The Origins of Phenotypic Heterogeneity in Cancer

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ABSTRACT

Heterogeneity is a pervasive feature of cancer, and understanding the sources and regulatory mechanisms underlying heterogeneity could provide key insights to help improve the diagnosis and treatment of cancer. In this review, we discuss the origin of heterogeneity in the phenotype of individual cancer cells. Genotype–phenotype (G–P) maps are widely used in evolutionary biology to represent the complex interactions of genes and the environment that lead to phenotypes that impact fitness. Here, we present the rationale of an extended G–P (eG–P) map with a cone structure in cancer. The eG–P cone is formed by cells that are similar at the genome layer but gradually increase variability in the epigenome, transcriptome, proteome, metabolome, and signalome layers to produce large variability at the phenome layer. Experimental evidence from single-cell-omics analyses supporting the cancer eG–P cone concept is presented, and the impact of epimutations and the interaction of cancer and tumor microenvironmental eG–P cones are integrated with the current understanding of cancer biology. The eG–P cone concept uncovers potential therapeutic strategies to reduce cancer evolution and improve cancer treatment. More methods to study phenotypes in single cells will be the key to better understand cancer cell fitness in tumor biology and therapeutics.

The Extended Genome–Phenome Cone in Cancer

Cancer is an intrinsically heterogeneous disease and its tackling requires the understanding of different levels of heterogeneity and their origins (**Fig. 1A**; refs. 1, 2). Cancers stemming from different tissues are diverse, although the same tissue can also give rise to cancers with different characteristics (3). Each patient has a unique cancer evolutionary history leading to a complex clonal composition of primary tumors and metastasis. Initial neoplastic lesions usually derive from a single clone of genetic lineage, meaning that the genomic space occupied by these cells is small. With time, genomic instability leads to the accumulation of somatic chromosomal aberrations and genomic doublings that precedes malignant progression (4). In metastasis, the genomic composition shifts during the outgrowth and most of the alterations observed already exist in the primary tumor with a dominance of specific clones been observed in multiple organs (5). These clones harbor different genetic driver gene alterations and within them, single tumor cells possess large heterogeneity (**Fig. 1A**; refs. 6–8). These sources of heterogeneity were recently covered by excellent reviews (7–13) and will not be the topic of the present review.

The concept of genotype–phenotype (G–P) map is widely used in evolutionary biology to represent the complex interactions of genes and environment that lead to phenotypes that impact fitness and ultimately selection of the fittest (1). In cancer, the traditional view is that the G–P map is impacted by genetic alteration of cancer genes, many of which have complex pleiotropic effects on the phenotype. However, several layers can be interposed between the genome and the phenome, which produce an extended G–P (eG–P) map (**Fig. 1B**). Here, the phenome will be used to represent cell characteristics and behaviors (e.g., migration, division, survival, among others), whereas the expression of RNAs, proteins and other features are represented by the remaining layers of the eG–P map.

A clone of cancer cells will occupy a limited genome space due to the genetic similarity. However, clonal cells are heterogeneous in the other layers of the eG–P map, eventually occupying a considerable space on the phenome layer, to form a cone-like structure. Here we propose the eG–P cone concept, encompassing the volume occupied throughout all layers of genetically similar cells (**Fig. 1B**), which will be the guiding concept throughout this review. An eG–P cone can also be formed by an individual cell over time, as cell-cycle–dependent changes (14) and time-dependent fluctuations alter the cell at the different layers while not altering their genome (15).

The further down on the eG–P map, the larger is the heterogeneity, as translation, metabolism, signaling networks, among others, add variability to the phenome. If the main contributor to the enlarged bottom of the eG–P cone is time or mitosis is still a matter of very active research as both were described to add heterogeneity to several of the eG–P layers (16, 17).

Cancer is a disease of deregulated fitness, defined by the capacity to survive and leave descendants (18). Here we express cell fitness only in the phenome layer, although this is the result of the integration of all the other layers of the eG–P map. Although the presence of a driver mutation in an oncogene or the epigenetic silencing of a tumor suppressor gene (TSG) in a given cell can indicate that this cell has an increased fitness when compared with its peers, this will only be determined when these alterations change the fitness of the cell (19). Therefore, all layers are represented by flat surfaces, except the phenome layer, in which the hills and valleys represent different levels of cell fitness (**Fig. 1B**; ref. 20).

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Cancer Res 2022;82:3–11

doi: 10.1158/0008-5472.CAN-21-1940

Experimental Evidence That Supports the eG–P Cone in Cancer

Fundamental for supporting the eG–P cone concept are techniques that evaluate more than one eG–P layer in individual cells, which were introduced for most of the combination of layers (**Fig. 1C**; refs. 21–26). Most importantly, recent bioinformatic analyses allowed the integration of single-cell data obtained with different techniques. This was used, for example, to integrate scATAC-seq profiles with scRNA-seq of the mouse visual cortex, demonstrating that at the RNA levels, cells occupy a larger area and more clusters in uniform manifold approximation and projection (UMAP) when compared with the chromatin accessibility layer, in support of the eG–P cone (21, 27).

Live-cell image tracking coupled with scRNA-seq and/or microfluidic provided information on how signaling network dynamics relate with transcriptome and phenome layers. Under lipopolysaccharide exposure, macrophages display distinct patterns of NF-κB activation, each one resulting in specific profiles of cytokine expression. A pre-exposure of these cells to TNFα altered the proportion of observed NF-κB patterns (28), indicating that subpopulations of genetic similar cells can have different signaling responses depending on the signaling molecules they are in contact with, which indicates a larger area occupied by the signalome in relation to the transcriptome (**Fig. 1D**).

Phenotypic stability was first experimentally addressed by Luria and Delbrück in 1943 (29), whose experimental design is based on the premise that sensitive cells can transform in resistant ones, but not the contrary and that this transformation is permanent (**Fig. 1E**). Therefore, this assay is best suited for differentiating stable (genetic), or long lasting (epigenetic), alterations from very dynamic ones such as posttranslational modifications. Variations of this experimental design were applied to account for the reversible nature of several key molecular events involved in tolerance and resistance. These strategies were applied to study the stability of epigenetic marks and mRNAs levels. RNA from clones of about 100,000 cells (~16.6 generations) was sequenced and the distribution of reads in these clones was compared with a similar number of randomly grouped cell. As expected, all genes in randomly sampled groups of cells had a Gaussian distribution with a small variance. In colonies, however, two groups of genes could be observed—the majority of them had a small variance of reads, suggesting that their expression in colonies is not different from randomly sampled cells. However, around 200 genes had a large variance in reads among clonal cells, indicating a memory in expression levels over the generations that passed from the original cell to the cells in the

