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Intratumoral heterogeneity: more than just mutations

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Abstract

Most human tumors are composed of genetically and phenotypically heterogeneous cancer cell populations that poses a major challenge for the clinical management of cancer patients. Advances of single cell technologies have allowed the profiling of tumors at unprecedented depth, which in combination with newly-developed computational tools enable the dissection of tumor evolution with increasing precision. However, our understanding of mechanisms that regulate intratumoral heterogeneity and our ability to modulate it has been lagging behind. Recent data demonstrate that epigenetic regulators, including histone demethylases, may control the cell-to-cell variability of transcriptomes and chromatin profiles and they may modulate therapeutic responses via this function. Thus, the therapeutic targeting of epigenetic enzymes may be used to decrease intratumoral cellular heterogeneity and decrease treatment resistance when used in combination with other types of agents.

Keywords

transcriptomic heterogeneity; epigenetic; histone demethylase

Introduction

DNA is called the blueprint of life, but it is now widely accepted that epigenetic modulation of chromatin plays critical roles in regulating when and where genes are expressed during cell fate determination and tumor development [1]. In contrast to genetic variation, epigenetic programs are reversible, and thus they can more directly regulate phenotypic plasticity. There are now many examples demonstrating the importance of phenotypic plasticity in the context of genetic homogeneity. Studies analyzing phenotypic discordance between monozygotic twins determined that epigenetic differences exist even between genetically identical twins and this could explain why only one twin gets a specific disease [2]. Similarly, cloned cats are not truly identical as the coat color of the cloned kitten is

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notably different from its genetic mother's due to epigenetic differences in the inactivation patterns of coat color genes [3, 4]. Honeybees have become a great model for studying phenotypic plasticity, as queens and workers develop from genetically identical larvae yet as adults they are strikingly different [5]. In larvae treated with siRNA targeting DNMT3 DNA methyltransferase the majority of emerging adults were queens in contrast to the larval group injected with a control siRNA where the majority of adults were workers [6]. Thus, epigenetic mechanisms are key determinants of cellular phenotypic variability during development.

In tumorigenesis, cellular heterogeneity for phenotypic features is a key mechanism underlying disease progression and therapeutic resistance, yet its regulation is poorly understood at the molecular level. In recent years technological advances enabled studies directed toward elucidating molecular mechanisms underlying phenotypic plasticity in cancer with the aim of utilizing this knowledge for the development of more effective therapies. Unraveling tumor heterogeneity is clinically valuable, as therapeutic responses are largely determined by the evolution of resistant subpopulations and changes in cellular phenotypes. In this review, we will summarize research efforts aimed at characterizing cellular phenotypic heterogeneity. We will also highlight recent work striving to connect epigenetic control of heterogeneity for gene expression to biological phenomena.

Sources of Intratumoral Heterogeneity

The majority of tumors develop from a single mutated cell and they accumulate additional mutations as they progress to advanced disease via Darwinian evolution [7, 8]. Recent advances in sequencing technologies enable the comprehensive understanding of cellular genetic heterogeneity within tumors [9]. The identification of tumor evolutionary trajectories has important implications for the design of effective cancer therapeutic strategies as it guides the selection of the most appropriate drug combinations based on the genetic composition of the cells. Rare mutant cells resistant to therapy can be pre-existing within tumors prior to treatment or can emerge during the course of the disease sometime due to mutations directly or indirectly triggered by the anti-cancer agents themselves [10, 11]. For example, profiling of 20 triple-negative breast cancer (TNBC) before and after neoadjuvant chemotherapy by single-cell DNA-seq revealed that genetically distinct resistant cells are pre-existing, and resistance to neoadjuvant chemotherapy is due to selection for these subpopulations [12]. Similarly, analysis on metastatic estrogen receptor positive (ER⁺) breast cancer patients showed that pre-existing minor subclones become dominant after chemotherapy [13]. Genetically distinct fulvestrant and tamoxifen-resistant subclones also pre-exist in ER⁺ breast cancer cell lines and they get selected for during treatment [14]. Mutations enabling tumor progression and therapeutic resistance may already be present in early preinvasive or even premalignant lesions [15]. In line with this, topographic single cell sequencing applied to *in situ* and invasive regions within the same breast tumor tissue sections determined that multiple mutated clones evolved within the ducts prior to invasion and some subclones were more prevalent in the invasive regions suggesting multiclonal invasion [9]. Another report described that the ability to invade was already acquired during the formation of DCIS (ductal carcinoma *in situ*) followed by the development of multiple genetically heterogeneous DCIS subclones [16]. However, as most DCIS never progress to

