Orthotopic tumour growth. For orthotopic tumour growth, 50,000 tumour cells in a mix of medium and matrigel (1:1 ratio) were injected into the caecal wall of nude mice using laparotomy under anaesthetic. Metacam was subcutaneously administered as pre-operative analgesia. Mice were monitored daily during recovery. Growth curves and clone dynamics of chemotherapy-treated Co100 xenografts and CO100-OPN xenografts were compared to parental Co100 tumours (Fig. 3d).

Tumour isolation. Mice were killed at various time points to isolate tumours. Immediately after isolation, tumours were fixed using 4% paraformaldehyde. Then, 20-µm-thick frozen tissue sections were taken from different locations within the tumour at intervals of >100 µm, to prevent doublesectioning of clones. For immunohistochemistry purposes, representative tumours were embedded in paraffin.

Limiting dilution assay. Cells were dissociated and plated in 96-well plates (Corning) using an SH800 Cell Sorter (Sony) in a limiting dilution fashion at 1, 2, 4, 8, 16, 24, 32, 64, 128 and 256 cells per well. Clonal frequency and significance were analysed using the Extreme Limiting Dilution Analysis (ELDA) ‘limdil’ function35. For limiting dilution assays with tumour cells from freshly isolated xenografts, the outside (~1 mm) and centre of the tumour were first separated using razor blades and cut into small pieces. Both populations were dissociated in medium containing collagenase (Roche) and hyaluronidase (Sigma) at 37°C for 1 h. Cells were filtered through a 70 µm filter. Dead cells were excluded by 7-AAD staining (BD Biosciences).

In vivo transplantation assay. freshly isolated subcutaneous xenografts were separated in centre and edge fractions and dissociated as described above. Of each fraction, 1,000 cells were subcutaneously injected into the flanks of nude mice (n = 5) and tumour outgrowth was monitored.

Flow cytometry analysis of freshly isolated xenografts. Dissociated tumour cells were washed in FACS buffer (PBS + 1% FCS, Life Technologies). Cells were incubated for 1 h at 4°C with either mouse anti-AC133-biotin (130-090-664, Milteny, 1:200), or isotype controls (mouse IgG1-biotin, ebioscience, 1:500). As secondary antibody, streptavidin-APC (BD Pharmingen, 1:500) was used. Dead cells were excluded by 7-AAD staining (BD Biosciences). Cells were analysed using FACSBag (BD Biosystems).

In vivo validation of inducible reporter expression. To determine the optimal 4-OH-tamoxifen dose for sporadic label induction, mice were intraperitoneally injected with decreasing concentrations of 4-OH-tamoxifen. One week after injection, tumours were isolated. Tissue culture was dissociated with collagenase/ hyaluronidase, filtered through a 70 µm cell strainer (Corning) and mStrawberry expression was measured using FACSBag (BD Biosystems).

Frozen tissue section imaging. Frozen tissue sections were analysed using an SP8-X confocal microscope (Leica). Sections were counterstained with Hoechst 33342 (Sigma) to detect nuclei (405 nm laser) and eventually Actin-Green-488 (phalloidin) ready probe (ThermoFisher) to detect F-actin (488 nm laser). Whole tumour sections were scanned for mStrawberry expression (573 nm laser). For immunofluorescence the following primary antibodies were used: rabbit anti-Ki67 (SAB500134, Sigma, 1:200), mouse anti-Ki67 MIB-1 (M724029-2, Agilent/DAKO, 1:200), rabbit anti-cleaved caspase-3 (Asp175, Cell Signaling 1:600), rabbit anti-alpha-SMA (ab5694, Abcam 1:100), mouse anti-alpha-SMA (A-2547, Sigma, 1:100), rabbit anti-AC133 (Milteniy, 1:100), rabbit anti-Mucin2 (H-300, 1:1534, Santa Cruz, 1:100), mouse anti-Ki67 (926-68073, Li-Cor Biosciences 1:500), rabbit anti-angiogenic alkaline phosphatase (IAP, GTX227232, Genetex, 1:100), rabbit anti-lysozyme E (A0099, Dako, 1:100), rabbit anti-CD31 (AB28369, Abcam, 1:100), rabbit anti-osteopontin (ab91655, Abcam, 1:100) and mouse anti-alpha-Defensin-5 (A9C8, Abcam, 1:100). As secondary antibody goat anti-rabbit-Alexa488 (A11008, Invitrogen 1:500), goat anti-mouse-Alexa488 (A11035, Invitrogen 1:500), donkey-anti-rabbit-Irdeyeb0 (926-68073, Li-Cor Biosciences 1:500) or donkey-anti-mouse-Irdeyeb0 (926-32222, Li-Cor Biosciences 1:500) was used. For imaging, Leica Application Suite-Advanced Fluorescence was used. For image analysis ImageJ was used.