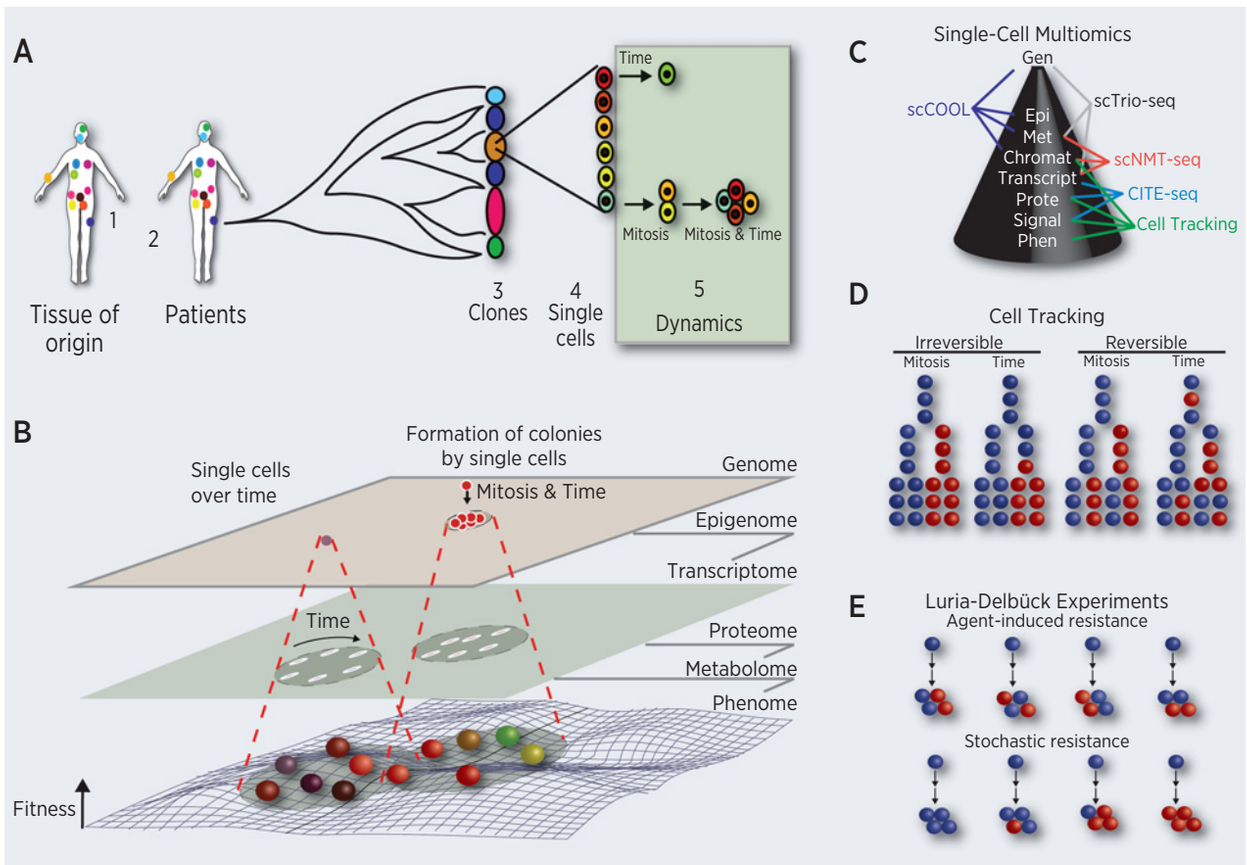

Figure 1.

Levels and origins of cancer phenome heterogeneity. **A,** Sources of tumor heterogeneity: (**1**) tissue-specific tumor types; (**2**) same tumor type from different patients; (**3**) intratumor clonal genetic differences; (**4**) single-cell genetic heterogeneity; and (**5**) single-cell fluctuation over time and/or mitosis. **B,** The eG–P cone, formed by the characteristics of clonal cells or a single cell over time at the layers of the eG–P map. **C,** Experimental strategies that support the eG–P cone. Single-cell direct measurement of multiple layers; cell tracking (**D**) and Luria–Delbrück-type assays (**E**).

colony (30). Mathematical modeling of the differences among clones in reads of scRNA-seq of these genes indicates an ON time between 5 and 10 generations, depending on the gene. However, as only the final colony was analyzed, no direct description of the dynamics of ON/OFF status could be obtained.

A similar study evaluating colonies of 500 to 1,000 cells (9–10 generations) found that between 30% and 40% of the about 2,000 sequenced mRNAs were clonally stable in their expression level. Long-term following of several clones indicated that some clones maintained their epithelial–mesenchymal expression status for up to 168 days, whereas other clones slowly drifted to a more mesenchymal transcriptomic profile, with a concomitant increase in the variability of the expression of vimentin in single cells of these clones indicating a broadening of the transcriptomic layer of the eG–P cone with time (15).

The strategies based on the similarity or the variance of different cell characteristics in colonies clearly indicate the degree of nongenetic heterogeneity at the epigenome (15), transcriptome (30), and phenome layers (31–34). At the latter, cells from small colonies are desynchronized in the cell cycle, possess different DNA-repair capabilities (32) and have considerable differences in the sensitivity to drugs, experiencing fractional killing (31). This variation in response to a drug can be explained by the heterogeneity produced by any of the layers interposed between the genome and the phenome, in the levels of biomolecules, specially proteins involved in apoptotic priming (31).

Cell fitness, as it integrates several phenotypes, can be considered one of the most complete ways of assessing the phenome layer, although measuring fitness will not indicate the relative contribution of the several phenotypes that impact the capacity to survive and leave descendants. Measuring fitness requires long-term experiments, as fitness can only be measured after at least one average duplication time. A broad assessment of the dynamics of fitness in cell culture indicated that normal and cancer cells have dynamic fitness that fluctuate over a few days and three to four cell divisions (32).

The collection of genome, epigenome, transcriptome and proteome data from single cells supports the concept of the eG–P cone, but the complexity of the layers and mainly the multiple interactions among them still does not allow the description of the relative contributions of the individual layers to cancer functional heterogeneity (35, 36).

Molecular Mechanisms That Shape the eG–P Cone

Copying information correctly to the offspring is one of the basic principles of life, but making it with a few errors is central for evolution (20, 37). However, a close to perfect copy of DNA is only one part of the equation, as building and maintaining a cell in a defined phenotypic space requires biological processes counteracting the stochasticity of most molecular events governing the cell.

Defects or fluctuations in epigenetic mechanisms

Epigenetics, defined here as DNA methylation in CpG dinucleotides, covalent modifications in histone proteins, noncoding RNAs (ncRNA), and other complementary mechanisms that control chromatin organization, can both provide the dynamics and the stability that underly several processes. Methylation status of DNA, posttranslational modification of histones, 3D organization of the chromatin are involved in transmitting epigenetic-only information over several generations (38). Heterochromatin and euchromatin tend to self-organize in complex 3D structure, forged by the phase separation of chromatin-associated proteins that preferentially undergo homotypic rather than heterotypic interactions (39). However, this self-organization is far from representing a stable structure and these phase-separated regions undergo extensive dynamics as they are destroyed and reconstructed at each cell division (40).