life-threatening metastatic disease, these findings indicate that the presence of mutant clones may not be sufficient to drive disease progression and highlight the role of the microenvironment, particularly immune cells, in this process [17, 18]. Immune editing by tumor-specific T cell infiltrate can eliminate the majority of tumor cells resulting in a more clonal tumor, thus, shaping tumor evolution [19]. Heterogeneity for neoantigens can also influence the efficacy of immunotherapies. A recent report demonstrated that clonal expression of neoantigens rather than total neoantigen burden determines the sensitivity to immune checkpoint blockade in patients with advanced non-small-cell lung cancer (NSCLC) and melanoma [20].

Microenvironmental factors including hypoxia, tissue stiffness, and chronic inflammation can both directly and indirectly influence cancer cell epigenetic and phenotypic heterogeneity [21]. Hypoxia regulates the activity and protein levels of histone and DNA modifying enzymes including the G9a histone H3 lysine 9 (H3K9me) methyltransferase [22, 23], KDM4C and other histone demethylases [24, 25], and the TET family of DNA demethylases [26], which could explain hypoxia-induced epigenetic alterations within tumors. Blocking this hypoxia response by BET bromodomain inhibitors can reduce tumor growth and may also enhance to efficacy of anti-cancer therapies [27]. Cancer cell interaction with fibroblasts and the extracellular matrix (ECM) produced by them also trigger epigenetic alterations that could explain the distinct epigenetic profiles of cancer cells at the tumor-stroma interphase [28]. Some of this could be due to the regulation of expression or activity of histone modifying enzymes including JMJD1a by cancer cell attachment to different types of ECM [29]. Chronic inflammation triggered by smoking or immune dysfunction such as in psoriasis and ulcerative colitis alters the epigenetic profiles of the cells and may play a role in tumor initiation and progression [30–32]. Anti-inflammatory agents, including healthy diet rich in fruits and vegetables, may decrease cancer incidence by interfering with this process [33].

Other sources of intratumoral heterogeneity is differentiation hierarchy-related epigenetic heterogeneity that is already present in normal tissues. The differentiation state of the transformed cell, normal cell-of-origin of cancer, is one of the determinants of tumor molecular subtypes. For example, in breast cancer all three major tumor subtypes, luminal A, luminal B, basal-like, and HER2+ [34], thought to arise from distinct luminal and basal progenitors. However, detailed molecular and functional analysis of normal breast tissue and breast tumors in *BRCA1* germline mutation carriers in combination with engineered mouse models revealed that basal-like breast cancer could arise from luminal progenitors [35, 36]. Furthermore, nanogrid single-nucleus RNA-seq in a TNBC determined that most cells had basal-like subtype, while a significant fraction of single tumor nuclei were HER2+, luminal A, luminal B, and normal like, indicating diverse cancer phenotypes reflecting distinct breast tumor subtypes even within a single tumor [37]. Further in-depth analysis is required to understand how heterogeneous tumors arise from different cells-of-origin.

Epithelial-mesenchymal transition (EMT) has also been identified as a regulator of cellular heterogeneity and cells undergoing EMT acquire stem cell-like features [38–40]. However, despite large amounts of data on the role of EMT in cell culture and animal models, its applicability in human cancer diagnosis and treatment has remained elusive, in part due to

difficulties with assessing EMT and differentiation state-related heterogeneity in clinical samples. A recent study described that rare breast tumor cells express both epithelial and mesenchymal markers and circulating tumor cells (CTCs) are highly enriched in mesenchymal cells [41]. Interestingly, in one patient the phenotype of the CTCs shifted between epithelial and mesenchymal during each treatment cycle, suggesting epigenetic heterogeneity within the tumor or switching of epigenetic states. An experimental mouse model of pancreatic cancer using tracking of lineage-labeled cells revealed that EMT can be detected in premalignant lesions and it may contribute to early dissemination even before tumor formation [42], further demonstrating the link between EMT and intratumor cellular heterogeneity and tumor progression.

