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Novel single-cell technologies in acute myeloid leukemia research

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Abstract

Acute myeloid leukemia (AML) is a lethal malignancy because patients who initially respond to chemotherapy eventually relapse with treatment refractory disease. Relapse is caused by leukemia stem cells (LSCs) that reestablish the disease through self-renewal. Self-renewal is the ability of a stem cell to produce copies of itself and give rise to progeny cells. Therefore, therapeutic strategies eradicating LSCs are essential to prevent relapse and achieve long-term remission in AML. AML is a heterogeneous disease both at phenotypic and genotypic levels, and this heterogeneity extends to LSCs. Classical studies in AML have aimed at characterization of the bulk tumor population, thereby masking cellular heterogeneity. Single-cell approaches provide a novel opportunity to elucidate molecular mechanisms in heterogeneous diseases such as AML. In recent years, major advancements in single-cell measurement systems have revolutionized our understanding of the pathophysiology of AML and enabled the characterization of LSCs. Identifying the molecular mechanisms critical to AML LSCs will aid in the development of targeted therapeutic strategies to combat this deadly disease. (Translational Research 2017;189:123–135)

INTRODUCTION

Acute myeloid leukemia (AML) is the most common acute leukemia in adults and is characterized by the accumulation of poorly differentiated myeloid cells.¹ With an overall 2-year survival rate of less than 50%, AML is an aggressive hematological malignancy.² Although 60%–80% of patients achieve complete remission after standard chemotherapy, more than 50% patients experience relapse.² Despite enormous progress in the understanding of leukemia pathophysiology, prognosis after AML relapse is extremely poor. AML exhibits a hierarchical cellular organization, with a minor fraction of leukemia stem cells (LSCs) at the apex of the hierarchy which are capable of self-renewal and disease maintenance.³ Although genetically abnormal, LSCs share several characteristics with normal hematopoietic stem cells (HSCs), including quiescence, multipotency, and self-renewal. Genomic analyses led to the identification of preleukemic mutations in HSCs that

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drive clonal evolution in AML and survive standard induction chemotherapy, contributing to disease relapse.^{3,4} Therefore, elucidation of LSC biology and development of therapeutic strategies to target them are fundamental for achieving long-term remission in AML.

Traditional methods for understanding the mechanisms of AML have aimed at characterization of bulk cell populations. However, bulk analysis approaches mask cellular heterogeneity and provide limited insight into rare and heterogeneous cell populations such as LSCs, thereby presenting major challenges in drug discovery and development. Recently, several technologies have emerged that can comprehensively analyze the single-cell at the molecular level. Examples of such approaches include microfluidic-based single-cell sorting methods,⁵ high-throughput multiplexed single-cell quantitative PCR (qPCR),^{6,7} single-cell genome^{8–10} and transcriptome^{11,12} sequencing approaches, mass cytometry-based proteomic strategies,¹³ and data analysis methods^{14,15} (Table I). Single-cell techniques have provided an unprecedented opportunity to identify rare cell types such as cancer stem cells and to investigate the dynamic processes in cell fate transitions. In addition, multiomics approaches integrating measurements of genes and transcripts,²⁵ proteins and transcripts^{26,27} and genes,²⁸ panels of proteins and metabolites²⁹ from single cells have also been reported that could provide a more detailed picture of the interplay of biomolecules within single cells. The advent of single-cell technologies has opened up new avenues to study tumor heterogeneity, identify rare cell types, and ultimately, guide diagnosis and treatment.

AML is a heterogeneous disease.

In a malignancy such as AML, which exhibits enormous molecular heterogeneity, application of single-cell technologies could provide powerful insights into initiation, evolution, and relapse of the disease. This review summarizes recent reports of the utilization of single-cell approaches in unraveling the biology of AML with emphasis on LSCs.

Understanding cellular heterogeneity has immense clinical implications in AML. LSCs, which initiate AML, are capable of self-renewal and differentiation into the heterogeneous lineages composing the bulk tumor. AML cells have been reported to exhibit variability in a wide range of parameters including phenotype, genotype, intracellular signaling, and therapeutic response. Thus, it is essential to dissect cellular heterogeneity in AML and identify pathways critical for functioning of LSCs.

Single-cell cytometric analyses to investigate phenotypic and functional heterogeneity in AML.

Phenotypic heterogeneity.—Fluorescence-based flow cytometry has been instrumental in identifying cell types based on cell surface marker expression patterns. Like normal hematopoiesis, AML is organized in a hierarchy of subpopulations that have been characterized by their cell surface proteins. Although initial studies suggested that a rare subset of cells resembling hematopoietic stem/progenitor cells (HSPCs) has LSC property,³⁰ it is now evident that tumor-initiating activities can be found in immunophenotypically distinct compartments of AML.^{31–34} Homeobox genes are important for hematopoietic development and stem cell renewal, and ectopic expression of Hoxa9 and Meis1 (H9M) in

hematopoietic progenitors leads to rapid onset of AML.³⁵ In a H9M mouse model of AML, Gibbs et al. used fluorescence-based flow cytometry for immunophenotypic characterization of LSCs.¹⁶ The study demonstrated that LSCs exist in 3 phenotypically distinct compartments corresponding to different lineages on the normal hematopoietic hierarchy: stem/progenitor cells (Lin-kit+) and committed progenitors of the myeloid (Gr1+kit+) and lymphoid (Lym+kit+) lineages. Gene expression microarray data showed that the 3 different LSC populations had overlapping enrichment of leukemogenesis and HSPC-associated gene signatures. Mass cytometric analysis of the phosphorylation status of 14 intracellular proteins demonstrated that the 3 distinct LSC subsets had conserved signaling and survival networks (DNA methylation, and MAPK/Erk). Targeting such shared survival pathways significantly increased survival in vivo. Therefore, cancer stemness could represent a cellular state rather than a defined cell phenotype.