Immunohistochemistry. Frozen tissue sections were stained with either haematoxylin and eosin or Alcian blue. Immunohistochemistry was performed on paraffin-embedded tumour sections to stain for HIF-1α (mouse anti-HIF1α 610959, BD, 1:100 after antigen retrieval in Tris EDTA/PH9 for 15 min at 98°C). To visualize immunostaining, goat anti-mouse/rabbit/rat Power Vision Polycl-HRP (dvsp110HRP, Immunologic) was used as secondary antibody together with the Novared peroxidase HRP substrate kit (sk4800, Vector). As a counterstain, haematoxylin was used.

RNA–in situ hybridization tumour sections. RNA–in situ hybridization (ISH) was performed on fixed frozen tumour tissue sections to stain for LGR5. The procedure was performed according to the manufacturer’s instructions (RNA scope 2.5 HD Reagent Kit Brown, 322371, ACD, user manual no. 320534, 322300-UAM, ACD). In short, the fixed frozen sections were pre-treated with boiling in target retrieval solution for 5 min to allow access to target RNA. Next, RNAscope Protease Plus was incubated for 30 min at 40°C. To allow probe hybridization to RNA targets, probe incubations were performed in the HybEZ oven (ACD) for 2 h at 100°C.
40 °C (RNAscope Probe–Hs-LGR5, 311021, ACD). Probes Hs-PPIB and dapB were used as positive and negative control, respectively (RNAscope, ACD). After this, the slides were washed and incubated with a series of signal amplification solutions. 3'-diaminobenzidine (DAB) was used for visualization (ACD). Finally, the slides were counterstained with haematoxylin and mounted in Pertex. Whole tumour slides were automatically imaged with a Philips IntelliSite Ultra Fast 1.6 slide scanner. For image analysis, ImageJ was used.

**Tumour xenograft clearing.** Tumour xenograft slices were fixed for 24 h in 4% paraformaldehyde (PFA) (pH 7.5) at 4 °C and then washed in PBS at room temperature. At this point we started chemical clearing following the CUBIC protocol. Each piece of tissue was immersed in 10% of ScaleCUBIC-1 reagent (+2DAP 10 µM) at 37 °C with gentle shaking for 1 day. Reagent 1 was then changed every 3 days until the tissue reached full transparency. The tissue was then washed with PBS several times for 24 h with gentle shaking, immersed in 20% (wt/vol) sucrose in PBS following immersion of reagent 2 for 2 days at room temperature while shaking. The tissue was washed with PBS multiple times for 24 h and then immersed overnight in Rapiclear 1.52 (Sunjin Labs). Tissue was mounted using the same Rapiclear solution in a 3 mm imaging spacer (Sunjin Labs).

**Cleared tumour xenograft imaging.** Tumour xenograft 3D images were acquired with an inverted confocal microscope (Leica TCS S5P) using 405 nm (DAPI) and 543 nm (red) lasers with a ×10 objective (NA = 0.4). Z planes (Z step = 5 µm) were merged using the default Leica SPS software (automatic merging) and saved as LIF files. 3D rendering, clonal volume and sphericity analysis were performed using the 'Surface' algorithm from IMARIS software (version 8.3.0, Bitplane).