A large number of cancer genome sequencing studies have identified genetic alterations associated with tumor progression and therapeutic resistance [43–45]. However, since selection works on phenotype, genetic alterations drive tumor evolution only if they have phenotypic consequences. Determining this requires the combined analysis of phenotypic and genetic heterogeneity coupled with functional screens in experimental models and repeated longitudinal assessment of the tumors during the course of the disease. A recently developed microfluidic-based serial suspended microchannel resonator (SMR) platform enables the direct measurement of single-cell growth rate immediately upstream of single-cell RNA-seq (scRNA-seq) [46]. Study on a glioblastoma cell line have demonstrated that there was considerable heterogeneity in response to MDM2 inhibitor as some cells continued to grow whereas the majority of cells showed a marked reduction in growth rates after drug treatment. Analysis of transcriptional signatures that may underlie this heterogeneity determined that cells with a higher growth rate had a lower expression of genes related to apoptotic signaling orchestrated by p53 in the drug-treated population. Thus, these linked functional-molecular measurements offer an opportunity to explore the molecular mechanisms that drive drug resistance at the single-cell level, especially when viable single cells are available.

Drug-tolerant persisters (DTPs) within heterogeneous cell populations pose a major obstacle for the treatment of various diseases, including bacterial infections and cancer (Figure 1). In bacteria, some subpopulations are capable of surviving antibiotic treatment due to the presence of persisters, which exhibit reversible antibiotic-tolerant properties without specific genetic alterations [47–49]. There is strong evidence to suggest that persisters are pre-existing in bacterial populations [50], however, other evidence suggests that antibiotics can actually induce the formation of persister cell populations [51]. In cancer, DTPs provide a temporary pool for selection during treatment and facilitate the outgrowth of drug-resistant mutants as demonstrated by the emergence of EGFR(T790M)-positive clones from drug-tolerant subpopulations of lung cancer cells [10]. Because the DTP state is reversible, it is thought to be defined by epigenetic mechanisms. By modeling the response to EGFR TKI (tyrosine kinase inhibitor) in an EGFR mutant NSCLC cell line, DTPs were found to have higher levels of the KDM5A histone H3 lysine 4 demethylase as well as lower levels of histone H3 lysine 4 trimethyl (H3K4me3) [52]. Knockdown of *KDM5A* reduced the number of DTPs following EGFR TKI treatment. Similarly, HDAC (histone deacetylase) inhibitors efficiently decreased the frequency of DTPs through IGF-1 receptor and downstream chromatin modifications mediated by KDM5A, implicating the epigenetic machinery in the

regulation of cellular phenotypic heterogeneity in drug tolerance. Histone mass spectrometry analysis of EGFR-mutant NSCLC cells revealed that DTPs showed a global decrease in acetylated histone H3 (H3KAc) associated with active chromatin together with a global increase in H3K9me3 (histone H3 lysine 9 trimethyl) and H3K27me3 (histone H3 lysine 27 trimethyl) repressive marks ([53]). In T cell acute lymphoblastic leukemia (TALL), γ -secretase inhibitor-tolerant persister cells were found to be pre-existing and showed an altered chromatin state and BRD4 dependency [54]. Analysis of DTPs after treatment with MEK inhibitor Trametinib and PI3K/mTOR inhibitor BEZ235 in basal-like breast cancer determined that BRD4, KDM5B, and EZH2 activity are increased in DTPs after treatment [55]. Together, these findings provide evidence that drug-resistant cells can be derived from both pre-existing genetically distinct populations and from epigenetically distinct DTPs.

Epigenetic Regulation of Transcriptomic Heterogeneity

Clonal cell populations can exhibit substantial phenotypic variation especially when phenotypic diversity could be beneficial [56]. Such diversity may arise not only from genetic diversity but also from transcriptomic heterogeneity or noise in gene expression [57, 58] (Figure 2). Intrinsic fluctuations due to the randomness inherent to transcription could affect gene expression noise, however, the regulatory circuits of transcriptomic heterogeneity dynamics are not well understood. Epigenetic regulators such as histone modifying enzymes are critical for the establishment of cell-type-specific gene expression patterns, and therefore they are also likely to play a role in modulating cell-to-cell variability in transcription. Thus, a key question is: how do epigenetic modifiers impact transcriptomic heterogeneity under various conditions? Although epigenetic heterogeneity could be a reflection of genetic heterogeneity on genes encoding epigenetic regulators, stochastic noise in gene expression is also regulated by epigenetic programs in genetically homogeneous backgrounds, as illustrated by the honeybees described above. Much remains to be discovered about relationships between epigenetics and phenotypic variability, but a few recent examples, highlighted below, describing epigenetic mechanisms for the underlying heterogeneity of cellular phenotypes have been reported.

