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Spatiotemporal Regulation of Clonogenicity in Colorectal Cancer Xenografts

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Cancer evolution is predominantly studied by focusing on differences in the genetic characteristics of malignant cells within tumors. However, the spatiotemporal dynamics of clonal outgrowth that underlie evolutionary trajectories remain largely unresolved. Here, we sought to unravel the clonal dynamics of colorectal cancer (CRC) expansion in space and time by using a color-based clonal tracing method. This method involves lentiviral red-green-blue (RGB) color-coding(12), which enabled us to track individual cells and their clonal outgrowth during tumor initiation and growth in a xenograft model. We found that clonal expansion largely depends on the location of a clone, as small clones reside in the center and large clones mostly drive tumor growth at the border. These dynamics are recapitulated in a computational model, which confirms that the clone position within a tumor rather than cell-intrinsic features, is crucial for clonal outgrowth. We also found that no significant clonal loss occurs during tumor growth and clonal dispersal is limited in most models. Our results imply that, in addition to molecular features of clones such as (epi-)genetic differences between cells, clone location and the geometry of tumor growth are crucial for clonal expansion. Our findings suggest that either micro-environmental signals on the tumor border or differences in physical properties within the tumor, are major contributors to explain heterogeneous clonal expansion. Thus, this study provides further insights into the dynamics of solid tumor growth and progression, as well as the origins of tumor heterogeneity in a relevant model system.

Significance

Colorectal cancer (CRC) is a heterogeneous disease, with significant variation in genotype and phenotype within each individual tumor. This intra-tumor heterogeneity emerges during tumor development due to clonal evolution and in part can explain therapy resistance in CRC. However, a detailed understanding of the spatiotemporal development of tumors underlying cancer evolution and intra-tumor heterogeneity remains absent. Here, we use lineage-tracing experiments of human CRC cells transplanted into immunocompromised mice, in combination with computational modeling, to study the growth mode of CRC. We found that the clonal position is crucial for clonal outgrowth. This demonstrates that, in addition to the genetic composition, the environment and the geometry of tumor growth play a significant role in shaping tumor evolution.
We found that the injection volume had an important impact on the overall neutrality of the integration events (expression of the LeGO vectors and resulting color, as well as the stochastic expression depending on the vector insertion site in the genome). Representative images of LeGO-transduced colon cancer cells with three different vectors. From left to right: human primary cell lines Co100, Co147, CC09 and serum-cultured cell line HT55. Scale bars, 100 µM.

Detailed clone size measurements, we conclude that the full clone size heterogeneity is defined by cell-extrinsic features, and thus no evidence of an intrinsic hierarchy was found in established CRC tissue. Additionally, we found that clonal dispersal is limited and that the number of clones remains constant during tumor growth. Taken together, these findings provide critical insights in the commonly employed subcutaneous xenograft assay and indicate that spatial location and time of emergence of a clone is an important but until now under-recognized force in colon cancer evolution and heterogeneity.

Results

Multicolor clonal tracing in colorectal cancer. In order to study the clonal dynamics that drive expansion of CRC tissue in situ, we transfected a series of three primary colon cancer cultures (Co100, Co147 and CC09) and one serum-cultured cell line (HT55) with the LeGO vector set (Fig. 1A–C). Following titration of the virus we obtained RGB (red, green, blue) marked cultures in which cells were labeled with a wide range of unique colors that allows for clonal tracking (Fig. 1D). Importantly, by monitoring a series (n=10) of single cell clones by fluorescence-activated cell sorting (FACS) in time, we confirmed the stability of the expression of the LeGO vectors and resulting color, as well as the overall neutrality of the integration events (SI Appendix, Fig. S1).

Next, we injected the cultures using different injection volumes of Matrigel subcutaneously in immunocompromised (nude) mice. We found that the injection volume had an important impact on the resulting clone configuration. Larger injection volumes (100 µl) resulted in diffuse clonal expansion within the Matrigel plug, and clones simply expanded until they made contact rather than being in direct competition early after injection (SI Appendix, Fig. S2A). Therefore, to resemble clonal growth dynamics of established colon cancer in our xenograft model, we selected the smallest injection volume (50 µl) for the follow-up experiments.