Advances in flow cytometry have enabled the study of a large number of parameters in single cells at high resolution. Identification of leukemia subpopulation requires simultaneous measurements of many cell surface proteins. To investigate the functional status of leukemic cells, measurements of functionally relevant proteins (such as phosphorylated, intracellular signaling intermediates) are essential. Modern fluorescence-based flow cytometers can measure up to 15 parameters simultaneously.³⁶ Mass cytometry, in which protein detection is based on mass spectrometry-based detection of metal tags measures up to 40 parameters simultaneously in single cells.³⁷ Mass cytometry, therefore, provides an opportunity to investigate complex physiological status (by measuring signaling intermediates) in well-defined immunophenotypic subgroups of cells (by measuring cell surface proteins).

Computational strategies to analyze high-parameter cytometric data.

Traditional methods examined single-cell data as a scatter plot, which displays the correlation of only two markers at a time. However, with increase in the number of parameters measured per single cell, it is difficult to visualize such a high number of dimensions in a meaningful way. Qiu et al. developed a novel computational approach for multiparametric data analysis, the Spanning-tree Progression Analysis of Density-normalized Events (SPADE).¹⁴ SPADE is an algorithm to cluster cells according to similarities in the expression of measured parameters. Density-dependent down sampling allows this tool to detect and represent rare populations of cells. The resulting cell clusters are organized and linked with a minimum spanning tree meaning that clusters that are similar to each other are placed close to each other in the 2-dimensional representation. Thus, SPADE provides a simple 2-dimensional visualization of multiple cell types in a branched tree structure displaying how measured markers behave across all cell types in the data (Fig 1A). Application of SPADE to cytometry data of normal mouse and human bone marrow organized cells in a hierarchy of related phenotypes and produced a map of intracellular signal activation across the landscape of human hematopoietic development. However, SPADE typically clusters cells and examines the average of each cluster, resulting in the loss of single-cell resolution of the data. To overcome this limitation, Amir et al. developed viSNE,¹⁵ a tool for visualization of high-dimensional single-cell data based on the t-Distributed Stochastic Neighbor Embedding algorithm.² viSNE provides 2-dimensional

representation of single-cell data, preserving its local and global geometry. The viSNE map is visualized as a scatter plot where the cell's location in the plot represents information from all the original dimensions. Conceptually similar to a principal component analysis, dots that are similar to each other across all the measured dimensions are arranged next to each other in 2-dimensional space (Fig 1B). Integrating mass cytometry data of a panel of surface markers with viSNE, healthy and leukemic human bone marrow samples were mapped. Healthy bone marrow was found to map into a canonical shape, while in leukemia, the shape was malformed. In a diagnostic AML bone marrow sample, viSNE highlighted leukemia as a continuum of states demarcated by gradients of phenotypic marker expression, rather than distinct subpopulations. When applied to a matched pair of samples from a patient with AML, 1 sample taken before chemotherapy and the other after relapse of the disease, viSNE identified phenotypes unique to the diagnosis sample that were eliminated by chemotherapy and new phenotypes that arose at relapse, thereby elucidating the progression from diagnosis to relapse. In addition, viSNE identified a population of cells representing minimal residual disease.

Mass cytometry provides a substantial increase in the number of proteins that can be measured simultaneously in single cells. Such multidimensional single-cell measurements have led to significant advancements in our ability to interrogate the biology of leukemia subpopulations.

Functional heterogeneity.—Intratumor heterogeneity is known to be functionally and clinically relevant.⁴⁰ AML manifests as phenotypically and functionally diverse cells, often within the same patient. Although LSCs are thought to be responsible for disease persistence and relapse in AML, no uniform phenotypic identifier for LSCs has been found across patients.⁴¹ Recognizing a disconnect between the tumor-initiating potential and surface phenotype of LSCs, Levine et al. used mass cytometry to profile 16 surface markers and 14 intracellular phosphoproteins simultaneously in healthy and primary AML cells subjected to perturbations (cytokines and chemical inhibitors).¹⁸ Phenograph is an analysis program that was developed to algorithmically partition high-parameter single-cell data into phenotypically distinct subpopulations. Phenograph revealed that surface phenotypes of leukemic blasts do not necessarily reflect their intracellular state. By using hematopoietic progenitors, a signaling-based measure of primitive cells was defined. Phenograph detected the presence of primitive cells in most AML samples at varying frequencies and led to the isolation of a gene expression signature that predicted survival in independent cohorts. Han et al. applied mass cytometry to primary AML samples harboring *FLT3-ITD* mutations for simultaneous assessment of surface markers and phosphoproteins.¹⁹ SPADE analysis showed phenotypic heterogeneities across patients with the same genetic makeup, with distinct patterns of signal activation between samples. The mTOR targets p-4EBP1 and p-S6 were exclusively expressed in *FLT3-ITD* stem/progenitor cells but not in their normal counterparts, suggesting both as novel targets in *FLT3*-mutated AML. To understand the difference in relapse rates across AML subtypes, Behbehani et al. measured immunophenotypic, cell cycle and intracellular signaling markers in bone marrow aspirates from 41 AML patients and 5 healthy donors using mass cytometry.¹ The study revealed immunophenotypic and intracellular signaling heterogeneity among AML LSCs depending

