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Perspective

Investigating evolutionary perspective of carcinogenesis with single-cell transcriptome analysis

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Abstract

We developed phase-switch microfluidic devices for molecular profiling of a large number of single cells. Whole genome microarrays and RNA-sequencing are commonly used to determine the expression levels of genes in cell lysates (a physical mix of millions of cells) for inferring gene functions. However, cellular heterogeneity becomes an inherent noise in the measurement of gene expression. The unique molecular characteristics of individual cells, as well as the temporal and quantitative information of gene expression in cells, are lost when averaged among all cells in cell lysates. Our single-cell technology overcomes this limitation and enables us to obtain a large number of single-cell transcriptomes from a population of cells. A collection of single-cell molecular profiles allows us to study carcinogenesis from an evolutionary perspective by treating cancer as a diverse population of cells with abnormal molecular characteristics. Because a cancer cell population contains cells at various stages of development toward drug resistance, clustering similar single-cell molecular profiles could reveal how drug-resistant sub-clones evolve during cancer treatment. Here, we discuss how single-cell transcriptome analysis technology could enable the study of carcinogenesis from an evolutionary perspective and the development of drug-resistance in leukemia. The single-cell transcriptome analysis reported here could have a direct and significant impact on current cancer treatments and future personalized cancer therapies.

Key words Single-cell transcriptome, cancer molecular pathways, leukemia

Our laboratory applies nanotechnology for molecular characterization of individual cells[1-4]. We study the molecular profiles of many individual cells at various developmental stages, in contrast to the traditional population study approach using cell lysates, to investigate gene regulation of cell fate. A collection of molecular profiles is the molecular counterparts of time-lapse images used for describing stepwise developmental events[5]. As fluorescent tags facilitate the study of cell movement and cell development, collections of single-cell molecular profiles will allow predicting the effects of genetic manipulations, as well as applying the theory of evolution to cancer research[4].

Studying gene regulation at the single-cell level is necessary and effective for understanding normal or cancer cell development[2,6,7]. By fluorescently tagging stage-specific proteins, cell biologists can visualize protein movements within a cell to determine their functions and study the developmental stages at the tissue level. However, biomarkers from previous studies must be used in such approaches. In contrast, single-cell transcriptome analysis can overcome the limitation of using empirical approaches that rely on previously published studies and potentially provide a method for studying biological events without known biomarkers[4].

Single-cell Transcriptome Analysis

Whole genome microarrays and RNA-sequencing are commonly used to determine the expression levels of genes and to infer gene functions. However, cellular heterogeneity becomes an inherent noise in the measurement of gene expression[8-10]. Although cell lysates provide a large amount of RNA for analysis, the temporal and quantitative information of gene expression in individual cells are lost when averaged among all cells in a lysate. When you lose your keys, it is best to look for them where you lost them, not where the light is better. Gene-gene interaction occurs inside mammalian cells, and regulatory relationships of genes are best preserved within a single cell. The “lost key” in the investigation of gene regulatory relationships will be found in single-cell transcriptome analysis, not the analysis of cell lysates (where the light is better). Because of the

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heterogeneity and asynchrony among cells, a cell population usually contains cells at various developmental stages. Therefore, we can infer gene regulatory relationships from a collection of single-cell transcriptomes because the temporal and quantitative levels of mRNA are best preserved at the single-cell level. The small amount of RNA within a mammalian cell (approximately 20–40 pg of total RNA[11]) presents a challenge for single-cell transcriptome analysis. Our group has overcome the technical hurdles of single-cell molecular analysis by using microfluidic technology[1-4,12]. Traditional molecular analysis methods are performed on a microliter scale, whereas a typical mammalian cell has a volume of approximately 0.065 pL. Therefore, traditional techniques dilute cellular RNA more than 10^6-fold[12,13]. Using microfluidic devices, we have successfully obtained whole genome transcriptomes from individual cells for microarray analysis. The quality of single-cell data from our platform is similar to that from cell population experiments in which an input of 1 ng cDNA was used[2]. Obtaining single-cell whole transcriptome results in a technologic advancement that leads to a fundamental change in molecular biology and cancer research, and enables us to investigate cancer from an evolutionary perspective.

Intratumor Heterogeneity

Single-cell transcriptomes are of value in two settings: 1) some cells of interest, such as circulating tumor cells (CTCs), are very rare and only obtainable in very small numbers, and 2) when cells are aggregated for analysis, information on the extent or nature of heterogeneity in the cell population being studied is lost, along with the ability to identify and characterize sub-populations, such as stem cells, which may be the most relevant. Similar to a human population, which contains people in various ages, within a cell population, there are cells in various developmental stages because cellular events are occurring continuously and often asynchronously. Recent single-cell DNA sequencing has indicated that a tumor contains cells with various mutations[14-16]. With random sampling, gene expression profiles from various developmental stages can be obtained using our single-cell transcriptome analysis technology. Because cellular events are determined by sequential gene expression, cells at sequential developmental stages are more similar than these from further separated developmental stages. Therefore, organizing single-cell transcriptomes by similarity can reconstruct the stepwise developmental events at the molecular level. In addition, with landscape view of the cell expression profiles, gene-gene interaction can be better studied. One such example is our study on trans-differentiation of human cells into neural progenitors. By examining the entire p53 gene network instead of only the p53 gene itself, we identified regulatory relationships among genes that manipulate the fate of human skin cells[17,18]. The single-cell approach we have developed can be applied to various studies involving heterogeneous cell populations, such as understanding the leukemia development.