Analysis of small xenografts of ~300 mm³ showed a clear demarcation of individual clones in all evaluated models (Fig. 2A). We define a clone as a region of identical color representing the offspring of an individual injected cell. Although multiple clones can represent with similar colors, we estimate we can visually separate ~96 hues, and combining the RGB marking with spatial information allows for robust identification of clones originating from the moment of injection. The various tumor models presented distinct morphologies. Whereas Co100 and HT55 xenografts showed a well-differentiated morphology with evident glandular structures separated by stromal matrix, Co147 and CC09 instead were moderate-/poorly differentiated presenting with large tissue regions without glandular differentiation (Fig. 2B). In all models, clonal dispersal was limited, and only rarely were regions with a mixture of multiple clones in 2D sections detected (Fig. 2B and 2C). While the fraction of mixed clones is probably higher if all three dimensions are considered, it indicates that competition between clones in CRC is mostly the result of parallel expansion at distinct rates rather than the result of direct competition within glandular structures, something that remained elusive before. Also, the limited spread of clonally related cells throughout larger cancers revealed that the motility of cancer cells within xenografts is rather limited. These findings are in line with the results of multi-region sequencing analysis that indicate that private mutations are often detected in separate tumor regions and show that the LeGO xenografts are appropriate model systems to study colon cancer growth and progression (11, 15, 16).

Effective population size of colorectal cancers. Previous work has revealed that not all CRC cells have an equal ability to initiate tumor growth in immunocompromised mice. It has been established that cells that express markers of immature cell types such
as 4CI33, or that present with high Wnt pathway activity, have a superior capacity to induce colon cancer xenografts (17-19). These studies have mostly been performed using limiting dilution assays, in which a decreasing number of cells is injected, which then allows for the calculation of the proportion of cells within a population capable of initiating xenograft growth. However, it remains unclear how the reduced cell numbers impact on the ability to initiate tumors, for example due to the lack of paracrine and juxtacrine signaling input. Using our model system, we now have the ability to directly determine the proportion of injected cells that contributes to tumor initiation using an equal number of injected cells (Fig. 3A). By high-resolution analysis of xenograft tissue, and quantification of the number of clones, we could estimate the number of injected cells that actively grew out. We found that in vivo dispersal of cells is limited (Fig. 2), and this allowed us to identify each clone that resulted from the expansion of an injected cell as a connected region of cells with the same color. We found that the percentage of injected cells contributing to tumor initiation ranged between ~2-20%. The highest proportion of tumor initiating cells (TICs) was detected in the serum cultivated cell line (HT55), and the primary cell cultures displayed more limited clonogenic potential during the initiation phase (Fig. 3B). Additionally, comparison of the TIC frequency in the LeGO model versus the in vitro limiting dilution assay revealed only a weak correlation between both methods for determining the clonogenicity of tumor cells (Fig. 3C). We found that the limiting dilution assay could both under- and overestimate the clonogenic cell frequency. This indicates that in some models, injection of a larger cell number suppresses outgrowth of cells, as for example in Co147, while in another model the co-injected cancer cells promote outgrowth of cells (e.g. Co100). Hence, we suggest that the interpretation of data derived from an in vitro assay about the clonogenic capacity of tumor cells should be done with caution. Importantly, the estimated proportion of cells initiating tumor growth was independent of the tumor volume analyzed (Fig. 3D) and actual number of cells injected (Fig. 3E), indicating that clones that contribute to tumor initiation permanently reside in the tumor tissue, and are not lost due to competition for example, making this assay robust to analyze different time points or tumor volumes.

