Single-Cell RNA Sequencing of Ovarian Cancer: Promises and Challenges

7

Shobhana Talukdar, Zenas Chang, Boris Winterhoff, and Timothy K. Starr

7.1 Introduction

Ovarian cancer (OC) is the most lethal gynecologic malignancy in the developed world, accounting for more than 14,000 estimated new deaths in the United States, 2018 [1]. Despite improvement in overall survival from 37% to 46% [2] in the last three decades with the use of radical surgery and cytotoxic chemotherapy, the mortality rate for patients with advanced ovarian cancer, which is greater than 50% [3], continues to be a growing concern. The inability to cure and halt the progression of this disease has prompted researchers to explore the unique cellular and molecular characteristics of these tumors in an effort to develop tailored treatment for individual patients based on molecular phenotyping of these tumors [4–7]. Over the past several decades, with the use of -omics level technology, we have a much better understanding of the molecular complexity of ovarian cancer, including the systematic analyses of transcriptome, genome, proteome, and epigenome in hundreds of patient samples [8–12]. Based on these -omics level studies, it has been increasingly recognized that ovarian cancer represents a disease that is characterized by cellular heterogeneity caused by multiple molecular and environmental factors such as intratumor evolution, cellular plasticity, and multiple sources of stochastic variability [13]. Profound chromosomal instability, which promotes intratumor heterogeneity often portends poor prognosis [14] and patients harboring such diverse tumor clones have a higher chance of relapse [15]. A key challenge in cancer therapeutics is the detection of rare subpopulations of cells with clonal diversity and genomic instability which can lead to possible drug resistance. High-resolution microarrays and next-generation

S. Talukdar · Z. Chang
Division of Gynecologic Oncology, Department of Obstetrics, Gynecology and Women's Health, University of Minnesota School of Medicine, Minneapolis, MN, USA

B. Winterhoff
Division of Gynecologic Oncology, Department of Obstetrics, Gynecology and Women's Health, University of Minnesota School of Medicine, Minneapolis, MN, USA

Masonic Cancer Center, University of Minnesota, Minneapolis, MN, USA

T. K. Starr (✉)
Division of Gynecologic Oncology, Department of Obstetrics, Gynecology and Women's Health, University of Minnesota School of Medicine, Minneapolis, MN, USA

Masonic Cancer Center, University of Minnesota, Minneapolis, MN, USA

Institute of Health Informatics, University of Minnesota, Minneapolis, MN, USA
e-mail: star0044@umn.edu

H. Schatten (ed.), *Ovarian Cancer: Molecular & Diagnostic Imaging and Treatment Strategies*, Advances in Experimental Medicine and Biology 1330, https://doi.org/10.1007/978-3-030-73359-9_7

sequencing technologies have rapidly increased our understanding of the molecular landscape of epithelial ovarian cancers. However, the vast majority of these studies perform analysis on bulk tissue samples, consisting of thousands to millions of cells, and the resulting data represents an average of all of these cells. Rare cell subpopulations are often undetectable in these analyses. Single-cell sequencing technologies provide means for high-resolution molecular phenotyping of large numbers of individual cancer cells and also cancer-associated stromal and immune cells and enables detailed characterization of intratumor heterogeneity in ovarian cancer patients.

This chapter highlights the contributions of single-cell RNA sequencing technologies in possible clinical management of ovarian cancer we discuss the challenges and future implications.

7.2 Why Consider scRNA-Seq in Ovarian Cancer?

The cancer environment consists of an amalgamation of cell types that coordinately result in growth and metastasis leading to morbidity in the patient. Cancer cells evolve and the cancer environment causes changes in supporting stroma, vascular machinery, and recruitment of various immune cell types. Each cell in this environment is bestowed with its unique genome, transcriptome, epigenome, and proteome. Even genetically identical cells will undergo random fluctuations in the mechanisms driving and regulating transcription and translation leading to stochastic gene expression [16, 17]. This underlying heterogeneity is evident in ovarian cancer and single-cell sequencing analysis now makes it possible to study each cell individually at the transcriptome level.

Previous attempts to analyze the molecular complexity of the tumor environment were based on bulk RNA sequencing (RNA-Seq) methods. This technique measures aggregate molecular abundance of RNA levels across several thousands of cells contained in a cancer biopsy. Lost is the information about what types of cells exist in the tumor, what genetic structural diversity exists, and what functional behavior (e.g., gene expression or protein abundance/function) they exhibit at the individual cellular level, and what interactions they have with the host (e.g., with the host immune system). Some of these limitations can now be overcome using single-cell RNA sequencing (scRNA-Seq), which performs transcriptome-wide RNA analysis on several thousand individual cells present within the cancer microenvironment, with the ability to individually interrogate rare cell types from a single tumor sample. ScRNA-Seq has emerged as a powerful tool to dissect tumor tissues at the single-cell level into various cell types and/or cell states (Fig. 7.1) enabling a cell-by-cell molecular characterization of thousands of cells within a tumor specimen thus capable of providing new insights into the mechanism of platinum resistance, with the potential to discover new therapeutic targets.