on cytogenetic and molecular subtypes of the disease. The core binding factor AML, which has a clinically favorable outcome, had a high percentage of proliferating stem cells in S-phase of the cell cycle, whereas *FLT3-ITD* AML, which has a poor clinical outcome, showed a dramatic reduction in LSCs in the S-phase. This study suggested that the difference in chemotherapeutic response of AML risk groups could be attributed to the cell cycle status of LSCs. High-parameter simultaneous analysis of phenotypic markers and signaling behavior allows us to build cell-type specific connectivity maps using Bayesian network analysis.^{42–44} Such signaling connectivity maps provide a system-wide understanding of the signaling state in leukemia cells, which could be used to rationally develop targeted therapies. Using mass cytometric analysis, Fisher et al. demonstrated the constitutive activation of NFkB and hyperactivation of JAK-STAT, MAPK, and PI3K pathways in secondary AML.⁴⁵

The aforementioned studies using mass cytometry to define the functional status of AML cell subtypes provided critical insights into the molecular mechanisms of LSC behavior.

Single-cell transcriptional analysis to study heterogeneity in AML.

Saadapour et al. characterized heterogeneity in leukemic cells using the MLL-AF9 driven mouse model of AML.¹⁷ The study involved fluorescence-activated cell sorting analysis of 7 surface markers in leukemic cells followed by single-cell qPCR to assay the transcriptional profile of 175 genes in 71 AML cells. The analysis revealed striking variation of gene expression within leukemic cells and identified 2 distinct subtypes of leukemic cell, one similar to granulocyte/monocyte progenitors (leukemia1) and the other to macrophage and dendritic cells (leukemia 2). Leukemia 1 overexpressed leukemic regulators like *Runx1*,⁴⁶ *Etv6*,⁴⁷ and the chromatin regulator *Brd3*, an emerging target for cancer therapy.⁴⁸ In colony-forming assays, leukemia 1 exhibited higher proliferation rates and differentiation capabilities. This study provided new insights into molecular heterogeneity in AML and revealed new functional subgroups that correspond to unique transcriptional programs.

Corces et al. defined chromatin accessibility and transcriptional landscapes in 13 human primary blood cell types that span the hematopoietic hierarchy.²⁴ By employing single-cell assay for transposase-accessible chromatin using sequencing (ATAC-seq), a method capable of measuring chromatin accessibility in rare cellular populations, the authors demonstrated that normal myeloid progenitors (LMPP) and monocytes reveal chromatin accessibility patterns similar to their bulk counterparts. In contrast, single AML cells showed chromatin accessibility patterns that were either intermediate between 2 developmental hematopoietic states or showed a combination of both intermediate and normal states. These findings indicate that, at the single-cell level, gene expression regulatory patterns (as evidenced by chromatin accessibility) of leukemic blasts does not follow the normal hematopoietic trajectory.

The DNA methyltransferase, DNMT3A, is a critical epigenetic modifier and tumor suppressor. Somatic mutations in *DNMT3A* are frequent in hematologic malignancies including AML, with *DNMT3A R878H* being the most common mutation. Dai et al. showed that by using a *Dnmt3a R878H* conditional knockin approach, mice developed AML enriched in hematopoietic stem/progenitor Lin[−]Sca1⁺cKit⁺ (LSK) cells.²² To obtain insight

into the biological signature of leukemic progenitors, gene expression profiles of LSKs between *Dnmt3a*^{WT/WT} and *Dnmt3a*^{WT/R878H} were compared using single-cell RNA sequencing. The expression of genes involved in cell cycle regulation and transition was more heterogeneous in *Dnmt3a*^{WT/R878H} mice than that in *Dnmt3a*^{WT/WT}. Moreover, genes involved in leukemogenesis such as *Meis1*, *Hlf*, *Mpl*, *Hoxa*, *Hoxa10*, and *Gata3* were upregulated in LSKs *Dnmt3a*^{WT/R878H} mice. *Dnmt3a* R878H mutation caused accumulation of leukemic cells in G2/M phase through upregulation of CDK1 due to activation of mTOR.

Computational strategies to analyze single-cell transcriptional data.

Single-cell RNA sequencing (sc RNA-seq) is a powerful method to investigate cell-to-cell transcriptomic variation. Through the combination of high-throughput sequencing and bioinformatic tools, sc RNA-seq can distinguish between cell subsets, identify new cell types and provide insights into dynamic cellular processes. Sc-RNA seq is being increasingly used in the areas of cancer research,⁴⁹ embryology,⁹ and developmental biology.¹² Although sc RNA-seq provides an unprecedented opportunity to study global gene expression at the single-cell resolution, there are technical hurdles that need to be resolved. Compared to bulk RNA-seq, sc RNA-seq data are affected by higher noise levels. Amplification of the tiny amount of RNA in a single cell to a quantity suitable for sequencing may lead to amplification biases or dropout events. Bimodal gene expression distribution is a characteristic of sc RNA-seq data in which expression is either strongly non-zero or undetectable. Another problem is analyzing the enormous amount of data that result from sc RNA-seq. Bulk-based normalization methods are widely applied to sc RNA-seq data sets. However, a variety of methods have been recently proposed to analyze differential gene expression from sc RNA-seq data. These overcome the limitations of bulk-based analysis methods by modeling the probability of dropout events, considering the multimodal nature of sc RNA-seq or including a model of transcriptional bursts seen in single-cell transcriptional data.¹⁰