During the formation of colonies, methylation profiles revealed that most loci are stable over up to 10 generations, whereas others are very close to the variability of loci from randomly sampled cells, thus not having any memory of their origin. Therefore, DNA methylation can both be part of the stability in expression of some, while being involved in the dynamics of other genes and phenotypes (15). The eG–P cone in this case will be an integration of the impact of memory and nonmemory genes. Although for memory genes, the level of mRNA, protein, and impact on the cell's phenotype is homogeneous in clones, for nonmemory genes this is heterogeneous and therefore memory and nonmemory genes can be depicted as eG–P cones with a thin and thick shape, respectively. The important question to ask is which gene, memory or nonmemory, has the largest impact on the cancer cell phenome, especially its fitness (**Fig. 2A**). In this case, the interdependence of the gene functions has to be considered. For example, in a metabolic pathway, heterogeneity in the levels of a rate-limiting enzyme has much more impact than in all remaining enzymes in the pathway and therefore, the cone shape of the rate limiting enzyme will be determinant for the shape of the integrated eG–P cone, especially on the phenome layer (**Fig. 2B**). In cancer, complex networks control the phenome and fitness (41) and determining which heterogeneity matters will require integrations of multiple data and systems biology analysis.

The eG–P cone concept can help to determine the contribution of the heterogeneity from the different layers of the cone to cancer cell fitness. Three broad groups of heterogeneity can be considered, as heterogeneity that does not impact the phenome or fitness, heterogeneity that impacts the phenome but does not contribute to fitness and the heterogeneity that changes the cell fitness. Determining which heterogeneity matters requires carefully planned experiments, possibly involving measuring the impact of the variability in a given layer on the fitness.

The transcriptional expression of a given locus is largely determined by chromatin conformation and the homeostatic network is determined by the close interaction of the polycomb repressive complexes (PRC). Polycomb repression, mediated mainly by the methylation of lysine 27 of histone 3 (H3K27me), is heritable through cell division, but can be dynamically reversed and represents one of the mechanisms of epigenetic memory (42). In line with the importance of this mechanism in cell identity is the high frequency of mutation in histone H3 in cancer, specially the substitution of lysine 27 by methionine (H3K27M) in a highly dominant manner (42). Mutations in the genes that encode writers, readers, and erasers of epigenetic marks strongly modifies the epigenetic homeostasis, which is why many tumors end up exhibiting destabilized developmental programs.

In light of the eG–P cone concept, epigenomic changes can lead to the occupation of a large phenome space thus priming the cell population for genomic events (43). Gene silencing through promoter methylation of the mismatch repair protein MLH1 is a founding event in colon and endometrial cancer, which, in the latter, is a primary cause of genomic events that drive cancer development (44, 45). The observation that epimutations are common in cancer is an indication that enlarging the epigenome and transcriptome increases fitness of the cancer cell population, possibly through the overall broadening of the millions of clones that form the overlapping eG–P cones from cancer cell and normal cells that compose a cancer (**Fig. 2C**).

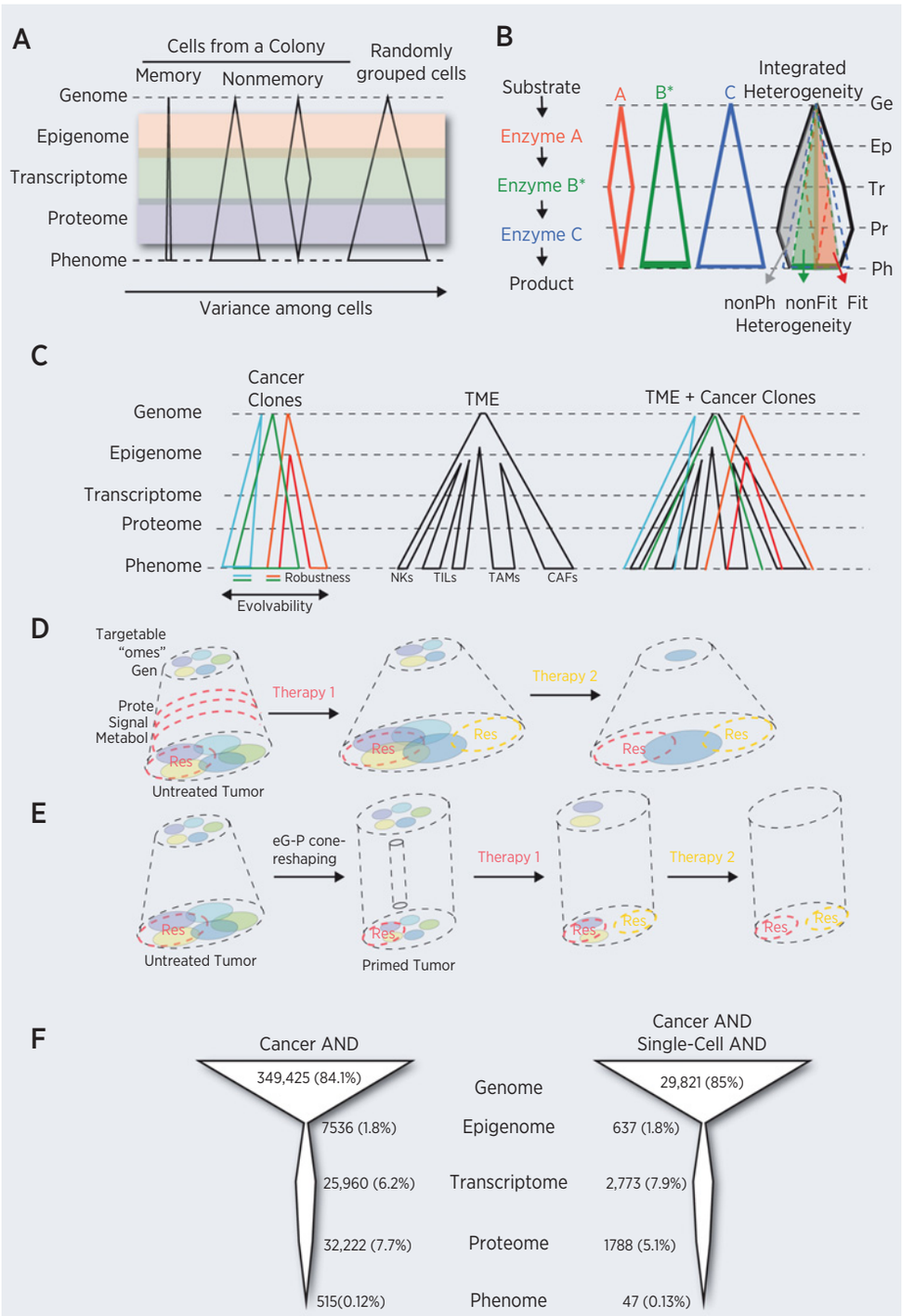

Figure 2.