7.3 Evolution of RNA Sequencing Techniques

The history of single-cell experiments can be traced back to the late 1900s when the pioneering experiments on cell staining techniques and cytological methods allowed scientists to directly visualize genetic differences on chromosomes in single cells. However, these cytogenetic and immunostaining methods were limited to measuring targeted genes and proteins and gross structural anomalies. Starting in the 1980s, quantitative microarray technologies were developed for measuring genome-wide DNA and RNA information, although these methods suffered from certain inherent drawbacks of requiring too much input material for single-cell analysis. At the same time, PCR technologies were capable of amplifying small targeted regions of the genome using small sample sizes. In an effort to overcome these limitations, whole-transcriptome amplification (WTA) [18] and whole-genome-amplification (WGA) [19, 20] methods were developed to amplify genome-wide DNA and RNA which was an important milestone in the

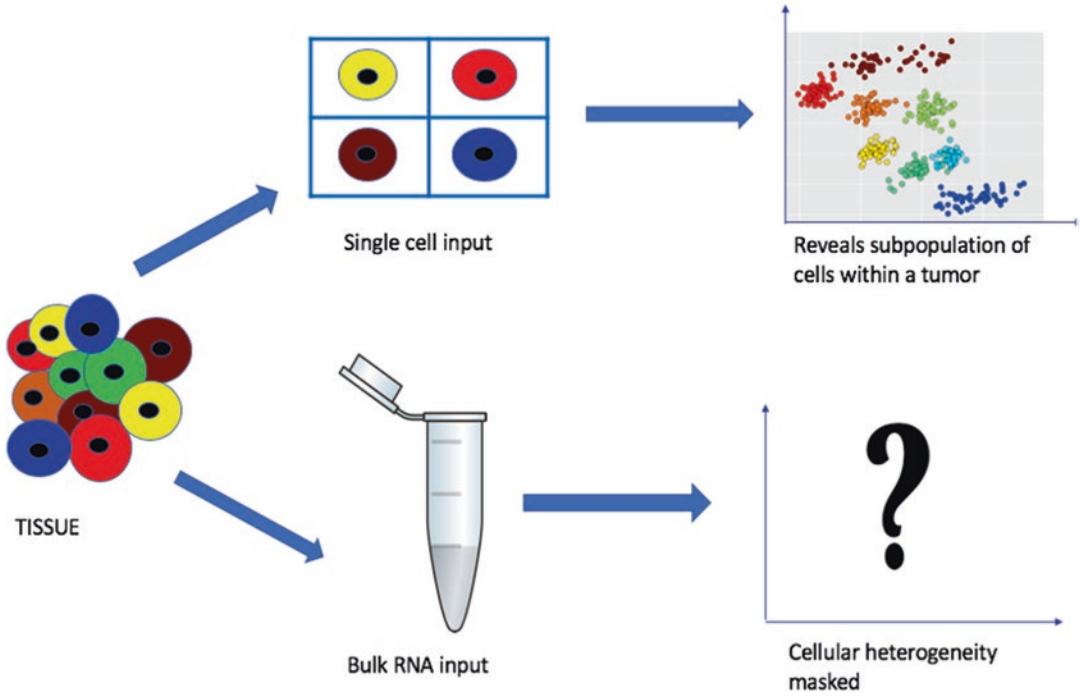

Fig. 7.1 Bulk RNA versus scRNA-seq genomic analysis: scRNA-seq provides the expression profile of individual cells whereas conventional bulk sequencing can provide only the average expression signal for an ensemble of cells, while masking the biologic heterogeneity

field of genomics. The next significant milestone occurred in 2005 with the development of the first next-generation sequencing (NGS) technologies, which enabled genome-wide sequencing of DNA and RNA [21]. It was these pioneering studies that opened the gateway to the new field of single-cell genomics (Fig. 7.2). The first transcriptomes generated via single-cell RNA sequencing (scRNA-seq) were published in 2009, when Tang et al. reported an analysis of the mouse blastomere transcriptome at a single-cell resolution [22]. Two years later, Navin et al. [23] generated the first single-cell genomes from breast cancer samples. Since then, a plethora of scRNA-seq technologies has been developed, providing an unbiased measurement of expression profiles at a single-cell resolution.

7.4 Basic Framework of Single-Cell Isolation Protocols

Each scRNA-seq protocol involves several fundamental steps: (1) sample acquisition from patient; (2) creation of single-cell suspension; (3) cell lysis, mRNA capture, and reverse transcription into cDNA; (4) cDNA amplification by PCR; (5) sequencing of PCR amplicons; and (6) bioinformatic analyses of the sequence data. Capturing single cells may seem trivial but capturing these cells quickly and accurately with high efficiency is one of the main challenges of single-cell sequencing.

Several different protocols have been developed to perform these required steps. Current technologies generally require that the cells be alive immediately preceding step (3), which means that the time required for sample acquisition and creation of the single-cell suspension should be minimized to avoid cell death. Samples are normally acquired from freshly obtained