Among the most popular sc RNA-seq analysis methods, Model-based Analysis of Single-Cell Transcriptomics (MAST)²⁵ employs a generalized linear hurdle model to simultaneously account for stochastic dropouts and characteristic bimodal expression distributions. The rate of expression and the level of expression are modeled for each gene, indicating whether the gene is expressed in a cell. The fraction of genes that are expressed and detectable in each cell, called cellular detection rate, is modeled as a covariate. To improve the inference of genes with sparse expression, the model parameters are fitted using an empirical Bayesian framework. Differential gene expression is determined using the likelihood ratio test. Application of MAST to sc RNA-seq data of primary human unstimulated and cytokine activated mucosal-associated invariant T (MAIT) cells, MAST identified novel expression signatures of activation and highlighted a population of MAIT cells showing partial activation but no induction of effector function.

Sc RNA-seq can be employed to study the transcriptional dynamics of a temporal process such as cell differentiation during which a high degree of variability in gene expression is observed between individual cells. Trapnell et al. developed Monocle, an unsupervised algorithm that increases the temporal resolution of transcriptome dynamics using single-cell

RNA-seq data collected at multiple time points.⁵⁰ Monocle orders single-cell expression profiles in pseudotime, a quantitative measure of progress through a biological process. Monocle constructs a minimum spanning tree on cells and finds the longest sequence of transcriptionally similar cells to produce a trajectory of an individual cell's progress through differentiation. As cells differentiate, they may diverge into separate paths. Monocle can reconstruct branched biological processes in which a precursor cell gives rise to multiple lineages, therefore ordering cells by their progress through differentiation. Application of monocle to sc RNA-seq data from differentiating primary human myoblasts organized them into a 2-phase trajectory and isolated a branch of nondifferentiating cells. It revealed switch-like changes in the expression of key regulatory factors, sequential waves of gene regulation and the expression of regulators that were previously unknown to act in differentiation.

Setty et al. presented Wishbone, an algorithm for positioning single cells along bifurcating developmental trajectories with high resolution.²⁶ Using multi-dimensional single-cell data such as mass cytometry or RNA-seq data as input, Wishbone orders cells according to their developmental progression. Using 30-channel mass cytometry data measuring T-cell development in mouse thymus, Wishbone recovered the known stages in T-cell development with high accuracy and developmental resolution, including the bifurcation point. In addition, Wishbone identified maturation and branch points in myeloid development when applied to human myeloid differentiation data generated by mass cytometry and mouse myeloid differentiation data generated using sc RNA-seq. Thus, Wishbone can be broadly used in systems with bifurcating trajectories across diverse single-cell technologies.

These tailored sc RNA-seq analysis methods outperform bulk strategies and have the potential to aid in the development of more precise targeted therapies.

Single-cell genotyping to study clonal diversity in AML.

AML is known to harbor subclonal populations.⁵¹ Selection for resistant clones within a neoplasm could lead to relapse and therapeutic failure in cancer.^{52,53} Clonal genetic diversity has been shown to predict progression in AML.^{4,10,54} Studies tracking clonal composition from diagnosis to relapse mostly rely on next generation sequencing (NGS) of bulk tumor samples. Such methods predict the population frequency of individual clones, their genetic composition and evolutionary relationships. Hughes et al. performed single-cell sequencing on individual cells from 3 subjects initially diagnosed with myelodysplastic syndrome, which progressed to AML, each of whom had been previously characterized by whole-genome sequencing of bulk tumor samples.²¹ Although single-cell genotyping strongly supported the clonal architecture revealed by analysis of bulk samples, several modifications were suggested. It resolved the clonal assignment of single nucleotide variants that had been initially ambiguous. Although a vast majority of single nucleotide variants predicted to co-occur in a clonal population were present in at least 1 cell (supporting linear evolution), a small set of variants was identified to be mutually exclusive across multiple cells in each subject, suggesting subclonality. Thus, single-cell genotyping identified areas of clonal complexity previously unappreciated by NGS. By genotyping a large number of cells (n = 95) for 10 clonal markers, Klco et al. showed that single-cell genotyping supports tumor clonality predicted from bulk AML samples.⁵⁵ To test the accuracy of current models

of clonal diversity in AML, Paguirigan et al. developed protocols to directly genotype single AML cells for two common mutations, *FLT3-ITD*, and *NPM1*.⁵⁶ Single-cell genotyping detects co-occurring mutations, thereby revealing the sequence of acquisition of mutations during clonal evolution. The analysis demonstrated that mutations of *FLT3* and *NPM1* occur in both homozygous and heterozygous states in AML samples, and these mutations may arise through independent clonal events, leading to convergent evolution.

As most bone marrow cells are short lived, it is difficult for multiple leukemogenic mutations to accumulate in a single clonal lineage. Jan et al proposed that serial mutations occur in self-renewing HSCs.³ To investigate this model, genomic analysis was performed on the HSCs of 6 AML patients with *FLT3-ITD*. Using exome sequencing, mutations in 57 genes were identified in these samples including *FLT3*, *NPM1*, *TET2*, and *SMC1A*, which frequently occur in AML. Residual HSCs were isolated from the samples by fluorescence-activated cell sorting. Although the residual HSCs were functionally normal, several mutations recurrently found in AML (such as *FLT3*, *NPM1*, and *SMC1A*) were detected in these cells, identifying them as preleukemic HSCs. Single-cell genotyping identified sequential acquisition of mutations in these preleukemic HSCs, suggesting the clonal evolution of AML genomes from founder mutations. Therapeutic targeting of such preleukemic HSCs may lead to more durable remissions in AML.