Interactions among layers and genes in the eG–P cone. **A,** Memory genes are characterized by the maintenance of expression level of the founder cell in all cells in the colony, thus producing homogeneous colonies, whereas nonmemory genes vary among cells in colonies and become as variable as randomly grouped cells. **B,** Integration of the eG–P cones of genes part of a pathway. The variability in the product will be determined by the variability in the rate limiting enzyme (*). Heterogeneities with no impact on the phenome (nonPh) or fitness (nonfit) and heterogeneities that impact fitness (Fit). **C,** eG–P cones of cancer cells and cells from the TME. Hypothetical eG–P cone of cancer cells and TME. Robustness represented by the overlap of clones in the phenome layer and evolvability represented by the overall area occupied on the phenome. **D,** Impact of treatment on the eG–P cone and role of the phenome size in resistance (Res). **E,** Reshaping of the eG–P cone and its impact on combination therapy. **F,** PubMed results of searches for the words "cancer" and "single-cells" and the indicated words for components of the layers and the different "omics" (search date: May 25, 2021).

Asymmetric mitosis

Asymmetric mitosis (AM) is an event observed both in prokaryotes and eukaryotes and is responsible for the generation of two daughter cells with different characteristics (46). In unicellular organisms, AM is responsible for increasing fitness of one of the daughter cells in detriment of its sister (47, 48). In mammals, AM is classically described in multicellular organisms as regulating tissue homeostasis, during the production and maintenance of stem cells (49) or during development, when mitochondria are retained in the oocyte during the extrusion of the polar body (50). In mature tissues, epithelial cells with stem-cell characteristics asymmetrically segregate aged mitochondria (51) and components responsible for cell degradation processes (16).

AM was observed in tumor cells (52, 53), potentially increasing cellular variability and having a negative impact on the effectiveness of drugs (54). Asymmetric segregation of molecules, such as EGFR and neurotrophin receptor (p75NTR), for instance, resulted in increased resistance of glioblastoma cells to therapy targeting this receptor (55). Unequal distribution of extrachromosomal oncogenes contribute to the heterogeneity and tumor evolution of 17 different cancer types (56), indicating that AM plays a role in generating variability in clones thus broadening the shape of the eG–P cone in several cancer types.

Stochastic fluctuations in cellular processes

Most cells are in steady state, in which production and degradation of the components of the cell are at similar rates to maintain stable concentrations. This is true not only for RNAs, proteins, lipids, and most metabolites, but also for posttranslational modifications used in signal transduction networks. Half-lives of these molecules vary over several orders of magnitude, from the millisecond range of some signal transduction mechanisms to several months for a few proteins (57). Notwithstanding, small alterations in either the ON or the OFF rate of the steady state can produce considerable consequences on the level of the component after several half-lives. The relative ON or OFF rates also impacts the dynamics of processes. Binding of transcription factors to DNA has a high OFF rate, thus remaining more that 95% of their time in the unbound state. Therefore, short stabilization of the transcription initiation machinery leads to transcriptional bursts, which are major contributors to the transcriptional stochasticity in single cells (58).

p53 mediates the activation of several stress-activated responses that control key processes for cancer biology. Under different stress stimuli, p53 dynamics is largely heterogeneous in single cells (59, 60), which is involved, for example, in the fractional killing observed in colon cancer cells after chemotherapy (59). After stress, acute p53 pulses can lead to temporary cell-cycle arrest, whereas sustained p53 pulses commonly induce cell senescence, showing that the dynamics of p53 levels are an important event in the regulation of cellular fate (17, 61). The heterogeneity in keeping cells arrested in the cell cycle varies upon the mean amplitude of p53 pulses in single cells after irradiation (62), making the relationship between p53 dynamics observed in a single-cell and the global expression of p53 even more complex. In addition to p53 dynamics itself, the stability of mRNA from target genes determines which signaling pathways will be activated by a given stress stimulus (61). Conformational changes, such as p53 tetramerization, can also modulate p53 response pathways, even if p53 levels remain unchanged in individual cells (63).

The examples above clearly show that small stochastic events can potentially impact processes central for the fitness of a cell such as the outcomes to progress through the cell cycle or survive to a therapeutic intervention. As dynamic states are much more sensitive to stochastic fluctuations in their components, it can be hypothesized that these dynamic states contribute to the generation of nongenetical heterogeneity that produced the base of the cone.

Interaction of the Normal and Tumoral eG–P Cones

A multicellular organism can be considered a very large eG–P cone, formed by cells who share the same or a very similar genome but adopt very different phenotypes through an orderly development program (64). Of note is that some tissues, such as the brain, obtain a considerable portion of their variability from chromosomal aneuploidy (65), thus representing a cone with an enlarged circumference at the top, but still with a top much smaller than the bottom. Multiple clones of B or T lymphocytes exhibiting a similar antigen recognition phenotype have an enlarged genome layer, due to their V(D)J recombination strategy for generating heterogeneity (66), thus having an inverted cone shape.

Cells from the tumor microenvironment (TME), despite being genetically more homogeneous than cancer cells, add enormous heterogeneity to tumors. Even spatial compartmentalization of cells in the tumor adds proteomic heterogeneity to the TME (67). In this sense, the TME is the largest eG–P cone in cancers, as cells with normal genome from different tissues are part of the TME and produce considerable transcriptome (68) and proteome (69) variability. The multiple eG–P cones of cancer cells will be combined with the TME cones producing an integrated cancer cone determined by the complex interactions of these cones (**Fig. 2C**).

The best source of TME eG-P cone comes from T-cell clones identified by its T-cell receptor sequence. Even within TCR clonotypes where the distribution of phenotypic states is significantly constrained, a continuity of intratumoral T-cell activation and phenotypic diversity is still observed (68). These clones show considerable differences in the expression of key genes of the immune response, such as IL17 and FOXP3, while being homogeneous in the expression of other genes such as TGFß and IL10 (70). High intratumor heterogeneity in the lymphocyte population occurs in diverse types of cancer. Interestingly, a higher phenotypic diversity is observed in the T cells population in breast cancer microenvironment when compared with normal breast tissues. A phenotypic expansion is also observed in lung cancer, notably for cytotoxic T cells and T regulatory cells (71, 72). Specific effects mediated by immunotherapies in tumor-infiltrating cells showed that anti-PD1 and anti-CTLA-4 therapies induce the expansion of different T cells thus amplifying the TME cone, while shrinking the cancer cone (73).

The TME is also composed of a large fraction of myeloid cells, dominantly macrophages, dendritic cells, and neutrophils at varying stages of differentiation. Despite the classical notion that tumor-associated macrophages (TAM) polarize in antitumoral (M1) or protumoral (M2) phenotypes in the TME (74, 75), recent single-cell analysis revealed a large number of TAM subpopulations do not correspond to M1/M2 polarization. Instead, poor separation of TAM clusters in the TME population indicates a degree of diverse cell states phenotypes (76) or even mixed expression of both protumoral- and antitumoral-associated genes in the same cells (68), producing a large TME cone bottom of TAMs.