In breast cancer, the majority of tumors are luminal ER⁺ and require estrogen and ER for their growth [59]. While the majority of ER⁺ breast cancer patients respond to endocrine therapies, a significant fraction develops resistance and progresses to metastatic disease [60]. *KDM5B* was identified as an oncogene commonly amplified and overexpressed in luminal ER⁺ breast tumors and higher KDM5B activity was associated with poor outcome in ER⁺ breast cancer patients treated with endocrine therapy [61]. These findings suggest that KDM5B may have a role in regulating the response and resistance to endocrine therapies. Since KDM5B is a H3K4me3 demethylase and broad H3K4me3 domains mark cell identity genes associated with high transcriptional consistency [62], higher KDM5B activity may increase cellular transcriptomic heterogeneity by decreasing H3K4me3 peak broadness. Indeed, a recent study reported increased promoter H3K4me3 peak broadness following KDM5 inhibitor treatment in luminal ER⁺ breast cancer, which was associated with an increase in the fraction of cells expressing the associated genes resulting in more uniform cellular gene expression patterns [14]. Inhibition of KDM5 also decreased cell-to-cell cellular heterogeneity and increased sensitivity to fulvestrant even in previously fulvestrant-

resistant cells. Higher *KDM5B* expression in ER⁺ luminal breast tumors is correlated with higher transcriptomic, but not subclonal genetic heterogeneity. Besides *KDM5B*, higher expressions of several other histone demethylases also correlated with higher transcriptomic heterogeneity in human tumors, implying that histone demethylases in general may play a role in regulating transcriptomic heterogeneity [14]. While this heterogeneity might in part be due to heterogeneity for *KDM5B* copy number gain within cancer cell populations as *KDM5B* expression itself displays marked cellular variability, it is plausible that changes in H3K4me3 broadness regulate the stochastic noise in gene expression.

Gene expression is also variable due to short, rare events of active transcription [58, 63], and the presence or absence of active sites of transcription are correlated with transcriptional bursts [64]. Thus, the prevalence of transcriptionally active chromatin marked with H3K4me3 would result in rapid events of gene activation and inactivation, leading to smaller fluctuations than in the transcriptionally silenced state (Figure 3). However, it is also possible that more active chromatin facilitates spurious or less stringently-controlled transcription leading to higher transcriptomic heterogeneity. This hypothesis is supported by findings in colon cancer describing the presence of large blocks of contiguous hypomethylation in cancer compared to normal cells, and genes located within hypomethylated blocks displayed increased variability in gene expression among tumors [65]. Other candidate regulators of transcriptomic heterogeneity are chromatin remodeling factors that rearrange the chromatin state by promoting either an open or a closed configuration. Chromatin remodeling may therefore be responsible for genes transitioning between active and inactive states. In line with this, single-cell gene expression profiling of mouse pluripotent stem cells (PSCs) determined that while Polycomb target genes exhibited greater expression variability, genes involved in housekeeping and metabolic functions displayed relatively uniform expression [66]. Knocking out PRC2 (polycomb repressive complex 2) function through deletion of the polycomb-group protein EED resulted in increased transcriptomic heterogeneity. Characterization of the sources of transcriptional variability at gene-level resolution in induced pluripotent stem cell (iPSC) lines also revealed that gene expression variation was derived from PRC2 and H3K27me3-mark-associated genes [67]. Conversely, DGCR (DiGeorge syndrome chromosome region) knockout mouse embryonic stem cells (mESCs), which lack mature miRNAs due to loss-of-function of miRNA processing factors, showed decreased transcriptomic heterogeneity [66]. As these examples demonstrate, changes in both activated and repressed chromatin can lead to increase in cellular transcriptomic heterogeneity implying that perturbation of the steady-state levels rather than the directionality of the change is what is important. However, a central unanswered question in epigenetics is whether transcriptional silencing precedes epigenetic repression or it is a consequence of it. DNA methylation plays a key role in imprinting and silencing repetitive elements, but experimental data in various organisms as well as the phenotype of humans with mutations in the DNA methylation pathway suggest that it is not a common regulator of gene expression [68]. The same observations have also been made for the polycomb repressive complex 2 (PRC2) in embryonic stem cells – PRC2 is recruited to loci after loss of transcription [69]. Thus, cellular variability for the expression of transcription factors is likely a major contributor to transcriptomic heterogeneity.