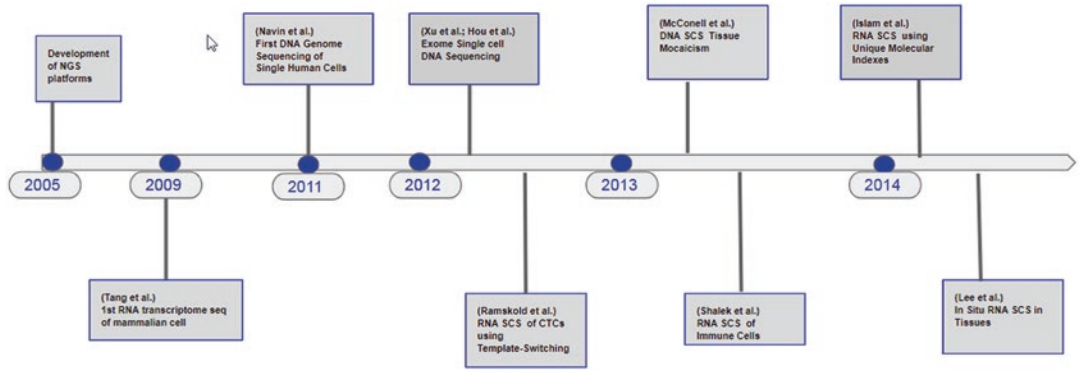

Fig. 7.2 Milestones in single cell sequencing research

biopsies or waste tissue from debulking surgery after clinicopathologic examination. It is helpful to have a trained pathologist identify sections of the tumor that are free from necrosis. The sample to be profiled is dissociated by enzymatic digestion and suspended in a buffer using numerous protocols. Cell dissociation can be very challenging in some cases, as enzymatic treatment of a tissue with trypsin or collagenase may impact cell viability and incubation time required will affect transcriptional activity, potentially adding confounding variables to gene expression. An alternative approach is to perform sequencing on single nuclei, instead of single whole cells, which can reduce artifacts introduced by single-cell dissociation [24].

Isolation of individual cells is the most critical part of the entire process as it is a primary determinant of the throughput of the method. This technique uses several different protocols in order to profile enough cells to capture infrequent or rare cells. Initial methods used manual pipetting of cells into wells, Flow Activated Cell Sorting (FACs) to isolate single cells into plates or microfluidic chips (e.g., Fluidigm C1) to capture single cells in nanoliter chambers which used to be laborious and were prone to error [25–27]. More recent technologies such as droplet-based microfluidics and nanowell-based technologies to randomly capture single cells into isolated nanoliter compartments (droplets or nanowells) are more powerful increasing throughput to tens of thousands of cells thus reducing manual labor to a significant extent [28, 29].

Once cells are isolated, they are lysed to capture as many RNA molecules as possible. A single-cell can only supply very limited starting material (about 0.1 pg of mRNA in each cell), so amplification methods are needed to produce high fidelity, high coverage, and reliable data [18]. In order to specifically analyze polyadenylated mRNA molecules, and to avoid capturing ribosomal RNAs, poly[T]-primers are commonly used. Analysis of non-polyadenylated mRNAs is typically more challenging and requires specialized protocols [30, 31]. Next, poly [T]-primed mRNA is converted to complementary DNA (cDNA) by reverse transcriptase. The poly[T]-primers usually contain one or more barcodes (short unique sequences of DNA), which serve as molecular tags for the amplified mRNA, and can be used to assign sequences to individual cells and individual transcripts. For example, droplet-based sequencing uses a bead coated with millions of poly[T]-primers. All of the poly[T]-primers attached to a single bead will have a cell barcode that is the same on all primers, and then a second barcode called a Unique Molecular Identifier (UMI) which is unique to each primer on the bead. The first barcode is used to assign the sequence to the cell and the UMI barcode is used to assign the sequence to a specific mRNA transcript. This technique allows for digital molecule quantification. Incorporation of UMI also reduces amplification bias such as overrepresentation or underrepresentation of certain regions of cDNA [32]. The minute amounts of cDNA are then amplified either by

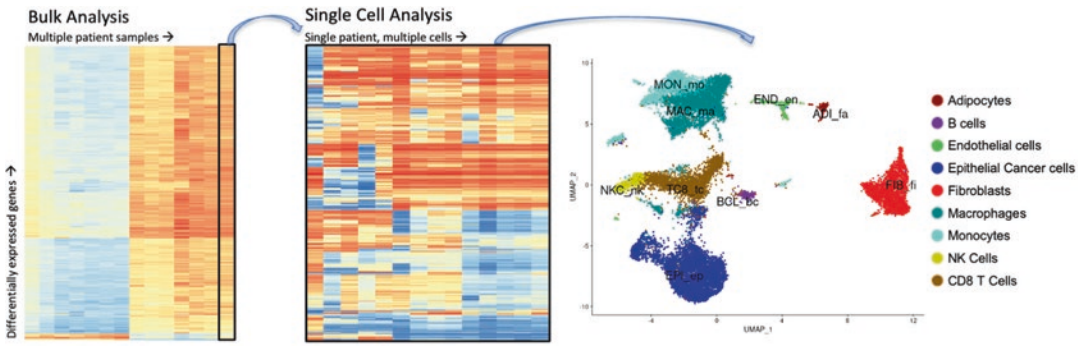

Fig. 7.3 Real-life single cell analyses data from a viable omental metastases obtained during primary surgery from an ovarian cancer patient treated at the University of Minnesota. The detailed information about the number, gene function, pathway function, of individual cells and cell types, as well as their interactions is preserved in scRNA-seq, compared to the bulk sample

PCR or, in some instances, by in vitro transcription followed by another round of reverse transcription. After barcode tagging and PCR amplification, the PCR amplicons are sequenced using Next-Generation Sequencing (NGS) platforms. Single-cell transcriptome data are then analyzed using statistical techniques for clustering cells and identifying cell types, cell states, and signaling pathways (Fig. 7.3).