**Growth dynamics of colorectal cancer tissue.** To elucidate the underlying dynamics of colon cancer tissue expansion we mixed LeGO cultures with non-transduced cultures. This had the benefit that LeGO clones were better separated and allowed us to use a semi-automated image analysis pipeline to quantify the clone sizes within the whole xenograft tissues (Fig. 4A and Material and Methods). Analysis of hundreds of clones within tumors of different sizes revealed that on average the median clone size increased as expected in an expanding tissue (Fig. 4B). More interestingly, we detected that the heterogeneity in clone sizes was very large, and many clones remained small and did not seem to significantly contribute to tumor expansion (Fig. 4B and SI Appendix, Fig. S3). When plotting the relation between the proportion of clones that contribute to which fraction of tumor volume, we indeed detected that a small number of clones is responsible for the majority of the tumor growth (Fig. 4C). Furthermore, in larger tumors, the trend towards a relatively small number of clones driving tumor expansion is increased (Fig. 4C). It has been observed previously, by using genetic clonal tracing strategies in solid tumors, that not all cells contribute equally to cancer growth. In those studies, this heterogeneity was attributed to the intrinsic differences in clonogenic potential of cells, resulting from different cell states, i.e. stem cells vs. differentiated cells (13, 14). We now have the ability to evaluate this by studying the configuration of clones within the tissue. As is immediately apparent from the images from whole LeGO xenograft sections, there is a clear relationship between the position of the clone and its size in all cancer models studied (Fig. 4A and D, SI Appendix, Fig. S2-A-D). Larger clones are predominantly located at the xenograft edges, implying that competition for an optimal location instead of the intrinsic properties of clones defines which clones drive expansion in this model. This implies that before clones get into direct competition, i.e. before an established tumor has formed from the injected cells, the heterogeneity in clone sizes is much smaller. Indeed, in very small tumors, where clones are not yet in contact, all clones appear to expand equally (SI Appendix, Fig. S2A). These results are in line with two recent studies that used short-term footprinting clonal tracing in an integrative approach to study the tumor growth and dynamics.
lineage-tracing strategies to confirm that clonal proliferation is most abundant at the leading edge of cancers (7, 8). Additionally, we have generated pancreatic ductal carcinoma (PDAC) xenografts from LeGO transduced cultures, and confirmed that PDACs show very similar growth dynamics as the CRC xenografts (SI Appendix, Fig. S4). To test if the leading edge might differ intrinsically from the center, we first confirmed that our cell lines contain genetic variations on the copy number level as described recently for other lines (20, 21). Significant genetic variations were detected between various xenografted tumors as well as the parental line (SI Appendix, Fig. S5A). This shows that genetic diversity is maintained in primary CRC cell cultures employed here, albeit potentially less as compared to in situ human cancers. In contrast, we found no significant differences in copy number between the edge and center of the same tumor (SI Appendix, Fig. S5B). Therefore, spatial difference in growth does not coincide with the observed genetic heterogeneity. Moreover, when cells derived from the center or edge of one tumor are re-transplanted, no difference in the growth rate was observed (SI Appendix, Fig. S5C) confirming that larger clones residing at the tumor edge are not intrinsically fitter.

**Modeling colorectal cancer growth.** To further support the notion that locations of founding cells rather than cell intrinsic features determine the in vivo clonogenic potential, we developed a cellular automaton model to simulate xenograft expansion with growth either confined to the surface or throughout the whole tumor (Fig. S4 and SI Appendix, Movies S1-3). If neither of the models (volume or surface growth) could explain the data, this would argue in favor of intrinsic differences between cells, for example as proposed by the cancer stem cell theory. We model tumor growth in 3D as a population of cells that stochastically replicate when they have sufficient free space available for the offspring (SI Appendix, Computer models), similar to our previous work (6). In the surface growth model, cells replicate only on the surface. In the volume growth model, as the tumor expands, free space is created inside the tumor which causes it to grow exponentially. The initial tumor conditions are taken to match the xenograft experiments: 10,000 uniquely labeled cells in a volume of 50 μl. Tumor growth is simulated until the maximum size of 1.3 billion cells, which corresponds to a tumor volume of 1.3 cm³, which is well above the maximum tumor volume in our xenograft experiments. For direct comparison with the experimental data we take virtual 2D sections from the simulated tumors at various positions and quantify clone sizes (SI Appendix, Computer models). With growth confined to the surface we found excellent agreement between the simulated clone sizes as a function of the overall tumor volume, and the experimentally observed clone sizes (Fig. S5B). Moreover, the clone size distributions and patterns from the simulated environment-instructed tumor growth were highly similar to the clone patterns observed in the xenografts (Fig. S5C). In contrast, volume growth results in exponentially growing tumors with a different pattern of clonal expansion that does not explain the experimental data (Fig. S5C and S5D). The spatiotemporal localization of growth is therefore crucial to explain the data and results in a large heterogeneity of clone sizes even if all clones have an equal growth rate (neutral dynamics). To assess the effect of non-neutrality we modeled tumor growth with non-uniform growth rates of clones (normal distributed growth rates, mean = 1, standard deviation = 0.05, 0.1 or 0.2). We find that the clone size distribution is very similar to the neutral case (SI Appendix, Fig. S6A). Other biologically realistic alterations of the model (a stem cell hierarchy or extensive cell death during growth) also only induce subtle changes in the growth pattern as revealed by the distribution of clone sizes (SI Appendix, Fig. S6B). Together these results indicate that *in situ* clonogenicity for clones of equal age is the result of spatial organization of the tissue.