Transcriptomic Heterogeneity and Cellular Fitness

Most studies analyzing intratumoral heterogeneity have focused on genetic alterations and in many cases therapeutic resistance is due to mutations in genes and pathways targeted by the treatment [44]. However, non-genetic variability such as epigenetic heterogeneity also contributes to therapeutic resistance by multiple different mechanisms [70]. Thus, manipulation of the epigenetic landscape in cancer cells would be a powerful therapeutic tool as it could be used to improve the efficacy of targeted or chemotherapies. For example, inhibition of KDM5 histone demethylases increases sensitivity to endocrine and HER2-targeted therapies in breast cancer, and EGFR-targeting and chemotherapies in lung carcinomas [14, 71, 72]. Similarly, in melanoma a slow cycling JARID1B (KDM5B) high subpopulation has been shown to be required for tumor maintenance and is associated with resistance to chemo- and BRAF-targeting therapies [73, 74]. Clonal subpopulations of a breast cancer cell line exhibiting increased cell-to-cell transcriptomic variability were also shown to display enhanced metastatic capacity and drug resistance [75]. Furthermore, a recent study showed that genes involved in SWI/SNF chromatin remodeling complex, including *ARID1A*, *ARID1B*, and *ARID2*, were commonly wild type in the primary tumors but mutated in metastatic recurrences of treatment-resistant breast cancer [76], suggesting that the altered epigenetic state due to these mutations increases cellular fitness in these conditions. Gene expression noise has also been recognized as an underlying mechanism of HIV latency [77]. By screening a library of 1,600 bioactive small molecules in an isoclonal Jurkat cell line containing one integrated copy of the HIV LTR promoter expressing a short-lived green fluorescent protein (GFP) reporter (d2GFP) several epigenetic modulators were identified as noise enhancers in HIV gene expression. These included DNMT, HDAC, and BET bromodomain inhibitors, which increased gene expression noise and enhanced HIV reactivation [78]. This observation suggests that chromatin modifiers can regulate noise in gene expression to establish persistent states (e.g., HIV latency).

Another example for a close relationship between gene expression noise and cellular fitness has been observed in yeast *Saccharomyces cerevisiae* mating pathway in which a mating-specific transcription factor Ste12 is inhibited by two MAPK-responsive regulators, Dig1 and Dig2 displaying largely overlapping functions implying redundancy. However, deletion of Dig1 (and not Dig2) resulted in a specific increase in gene expression noise in the mating pathway, as determined using reporter genes, change in the nuclear localization of Ste12, and increased interchromosomal interactions of Ste12-dependent genes resulting in decreased cell growth. These data suggest that long-range interactions, while a common regulatory mechanism of gene expression, may come at the cost of increased variability, thus, yeast cells evolved ways to suppress this noise by controlling Dig1 activity [79]. It would be interesting to see if similar noise-regulatory mechanisms also exist in mammalian cells and if their alterations play a role in tumorigenesis.

Recent work in yeast studying how stochastic gene expression variability influences evolutionary adaptation to a stressful environment under fluconazole treatment. Bimodal expression of PDR5, a natural multidrug transporter, enhanced the survival of yeast cells under treatment, suggesting that phenotypic heterogeneity had a major impact on cellular sensitivity to fluconazole stress [80]. Moreover, a cell population with bimodal expression of

PDR5 reached a higher level of drug resistance after long-term fluconazole treatment and increased the adaptive value of beneficial mutations. At the same time another cell population with unimodal expression of PDR5 showed a lower level of fluconazole resistance despite the similarity of acquired mutational profiles in both cell populations. These findings demonstrate that phenotypic heterogeneity facilitates evolutionary rescue from deteriorating environmental stress by generating individual cells with exceptionally high cellular fitness.

