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Perspective

Single-Cell RNA Sequencing in Cancer: Lessons Learned and Emerging Challenges

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Bulk genomic analyses and expression profiling of clinical specimens have shaped much of our understanding of cancer in patients. However, human tumors are intricate ecosystems composed of diverse cells, including malignant, immune, and stromal subsets, whose precise characterization is masked by bulk genomic methods. Single-cell genomic techniques have emerged as powerful approaches to dissect human tumors at the resolution of individual cells, providing a compelling approach to deciphering cancer biology. Here, we discuss some of the common themes emerging from initial studies of single-cell RNA sequencing in cancer and then highlight challenges in cancer biology for which emerging single-cell genomics methods may provide a compelling approach.

Although cancer is a genetic disease, malignancies are associated with aberrations in gene expression related to both cancer cell-intrinsic and extrinsic factors. Genomic profiling of biological samples has revolutionized our understanding of cancer in patients. Yet most datasets and analyses of gene expression are dominated by bulk profiling, in which an entire biological sample (e.g., tumor) is profiled as a single entity, thus averaging the expression profiles of the constituent cells. Expression intra-tumor heterogeneity (eITH) governs many critical facets of tumor biology that are driven by subsets of tumor cells, such as tumor growth, metastasis, and resistance to treatment. eITH of malignant cells is governed by at least three determinants: (1) genetic heterogeneity, due to subclonal mutations that arise during tumor evolution, drives diverse cellular programs; (2) epigenetic and developmental programs, such as those of tissue stem cells and their differentiated progeny, ascribe cancer cells with an (often aberrant) cell identity and in some cases enable cellular plasticity; and (3) extrinsic and spatial factors that determine oxygen, nutrient availability, and cell-cell interactions. Single-cell RNA sequencing (scRNA-seq) techniques measure the transcriptional output of cells directly in tumor samples; the measured cellular programs represent an integration of its genetic, epigenetic, and environmental cues and determine cancer cells fitness, behavior, and response to therapies. As such, scRNA-seq studies place cellular biology at the center of cancer biology and offer both different resolution and perspective to bulk genomic studies. We have recently reviewed initial studies of single-cell expression profiling in human cancer (Tirosh and Suvà, 2019). Here, we focus on emerging themes and on future challenges for the field.

Cell Types, Cell States, and Malignant and Non-malignant Cells

Analysis of the expression diversity within tumors (or other tissues) typically reveals two layers: highly distinct clusters that may be considered as "cell types" (e.g., malignant cells, T cells, and fibroblasts) and further diversity within each of those clusters that may be considered as "cell states," such as progression along the cell cycle, different metabolic states, and other dynamic programs. Cell types and states may then be annotated based on the identity of preferentially expressed genes (in each cluster or subcluster) along with genetic classification, including large-scale copy number alterations (CNAs), point mutations, and fusion proteins, that can be inferred from scRNA-seq data and help to resolve malignant from non-malignant cells (Filbin et al., 2018; Jerby-Arnon et al., 2018; Patel et al., 2014; Puram et al., 2017; Tirosh et al., 2016a, 2016b; Venteicher et al., 2017). An emerging theme from recent scRNA-seq studies is that malignant cells tend to cluster in their expression profiles primarily by patient sample, and non-malignant cells cluster in their expression profiles by cell type, somewhat independently of the patient of origin (Filbin et al., 2018; Jerby-Arnon et al., 2018; Tirosh et al., 2016a, 2016b; Venteicher et al., 2017). This indicates that (1) inter-tumor heterogeneity is typically larger for malignant cells than for any particular type of non-malignant cells. (2) For malignant cells, inter-tumor heterogeneity is much larger than intra-tumor heterogeneity. These results highlight the considerable degree of inter-tumor heterogeneity and might be misinterpreted as reflecting a limited role of intra-tumor heterogeneity of cancer cells. However, the power of single-cell methods is that they now further allow us to interrogate the patterns of intra-tumor heterogeneity, which have been difficult to assess with previous approaches and are often continuous rather than discrete.