**Discussion**

Using a color-based clonal tracing method in combination with primary human CRC cultures, we obtained important insights in the dynamics of colon cancer xenograft growth and clonal heterogeneity. Firstly, we revealed that the initiation phase of xenograft growth is dependent on the volume used for injection, and the number of cells injected. Controlling these variables is essential for accurate interpretation of TIC assays and clonal competition studies. Secondly, the xenografts from LeGO-transduced primary cultures revealed that clonal dispersal and clonal mixing is limited in colon cancer xenografts. This observation has important implications, as it strengthens the notion that clones in colon cancer tissue expand in parallel when residing in an identical environment. Also, this provides geometrical support to the idea that a large proportion of CRCs display predominantly neutral
evolution and that no large selective sweeps follow emergence of novel, more aggressive clones in established cancers.

Previous short-term lineage tracing experiments by us and others indicated that clonal expansion is spatially regulated in established CRC xenograft tissue(7, 8). In these studies clones expressing one or a few different colors were induced in established tumors. The small number of colors in these experimental systems prevented long-term tracing of individual clones because growth and merging of neighboring clones are hard to distinguish with a limited resolution. In the current study we have overcome this limitation, which has enabled us to study long-term clonal dynamics in xenografts of established CRCs. The combination of long-term lineage tracing and computational modeling revealed that clone size heterogeneity can be fully explained by externally driven growth at the leading edge of the tumor. In contrast, previous barcoding studies in human xenografts indicated that distinct types of stem-like cells in colon cancer xenografts exhibit distinct repopulating features caused by intrinsic functional differences in the self-renewal and tumor forming capacity of tumor cells(13, 14).

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Materials and Methods

Cell culture. Human primary colon cancer cultures were established as described previously(18). Cultures were isolated from patients with colorectal cancer with approval of the medical ethical committee of the AMC and University of Palermo. Primary cell lines are cultured in polyHEMA (Poly(2-hydroxyethyl methacrylate, Sigma) coated flasks (Corning) to allow spheroid formation. Advanced DMEM/F12 (Life Technologies), culture medium is used which is supplemented with N-2 (Life Technologies), L-glutamine, glucose, HEPES, heparin, insulin, epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) as described previously(18). The primary human PDAC culture 067 was established as described previously(22) and cultured in IMDM (Life Technologies) supplemented with 8% FBS and L-glutamine. DLD1 (ATCC) and HT55 (Sanger institute, UK) cells were cultured in DMEM/F12 (Life Technologies) supplemented with 8% FCS (Life Technologies). Capan-2 (ATCC) was cultured in DMEM (Life Technologies) supplemented with 8% FCS. Cell lines were authenticated by STR profiling in combination with mutation analysis and have been regularly tested for mycoplasma infection.

Multicolor marking. Cell lines were simultaneously transduced with three different constructs according to a previously published protocol(12). The following lentiviral gene ontology (LeGO) vectors were used; LeGO-C2 (27339), LeGO-v2 (27340) and LeGO-C2r (27338) (Addgene). In short, 50,000 single cells were seeded in a 24-well plate in 500 µl culture medium in the presence of 8 µg ml⁻¹ polybrene (Sigma). Lentivirus containing the three vectors was added in a volume that ensured ~60% transduction rate of each vector. Plates were centrifuged for 1 hour at 24°C and incubated overnight at 37°C. Transduction rates were analyzed by flow cytometry after three days. After transduction, cell lines were passaged in a low dilution and for a maximum of five passages before in vivo use.

Flow Cytometry. Flow cytometry was performed on a Fluorescence Activated Cell Sorting (FACS) Aria SORP (BD Biosciences) machine with 405-, 488- and 561-nm lasers. Data was analyzed with the FlowJo (FlowJo LLC) software.

Vector expression. Vector integration stability was analyzed by FACS. DLD1 cells were transduced with the LeGO system and then single-cell sorted. Single-cell clone cultures were expanded and passaged twice a week. Upon passing the expression of Cerulean, Venus and mCherry was analyzed by FACS. Every cell line was analyzed at least twice in a 12-week follow-up period.