Synergies between Epigenetic and Targeted Therapies

As the above examples demonstrate, there is a clear scientific rationale for combining epigenetic agents with chemo- or targeted therapies to improve treatment efficacy. One of the earliest examples for this is the combination of HDAC and DNA methyltransferase inhibitors (HDACi and DNMTi) that induces robust re-expression of hypermethylated genes including tumor suppressors leading to reduced growth [81]. Remarkably, combinatorial treatment of DNMTi and HDACi followed by immune checkpoint therapy achieved objective response in multiple NSCLC patients, including one who had a complete response and was alive with no evidence of disease progression even two years after therapy [82]. This impressive response to immunotherapy after DNMTi and HDACi treatment suggests that combined epigenetic therapy changes the expression of immune-related genes or reactivates repressed endogenous retroviral elements that then trigger an IFN-mediated immune response [83, 84].

Recent work demonstrated that the combination of DNMTi and HDACi *in vivo* strongly induced the upregulation of CCL5, a chemokine involved in lymphocyte attraction, and this may contribute to the increased number of CD8⁺ T cells in tumors after treatment [85]. A study in a murine model of ovarian cancer also showed that the combination of HDACi and DNMTi increased T and NK cell activation, and DNMTi/HDACi plus immune checkpoint inhibitor anti-PD-1 resulted in great antitumor effect and increased overall survival [86]. HDACi was also shown to cooperate with mTOR inhibitors triggering catastrophic oxidative stress and tumor regression in NF1-deficient malignant peripheral nerve sheath tumors (MPNST) as well as in *NF1*-mutant and *KRAS*-mutant NSCLC [87]. Combining DNMTi with FLT3 inhibition increased therapeutic response of *TET2*-mutant acute myeloid leukemia (AML) cells [88]. A screen of small molecule inhibitors in neuroblastoma to discover synergistic drug combinations identified PI3K and BET bromodomain inhibitors (PI3Ki and BETi) [89]. The combination treatment of PI3Ki and BETi delayed tumor progression and increased overall survival compared with either agent alone in a xenograft model. Analysis of a large panel of cancer cell lines revealed the correlation between cell sensitivity to EZH2 inhibitors and reciprocal upregulation of H3K27Ac, thus combinatorial treatment with EZH2i and BETi efficiently blocked tumor growth in diverse cancer types [90]. In addition, in those insensitive to EZH2-BET inhibitor combination, MAPK pathway was ubiquitously activated upon the treatment, and thus a triple combination of EZH2i, BETi and ERKi achieves robust efficacy with acceptable toxicity.

Epigenetic therapy has also proved effective for the re-expression of transcriptionally repressed genes. In renal cell carcinoma, expression of organic cation transporter *OCT2* that

enhances the cellular uptake and cytotoxicity of platinum agent was repressed due to promoter DNA hypermethylation, and therefore DNMTi treatment induced OCT2 expression and sensitized cancer cells to oxaliplatin [91]. Elegant example of a link between epigenetics and drug sensitization connected epigenetic perturbation to synthetic lethality in the DNA repair network [92]. Although homologous recombination (HR)-deficient tumors, such as those with mutant *BRCA1*, have been shown to be sensitive to PARP inhibitors (PARPi), there is a pressing need to develop strategies to sensitize HR-proficient tumors to PARP inhibitors as the majority of tumors are HR-proficient. Pharmacologic inhibition or genetic deletion of BET bromodomain proteins impaired transcription of *BRCA1* and *RAD51*, two genes essential for homologous recombination, thus, increasing sensitivity to PARPi, oraparib, in animal models of HR-proficient breast and ovarian cancers [92]. BET inhibitors have also been tested in numerous other drug combinations in various cancer types with promising preclinical data and several clinical trials ongoing [93].

Although none of these studies have analyzed changes in cellular heterogeneity following treatment with epigenetic agents, it is conceivable that many of these drugs may make cellular expression patterns more uniform resulting in lower heterogeneity and decrease risk of resistance; a hypothesis that is relatively easy to test with the use of scRNA-seq and related single cell methods.

Concluding Remarks

Advances in sequencing technologies now allow for the comprehensive profiling of gene expression, genetic, and epigenetic patterns of single cells enabling deeper understanding of cellular heterogeneity within normal and neoplastic tissues. Similarly, the development of cellular barcoding methods coupled with CRISPR screens allow for the assessment of the consequences of genetic perturbations at the single cell level. Thus, genetic screens that used to be feasible only in single cell organisms such as yeast can now be performed in mammalian cells. The application of these technologies to cancer has revealed intratumoral heterogeneity at unprecedented depth. However, to what degree this heterogeneity is regulated and can be regulated versus being just a reflection of biological noise remains to be determined (see Outstanding Questions). We anticipate that the combination of these single cell experimental data and mathematical modeling will make rapid progress in this area and further our understanding of drivers of tumor evolution and regulators of cellular heterogeneity. This knowledge will serve as basis for clinical translation of these findings for the more efficient treatment of cancer.