Recurrent Patterns of eITH among Cancer Cells

What then are the patterns of heterogeneity observed among the malignant cells in an individual tumor? First of all, the level of any individual gene varies between cells, partially due to the stochastic and noisy nature of gene expression regulation. In addition to

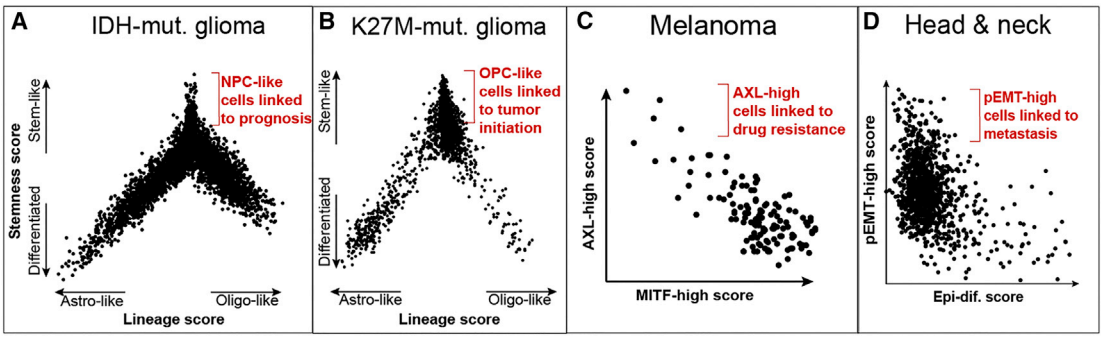

Figure 1. Recurrent Patterns of eITH among Malignant Cells

(A–D) IDH mutant glioma (A; Tirosh et al., 2016b; Venteicher et al., 2017), H3-K27M glioma (B; Filbin et al., 2018), melanoma (C; Tirosh et al., 2016a), and head and neck cancer (D; Puram et al., 2017). Images represent a scatter-plot in which cells (dots) are scored by the expression of particular programs identified in that cancer type (x and y axes). In each case, a similar pattern of eITH was identified across multiple patients. Plots were either reproduced (A–C) or generated from the data (D) of the original publication. Also highlighted in red are the associations between certain subpopulations and important clinical features, as described in the main text and the original publications.

this stochasticity, however, sets of genes vary coherently between cells, reflecting different cellular states that might have a more significant biological implication. For example, concomitant upregulation of dozens of cell-cycle-related genes indicates that certain cells have entered the cell cycle and others were not cycling at the moment in which they were profiled (Tirosh et al., 2016a, 2016b). Similarly, concomitant upregulation of many genes associated with hypoxia and/or stress responses indicate that certain cells respond to their immediate microenvironment and to the lack of nutrients or oxygen by mounting such responses (Patel et al., 2014; Tirosh et al., 2016a, 2016b). These two processes—cell cycle and stress responses—reflect common patterns of cellular heterogeneity within tumors, which are observed within different patients and across different cancer types.

In addition to those "generic" patterns of heterogeneity, there are also patterns that are context specific and hence may be more informative about the specific biology of a given tumor type. Multiple previous studies found that apart from cell cycle and stress there are particular intra-tumor expression programs (i.e., sets of genes that coherently vary among malignant cells in the same tumor) that are found within multiple patients of a particular cancer type, but not in other cancer types (Figure 1; Tirosh and Suvà, 2019). Strikingly, in each of those cases, these were large-scale expression programs that were uncovered by unbiased analysis, and examination of the associated genes suggested that they impact on crucial aspects of tumor progression, including drug resistance, metastasis, and self-renewal. In melanoma, those programs highlighted distinct MITF and AXL expression levels of cells within the same tumor, with suggested implications for resistance to BRAF inhibition (Rambow et al., 2018; Shaffer et al., 2017; Tirosh et al., 2016a). More recently, a cancer cell state in melanoma that promotes immune evasion and resistance to immune checkpoint inhibitors was identified by scRNA-seq analysis of 33 melanoma samples (Jerby-Arnon et al., 2018). In head and neck cancer, malignant cells differed in their expression of epithelial differentiation programs and in the expression of epithelial-to-mesenchymal transition programs, with important implications for metastasis (Puram et al., 2017). In glioblastoma, IDH mutant gliomas, and histone H3-K27M mutant gliomas, eITH patterns were dominated by glial differentiation and neurodevelopmental programs. These programs highlighted putative differentiation hierarchies, with stem-like cells that were linked to self-renewal and increased aggressiveness (Filbin et al., 2018; Patel et al., 2014; Tirosh et al., 2016b; Venteicher et al., 2017).