CBL encodes a member of the Cbl family of proteins, which functions as an E3 ubiquitin ligase. Recently, the development of an inv(16) AML in an adult with CBL syndrome caused by germline *CBL* mutation was described.⁵⁷ Together with the acquisition of inv(16), progression to AML was accompanied by mutations in 12 genes (eg, *CAND1*, *NID2*, *ARF3*, *PTPRT*, and *DOCK6*). However, which of these mutations were essential for the development of AML remained unclear. Niemöller et al. further characterized the clonal composition and evolution of AML based on mutations in *CAND1*, *PTPRT*, and *DOCK6*⁵⁸ because these mutations had been previously reported to have deleterious effects on cells.^{59–61} Single-cell genotyping confirmed the co-occurrence of *CAND1*, *PTPRT*, and *DOCK6* mutations in the same AML clone and revealed a clonal hierarchy, as *PTPRT* mutation was likely acquired after *CAND1* and *DOCK6* mutations. *PTPRT*, which encodes a STAT3-inhibiting protein tyrosine phosphatase, contributed to AML development at a later stage by stimulating proliferation.

Internal tandem duplication (ITD) of the *FLT3* gene occurs in upto one-third of patients with AML and is associated with more aggressive disease and failure of standard treatment. Quizartinib, a highly selective tyrosine kinase inhibitor of *FLT3-ITD* has demonstrated a composite complete remission rate of approximately 50% in relapsed or refractory AML patients with *FLT3-ITD* mutations.⁶ However, responses to quizartinib are largely short-lived, and relapses have been exclusively associated with acquired resistance mutations in the *FLT3-ITD* kinase domain.⁷ Smith et al. used targeted *FLT3* sequencing of single cells and colonies from patient samples to demonstrate tremendous clonal diversity in the majority of *FLT3-ITD* AML patients with acquired resistance to quizartinib.¹¹ Previously, substitutions at *FLT3-ITD D835* residue have been reported to confer resistance to quizartinib.⁷ Single-cell genotyping of *FLT3* for both *ITD* and *D835* mutations identified 8–

18 subpopulations per patient. Such high degree of mutational heterogeneity within *FLT3* suggests that clinical resistance to quizartinib is more complex than previously appreciated.

The aforementioned studies highlight the effectiveness of single-cell approaches in identifying preleukemic driver mutations, understanding clonal composition and tracking clonal evolution in AML.

Single-cell approaches to investigate molecular mechanisms of drug response and cancer-associated pathways in AML.

Therapeutic response.—Single-cell network profiling (SCNP) uses multiparametric flow cytometry to simultaneously measure changes in extracellular surface markers and intracellular signaling proteins on exposure of cells to extracellular modulators (eg., stimulators, inhibitors).^{43,62–64} SCNP facilitates interrogation of a wide array of biological phenomena by measuring signaling networks in millions of cells at the single-cell level. Rosen et al. used SCNP to characterize biological pathways associated with in vitro resistance or sensitivity to chemotherapeutics commonly used in AML (ie, cytarabine/daunorubicin [Ara-C/Dauno], gemtuzumab ozogamicin [GO], decitabine [DEC], azacitidine [AZA], clofarabine).⁶⁵ Simultaneous measurement of DNA damage, apoptosis, and signaling pathway responses in AML blasts treated with the chemotherapeutic drugs showed the distinct profile of each sample. The apoptotic responses of AML samples to Ara-C/Dauno and GO showed a high degree of correlation, suggesting cross-resistance/sensitivity between the 2 regimens. Resistance of cells to Ara-C/Dauno was found to be associated with high levels of intracellular p-Erk, p-Akt, and p-S6. Treatment of Ara-C/Dauno resistant cells with the epigenetic modulators DEC and AZA identified cells sensitive or resistant to either or both agents, highlighting mechanistic differences between these 2 agents. Moreover, differential DNA damage and apoptotic response was observed in AML samples when treated with the drugs. Treatment with the genotoxins Ara-C/Dauno, GO, and clofarabine induced DNA damage (pH2AX) before apoptosis, whereas the epigenetic modulators DEC and AZA caused simultaneous induction of DNA damage and apoptosis. This study provided an insight into biological pathways associated with Ara-C/Dauno resistance in AML and illustrated the potential of SCNP to assess functional heterogeneity between clinical samples. Kornblau et al. applied SCNP to predict the likelihood of response to induction chemotherapy in 2 cohorts of AML (n = 34 and 88) at diagnosis.⁶⁶ The study revealed that for patients aged less than 60 years, complete remission was associated with the presence of intact caspase-dependent apoptotic pathways. In patients aged more than 60 years, resistance was associated with FLT3 ligand-mediated increase in p-Akt and p-Erk. The results were independent of cytogenetics, *FLT3* mutational status, and diagnosis of secondary AML. This study emphasized the significance of SCNP as a basis for the development of tests highly predictive for response to induction chemotherapy. Cesano et al. developed a classifier for predicting response of elderly patients to induction chemotherapy based on SCNP analysis of AML patients aged greater than 55 years.⁵ The classifier which used cell survival, differentiation and apoptotic signaling pathways as inputs provided prognostic information for treatment planning in elderly AML.