7.4.1 Single-Cell RNA Sequencing Data Analysis

Numerous specialized software packages are available for single-cell data analysis: this data analysis consists of several tasks which can be completed using various bioinformatic approaches [33–37]. There has been an explosion of published methods for analyzing scRNA-Seq data. The software development website, Github, hosts a useful database of software tools developed for scRNA-seq (https://www.scrna-tools.org). The basic analyses tasks can be grouped into four broad phases: data acquisition, data cleaning, cell assignment, and gene identification. In Phase 1 (data acquisition) raw nucleotide sequencing reads are de-multiplexed and mapped to the transcriptome. Quantification based on read count or UMI is then used to calculate gene expression of each gene in each cell. The data is then "cleaned" (Phase 2), which involves removal of low-quality cells and uninformative genes. The data can also be scaled, normalized and missing values imputed during this phase. Phase 3 assigns cells, either in a discrete manner to known (classification) or unknown (clustering) groups or along a continuous trajectory from one cell type to another using selected genes with a high variation of expression in the dataset. There are many methods proposed for this phase and there is currently no consensus on the best method to use. In general, clustering can be based on principle components, imputation, and/or nonnegative matrix factorization. Machine learning techniques have also been incorporated. In Phase 4, informative genes (e.g., differentially expressed, markers, specific patterns of expression) are then identified to explain these groups or trajectories. Despite the availability of numerous tools, there is little agreement on which method is the best one to use for single-cell analysis [38].

7.5 Applications of Single-Cell RNA Sequencing in Ovarian Cancer

Single-cell RNA sequencing technology has been used in various cancer types to address a range of general and specific research questions related to etiology, evolution, and treatment. Most notable work using this technology has been carried out

in the field of melanoma, breast, and brain cancer [39–41]. Its applications in ovarian cancer are now emerging and will likely soon expand our knowledge in the understanding of the disease in the following key areas:

7.5.1 Characterizing the Heterogeneity of a Tumor Population and Identifying Cell Types

Intratumor heterogeneity poses a major challenge to cancer diagnosis and treatment and has been identified as a major cause of treatment failure and drug resistance in ovarian cancer [17]. Tumors evolve from normal cells. During this process, the cancer cells accumulate genomic and epigenomic alterations and evolve to form distinct lineages and subpopulations. This heterogeneity can be seen as variability between tumors across patients, wherein different stages, genetic lesions, or expression programs are associated with distinct outcomes or therapeutic responses [42–44]. In addition, cells from the same tumor are also very diverse, displaying different mutations or distinct phenotypic or epigenetic states [23, 45–47]. This heterogeneity is especially important to study as we recognize the significance of individual or subsets of clones to treatment resistance and tumor recurrence. Bulk sequencing of such tumor samples has inherent challenges, as small subpopulations of tumor cells are masked when analyzed alongside other low and high RNA expressing cells within a bulk tumor sample. Single-cell sequencing of tumor cells has been able to successfully address this issue by identifying subpopulations of cancer cells within a single patient thus facilitating the ability to characterize intratumor heterogeneity.

7.5.1.1 Identification of Cell Subtypes and Molecular Subtypes

We performed scRNA-Seq on 66 individual cells isolated from a tumor specimen obtained from a patient with high-grade serous ovarian cancer during primary debulking surgery [48]. Before sequencing, cells were sorted by FACS to remove cells with major immune markers including CD45 and CD3. After removing the immune cells, we performed scRNA-seq on the remainder using the Fluidigm C1 platform, which uses a microfluidics system to separate cells into 96 single cell wells. We were able to use the single-cell gene expression data generated in this experiment to classify cells as epithelial or stromal cells. Importantly, within each subgroup, we were able to identify individual cells with unique gene expression patterns that differed from the other cells. For example, we were able to identify a few cells that expressed genes associated with ovarian cancer stem cells.

Many groups have attempted to classify ovarian cancer patients based on bulk RNA-seq gene expression patterns [8, 11, 12, 48, 49]. Four consensus molecular subtypes have been defined based on this work. The subtypes, labeled Mesenchymal, Differentiated, Immunoreactive, and Proliferative, are defined by differential expression of a core group of ~1000 genes. Interestingly, when we used this set of genes to classify each single cell, we found that all four molecular subtypes were present within the patient's tumor sample. This finding indicates that molecular subtyping may need to be done at the single-cell level to have more reliable prognostic and predictive value.

7.5.1.2 Studying Immune Microenvironment of a Tumor

It has been increasingly recognized that ovarian cancer is an immunogenic tumor. Several immune cell types have been identified as key players which are associated positively or negatively with antitumor activity. The accumulation of tumor-infiltrating lymphocytes (TILs) in ovarian tumor is associated with antitumor activity, while several other immune cell types are correlated with evasion of immune surveillance and promotion of tumor growth, evasion, and metastases. These pro-tumor cells include tumor-associated macrophages (TAMs), regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and Tumor-associated dendritic cells (tDCs) [50]. Studying the make-up of the immune cell population within ovarian tumor using scRNA-seq will

be instrumental in finding more promising immunotherapeutic targets for cancers.

7.5.1.3 Evolution of Therapy Resistance

Although ovarian cancers show initial response to chemotherapy or targeted therapies, the majority eventually develop resistance which is a major clinical obstacle in the treatment of these patients. The precise mechanism by which tumors evolve into chemoresistance clones remains is difficult to ascertain in each individual patient. It is unclear whether resistant clones are rare subpopulations that are preexisting in the tumor mass and gradually evolved after therapy (adaptive resistance) or therapeutic agents directly cause new mutations that confer a resistant phenotype within a tumor (acquired resistance). Studies in other human cancers have shown that intratumor heterogeneity itself might play an important role in resistance evolution [51]. The role of epithelial-to-mesenchymal transition (EMT) and cell plasticity in conferring a resistance phenotype has also been proposed and recent studies have shown that tumor cells may switch to a mesenchymal phenotype in response to chemotherapy [52]. ScRNA-seq in ovarian cancer holds great potential for improving our understanding of resistance evolution in response to therapy.