These initial results suggest two important conclusions. First, the patterns of eITH are of considerable significance for the maintenance and progression of tumors, warranting further analysis and future approaches to target particular tumor subpopulations. Second, the consistency of these patterns across patients of the same cancer type or subtype suggests that these reflect fundamental properties of the corresponding cells from which the cancer originates. For example, gliomas likely originate from neural stem-like cells, which have an intrinsic capacity to differentiate toward astrocytes and oligodendrocytes; thus, the resulting cancer cells display an aberrant differentiation toward astrocyte-like and oligodendrocyte-like cells that dominate the cellular diversity within gliomas. Similarly, epithelial cells have the intrinsic capacity to activate mesenchymal programs that might normally be required during development and wound healing; thus, the resulting cancer cells may also activate those programs in response to cues, such as transforming growth factor β (TGF-β), produced by surrounding fibroblasts.

Thus, these patterns might be thought of as reflecting the "epigenetic landscape" of the cancer cells: only particular programs may be accessible to cells in each cancer type, based on the nature of the cell of origin or the impact of oncogenic transformation. Accordingly, cells may dynamically activate those "accessible" programs, resulting in their variability within an individual tumor. This model suggests that it will be important to define such programs of variability in each tumor type (or subtype) and subsequently examine its impact on the functional properties of the cancer cells and their vulnerabilities.

Integrating Bulk with Single-Cell Tumor Profiles

These initial studies provide a proof of concept for the utility of single-cell expression profiling of tumors. However, it is also

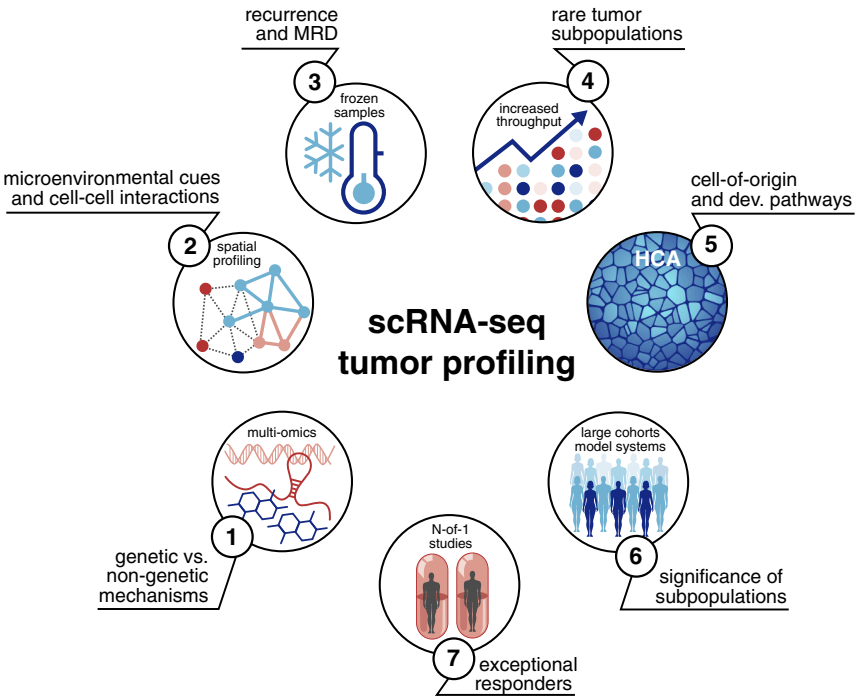

Figure 2. Questions in Cancer Biology Addressed by Emerging Directions with scRNA-Seq