**Image analysis.** Automated clone size quantification was performed on whole tumour cross-sectional slides imaged by confocal microscopy and converted to tiff file format. mStrawberry–positive areas were identified based on a signal above three times the local background level. The number of cells within clones was obtained by dividing the total clone area by the average cross-sectional area of a cell, as determined for each line using measurements on high-resolution images of tumour sections stained for Hoechst and phallidin, where the latter is used to demarcate cell borders. Because of irregular clone shape and potential migration, mStrawberry–positive areas separated by less than five cell diameters were considered clonal.

**mRNA profiling of tumour regions.** To generate RNA expression profiles of cancer cells located at the edge or centre of each tumour, we first mechanically separated the two regions and pooled the material obtained from 10 tumour slices (10 µm). Total RNA was isolated by incubating the tissues with proteinase K at 56 °C for 30 min. To revert the crosslink, the extracts were incubated at 80 °C for 15 min and then on ice for 3 min. After centrifugation at 20,000g for 15 min, supernatants were treated with DNaseI for 15 min at room temperature. Purification of total RNA was performed using RNAeasy FFPE columns (Qiagen). To generate sequencing libraries, the TrueSeq Stranded mRNA Library Prep Kit (Illumina) was used in combination with 4 µg of total RNA obtained from the edge or the centre of each tumour sample. Sequencing was performed on a HiSeq2500 (Illumina) in 50bp single-end mode. Reads were preprocessed, aligned and quantified using Trimomatic (0.36), HISAT2 (2.0.4), SAMtools (1.3.1), StringTie (1.2.4) and subread featureCounts (1.5.0–p1) with GRCm38.85 (v.85). DESeq2 (1.10.1) was applied for normalization, differential expression analysis and plotting. The results were annotated with org.Hs.eg.db (3.2.3) and used to rank genes according to the inverse Benjamin–Hochberg adjusted P value multiplied by the sign of the log, fold change, breaking ties based on log, fold change (edge versus centre). The ranked gene list was passed to GseaPeranked (2.2.3) for gene set enrichment analysis on stem cell marker, hypoxia and proliferation gene sets relevant for colorectal cancer. Separation of mouse and human RNA sequencing reads was based on the Xenolfiter-R method after alignment to GRCan3.87 and GRCh38.87 with STAR-2.5.2b. All RNA sequencing data are deposited in the Gene Expression Omnibus (GEO) database under accession no. GSE95499.

cDNA synthesis and quantitative RT–PCR. RNA from tumour sections was isolated as described above. RNA from cell lines was isolated using the Nucleospin RNA isolation kit (Macherey-Nagel). 1 µg of RNA was used to synthesize cDNA using SuperScript III according to the manufacturer’s protocol (Invitrogen). Quantitative RT–PCR was performed with LC480 SYBR green (Roche) in accordance with the manufacturer’s instructions on an LC480. The following primers were used:

| Name       | Sequence | Gene   | Product Size |
|------------|----------|--------|--------------|
| GAPDH      | 5′-CCACGAAAGCACAAGGAAAGG-3′ | GAPDH  | 396 bp       |
| Rev        | 5′-CAAGGGGCTGATGGCAAATGTG-3′ |         |              |
| GUSB       | 5′-TGGTGGAGGAGGTTCATTGTGA-3′ | GUSB    | 396 bp       |
| Rev        | 5′-GCACCTCTCGTGGTGGTACTGT-3′ |         |              |
| LGR5       | 5′-ACCAGACATGCGTCTTGGAAAC-3′ | LGR5    | 396 bp       |
| Rev        | 5′-TTCAGGAGGAGGTATCTTAT-3′ |         |              |
| GFP        | 5′-CTTAAGAGGAGGCAGGCAAC-3′ | GFP     | 396 bp       |
| Rev        | 5′-ATGCCCTTCTTCTGCTTGTC-3′ |         |              |
| OPN        | 5′-GAGGTTTCCAGGACAGGCAGCA-3′ | OPN     | 396 bp       |
| Rev        | 5′-AACGGGGATGCGCTTGTATG-3′ |         |              |

**Secreted OPN detection.** Secreted OPN levels were measured using the Human Osteopontin DuoSet ELISA kit (R&D Systems). Cells were counted before seeding and, after 3 days, supernatant was used for OPN determination.