7.5.2 Identification of Ovarian Cancer Stem Cells/Ovarian Cancer Stem like Cells

There is strong evidence that ovarian cancer is driven and sustained by cancer stem cells (CSC) [53, 54]. These cells frequently exhibit a slow cycling rate making them inherently resistant to standard chemotherapy [55–57] that, by definition, targets actively proliferating cells. Thus, the high frequency of relapse despite optimal cytoreduction and adjuvant chemotherapy in ovarian cancer might be accounted for by a subpopulation of quiescent CSC that survive treatment. These cells then become active in a later phase, playing a crucial role in cancer chemoresistance, metastasis, and tumor recurrence. Although CSCs constitute a few percent of the tumor mass, identifying them will be crucial for improving current cure rates of less than 50% for advanced stage patients. Studies have investigated these CSCs in ovarian cancer however molecular markers that can reliably identify these cells are not well-defined [53, 54, 58]. We analyzed the expression of cancer stem cell markers in our research and identified many cell subtypes, predominantly in the stromal subgroup that expressed markers associated with CSCs [48]. Future research in this area will be instrumental in reliably identifying and quantifying the frequency of CSCs in ovarian cancer. Our preliminary scRNA-seq analysis of viable primary ovarian cancer samples demonstrates the presence of small cancer stem like subpopulations (comprising 1–2% of all cells analyzed).

7.5.3 Circulating Tumor Cells and Metastatic Dissemination

Cancer metastasis is a complex biological process accounting for more than 90% of cancer-related deaths [59]. Several models of metastasis have been proposed (late dissemination, early seeding, and self-seeding) but remain unresolved. Our understanding of the lineage of metastatic cells is limited. The metastatic cascade is a multistep phenomenon that involves invasion into the bloodstream by cancer cells, their survival in the blood as circulating tumor cells (CTCs), followed by extravasation and seeding of metastatic CTCs into distant sites [59]. Ovarian cancer metastasis can also occur by disseminating tumor cells (DTCs) through the peritoneal cavity, independent of the vascular system, followed by attachment and seeding within the peritoneal cavity. In general, it is thought that the majority of CTCs and DTCs die in the bloodstream or upon arrival at a distant site, due to high shear forces and anoikis signals in circulation, immune defense, or limited capability to adapt to a foreign microenvironment [60]. It is therefore crucial to identify features that distinguish DTCs and CTCs that are able to survive and initiate metastasis.

ScRNA-seq has been used to investigate metastatic dissemination and CTCs in the blood. One study identified three distinct gene signatures in CTCs associated with metastasis in melanoma patients [61]. The dissemination of single CTCs and CTC clusters was also studied in metastatic breast cancer patients and mouse models [55] in which plakoglobin was identified as a key regulator of CTC clusters; CTC clusters were found to have increased metastatic potential relative to individual CTCs. Another study found that CTCs express their own extracellular matrix proteins in the blood in metastatic pancreatic cancer patients [62]. The use of single-cell RNA-seq technique to investigate cell lineage of metastatic cells or to study the relevance of CTCs in ovarian cancer has yet to be reported.

7.5.4 Studying Tumor-Derived Exosomes

Another emerging use of scRNA-seq is the study of tumor-derived exosomes, with the goal of gaining insight into several aspects of the tumor microenvironment. Exosomes are membrane bound vesicles, 50–100 nm in size that are secreted by tumor and immune cells for short- and long-distance intercellular communication and are also involved in mediating exchange of protein and genetic material between cells [63]. Thus, exosomes are thought to play an important role in mechanisms of therapeutic response and resistance [63, 64]. Recent studies have attempted to study several components of exosomes, including DNA, RNA, miRNA, and proteins [64]. ScRNA-seq can be adapted to single-exosome RNA sequencing and used as a powerful tool for longitudinally monitoring of RNA expression profiles in circulating exosomes to study changes in immune pathway genes during immunotherapy as well as to study differential patterns of expression between responders and nonresponders [63, 65].

7.6 Published and Ongoing Work in Ovarian Cancer Using ScRNA-Seq

There is a paucity of published literature on utilization of scRNA-seq to understand genetic complexity of high-grade ovarian cancer. Dr. Winterhoff et al. [48] published the first study on gene expression patterns in single cells from a patient with high-grade serous ovarian cancer using scRNA-seq technology. Analysis of the RNA expression patterns on 66 evaluable single cells from a primary tumor identified two major subsets of cells: epithelial and stromal cells. The epithelial group was characterized by proliferative genes including genes associated with oxidative phosphorylation and MYC activity, while the stromal group was characterized by increased expression of extracellular matrix (ECM) genes and genes associated with epithelial-to-mesenchymal transition (EMT). Neither of these groups displayed gene expression patterns associated with chemoresistance which was consistent with clinical history of the patient who showed no evidence of disease recurrence 19 months post-surgery. This study provided a first view of the application of single-cell gene expression analysis in ovarian cancer for understanding the etiology, progression, and drug resistance in ovarian cancer.