See main text for further description of each of the biological questions and the emerging approaches to address it. MRD, minimal residual disease.

important to realize the limitations of this approach. First, scRNA-seq provides only a partial sampling of the transcriptome of cells, with a bias for more highly expressed genes over mid-to-lowly expressed ones. Second, scRNA-seq is very sensitive to sample quality and as such is not suited for the profiling of sub-optimally preserved or handled clinical specimen. Third, high cost limits the ability to profile large cohorts of tumors, and only few to a few dozens of samples are typically profiled in each study. Given these challenges with single-cell approaches and the comprehensive bulk databases that already exist, such as The Cancer Genome Atlas (TCGA), we anticipate that single-cell datasets will not supplant bulk tumor profiling but instead will be integrated with existing and new bulk profiles to advance our understanding of tumor biology.

In the past, bulk profiles were analyzed in a somewhat naive way, ignoring the fact that each bulk profile reflects a composite of many distinct populations and sometimes misinterpreting signals of the tumor microenvironment as those of the cancer cells. Single-cell genomics, along with advances in cancer immunotherapies, have emphasized the significance of considering not just the cancer cells but also the various non-cancer populations. As a result, many recent methods and studies have focused on inferring the composition of tumors through deconvolution of their bulk profiles (Aran et al., 2017; Newman et al., 2015; Puram et al., 2017; Tirosh et al., 2016a). Such methods typically require a prior definition of potential tumor components (i.e., cell types), which can now be derived directly and accurately from scRNA-seq studies. In the next few years, we expect this trend to expand and that it will become standard to analyze bulk profiles in light of their measured or inferred composition. For example, if certain tumors have high expression of extracellular matrix (ECM) genes, the naive possibility of an aberrant ECM expression by the cancer cells would need to be contrasted with the alternative option of abundant ECM-producing fibroblasts. More generally, any subpopulation of cells that is identified within tumors through single-cell approaches could then be investigated for its estimated abundance and associations across large cohorts of bulk samples, thereby partially circumventing the limited size of single-cell cohorts.

Emerging Challenges and Directions in Single-Cell Cancer Analysis

In the next few years, the approach of investigating tumors through single-cell RNA-seq will be further integrated into cancer research and will be used to address an increasing number of questions about tumor biology (Figure 2). Below, we briefly describe some of the main questions that are likely to be addressed and point to emerging technologies in the area of scRNA-seq that would facilitate these studies.

Distinguishing the Contributions of Genetic versus Non-genetic Mechanisms: Single-Cell Multi-omics Analyses

Genetic heterogeneity is a prominent mechanism that generates diversity between and within tumors. However, an increasing amount of evidence points to the significance of non-genetic mechanisms. For example, in the context of eITH, we have recently shown that cellular hierarchies in IDH mutant and histone H3-K27M mutant glioma appear to be largely independent of genetics (Filbin et al., 2018; Tirosh et al., 2016b; Venteicher et al., 2017). Thus, an important challenge is to map both cell state and genetic state in the same cells and begin to distinguish between these mechanisms. Notably, therapeutic approaches for dealing with genetic versus non-genetic ITH may be quite distinct, underscoring the significance of this question. To date, most scRNA-seq studies either ignored the genetic states of cells or had a partial capacity to evaluate genetic states, by (1) detecting mutations in the RNA with limited sensitivity, (2) targeted amplification of individual mutations, or (3) inferring CNAs (Filbin et al., 2018; Giustacchini et al., 2017; Tirosh and Suvà, 2019). However, multiple technologies are being developed (Macaulay et al., 2015, 2016, 2017) and improved to enable such joint profiling of cell state and genetics, and these are expected to provide a more integrated view in the future (Nam et al., 2018; Rodriguez-Meira et al., 2019; Velten et al., 2018). For example, a recent study in acute myelogenous leukemia

(AML) highlighted that cell type composition correlated with prototypic genetic alterations (van Galen et al., 2019). More generally, "multi-OMICs" approaches are being developed to combine the measurement of mRNA levels with various other features, such as protein levels, DNA methylation, chromatin accessibility, clonal expansion, etc. These approaches will provide the ability to identify mechanisms that generate eITH and advance scRNA-seq toward more detailed mechanisms.