Hematopoietic Stem Cells and Leukemia

Hematopoietic stem cells (HSC) are capable of self-renewal and differentiating into different types of blood cells as well as immunocompetent cells. A group of primitive cells exist in hematopoietic tissues from which all blood cells arise[19]. Hematopoietic stem cell transplantation (HSCT) is the clinical application of cellular plasticity to restore hematopoietic and immunologic functions that have been depleted by chemotherapy used for treating leukemia or by various other diseases.

The discovery of developmentally plastic cells for HSCT was initiated after the atomic bomb detonations in the Japanese cities of Hiroshima and Nagasaki. The people exposed to the radiation from these bombs developed severe bone marrow and lymphatic failures. Since then, radiobiological studies on radiation injury treatments have emerged in many countries. In 1951, Brecher and Cronkite at the Brookhaven National Laboratory performed radiation shielding experiments on rats and made an important discovery: normal bone marrow that had not been exposed to radiation could cure severe bone marrow failure caused by a lethal radiation dose[20]. Soon afterwards, in the United States, Lorenz *et al*.[21] reported the first experimental results on bone marrow cell infusions into animals that had received a lethal level of radiation. They found that bone marrow allografts from healthy animal donors could replenish hematopoietic tissues of the recipients, which had been severely damaged, and hence proposed the scientific hypothesis of "transplantation." In 1957, Thomas *et al*.[22] at the University of Washington reported the world's first case of isogenic bone marrow transplantation (BMT) from an identical twin donor to successfully treat leukemia. However, among 200 reported cases of transplantations, over half failed due to immune rejection[23]. Thus, the importance of human leukocyte antigen (HLA) matching was recognized. In 1973, Speck *et al*.[24] reported an aplastic anemia patient who underwent a successful allogeneic BMT from an unrelated donor with matching HLA type. Allogeneic BMTs are BMTs occur between a donor and recipient of the same species but with different genotypes. The highly plastic nature and strong regenerative capacity which enable the regeneration of the blood system also render the hematopoietic system vulnerable to cancer development, such as leukemia.

There are 4 common types of leukemia: 1) chronic lymphocytic leukemia (CLL), which affects lymphoid cells and accounts for more than 15,000 new cases of leukemia each year in the United States; 2) chronic myeloid leukemia (CML), which affects myeloid cells and accounts for nearly 5,000 new cases; 3) acute lymphocytic (lymphoblastic) leukemia (ALL), which affects lymphoid cells, accounts for more than 5,000 new cases and is the most common type of leukemia in young children; and 4) acute myeloid leukemia (AML), which affects myeloid cells and accounts for more than 13,000 new cases (http://www.cancer.gov). Treatment of leukemia has advanced rapidly in the past several decades. Many drugs have been developed to target various types of leukemia, but relapse has been a major challenge in treating leukemia; one example is the treatment of CML.

Chronic Myeloid Leukemia

CML is caused by the abnormal proliferation of myeloid cells in

the bone marrow and the accumulation of these cells in the blood. CML is characterized by a reciprocal translocation of chromosomes 9 and 22, the so-called Philadelphia chromosome (Ph1). This Ph1 translocation generates the BCR-ABL fusion gene encoding a constitutively active tyrosine kinase in cells and transforms them into cancer stem cells. The tyrosine kinase inhibitor (TKI) Imatinib Mesylate (IM; formerly STI571, Gleevec®, Novartis, Basel, Switzerland) successfully inhibits the ATP-binding site of BCRABL1[25]. IM is a competitive inhibitor of the oncogenic BCR-ABL kinase that provides a strong response in most patients and is now a standard treatment of CML[26,27]. The estimated event-free survival rate was 81%, the freedom from progression to accelerated phase or blast crisis (BC) was 92%, and the overall survival rates was 85% when only CML-related deaths were considered and 93% when only deaths prior to stem cell transplantation were considered[28,29]. However, most patients continue to have low levels of residual disease after treatment with IM due to the development of drug-resistant sub-clones[30]. BCR-ABL–positive cancer stem cells have been identified and reported to be resistant to a wide range of pro-apoptotic agents, including tyrosine kinase inhibitors[31]. Second-generation TKIs, nilotinib (formerly AMN107; Tasigna®, Novartis), dasatinib (formerly BMS354825; Sprycel™, Bristol Myers Squibb, NY, USA) and bosutinib (formerly SKI-606, Pfizer, NY, USA), are capable of preventing some of these drug-resistant relapses[32,33]. The challenge of treating CML is the complete elimination of CML stem cells to prevent the development of drug-resistant sub-clones and disease relapse[34]. The development of drug-resistant sub-clones may involve *in vivo* microenvironments and the activation of multiple molecular pathways[35,36].

The key to prevent the development of drug-resistant CML is intervention of the stepwise development of such drug-resistant sub-clones. The single-cell transcriptome method we developed provides a method to obtain single-cell molecular profiles for investigating the clonal development of drug-resistant cells. Relapse is the major contributor to treatment failure of leukemia, and leukemia stem cells are considered the origin of disease relapse. Identifying the abnormal activities of leukemia stem cells from single-cell transcriptomes will aid our understanding of leukemia development and will eventually augment and benefit the understanding, diagnosis, and treatment of leukemia[37].

Concluding Remarks

Cancer development from an evolutionary perspective is the result of genetic mutations developed under suitable environment. One example is the drug-resistant CML. Various molecular pathways have been reported in the development of CML including Hedgehog[36], Wnt, BMI-1, Notch[38], PTEN[39], Alox5[40], and FoxO[41] signaling pathways. The development of treatment-resistant cancer cells could be a process similar to the development of drug-resistant bacteria, which is a stepwise selection process. A combination of different chemotherapeutic drugs may be the solution to prevent the relapse of leukemia. Single-cell molecular analysis could potentially reveal the stepwise development of these sub-clones and facilitate the development of personalized chemotherapeutic combination therapy.

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