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GLOSSARY

Histone demethylase

Histone demethylases are enzymes that remove a methyl group from histones.

Cellular state

the physiological condition of a cell defined by molecular profiles such as transcriptome. Cellular states may be transient and cells can switch from one state to another due to environmental conditions.

Persister cells

Persisters are dormant variants of regular cells that form stochastically in cell populations and are highly tolerant to drug treatment.

Serial SMR platform

The serial suspended microchannel resonator device can precisely measure growth rates of many individual cells simultaneously, as several different channels in the device give the cell time to grow. Single cells flow through a series of SMRs, followed by the collection of single cells for scRNA-seq. These linked measurements offer the potential of characterizing intratumoral cellular heterogeneity in the presence or absence of various agents.

Nanogrid single-nucleus RNA-seq

Nanogrid single-nucleus RNA-seq enables imaging, selection, and sequencing of thousands of single nuclei in parallel. This is a powerful tool for studying the transcriptional programs of single tumor nuclei from patient's tumor samples since most archival tissue samples have previously been flash frozen that ruptures the cell membranes.

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Highlights

Tumorigenesis is a process of Darwinian evolution driven by cellular heterogeneity for phenotypic features combined with selection.

Cancer cells display startling genetic and transcriptomic heterogeneity that increases the probability of therapeutic resistance.

Microenvironmental factors such as hypoxia and inflammation have major impact on cellular heterogeneity within tumors.

Drug tolerant persister cells are characterized by specific epigenetic states that are reversible. Cellular transcriptomic heterogeneity could be due to noise in transcription and is modulated by epigenetic regulators such as the KDM5 family of histone H3 lysine 4 demethylases.

Combination of epigenetic agents that decrease cellular transcriptomic heterogeneity with targeted or chemotherapies enhances treatment efficacy and reduces the emergence of resistant subpopulations.

Outstanding Questions Box

Are intratumoral cellular epigenetic and genetic heterogeneity independent features or just reflection of the same characteristic?

Is cellular transcriptomic heterogeneity a regulated heritable feature or just a reflection of biological noise in transcription?

What are the mechanisms by which microenvironmental factors influence cellular epigenetic heterogeneity and what roles do these play in tumor initiation and progression?

If the main function of epigenetic regulators is to regulate cellular heterogeneity and their inhibition only shows beneficial effects when used in combination with other agents, how can they be tested in clinical trials?

What is the best way to assess cellular transcriptomic heterogeneity in clinical samples and how can this be used to guide therapeutic decisions?

Figure 1. Intratumoral cellular genetic heterogeneity and drug-tolerant persisters.

Human tumors often display intratumoral heterogeneity for various biological features. Intratumoral heterogeneity can be due to the presence of multiple genetically distinct subclones within a single tumor (left). Drug-tolerant persisters (DTPs) constitute a subpopulation of genetically homogenous tumor cells (right). Epigenetically distinct tumor cell subpopulations allow for reversible transitions from drug-sensitive to drug-tolerant states.

Figure 2. Evolutionary trajectories and transcriptomic heterogeneity.

Tumors develop along several evolutionary trajectories. Driver mutations have a selective advantage during cancer progression, and each genetically distinct subclone can exhibit substantial phenotypic variation due to cellular transcriptomic heterogeneity. Transcriptomic heterogeneity can be a consequence of stochastic noise in gene expression.

Figure 3. Epigenetic regulation of transcriptomic heterogeneity.

Epigenetic modulators such as histone H3K4 demethylases can impact cellular transcriptomic heterogeneity directly. Broad H3K4me3 promoter peaks can lead to rapid events of active transcription leading to smaller fluctuations in gene expression (right), whereas narrow H3K4me3 peak could result in rare events of active transcription (left). Larger fluctuations in gene expression lead to more heterogeneous gene expression patterns within cancer cell populations, thus increasing the possibility that a subpopulation of cancer cells is resistant to anti-cancer therapies. Epigenetic agents may improve the efficacy of cancer therapies by modulating cellular transcriptomic heterogeneity.