Cell-Cell Interactions and Spatial Location: Spatially Resolved Single-Cell Technologies

Each tumor is analogous to an ecosystem, and thus, the state of each cell may be highly dependent on its exact spatial location and interaction with adjacent cells. These complex interactions and their impact on cancer cell biology remain poorly understood. Coupling transcriptomes with spatial information will dramatically improve the ability to predict and further characterize such interactions, and accordingly, many approaches are being developed to couple scRNA-seq with spatial information. To date, most scRNA-seq studies have measured the state of cells without preserving any information on their location. Other studies have measured or inferred the spatial location of cells but typically characterized the cells by either multiplexed markers (Chen et al., 2015) or in lower (non-cellular) resolution (Chen et al., 2017) or relied on a stereotypical tissue structure, which is a more effective strategy for normal than for tumor tissues (Halpern et al., 2017; Satija et al., 2015). Continuous improvements in spatially resolved technologies for tumor profiling, as well as the integration of data from disparate technologies, will dramatically advance these directions over the next few years and improve our understanding of cell-cell interactions and spatial effects.

Apart from spatial profiling, the study of cell-cell interactions within tumors will advance through increased focus on the expression of ligands and receptors as central mediators of cellular communication. Databases of ligand-receptor complexes, coupled with a definition of tumor cell subpopulations by scRNA-seq data, highlight potential cell-cell interactions, in which one population produces a ligand that signals to another population expressing the corresponding receptor (Puram et al., 2017; Vento-Tormo et al., 2018). The complexity of the tumor microenvironment implies that the number of such potential interactions would be large, warranting follow-up studies that will determine the significance of individual interactions.

Residual Disease and Recurrences: Leveraging Single-Nucleus Profiling (Frozen)

In many cancer types, initial therapeutic responses are typically followed by the recurrence of tumors with increased aggressiveness and drug resistance, highlighting the significance of both the residual disease and its evolution and growth toward establishing the recurrent tumors. Previous studies compared primary and recurrent tumors and revealed various genetic causes of drug resistance. Future studies will extend this approach to comparison by scRNA-seq and provide a more detailed view of recurrent tumors and their difference from the corresponding primary tumors (Brady et al., 2017; Kim et al., 2018). An important challenge is that current protocols require fresh tumor samples for scRNA-seq, creating a severe bottleneck for processing of matched primary and recurrent samples given the time until recurrence. However, multiple approaches may enable profiling of frozen samples and thereby open the door to characterizing collections of samples that are stored in hospitals and labs, including longitudinal cohorts. For example, frozen or fixed samples may be analyzed by isolation and sequencing of single nuclei (instead of single cells), as recently demonstrated (Gao et al., 2017; Habib et al., 2016, 2017). In addition to recurrent tumors, scRNA-seq provides a compelling approach to directly characterize the rare residual cells that survive through treatments and ultimately underlie tumor recurrence. Such rare cells, typically referred to as minimal residual disease (MRD), may be profiled in animal models (Rambow et al., 2018) and compared to both primary and recurrent tumors, providing additional insights to their capacity to evade treatments, remain dormant, and finally give rise to recurrent tumors.

Tumor Subpopulations: Their Detection, Functional Significance, and Origin

Tumor progression is an evolutionary process, and hence, only few cells are sufficient for a new phenotype to gradually emerge through selection. Consequently, critical subpopulations, such as cancer stem cells, drug-resistant cells, and migratory cells that underlie metastasis, might reflect a very rare subpopulation (e.g., less than 0.1%) that escapes detection by most methods, including current scRNA-seq approaches. Importantly, although previous scRNA-seq studies were performed with platforms that measure limited cell numbers (e.g., tens to hundreds), more recent and ongoing studies now leverage droplet microfluidics or other technologies (Gierahn et al., 2017; Jerby- Arnon et al., 2018; Cao et al., 2017) that enable an order of magnitude more cells in each batch (e.g., thousands), and future technologies are likely to further expand this capacity into tens of thousands to even millions of cells, which would help uncover very rare subpopulations.