Cellular heterogeneity in AML leads to differential drug response. Variations in the metabolic activity of cells could be one of the several factors contributing to such heterogeneity. Measurement of enzyme activity in drug-treated cells is beyond the scope of established single-cell analysis methods such as flow cytometry. Chemical cytometry provides a direct, quantitative readout of cell contents wherein individual cells are lysed and the released intracellular molecules are separated electrophoretically and detected by fluorescence or a similar sensitive method.⁶⁷ Kovarik et al. used a robust microfluidic platform to explore the heterogeneity of enzyme activity in single cells treated with Tosedostat,²⁰ an aminopeptidase inhibitor currently in clinical trial for AML. Chemical cytometry was used to measure degradation of a fragment of cyclin-dependent kinase 4 (cdk4) in the U937 AML cell line in the presence and absence of Tosedostat. The analysis of 99 untreated cells showed rapid and consistent degradation of the peptide reporter within 20 minutes of loading while drug-treated cells showed inhibited but ongoing degradation of the reporter. Monitoring this time-dependent peptide degradation in 498 individual cells demonstrated substantial cellular heterogeneity in peptide processing in response to drug treatment. Single-cell studies can, therefore, shed light on differential drug response and chemoresistance in AML.

Multidrug resistance.—The first line of treatment in AML is anthracycline-based combination chemotherapy. A major limitation to the success of combination chemotherapy is the resistance of leukemia cells to a spectrum of anti-cancer drugs that are structurally and mechanistically unrelated, a phenomenon known as multidrug resistance. A well-established cause of MDR is the increased efflux of cytotoxic drugs from cells mediated by ATP binding cassette (ABC) transporters or MDR proteins.⁶⁸ Till date, minimal information is available on the differences in MDR activity at the single-cell level. Khamenehfar et al. used a dielectrophoretic microfluidic chip-based assay for determination of drug accumulation in single cells of primary AML samples.⁶⁹ By measuring the accumulation of daunorubicin in AML cells in the presence or absence of MDR inhibitors, the dielectrophoretic chip successfully categorized leukemic blasts as MDR-positive and MDR-negative as well as distinguished them from benign white blood cells. Samples from patients who went into complete remission after combination chemotherapy were enriched for MDR-negative leukemic blasts, whereas MDR-positive blasts were predominant in patients who failed to achieve complete remission. However, pronounced heterogeneity in MDR was observed within leukemic blasts, with MDR-positive cells detected in complete remission samples. Comparison of MDR activity in samples taken at pretherapy and during subsequent relapse from 2 patients who went on to complete remission, demonstrated the presence of MDR-positive cells in both pretherapy and relapse samples, consistent with MDR as a mechanism of AML relapse. Such studies uncovering drug resistance heterogeneity may provide an improved understanding of chemoresistance in AML.

DNA damage response.—Impaired cellular capacity to repair DNA damage has significant clinical implications in the predisposition to cancer and response of cancer cells to cytotoxic therapies.⁷⁰ The 2 major mechanisms of DNA double strand break repair are homologous recombination repair (HRR) and nonhomologous end joining (NHEJ).⁷¹ HRR predominates in replicating cells while NHEJ is active in resting cells. Rosen et al. used

flow-cytometry-based SCNP to measure drug-induced activation of DNA damage response (DDR) in cell lines with defined HRR pathway mutations (*ATM*^{-/-}, *ATM*^{+/-}, *BRCA1*^{-/-}, *BRCA1*^{+/-}) and in primary AML samples.⁷² After treating cells with genotoxins having different mechanisms of action, both HRR and NHEJ were examined by measuring changes in DNA repair proteins. Etoposide was found to induce cell proliferation independent DDR in primary AML and *ATM*^{+/+}, but not *ATM*^{-/-} cell lines. Treatment with a PARP inhibitor induced DNA damage in proliferating cells in both primary AML and cell lines and distinguished cell lines deficient (*BRCA1*^{-/-}) or impaired (*BRCA1*^{+/-}) in HRR from *BRCA1*^{+/+} cell lines based on levels of pH2AX induction. This finding is consistent with preclinical data showing that PARP inhibition for cancer treatment is most effective in *BRCA1*-mutated or -deficient cell lines. Application of this assay to primary AML samples identified heterogeneous patterns of DDR activity in them. Although some samples showed low or robust activation of both HRR and NHEJ pathways, others activated NHEJ as the predominant DDR pathway.

Apoptotic response.—MCL1, a member of the BCL2 proto-oncogene family, has been reported to be overexpressed in AML cells compared to normal bone marrow cells.⁷³ Emerging evidence supports that aberrant activation of the receptor tyrosine kinase FLT3 may lead to MCL1 upregulation in AML.^{74,75} Nanochannel electroporation is a method of single-cell-based DNA/RNA delivery with excellent dosage control and minimal cell damage.^{76,77} Gao et al. demonstrated a time lapse effect of MCL1 siRNA on MCL1 mRNA and apoptosis in AML cells at the single-cell level²³ by integration of nanochannel electroporation with single-cell qPCR⁷⁸ and fluorescence resonance energy transfer-based molecular beacons (MBs detection).^{79,80} The effect of MCL1 siRNA on mRNA silencing and apoptosis was investigated in 2 AML cell lines: KG1a with wild-type *FLT3* and MV4–11 with *FLT3-ITD*. It was observed that more MCL1 siRNA was required to induce apoptosis in MV4–11 cells as compared to KG1a. These results were also validated in primary AML samples with wild-type *FLT3* and *FLT3-ITD*, partially explaining the basis for chemoresistance in patients with *FLT3-ITD*.²³ In KG1a cells, it required a single large dose of siRNA or multiple low doses with short-delivery intervals to maintain suppressed levels of MCL1 mRNA in cells and trigger apoptosis. Understanding the mechanisms underlying differential apoptotic resistance in AML can lead to the development of novel therapeutic strategies for improving clinical outcome.