Later, Shih et al. studied primary and metastatic tumor tissue samples from women with HGSOC using high-throughput single-cell RNA-seq analysis and found that while there was considerable heterogeneity among primary tumor cells from different patients, the expression profiles of metastatic lesions from different patients were remarkably similar, and were distinct from the primary lesions [66]. The group identified 16 distinct cell populations with specific cells correlated to high-grade tumors, low-grade tumors, benign, and one population unique to a patient with a breast cancer relapse. The proportion of these populations changed from primary to metastatic in a shift from mainly epithelial cells to leukocytes with few cancer epithelial cells in the

metastases. Differential gene expression showed myeloid lineage cells were the primary cell group expressing soluble factors in primary samples while fibroblasts did so in metastatic samples.

Izar et al. in their recent work analyzed ascitic fluid samples from high-grade ovarian cancer patients using single-cell RNA-seq technology and explored potential role of JAK/STAT pathway inhibition as a therapeutic option for these women [67].

A European consortium initiated the HERCULES project in 2016. The stated goals are to find solutions to drug resistance in high-grade ovarian cancer via single-cell analysis [68].

7.7 Conclusion

The field of single-cell sequencing is rapidly developing and will undoubtedly play an increasingly important role in the field of precision medicine by shedding light on intratumor heterogeneity, development of treatment resistance, and genetic drivers of metastasis and tumor evolution in ovarian cancer patients. This unprecedented understanding of tumor biology at the single-cell level will be instrumental for developing novel therapies in the future.

Key Points

- Intratumor heterogeneity is an inherent property of ovarian cancer which impacts clinical outcomes.
- Single-cell sequencing technologies enable characterization of intratumor heterogeneity in ovarian cancer.
- To date, single-cell studies on ovarian cancers are scarce, which limits the opportunity to investigate effects on clinical outcomes.
- Larger data sets are needed to establish potential associations between the unique information captured by single-cell sequencing and clinically relevant outcomes in ovarian cancer.

References

1. Siegel, R. L., Miller, K. D., & Jemal, A. (2018). Cancer statistics, 2018. *CA: A Cancer Journal for Clinicians, 68*(1), 7–30.
2. Howlader, N., Noone, A. M., Krapcho, M., Miller, D., Bishop, K., Kosary, C. L., Yu, M., Ruhl, J., Tatalovich, Z., Mariotto, A., Lewis, D. R., Chen, H. S., Feuer, E. J., & Cronin, K. A. (Eds.). *SEER cancer statistics review, 1975–2014*. Bethesda, MD: National Cancer Institute. Retrieved from https://seer.cancer.gov/csr/1975_2014/, based on November 2016 SEER data submission, posted to the SEER web site, April 2017.
3. Ng, J. S., Low, J. J., & Ilancheran, A. (2012). Epithelial ovarian cancer. *Best Practice & Research Clinical Obstetrics & Gynaecology, 26*, 337–345.
4. Garraway, L. A., Verweij, J., & Ballman, K. V. (2013). Precision oncology: An overview. *Journal of Clinical Oncology, 31*(15), 1803–1805.
5. Werner, H. M., Mills, G. B., & Ram, P. T. (2014). Cancer systems biology: A peek into the future of patient care? *Nature Reviews. Clinical Oncology, 11*(3), 167–176.
6. Rubin, E. H., et al. (2014). Developing precision medicine in a global world. *Clinical Cancer Research, 20*(6), 1419–1427.
7. Schwaederle, M., et al. (2015). Impact of precision medicine in diverse cancers: A meta-analysis of phase II clinical trials. *Journal of Clinical Oncology, 33*(32), 3817–3825.
8. Cancer Genome Atlas Research Network. (2011). Integrated genomic analyses of ovarian carcinoma. *Nature, 474*, 609–615.
9. Patch, A. M., Christie, E. L., Etemadmoghadam, D., Garsed, D. W., George, J., Fereday, S., Nones, K., Cowin, P., Alsop, K., Bailey, P. J., et al. (2015). Whole-genome characterization of chemoresistant ovarian cancer. *Nature, 521*, 489–494.
10. Verhaak, R. G., Hoadley, K. A., Purdom, E., Wang, V., Qi, Y., Wilkerson, M. D., Miller, C. R., Ding, L., Golub, T., Mesirov, J. P., et al. (2010). Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell, 17*, 98–110.
11. Verhaak, R., Tamayo, P., Yang, J. Y., Hubbard, D., Zhang, H., Creighton, C. J., Fereday, S., Lawrence, M., Carter, S. L., Mermel, C. H., et al. (2013). Prognostically relevant gene signatures of high-grade serous ovarian carcinoma. *Journal of Clinical Investigation, 123*, 517–525.
12. Tothill, R. W., Tinker, A. V., George, J., et al. (2008). Novel molecular subtypes of serous and endometrioid ovarian cancer linked to clinical outcome. *Clinical Cancer Research, 14*, 5198–5208.
13. Lee, J. Y., Yoon, J. K., Kim, B., Kim, S., Kim, M. A., Lim, H., et al. (2015). Tumor evolution and intratu-