Once unique subpopulations have been uncovered, their functional and clinical significance remains difficult to ascertain. Two main approaches enable further analysis of such subpopulations. First, studies of large cohorts would uncover statistical associations between the presence and/or frequency of subpopulations and clinical features, such as survival, metastasis, and drug responses. Given the cost and labor associated with scRNA-seq of large cohorts, we anticipate that many of these analyses will leverage available bulk RNA-seq datasets and use computational approaches for their deconvolution and the inference of tumor composition, as described above. However, whether these would be based on single-cell or bulk RNA-seq profiles, such correlative studies will require mechanistic follow-ups. Thus, a second approach will rely on the detailed characterization of such subpopulations by scRNA-seq to identify markers that would enable isolation of these subpopulations and further functional studies in animal and culture models.

As described above, recent scRNA-seq studies uncovered remarkable similarities between tumor expression profiles and those of normal developmental cell types, raising specific hypotheses about the origin of cancers and the ongoing differentiation processes that occur within them. Through extensive

scRNA-seq of tumors, of normal tissues, and of developmental processes, for example, through the human cell atlas (HCA) initiative, the number of such observations is expected to dramatically increase over the next few years, including refinement of many previous observations. For example, scRNA-seq of H3-K27M midline gliomas (Filbin et al., 2018) highlighted the similarity of malignant cells to oligodendrocytic precursor cells (OPCs), supporting previous literature that suggested that OPCs are a candidate cell of origin for this tumor type (Gibson et al., 2018). A caveat with these inferences is that scRNA-seq of an established tumor cannot identify the cell in which the mutation first occurred ("cell of mutation"), which can be distinct from the cell that gives rise to the tumor. Additionally, comparisons between malignant cells and normal cells not only highlight similarities but also provide a more detailed view of the differences between tumor cells and their presumed cells of origin and thereby increase our understanding of the oncogenic process.

Unique Tumor Phenotypes and Exceptional Responders: Single-Cell Analysis in N = 1 Cohorts

Although therapeutic decisions are based on statistical patterns in large cohorts, outliers are quite common and present important conundrums. For example, "exceptional responders" illustrate our limited understanding of treatment response and the theoretical possibility to improve patient stratifications (Ferreri et al., 2010). In the past, it has been difficult to understand the unique phenotypes of such cases, which were studied primarily by bulk genetic analysis. However, scRNA-seq, along with the associated technological improvements described above (e.g., spatial profiling and multi-OMIC techniques), enable characterization of tumor ecosystems at unprecedented depth and breadth. We propose that these would be ideal to begin to identify the basis for unique phenotypes and accordingly that N-of-1 scRNA-seq studies may be particularly informative. Because extreme phenotypes are often detected late, this approach will also likely depend on analysis of frozen samples that have been archived.

Concluding Remarks

Tumor heterogeneity has puzzled cancer biologists and clinicians for a long time. As genomic tools were lacking to accurately measure individual cells in a tumor, we relied on average measurements or single markers, masking many critical aspects of ITH and hindering our understanding of tumor biology. The precise characterization of single-cell programs in tumors places cellular biology at the center of cancer biology. By measuring the transcriptional output as a function of both cell type and genetics, these efforts lay the foundation for renewed understanding of cancer initiation and evolution. The comprehensive profiling of single-cell programs in cancer also sheds light on tumor classification based on bulk measurements by enabling the deconvolution of signal and highlighting unanticipated patterns of ITH. Although the increased understanding of ITH might at first seem daunting, common cellular states and programs are emerging and are offering new candidate vulnerabilities that could be exploited for therapeutics. The initial studies discussed here are the beginning of an exciting era of single-cell genomics of human cancer; they are paving the way for many more discoveries that will come from improved technologies, resolution, computational methods, and broader clinical settings.

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