**Statistics and reproducibility.** Sample sizes, statistical tests and definitions of error bars are indicated in the figure legends and calculated using Graphpad Prism 7 or MATLAB. All statistical tests were two-sided. Limiting dilution assays were analysed using the Extreme Limiting Dilution Analysis (ELDA) ‘limdil’ function. Between-group variances were similar and data were normally distributed. A P value of <0.05 was considered significant. We performed lineage tracing experiments in three independent cell lines with similar results.

**Data availability.** Source data for Figs. 1, 2, 6 and 7 and Supplementary Figs. 3 and 6 are provided in Supplementary Table 1. RNA sequencing data have been deposited in the GEO under accession no. GSE95499. Programming code has been deposited in GitHub (https://github.com/dmmiedema/Tumor-Growth-Model). Additional theoretical information regarding the modelling can be found in Supplementary Note 1. All other data supporting the findings of this study are available from the corresponding author upon reasonable request.

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| ☐   | ☒ For null hypothesis testing, the test statistic (e.g. \(F\), \(t\), \(r\)) with confidence intervals, effect sizes, degrees of freedom and \(P\) value noted |
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| ☐   | ☒ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |
| ☐   | ☒ Estimates of effect sizes (e.g. Cohen's \(d\), Pearson's \(r\)), indicating how they were calculated |
| ☐   | ☒ Clearly defined error bars |
| ☐   | ☒ State explicitly what error bars represent (e.g. SD, SE, CI) |

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

| Data collection | Leica LAS-X v3.1.5.16308, BD FACSDIVA |
|-----------------|---------------------------------------|
| Data analysis   | IMARIS (version 8.3.0); Extreme Limiting Dilution Analysis (ELDA) ‘limdil’ function (http://bioinf.wehi.edu.au/software/elda/); Graphpad Prism 7; MATLAB; Flowjo v10; ImageJ |
| Programming code has been deposited in GitHub: (https://github.com/dmmiedema/Tumor-Growth-Model) |

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- A description of any restrictions on data availability

Source data for Figures 1, 2, 6 and 7 and Supplementary Figures 3 and 6 have been provided in Supplementary Table 1. RNA sequencing data have been deposited.
in the Gene Expression Omnibus (GEO) under accession number GSE95499. Programming code has been deposited in GitHub: [https://github.com/dmmiedema/Tumor-Growth-Model]. Additional theoretical information regarding the modelling can be found in Supplementary Note 1. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Animal sample sizes were estimated on the basis of previous work (Vermeulen, L. et al. Science 342, 995-998 (2013) in which we performed a similar study in mouse intestine. Pilot experiments were used to estimate the amount of clones per tumour. |
|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | Animals were only excluded from analyses when no tumours appeared. |
| Replication | All experiments were repeated at least three times. Clonal data was analysed in three independent xenograft models, with similar results. All attempts at replication were successful. |
| Randomization | Animals were randomly assigned to experimental groups. |
| Blinding | No blinding was performed during in vivo experiments, since our study is descriptive and data was analysed using automated image analysis, blinding is not relevant. |

Reporting for specific materials, systems and methods

| Materials & experimental systems | Methods |
|---------------------------------|---------|
| Involved in the study | Involved in the study |
| n/a | n/a |
| - Unique biological materials | - ChIP-seq |
| - Antibodies | - Flow cytometry |
| - Eukaryotic cell lines | - MRI-based neuroimaging |
| - Palaeontology | | |
| - Animals and other organisms | | |
| - Human research participants | | |