mor heterogeneity of an epithelial ovarian cancer investigated using next-generation sequencing. *BMC Cancer, 15*, 85.
14. Donati, G. (2016). The niche in single-cell technologies. *Immunology and Cell Biology, 94*, 250–255.
15. Van Dijk, E. L., Auger, H., Jaszczyszyn, Y., & Thermes, C. (2014). Ten years of next-generation sequencing technology. *Trends in Genetics, 30*, 418–426.
16. Li, G. W., & Xie, X. S. (2011). Central dogma at the single-molecule level in living cells. *Nature, 475*, 308–315.
17. Raj, A., & van Oudenaarden, A. (2008). Nature, nurture, or chance: Stochastic gene expression and its consequences. *Cell, 135*, 216–226.
18. Van Gelder, R. N., von Zastrow, M. E., Yool, A., Dement, W. C., Barchas, J. D., & Eberwine, J. H. (1990). Amplified RNA synthesized from limited quantities of heterogeneous cDNA. *Proceedings of the National Academy of Sciences of the United States of America, 87*, 1663–1667.
19. Dean, F. B., Hosono, S., Fang, L., Wu, X., Faruqi, A. F., Bray-Ward, P., Sun, Z., Zong, Q., Du, Y., Du, J., et al. (2002). Comprehensive human genome amplification using multiple displacement amplification. *Proceedings of the National Academy of Sciences of the United States of America, 99*, 5261–5266.
20. Telenius, H., Carter, N. P., Bebb, C. E., Nordenskjold, M., Ponder, B. A., & Tunnacliffe, A. (1992). Degenerate oligonucleotide-primed PCR: General amplification of target DNA by a single degenerate primer. *Genomics, 13*, 718–725.
21. Mardis, E. R. (2011). A decade's perspective on DNA sequencing technology. *Nature, 470*, 198–203.
22. Tang, F., Barbacioru, C., Wang, Y., Nordman, E., Lee, C., Xu, N., Wang, X., Bodeau, J., Tuch, B. B., Siddiqui, A., et al. (2009). mRNA-Seq whole-transcriptome analysis of a single cell. *Nature Methods, 6*, 377–382.
23. Navin, N., Kendall, J., Troge, J., Andrews, P., Rodgers, L., McIndoo, J., et al. (2011). Tumour evolution inferred by single-cell sequencing. *Nature, 472*, 90–94.
24. Lake, B. B., Codeluppi, S., Yung, Y. C., Gao, D., Chun, J., Kharchenko, P. V., et al. (2017). A comparative strategy for single-nucleus and single-cell transcriptomes confirms accuracy in predicted cell-type expression from nuclear RNA. *Scientific Reports, 7*, 6031.
25. Gawad, C., Koh, W., & Quake, S. R. (2016). Single-cell genome sequencing: Current state of the science. *Nature Reviews. Genetics, 17*, 175–188.
26. Navin, N. E. (2014). Cancer genomics: One cell at a time. *Genome Biology, 15*(8), 452.
27. Gierahn, T. M., Wadsworth, M. H., et al. (2017). Seq-Well: Portable, low cost RNA sequencing of single cells at high throughput. *Nature Methods, 14*, 395–398.
28. Vitak, S. A., Torkenczy, K. A., Rosenkrantz, J. L., et al. (2017). Sequencing thousands of single-cell genomes with combinatorial indexing. *Nature Methods, 14*, 302–308.
29. Cao, J., Packer, J. S., Ramani, V., et al. (2017). Comprehensive single-cell transcriptional profiling of a multicellular organism. *Science, 357*(6352), 661–667.
30. Sheng, K., Cao, W., Niu, Y., Deng, Q., & Zong, C. (2017). Effective detection of variation in single-cell transcriptomes using MATQ-seq. *Nature Methods, 14*, 267–270.
31. Fan, X., Zhang, X., Wu, X., Guo, H., Hu, Y., Tang, F., et al. (2015). Single-cell RNA-seq transcriptome analysis of linear and circular RNAs in mouse preimplantation embryos. *Genome Biology, 16*, 148.
32. Kivioja, T., Vähärautio, A., Karlsson, K., Bonke, M., Enge, M., Linnarsson, S., et al. (2011). Counting absolute numbers of molecules using unique molecular identifiers. *Nature Methods, 9*, 72–74.
33. Bacher, R., & Kendziorski, C. (2016). Design and computational analysis of single-cell RNA-sequencing experiments. *Genome Biology, 17*, 63.
34. Wagner, A., Regev, A., & Yosef, N. (2016). Revealing the vectors of cellular identity with single-cell genomics. *Nature Biotechnology, 34*, 1145–1160.
35. Miragaia, R. J., Teichmann, S. A., & Hagai, T. (2017). Single-cell insights into transcriptomic diversity in immunity. *Current Opinion in Systems Biology, 5*, 63–71.
36. Poirion, O. B., Zhu, X., Ching, T., & Garmire, L. (2016). Single-cell transcriptomics bioinformatics and computational challenges. *Frontiers in Genetics, 7*, 163.
37. Rostom, R., Svensson, V., Teichmann, S. A., & Kar, G. (2017). Computational approaches for interpreting scRNA-seq data. *FEBS Letters, 591*(15), 2213–2225.
38. Freytag, S., Tian, L., Lönnstedt, I., Ng, M., & Bahlo, M. (2018). Comparison of clustering tools in R for medium-sized 10x Genomics single-cell RNA-sequencing data. *F1000Res, 7*, 1297.
39. Ramskold, D., Luo, S., Wang, Y. C., Li, R., Deng, Q., Faridani, O. R., Daniels, G. A., Khrebtukova, I., Loring, J. F., Laurent, L. C., et al. (2012). Full-length mRNA-Seq from single-cell levels of RNA and individual circulating tumor cells. *Nature Biotechnology, 30*, 777–782.
40. Aceto, N., Bardia, A., Miyamoto, D. T., Donaldson, M. C., Wittner, B. S., Spencer, J. A., Yu, M., Pely, A., Engstrom, A., Zhu, H., et al. (2014). Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis. *Cell, 158*, 1110–1122.
41. Patel, A. P., Tirosh, I., Trombetta, J. J., et al. (2014). Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science, 344*(6190), 1396–1401.
42. Yachida, S., et al. (2010). Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature, 467*, 1114–1117.
43. Eppert, K., et al. (2011). Stem cell gene expression programs influence clinical outcome in human leukemia. *Nature Medicine, 17*, 1086–1093.