CONCLUSIONS

Despite achieving complete remission after standard chemotherapy, most AML patients die of relapsed refractory disease. Conventional treatment strategies in AML target the bulk tumor population, relying on clinical phenotypes that have been determined by averaging the characteristics of thousands of cells. Understanding the information contained in a single cell is the key to therapeutically target every cell in a tumor. Single-cell analysis is transforming our understanding of the biology of AML (Table II). Single-cell methods provide a unique opportunity to unravel intratumor heterogeneity, identify rare cell populations such as LSCs, and track clonal evolution in AML. Recent reports of single-cell studies in AML have revealed a high degree of heterogeneity in the disease, pertaining to

diverse cellular aspects such as phenotype, genotype, gene expression, signaling, therapeutic response, and apoptotic resistance (Fig 2). A wide range of single-cell techniques are being developed to dissect heterogeneous tumors into well-defined subsets of cells. The choice of the technique depends on the specific biological question being addressed. Despite its various advantages, significant challenges still remain in the analysis, interpretation, and integration of single-cell data. However, it is expected that as single-cell technologies make progress, the experimental procedures and data interpretation will become more standardized. In future, single-cell biology will provide an improved understanding of the molecular mechanisms underlying drug resistance and relapse in AML, guiding the development of targeted therapies. Individualized treatment strategies will prolong patient survival and mark the beginning of a new era of translational research in AML.

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Abbreviations:

AML	acute myeloid leukemia
LSCs	leukemic stem cells
HSCs	hematopoietic stem cells
qPCR	quantitative polymerase chain reaction
FACS	fluorescence-activated cell sorting
NGS	next generation sequencing
SCNP	single-cell network profiling

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Fig 1.

Analysis of mass cytometry data reveals functional heterogeneity in AML. *Mll/AF9-NRAS^{G12V}* murine AML cells^{38,39} stained with a panel of cell surface markers and intracellular signaling intermediates were analyzed by mass cytometry. (A) SPADE plot revealing the immunophenotypic architecture of cells. Each circle on the plot represents a group of cells with similar expression levels of the measured cell surface markers. The size of the circle represents the number of cells in each cluster. The color of the circles is representative of the level of CD11b of each cluster. (B) viSNE plot of murine AML cells retaining single-cell granularity. Each point on the plot represents a cell. Cells that are similar in all the measured dimensions are aligned closely together in the plot. In this plot, the color represents levels of CD11b. Both SPADE and viSNE plots can be colored according to each of the measured epitopes. CD11b^{Low} cells are indicated by a circle on the viSNE plot. These cells harbor the leukemia stem cell (LSC)-enriched compartment. (C) The color of the viSNE or SPADE plot can be changed to reflect the levels of any of the proteins measured. In this experiment, a panel of signaling intermediates were measured including pErk, pAkt, p4EBP1, pSTAT5, and total levels of Myc and β -catenin. Using CD11b, we gated on the LSC-enriched compartment in panel B. In panel C, the levels of pSTAT5 and Myc are displayed within this CD11b^{Low} population. This data allows us to conclude that CD11b^{Low} cells (enriched for LSCs) are high in Myc and low in pSTAT5.

Fig 2.

A diagrammatic representation summarizing the application of single-cell approaches to address different biological questions in AML.

Table I.

Different approaches for single-cell analysis

Technique	Advantages	Disadvantages	Ref
Flow cytometry	Measures up to 15 parameters/cell, sorting of live cells	Cellular autofluorescence, spectral spillover between fluorophores	16,17
Mass cytometry	Measures up to 40 parameters/cell, no autofluorescence or spectral overlap	Slow acquisition rate, limited commercial availability of metal-tagged antibodies	18,19
Chemical cytometry	Biochemical analysis of single cell contents revealing metabolic status of cells	Capable of analyzing only a small number of cells	20
Single-cell DNA-seq	Allows sequencing of most of the genome; identification of mutations	High amplification bias	21
Single-cell RNA-seq	Detects more than 10,000 transcripts/cell	Dropouts, bimodality of gene expression, lack of standardized methods to normalize data	22
Single-cell q-PCR	High acquisition rate, relative quantification	Measures the expression of up to 96 genes per cell	17,23
3ATAC-seq	Rapid, high-quality measurements, low quantities of starting material	Requires an optimal number of cells for reliable results	24

Table II.