Cancer-associated fibroblasts (CAF) are one of the most abundant cells in stroma and play important roles in cancer pathogenesis through the building and remodeling of extracellular matrix, the secretion of growth factors, chemokines, and cytokines. They are a naturally heterogeneous cell population because many cellular sources might give rise to CAFs through activation or transdifferentiation (77).

This cell-of-origin heterogeneity has been demonstrated in a variety of studies where subpopulations of CAFs with a wide range of biological molecular markers were identified and their role in tumor promotion or prevention is heterogeneous (78–80). Single-cell analysis has supported the identification of CAFs subsets that are distinguishable by their transcriptome instead of biological markers, which could reflect their functional subtypes (81).

The presence and the abundance of CAFs play a role in cancer cells phenome. Pancreatic cancer cells shift the gene expression profile to either proliferative or EMT when cocultured with CAFs (82). Interaction with CAFs lead cancer cells to migratory and invasive phenotypes (83, 84). The presence of CAFs also affect response to therapy to several anticancer drugs due to modulation of mitochondrial priming to apoptosis (85) or alter cancer cells transcriptome favoring drug resistance (86). The presence of specific CAFs subtypes in the TME promotes the attraction and longer retention of Tregs, which lead to their increased survival and differentiation (87), which makes CAF subsets and its heterogeneity key players in cancer immunosurveillance and immunosuppression.

A vast body of knowledge was produced about TME heterogeneity, but little is known about the plasticity of the subpopulations over time (12). The diversity of cell states in the TME is significantly expanded when compared with normal tissue and a continuum spectrum of cell states is observed for some cells lineage. However, how these TME cellular states interact with tumor cells and change over time is still an open question. It is reasonable to suppose that the junction of the TME and multiple tumor eG–P cones may add or even synergize with regards of their variability, in which subclones of cancer cells that would otherwise have a small phenome area through interaction with different niches of the TME will occupy a larger phenome area and therefore produce an overall large eG–P cone bottom (**Fig. 2C**; ref. 88).

Therapeutic Strategies in Light of the eG–P Cone Concept

The dynamic nature of different phenotypes in cancer cells supports the concept that therapy resistance passes through a tolerant state, which is reversible but allows for initial survival until the occurrence and selection of a stable resistance (89). The deliberate increase of phenotypic diversity through the engineering of gene circuits to increase expression stochasticity of an antifungal resistance gene increased overall resistance to fluconazole in *Saccharomyces cerevisiae*, supporting the notion of the survival of the most dynamic (90). One possible explanation for this is the increased evolvability provided by phenotypic heterogeneity, suggesting that the size of the cone base of the cell population is an indicator of the evolvability capacity of this population. An overlapping of different clones on the phenome layer is an indication of the robustness of the population, as elimination of specific clones will maintain a given region of the phenome occupied (**Fig. 2C**; ref. 20).

Both preexisting and acquired resistance are found after chemotherapy, however the rate of acquired tolerance is generally greater than preexisting resistant clones (91). Of the genes known to contribute to resistance to vemurafenib in melanoma cells, only 5% were expressed prior to treatment whereas 66% of these genes were induced after 4 weeks of treatment (92), indicating a treatment-induced enlargement of the cone at the transcriptomic layer. In glioblastoma, single-cell phosphoproteomics analysis revealed that resistance to mTOR inhibitors occurs in a nongenetic way through signaling pathway rewiring (93). On the phenome layer, the transition from low to high multidrug resistance phenotype in leukemia cells challenged with vincristine was mediated by induction and not selection (94). Temozolomide treatment also increases the variability of clonal glioma cells in relation to DNA damage and cell fitness (32). In this sense, some therapies, despite eliminating a large number of cells and even clones, repopulate much of the phenome layer due to the presence of cells from different clones in the resistant phenotypic region prior or during treatment (**Fig. 2D**). It is important to remember that the cancer therapeutic repertoire works mainly on the genome, proteome, signalome, and metabolome and their impact on the phenome and cell fitness is what matters in cancer therapeutics (95). In addition, mutations or therapeutic interventions in individual genes or proteins affect pathways that control phenotypes. When pathways were considered in describing survival probability, the results were much better when compared with considering individual genes (96). This reinforces the importance of considering the eG–P cone, as the heterogeneity of the gene, RNA, or protein may be different from the heterogeneity of the integrated pathway that controls the phenotype, which in turn impacts fitness.

This raises the hypothesis that priming the tumors to reduce the capacity of generating heterogeneity will reduce the occupied phenome space producing less robustness and less evolvability. Targeting DNA methylation and histone acetylation reduced the diameter of the bottom of the cone in glioma cells and sensitized these cells to TMZ (32), although it is very challenging to separate the cone-shaping effects from the multiple other effects produced by a combination of epigenetic modulators. The combination of epigenetic modulators with chemotherapies is a very active research field and some evidence, mainly centered on the transcriptomic layer, is starting to emerge in support of the above mentioned hypothesis. The combination of a bromodomain and extra-terminal domain (BET) inhibitor with paclitaxel or a CDK4/6 inhibitor produced resistant populations derived from fewer clones in comparison to the population in the absence of the BET inhibitor (91), suggesting that reshaping the eG–P cone prior or during therapy may reduce evolvability due to a smaller occupation of the phenome layer (**Fig. 2E**).

Antagonistic pleiotropy selects a population of cells with one treatment that is more sensitive to a second treatment. Among the more than a thousand genes that impact antagonistic pleiotropy, transcriptional and epigenetic modifier genes were more frequent (97). Antagonistic pleiotropy can be used to design evolutionary traps, to direct populations towards specific traits with one treatment that produces a sensitization to a second treatment. It can be hypothesized that eG–P cones reshaped to a rod shape may be much more sensitive to evolutionary traps due to the lower probability of occupation of the resistance space of the antagonistic pleiotropic cancer drugs, due to the reduction in robustness and evolvability of this reshaped cones (**Fig. 2D** and **E**).

Gaps to be Filled and the Future of the eG–P Cone in Cancer Biology

One of the widely used arguments for sequencing the human genome was the promise to better understand cancer. Indeed, in the 20 years since the first draft of the human genome, science has made immense progress describing the genome of multiple cancers in thousands of patients as bulk sequences in large consortia (98). In the last years, single-cell genomics and specially transcriptomics have added complexity and understanding in several key aspects of cancer development and therapeutics. However, the "e-GP cone" formed by the papers in PubMed on the different layers of the eG–P map indicates

that genome is by far the most represented "ome" in cancer, with or without adding the term "single cells." In single-cell cancer biology, transcriptome is also very well represented, but the phenome is by far the term with fewer publications, both in cancer in general and in single-cell cancer biology in particular (**Fig. 2F**).

The methodologic availability for studying genomics and transcriptomics and even proteomics may explain this inverted eG–P cone, but it also shows the relative low knowledge about the phenome of cancer cells. More methods to study phenotypes in single cells, hopefully allowing the assessment of several phenotypes in a large number of single cells, will be key for changing this inverted cone shape and better translating the immense knowledge produced by the genomics and transcriptomics of single cancer cells to the phenome layer and ultimately to better understand cancer cell fitness in tumor biology and therapeutics.