44. Parsons, D. W., et al. (2008). An integrated genomic analysis of human glioblastoma multiforme. *Science, 321*, 1807–1812.
45. Gerlinger, M., et al. (2012). Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *The New England Journal of Medicine, 366*, 883–892.
46. Driessens, G., et al. (2012). Defining the mode of tumour growth by clonal analysis. *Nature, 488*, 527–530.
47. Schepers, A. G., et al. (2012). Lineage tracing reveals Lgr5+ stem cell activity in mouse intestinal adenomas. *Science, 337*, 730–735.
48. Winterhoff, B. J., Maile, M., Mitra, A. K., Sebe, A., Bazzaro, M., Geller, M. A., et al. (2017). Single cell sequencing reveals heterogeneity within ovarian cancer epithelium and cancer associated stromal cells. *Gynecologic Oncology, 144*, 598–606.
49. Konecny, G. E., Wang, C., Hamidi, H., Winterhoff, B., Kalli, K. R., Dering, J., et al. (2014). Prognostic and therapeutic relevance of molecular subtypes in high-grade serous ovarian cancer. *Journal of the National Cancer Institute, 106*, dju249.
50. Cai, D. L., & Jin, L. P. (2017). Immune cell population in ovarian tumor microenvironment. *Journal of Cancer, 8*, 2915–2923.
51. Navin, N. E. (2014). Tumor evolution in response to chemotherapy: Phenotype versus genotype. *Cell Reports, 6*, 417–419.
52. Almendro, V., Cheng, Y. K., Randles, A., Itzkovitz, S., Marusyk, A., Ametller, E., et al. (2014). Inference of tumor evolution during chemotherapy by computational modeling and in situ analysis of genetic and phenotypic cellular diversity. *Cell Reports, 6*, 514–527.
53. Shah, M. M., & Landen, C. N. (2014). Ovarian cancer stem cells: Are they real and why are they important? *Gynecologic Oncology, 132*, 483–489.
54. Burgos-Ojeda, D., Rueda, B. R., & Buckanovich, R. J. (2012). Ovarian cancer stem cell markers: Prognostic and therapeutic implications. *Cancer Letters, 322*, 1–7.
55. Chen, W., Dong, J., Haiech, J., Kilhoffer, M. C., & Zeniou, M. (2016). Cancer stem cell quiescence and plasticity as major challenges in cancer therapy. *Stem Cells International, 2016*, 1740936.
56. Nassar, D., & Blanpain, C. (2016). Cancer stem cells: Basic concepts and therapeutic implications. *Annual Review of Pathology, 11*, 47–76.
57. Takeishi, S., & Nakayama, K. I. (2016). To wake up cancer stem cells, or to let them sleep, that is the question. *Cancer Science, 107*, 875–881.
58. Bast, R. C., Hennessy, B., & Mills, G. B. (2009). The biology of ovarian cancer: New opportunities for translation. *Nature Reviews. Cancer, 9*, 415–428.
59. Fidler, I. J. (2003). The pathogenesis of cancer metastasis: The 'seed and soil' hypothesis revisited. *Nature Reviews. Cancer, 3*, 453–458.
60. Micalizzi, D. S., Maheswaran, S., & Haber, D. A. (2017). A conduit to metastasis: Circulating tumor cell biology. *Genes & Development, 31*, 1827–1840.
61. Ramsköld, D., Luo, S., Wang, Y. C., Li, R., Deng, Q., Faridani, O. R., et al. (2012). Full-length mRNA-Seq from single-cell levels of RNA and individual circulating tumor cells. *Nature Biotechnology, 30*, 777–782.
62. Ting, D. T., Wittner, B. S., Ligorio, M., Vincent Jordan, N., Shah, A. M., Miyamoto, D. T., et al. (2014). Single-cell RNA sequencing identifies extracellular matrix gene expression by pancreatic circulating tumor cells. *Cell Reports, 8*, 1905–1918.
63. Théry, C., Ostrowski, M., & Segura, E. (2009). Membrane vesicles as conveyors of immune responses. *Nature Reviews. Immunology, 9*, 581–593.
64. Liu, Y., Gu, Y., & Cao, X. (2015). The exosomes in tumor immunity. *Oncoimmunology, 4*, e1027472.
65. James Hurley, L. H., Brock, G., Sinclair, I., Sullivan, R. J., O'Neill, V. J., Skog, J., & Flaherty, K. (2015). Profiling exosomal mRNAs in patients undergoing immunotherapy for malignant melanoma. *Journal of Clinical Oncology, 33*, e22159.
66. Shih, A. J., Menzin, A., Whyte, J., Lovecchio, J., Liew, A., Khalili, H., et al. (2018). Identification of grade and origin specific cell populations in serous epithelial ovarian cancer by single cell RNA-seq. *PLoS One, 13*, e0206785.
67. Izar, B., Tirosh, I., Stover, E. H., Wakiro, I., Cuoco, M. S., et al. (2020). A single-cell landscape of high-grade serous ovarian cancer. *Nature Medicine, 26*(8), 1271–1279.
68. HERCULES Project: Comprehensive characterization and effective combinatorial targeting of high-grade serous ovarian cancer via single-cell analysis. Retrieved from http://www.project-hercules.eu.