Summary of single-cell studies in AML

Biological problem	Experimental model	Single-cell techniques	Conclusions	References
Phenotypic heterogeneity	H9M mice	Flow cytometry, mass cytometry	LSC activity found in different phenotypic compartments; LSC activity correlates better with global signaling activation status than with cell surface marker profile	Gibbs et al <i>Cell Stem Cell</i> . 2012 ¹⁶
Functional heterogeneity	Primary AML	Mass cytometry, phenograph	Surface phenotype does not reflect the intracellular signaling status of AML blasts; intracellular signaling status is a better predictor of self-renewal capacity	Levine et al <i>Cell</i> . 2015 ¹⁸
Functional heterogeneity	Human myeloid cell lines and <i>FLT3-ITD</i> primary AML	Flow cytometry, mass cytometry	Proliferative rate of LSCs of different AML subgroups correlates with risk stratification while the cycling rate of bulk leukemia cells did not	Han et al <i>Cytometry A</i> . 2015 ¹⁹
Functional heterogeneity	Primary AML	Mass cytometry	LSCs from different subtypes of AML vary in cell-cycle kinetics while the bulk leukemia cells from these samples do not; these variations correlate with prognostic subgroups	Behbehani et al <i>Cancer Discov</i> . 2015 ¹
Transcriptional heterogeneity	<i>MLL-AF9</i> mice	FACS, single-cell qPCR	Transcriptional profiling identified two subtypes of leukemia cells with differential proliferation and differentiation rates and distinct coexpression networks	Saadatpour et al <i>Genome Biol</i> . 2014 ¹⁷
Transcriptional heterogeneity	Primary human blood cells	ATAC-seq	Chromatin accessibility of AML blasts differed from that of normal myeloid progenitors and monocytes; single AML cells have regulome profiles corresponding to disparate hematopoietic developmental stages	Corces et al <i>Nat Genet</i> . 2016 ²⁴
Transcriptional heterogeneity	<i>Dnmt3a R878H</i> mice	Single-cell RNA-sequencing	<i>Dnmt3a</i> ^{R878H} mice developed AML enriched in LSCs; LSCs from <i>Dnmt3a</i> ^{WT/R878H} mice had more cells in G2/M phase and mTOR activated higher expression of CDK1 compared to wild type mice via single cell RNA sequencing.	Dai et al <i>Proc Natl Acad Sci USA</i> . 2017 ²²
Clonal diversity	Primary AML	Single-cell genotyping	Used single-cell sequencing to confirm the clonal evolution and architecture of secondary AML originally detected by bulk methods. Single-cell sequencing was used to resolve finer complexity of the clonal architecture.	Hughes et al <i>Plos Genet</i> . 2014 ²¹
Clonal diversity	Primary AML	Single-cell genotyping	Developed protocol for genotyping single AML cells for common mutations; revealed that clonal populations in AML can be homozygous or heterozygous for mutations of <i>FLT3</i> and <i>NPM1</i> pointing toward convergent evolution	Paguirigan et al <i>Sci Transl Med</i> . 2015 ²²
Clonal diversity	HSCs of primary AML	Single-cell genotyping	Identified preleukemic mutations in HSCs; determined clonal progression of multiple mutations in the HSCs of AML patients	Jan et al <i>Sci Transl Med</i> . 2012 ³
Clonal diversity	Primary AML	Single-cell genotyping	Characterized clonal composition and evolution of inv(16) AML (CBL) based on mutations in <i>PTPR</i> , <i>CAND1</i> , and <i>DOCK6</i> ; determined the co-occurrence of these mutations in the same AML clone; revealed clonal hierarchy as <i>PTPR</i> mutation was acquired after <i>CAND1</i> and <i>DOCK6</i>	Niemoller et al <i>Leuk Res</i> . 2016 ⁵⁴

Biological problem	Experimental model	Single-cell techniques	Conclusions	References
Clonal diversity	<i>FLT3-ITD</i> primary AML	Single-cell genotyping	Clonal heterogeneity underlies AML resistance to FLT3 inhibitor quizartinib; 8–18 subpopulations with mutations in <i>ITD</i> and <i>D835</i> identified in resistant <i>FLT3-ITD</i> AML patients	Smith et al <i>Blood</i> . 2017 ¹¹
Therapeutic response	Human cell lines and primary AML	Flow cytometry, SCNP	Differential response of AML cells to chemotherapeutic drugs; p-Erk, p-Akt associated with chemoresistance	Rosen et al <i>Leuk Res</i> . 2012 ⁶¹
Therapeutic response	Primary AML	Flow cytometry, SCNP	Predicted accuracy of response to induction chemotherapy in AML; in patients <60 y complete remission depends on intact apoptotic pathways; in patients >60 y chemoresistance is associated with FLT3-mediated increase in p-Akt and p-Erk signaling	Kornblau et al <i>Clin Cancer Res</i> . 2010 ⁶²
Therapeutic response	Human AML cell line	Chemical cytometry	Measured heterogeneity of enzyme activity in single cells treated with aminopeptidase inhibitor Tosedostat; detected substantial heterogeneity among individual cells in peptide processing in response to Tosedostat treatment	Kovarik et al <i>Anal Chem</i> . 2013 ²⁰
Multidrug resistance	Primary AML	Dielectrophoretic microfluidic chip	Determined differences in MDR activity at the single-cell level by measuring drug accumulation in the presence of MDR inhibitors; distinguished chemosensitive and resistant samples based on MDR activity	Khamenehfar et al <i>Anal Chem</i> . 2016 ⁶⁶
DNA damage response	Human cell lines and primary AML	Flow cytometry, SCNP	Measured drug induced DNA damage response in cells treated with genotoxins; identified heterogeneous DNA damage response in primary AML	Rosen et al <i>J Transl Med</i> . 2014 ⁶⁹
Apoptotic response	Human cell lines and primary AML	Nanochannel electroporation, single-cell qPCR	Studied differential apoptotic response in AML subtypes through the effect of MCL1 siRNA on mRNA silencing and apoptosis; more MCL1 siRNA was required to induce apoptosis in <i>FLT3-ITD</i> AML compared to AML with wild type <i>FLT3</i>	Gao et al <i>Mol Ther</i> . 2016 ²³