Authors' Disclosures

G. Lenz reports personal fees from CNPq during the conduct of the study and personal fees from CNPq outside the submitted work. G.R. Onzi reports grants from CNPq outside the submitted work and also reports employment with AstraZeneca. L.S. Lenz reports grants from CNPq outside the submitted work. J.H. Buss reports grants from CNPq outside the submitted work. J.A. Santos reports grants from CAPES outside the submitted work. K.R. Begnini reports grants from CAPES outside the submitted work.

Acknowledgments

G. Lenz, G.R. Onzi, L.S. Lenz, and J.H. Buss received CNPq fellowships; J.A. Santos and K.R. Begnini received CAPES fellowships. The authors wish to thank Juliano L. Faccioni, Eduardo C. Filippi-Chiela, Alexandre Bruni-Cardoso, and Nicolas C. Hoch for critical readings and suggestions.

Received June 16, 2021; revised September 14, 2021; accepted November 10, 2021; published first November 16, 2021.

References

1. Houle D, Govindaraju DR, Omholt S. Phenomics: the next challenge. Nat Rev Genet 2010;11:855–66.
2. Yi S, Lin S, Li Y, Zhao W, Mills GB, Sahni N. Functional variomics and network perturbation: connecting genotype to phenotype in cancer. Nat Rev Genet 2017;18:395–410.
3. Visvader JE. Cells of origin in cancer. Nature 2011;469:314–22.
4. Martinez P, Mallo D, Paulson TG, Li X, Sanchez CA, Reid BJ, et al. Evolution of Barrett's esophagus through space and time at single-crypt and whole-biopsy levels. Nat Commun 2018;9:794.
5. Echeverria GV, Powell E, Seth S, Ge Z, Carugo A, Bristow C, et al. High-resolution clonal mapping of multi-organ metastasis in triple negative breast cancer. Nat Commun 2018;9:5079.
6. Dagogo-Jack I, Shaw AT. Tumour heterogeneity and resistance to cancer therapies. Nat Rev Clin Oncol 2018;15:81–94.
7. McGranahan N, Swanton C. Clonal heterogeneity and tumor evolution: past, present, and the future. Cell 2017;168:613–28.
8. Schwartz R, Schäffer AA. The evolution of tumour phylogenetics: principles and practice. Nat Rev Genet 2017;18:213–29.
9. Baslan T, Hicks J. Unravelling biology and shifting paradigms in cancer with single-cell sequencing. Nat Rev Cancer 2017;17:557–69.
10. Berger MF, Mardis ER. The emerging clinical relevance of genomics in cancer medicine. Nat Rev Clin Oncol 2018;15:353–65.
11. Lawson DA, Kessenbrock K, Davis RT, Pervolarakis N, Werb Z. Tumour heterogeneity and metastasis at single-cell resolution. Nat Cell Biol 2018;20:1349–60.
12. Marusyk A, Janiszewska M, Heterogeneity PKI. The rosetta stone of therapy resistance. Cancer Cell 2020;37:471–84.
13. Castro LNG, Tirosh I, Suvà ML. Decoding cancer biology one cell at a time. Cancer Discov 2021;11:960–70.
14. Granada AE, Jiménez A, Stewart-Ornstein J, Blüthgen N, Reber S, Jambhekar A, et al. The effects of proliferation status and cell cycle phase on the responses of single cells to chemotherapy. Mol Biol Cell 2020;31:845–57.
15. Meir Z, Mukamel Z, Chomsky E, Lifshitz A, Tanay A. Single-cell analysis of clonal maintenance of transcriptional and epigenetic states in cancer cells. Nat Genet 2020;52:709–18.
16. Loeffler D, Wehling A, Schneiter F, Zhang Y, Müller-Bötticher N, Hoppe PS, et al. Asymmetric lysosome inheritance predicts activation of haematopoietic stem cells. Nature 2019;573:426–9.
17. Purvis JE, Karhohs KW, Mock C, Batchelor E, Loewer A, Lahav G. p53 dynamics control cell fate. Science 2012;336:1440–4.
18. Dawkins R. Climbing mount improbable. Penguin Books 1996.
19. Bowling S, Lawlor K, Rodríguez TA. Cell competition: the winners and losers of fitness selection. Development 2019;146:dev167486.
20. Payne JL, Wagner A. The causes of evolvability and their evolution. Nat Rev Genet 2019;20:24–38.
21. Stuart T, Satija R. Integrative single-cell analysis. Nat Rev Genet 2019;20:257–72.
22. Dey SS, Kester L, Spanjaard B, Bienko M, van Oudenaarden A. Integrated genome and transcriptome sequencing of the same cell. Nat Biotechnol 2015;33:285–9.
23. Hou Y, Guo H, Cao C, Li X, Hu B, Zhu P, et al. Single-cell triple omics sequencing reveals genetic, epigenetic, and transcriptomic heterogeneity in hepatocellular carcinomas. Cell Res 2016;26:304–19.
24. Clark SJ, Argelaguet R, Kapourani C-A, Stubbs TM, Lee HJ, Alda-Catalinas C, et al. scNMT-seq enables joint profiling of chromatin accessibility DNA methylation and transcription in single cells. Nat Commun 2018;9:781.
25. Stoeckius M, Hafemeister C, Stephenson W, Houck-Loomis B, Chattopadhyay PK, Swerdlow H, et al. Simultaneous epitope and transcriptome measurement in single cells. Nat Methods 2017;14:865–8.
26. Li L, Guo F, Gao Y, Ren Y, Yuan P, Yan L, et al. Single-cell multi-omics sequencing of human early embryos. Nat Cell Biol 2018;20:847–58.
27. Stuart T, Butler A, Hoffman P, Hafemeister C, Papalexi E, Mauck WM, et al. Comprehensive integration of single-cell data. Cell 2019;177:1888–902.
28. Lane K, Van Valen D, DeFelice MM, Macklin DN, Kudo T, Jaimovich A, et al. Measuring signaling and RNA-Seq in the same cell links gene expression to dynamic patterns of NF-κB activation. Cell Syst 2017;4:458–69.
29. Luria SE, Delbrück M. Mutations of bacteria from virus sensitivity to virus resistance. Genetics 1943;28:491–511.
30. Shaffer SM, Emert BL, Reyes Hueros RA, Cote C, Harmange G, Schaff DL, et al. Memory sequencing reveals heritable single-cell gene expression programs associated with distinct cellular behaviors. Cell 2020;182:947–59.
31. Inde Z, Forcina GC, Denton K, Dixon SJ. Kinetic heterogeneity of cancer cell fractional killing. Cell Rep 2020;32:107845.
32. Lenz LS, Faccioni JL, Bracco PA, Santos JAF, Pereira LC, Buss JH, et al. Cancer cell fitness is dynamic. Cancer Res 2021;81:1040–51.
33. Vo T-T, Ryan J, Carrasco R, Neuberg D, Rossi DJ, Stone RM, et al. Relative mitochondrial priming of myeloblasts and normal HSCs determines chemotherapeutic success in AML. Cell 2012;151:344–55.
34. Spencer SL, Gaudet S, Albeck JG, Burke JM, Sorger PK. Non-genetic origins of cell-to-cell variability in TRAIL-induced apoptosis. Nature 2009;459:428–32.
35. Keller L, Pantel K. Unravelling tumour heterogeneity by single-cell profiling of circulating tumour cells. Nat Rev Cancer 2019;19:553–67.
36. Ren X, Kang B, Zhang Z. Understanding tumor ecosystems by single-cell sequencing: promises and limitations. Genome Biol 2018;19:211.
37. McCulloch SD, Kunkel TA. The fidelity of DNA synthesis by eukaryotic replicative and translesion synthesis polymerases. Cell Res 2008;18:148–61.
38. Perez MF, Lehner B. Intergenerational and transgenerational epigenetic inheritance in animals. Nat Cell Biol 2019;21:143–51.
39. Misteli T. The self-organizing genome: principles of genome architecture and function. Cell 2020;183:28–45.
40. Bertero A, Fields PA, Ramani V, Bonora G, Yardimci GG, Reinecke H, et al. Dynamics of genome reorganization during human cardiogenesis reveal an RBM20-dependent splicing factory. Nat Commun 2019;10:1538.
41. Kuenzi BM, Ideker T. A census of pathway maps in cancer systems biology. Nat Rev Cancer 2020;20:233–46.
42. Phillips RE, Soshnev AA, Allis CD. Epigenomic reprogramming as a driver of malignant glioma. Cancer Cell 2020;38:647–60.
43. Vicente-Dueñas C, Hauer J, Cobaleda C, Borkhardt A, Sánchez-García I. Epigenetic priming in cancer initiation. Trends Cancer 2018;4:408–17.