Unique biological materials

Policy information about availability of materials

| Obtaining unique materials | All unique materials used are readily available from the authors. |

Antibodies

Antibodies used

- Rabbit monoclonal anti-Ki67, SAB5500134, Sigma, 1:200, lot#170407LV2D
- Mouse monoclonal anti-Ki67 (MIB-1) Agilent M7240, 1:200, lot#20052254
- Rabbit polyclonal anti-cleaved caspase3 (Asp175) Cell Signaling 9661, 1:600, lot#45
- Rabbit polyclonal anti-alpha-SMA, Abcam 5694, 1:100
- Mouse monoclonal, anti-alpha-SMA, A-2547, Sigma, 1:100, lot#076M4784V
- Rabbit anti-osteopontin ab91655, Abcam, 1:100, lot#GR64316-31
- Mouse anti-AC133-PE Miltenyi 130-080-801, 1:1000, lot#5170809431
- Mouse anti-CD133/1-Biotin (AC133) Miltenyi 130-090-664, 1:20, lot#5060531037
- Goat-anti-rabbit-Alexa488 Invitrogen A11008, 1:500, lot#1853312
- Goat-anti-rabbit-Alexa546 Invitrogen A11035, 1:500, lot#1810820
- Donkey-anti-mouse-IRDye680 U-Cor Biosciences 926-32222, 1:500, lot#C70419-08
- Rabbit polyclonal anti-CD31 Abcam ab28364, 1:20, lot#GR150486-9
Validation

All antibodies were used as validated by the manufacturer for their specific assay according to their datasheet. Rabbit anti-Ki67, mouse anti-ki67, anti-cleaved caspase3, anti-SMA, anti-osteopontin, anti-AC133, anti-CD133, anti-CD31, anti-HIF1a, anti-cytokeratin20, anti-intestine alkaline phosphatase, anti-lysozymeEC, anti-alpha-defensin5 were all published before. anti-Mucin2 was validated by the manufacturer for immunofluorescence. We performed primary and secondary antibody controls for all immunofluorescent stainings.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)  Human primary colon cancer cultures were established in our lab. HCT-15 (ATCC) and HT29 (Sanger) cells were purchased.

Authentication  Cell lines have been authenticated by STR profiling and mutation analysis

Mycoplasma contamination  All cell lines were tested negative for mycoplasm.

Commonly misidentified lines  No commonly misidentified cell lines were used

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals  Mouse: Hsd:Athymic Nude-Foxn1nu, female, 6-12 weeks, obtained from Envigo. Mouse: NOD-scid IL2rγnull mice (NSG; NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ), female, 6-12 weeks, were bred in our facility.

Wild animals  The study did not involve wild animals.

Field-collected samples  The study did not involve field collected samples.

Flow Cytometry

Plots  Confirm that:

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☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
☒ All plots are contour plots with outliers or pseudocolor plots.
☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation  Dissociated tumour cells were washed in FACS buffer (PBS + 1% FCS (Life Technologies)). Cells were incubated for 1 hour at 4°C with either mouse anti-AC133-biotin (130-090-664, Miltenyi, 1:20), or isotype controls (mouse IgG1-biotin (eBioscience, 1:500). As secondary antibody streptavidin-APC (BD pharmingen, 1:500) was used. Dead cells were excluded by 7-AAD staining (BD Biosciences).

Instrument  Cells were analysed using FACSCanto (BD Biosystems)

Software  Flowjo v10

Cell population abundance  mStrawberry+ cell population abundance was dependent on tamoxifen induction.

Gating strategy  Cells were selected in FSC/SSC dot plot to remove debris, single cells were gated using the FSC-H/FSC-W dot plot. GFP+, mStrawberry+ or PE+ cells were gated and compared with a control sample with no detectable fluorochrome expression.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.