In vivo experiments. The Animal Experimentation Committee at the Academic Medical Center in Amsterdam has approved all in vivo experiments (DEC103181) and all animal experiments were performed according to the national guidelines. Female nude (Hsd:Athymic Nude-Foxn1null) mice (6-12 weeks old) were obtained from Envigo. Animals were randomly assigned to experimental groups, no blinding was applied for these experiments. Animal exclusion was performed when no tumor growth appeared.

Fig. 5. Clonal expansion is highly dependent on colonocentric (A) tumor growth, (B) surface growth, (C) volume growth, (D) surface growth and volume growth. All experiments were performed in an体系 containing conditioned media. (A) Comparison of experimental surface growth (red lines) and surface growth (dashed blue lines).

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Xenograft studies. Xenograft tumors were generated by injecting 10,000 (CC09) or 50,000 (Co100, Co147 and HT55) human colon cancer cells in a mixture of medium and Matrigel (Corning) in a 1:1 ratio with a cell density of around 400-1,000 cells/µL. Cells were injected subcutaneously into the flanks of nude mice. Tumor growth was measured manually twice a week using a caliper. Mice were sacrificed based on tumor size at various time points to isolate tumors. After isolation tumors were fixed using 4% paraformaldehyde in phosphate buffered saline solution overnight at 4°C followed by preservation in a 20% sucrose solution for 12 hours at 4°C. Tumors were split into two equally sized parts and 10 µm-thick frozen tissue sections were collected from the tumor center.

In vivo transplantation assay. Center and edge (0.5 mm from tumor border) located cells were isolated from freshly collected xenografts by using razor blades. Immediately after tissue collection, cells were dissociated by using medium containing collagenase (Roche) and hyaluronidase (Sigma) at 37°C for 1 hour. Before injection, cells were filtered using a 70 µm cell strainer and dead cells were removed by 7-AAD staining (BD Biosciences) by using FACs. For each group, 1,000 cells were injected subcutaneously into the flanks of nude mice (n=3) and tumor growth was measured twice a week.

Copy number analysis. DNA was extracted using the NucléoTissu Tissue kit (Biokit) following the manufacturers procedure. To extract DNA from the in- and outside of tumors, we first mechanically separated the two regions. Shallow sequencing and data analysis were performed as previously described(23).

Limiting dilution assay. Cells were dissociated and plated in 96-wells plate (Corning) using SHED Cell Sorter (Sony) in a limiting dilution manner at 1, 2, 4, 8, 16, 24, 32, 64, 128, 256 cells per well. Clonal frequency and significance were determined using the Extreme Limiting Dilution Analysis (ELDA) Limdil function(24).

Imaging. Frozen tissue sections were imaged by an EVOS FL Cell Imaging System (Thermo Fisher Scientific). Sections were covered with ProLong Gold Antifade Mountant (Thermo Fisher Scientific) to ensure fluorescent signal and were stored at −80°C until used. Advanced Fluorescence software was used. For visualization of mCherry and Venus specific channels, images were collected at 40x and 100x magnification. The Leica Application Suite Advanced Fluorescence software was used.

Image analysis. Automated clone size quantification and localization was performed on whole tumor cross sectional slides imaged by fluorescence microscopy and converted to.tif file format with a custom written MATLAB script. The fluorescence microscopy data was analyzed by using the computer code. The software was written in MATLAB and used for quantification of clone size and position. Connected regions with the same color, but separated by >10 cell diameters were considered as separate clones. The number of mixed clones was identified manually.

Spatial model for tumor growth. We adapted the 3D spatial model we recently introduced for tumor evolution, for direct comparison with the xenograft data(6). In short, in this model tumors occupy sites of a regular 3D lattice. To simulate growth, iteratively a random cell which has at least one of the neighboring sites (von Neumann neighborhood) vacant, replaces it in a randomly chosen vacant neighbor site. A detailed description of the computational modelling, a description of the different model versions, and how numerical data is compared to the experimental data can be found in SI Appendix, Computer models.

Statistical analysis. 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. The between-group variances were similar and the data were normally distributed. P values of < 0.05 were considered significant.

Data availability. Source data for Figure 4 and 5 and Figure S3 is provided in Dataset S1.

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