44. Kane MF, Loda M, Gaida GM, Lipman J, Mishra R, Goldman H, et al. Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. Cancer Res 1997;57:808–11.
45. Simpkins SB, Bocker T, Swisher EM, Mutch DG, Gersell DJ, Kovatich AJ, et al. MLH1 promoter methylation and gene silencing is the primary cause of microsatellite instability in sporadic endometrial cancers. Hum Mol Genet 1999;8:661–6.
46. Venkei ZG, Yamashita YM. Emerging mechanisms of asymmetric stem cell division. J Cell Biol 2018;217:3785–95.
47. Lindner AB, Madden R, Demarez A, Stewart EJ, Taddei F. Asymmetric segregation of protein aggregates is associated with cellular aging and rejuvenation. Proc Natl Acad Sci USA. 2008;105:3076–81.
48. Higuchi-Sanabria R, Pernice WMA, Vevea JD, Alessi Wolken DM, Boldogh IR, Pon LA. Role of asymmetric cell division in lifespan control in Saccharomyces cerevisiae. FEMS Yeast Res 2014;14:1133–46.
49. Berika M, Elgayyar ME, El-Hashash AHK. Asymmetric cell division of stem cells in the lung and other systems. Front Cell Dev Biol 2014;2:33.
50. Dalton CM, Carroll J. Biased inheritance of mitochondria during asymmetric cell division in the mouse oocyte. J Cell Sci 2013;126:2955–64.
51. Katajisto P, Döhla J, Chaffer CL, Pentinmikko N, Marjanovic N, Iqbal S, et al. Stem cells. Asymmetric apportioning of aged mitochondria between daughter cells is required for stemness. Science 2015;348:340–3.
52. Lathia JD, Hitomi M, Gallagher J, Gadani SP, Adkins J, Vasanji A, et al. Distribution of CD133 reveals glioma stem cells self-renew through symmetric and asymmetric cell divisions. Cell Death Dis 2011;2:e200.
53. Wang L, Bu P, Ai Y, Srinivasan T, Chen HJ, Xiang K, et al. A long non-coding RNA targets microRNA miR-34a to regulate colon cancer stem cell asymmetric division. Elife 2016;5:e14620.
54. Qazi MA, Vora P, Venugopal C, Sidhu SS, Moffat J, Swanton C, et al. Intratumoral heterogeneity: pathways to treatment resistance and relapse in human glioblastoma. Ann Oncol 2017;28:1448–56.
55. Hitomi M, Chumakova AP, Silver DJ, Knudsen AM, Pontius WD, Murphy S, et al. Asymmetric cell division promotes therapeutic resistance in glioblastoma stem cells. JCI Insight 2021;6:130510.
56. Turner KM, Deshpande V, Beyter D, Koga T, Rusert J, Lee C, et al. Extrachromosomal oncogene amplification drives tumour evolution and genetic heterogeneity. Nature 2017;543:122–5.
57. Eden E, Geva-Zatorsky N, Issaeva I, Cohen A, Dekel E, Danon T, et al. Proteome half-life dynamics in living human cells. Science 2011;331:764–8.
58. Rodriguez J, Ren G, Day CR, Zhao K, Chow CC, Larson DR. Intrinsic dynamics of a human gene reveal the basis of expression heterogeneity. Cell 2019;176:213–26.
59. Paek AL, Liu JC, Loewer A, Forrester WC, Lahav G. Cell-to-cell variation in p53 dynamics leads to fractional killing. Cell 2016;165:631–42.
60. Lahav G. The strength of indecisiveness: oscillatory behavior for better cell fate determination. Sci STKE 2004;2004:pe55.
61. Hafner A, Reyes J, Stewart-Ornstein J, Tsabar M, Jambhekar A, Lahav G. Quantifying the central dogma in the p53 pathway in live single cells. Cell Syst 2020;10:495–505.
62. Reyes J, Chen J-Y, Stewart-Ornstein J, Karhohs KW, Mock CS, Lahav G. Fluctuations in p53 signaling allow escape from cell-cycle arrest. Mol Cell 2018;71:581–91.
63. Gaglia G, Guan Y, Shah JV, Lahav G. Activation and control of p53 tetramerization in individual living cells. Proc Natl Acad Sci USA 2013;110:15497–501.
64. Cao J, Spielmann M, Qiu X, Huang X, Ibrahim DM, Hill AJ, et al. The single-cell transcriptional landscape of mammalian organogenesis. Nature 2019;566:496–502.
65. Rehen SK, McConnell MJ, Kaushal D, Kingsbury MA, Yang AH, Chun J. Chromosomal variation in neurons of the developing and adult mammalian nervous system. Proc Natl Acad Sci U S A 2001;98:13361–6.
66. Huang H, Sikora MJ, Islam S, Chowdhury RR, Chien Y, Scriba TJ, et al. Select sequencing of clonally expanded CD8+ T cells reveals limits to clonal expansion. PNAS. National Academy of Sciences 2019;116:8995–9001.
67. Hunt AL, Bateman NW, Barakat W, Makohon-Moore S, Hood BL, Conrads KA, et al. Extensive three-dimensional intratumor proteomic heterogeneity revealed by multiregion sampling in high-grade serous ovarian tumor specimens. iScience 2021;24:102757.
68. Azizi E, Carr AJ, Plitas G, Cornish AE, Konopacki C, Prabhakaran S, et al. Single-cell map of diverse immune phenotypes in the breast tumor microenvironment. Cell 2018;174:1293–308.
69. Krieg C, Nowicka M, Guglietta S, Schindler S, Hartmann FJ, Weber LM, et al. High-dimensional single-cell analysis predicts response to anti-PD-1 immunotherapy. Nat Med 2018;24:144–53.
70. Han A, Glanville J, Hansmann L, Davis MM. Linking T-cell receptor sequence to functional phenotype at the single-cell level. Nat Biotechnol 2014;32:684–92.
71. Sharma A, Subudhi SK, Blando J, Scutti J, Vence L, Wargo J, et al. Anti-CTLA-4 immunotherapy does not deplete FOXP3+ regulatory T cells (Tregs) in human cancers. Clin Cancer Res 2019;25:1233–8.
72. Fridman WH, Pagès F, Sautès-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer 2012;12:298–306.
73. Wei SC, Levine JH, Cogdill AP, Zhao Y, Anang N-AAS, Andrews MC, et al. Distinct cellular mechanisms underlie anti-CTLA-4 and anti-PD-1 checkpoint blockade. Cell 2017;170:1120–33.e17.
74. Cassetta L, Pollard JW. Targeting macrophages: therapeutic approaches in cancer. Nat Rev Drug Discov 2018;17:887–904.
75. Ostuni R, Kratochvill F, Murray PJ, Natoli G. Macrophages and cancer: from mechanisms to therapeutic implications. Trends Immunol 2015;36:229–39.
76. Lambrechts D, Wauters E, Boeckx B, Aibar S, Nittner D, Burton O, et al. Phenotype molding of stromal cells in the lung tumor microenvironment. Nat Med 2018;24:1277–89.
77. Chen X, Song E. Turning foes to friends: targeting cancer-associated fibroblasts. Nat Rev Drug Discovery 2019;18:99–115.
78. Allinen M, Beroukhim R, Cai L, Brennan C, Lahti-Domenici J, Huang H, et al. Molecular characterization of the tumor microenvironment in breast cancer. Cancer Cell 2004;6:17–32.
79. Goetz JG, Minguet S, Navarro-Lérida I, Lazcano JJ, Samaniego R, Calvo E, et al. Biomechanical remodeling of the microenvironment by stromal caveolin-1 favors tumor invasion and metastasis. Cell 2011;146:148–63.
80. Zhang XH-F, Jin X, Malladi S, Zou Y, Wen YH, Brogi E, et al. Selection of bone metastasis seeds by mesenchymal signals in the primary tumor stroma. Cell 2013;154:1060–73.
81. Li H, Courtois ET, Sengupta D, Tan Y, Chen KH, Goh JJL, et al. Reference component analysis of single-cell transcriptomes elucidates cellular heterogeneity in human colorectal tumors. Nat Genet 2017;49:708–18.
82. Ligorio M, Sil S, Malagon-Lopez J, Nieman LT, Misale S, Di Pilato M, et al. Stromal microenvironment shapes the intratumoral architecture of pancreatic cancer. Cell 2019;178:160–75.e27.
83. Gaggioli C, Hooper S, Hidalgo-Carcedo C, Grosse R, Marshall JF, Harrington K, et al. Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. Nat Cell Biol 2007;9:1392–400.
84. Itoh G, Chida S, Yanagihara K, Yashiro M, Aiba N, Tanaka M. Cancer-associated fibroblasts induce cancer cell apoptosis that regulates invasion mode of tumours. Oncogene 2017;36:4434–44.
85. Landry BD, Leete T, Richards R, Cruz-Gordillo P, Schwartz HR, Honeywell ME, et al. Tumor-stroma interactions differentially alter drug sensitivity based on the origin of stromal cells. Mol Syst Biol 2018;14:e8322.
86. Tirosh I, Izar B, Prakadan SM, Wadsworth MH, Treacy D, Trombetta JJ, et al. Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. Science 2016;352:189–96.
87. Costa A, Kieffer Y, Scholer-Dahirel A, Pelon F, Bourachot B, Cardon M, et al. Fibroblast heterogeneity and immunosuppressive environment in human breast cancer. Cancer Cell 2018;33:463–79.
88. Marusyk A, Tabassum DP, Altrock PM, Almendro V, Michor F, Polyak K. Non-cell-autonomous driving of tumour growth supports sub-clonal heterogeneity. Nature 2014;514:54–8.
89. Sharma SV, Lee DY, Li B, Quinlan MP, Takahashi F, Maheswaran S, et al. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. Cell 2010;141:69–80.
90. Bódi Z, Farkas Z, Nevozhay D, Kalapis D, Lázár V, Csörgő B, et al. Phenotypic heterogeneity promotes adaptive evolution. PLoS Biol 2017;15:e2000644.
91. Ge JY, Shu S, Kwon M, Jovanović B, Murphy K, Gulvady A, et al. Acquired resistance to combined BET and CDK4/6 inhibition in triple-negative breast cancer. Nat Commun 2020;11:2350.
92. Shaffer SM, Dunagin MC, Torborg SR, Torre EA, Emert B, Krepler C, et al. Rare cell variability and drug-induced reprogramming as a mode of cancer drug resistance. Nature 2017;546:431–5.
93. Wei W, Shin YS, Xue M, Matsutani T, Masui K, Yang H, et al. Single-cell phosphoproteomics resolves adaptive signaling dynamics and informs targeted combination therapy in glioblastoma. Cancer Cell 2016;29:563–73.

94. Pisco AO, Brock A, Zhou J, Moor A, Mojtahedi M, Jackson D, et al. Non-Darwinian dynamics in therapy-induced cancer drug resistance. Nat Commun 2013;4:2467.
95. Niepel M, Spencer SL, Sorger PK. Non-genetic cell-to-cell variability and the consequences for pharmacology. Curr Opin Chem Biol 2009;13:556–61.
96. Wang S, Ma J, Zhang W, Shen JP, Huang J, Peng J, et al. Typing tumors using pathways selected by somatic evolution. Nat Commun 2018;9:4159.
97. Lin KH, Rutter JC, Xie A, Pardieu B, Winn ET, Bello RD, et al. Using antagonistic pleiotropy to design a chemotherapy-induced evolutionary trap to target drug resistance in cancer. Nat Genet 2020;52:408–17.
98. Cancer Genome Atlas Research Network, Weinstein JN, Collisson EA, Mills GB, Shaw KRM, Ozenberger BA, et al. The cancer genome atlas pan-cancer analysis project. Nat Genet 2013;45:1113–20.