5 Advances in the Characterization of Circulating Tumor Cells in Metastatic Breast Cancer: Single Cell Analyses and Interactions, and Patient-Derived Models for Drug Testing

Praveen K. Kujur, Bianca C. T. Flores, Naveen Ramalingam, Ludmilla T. D. Chinen, and Stefanie S. Jeffrey

Abstract

Metastasis is the major cause of breast cancer death worldwide. In metastatic breast cancer, circulating tumor cells (CTCs) can be captured from patient blood samples sequentially over time and thereby serve as surrogates to assess the biology of surviving cancer cells that may still persist in solitary or multiple metastatic sites following treatment. CTCs may thus function as potential real-time decision-making guides for selecting appropriate therapies during the course of disease or for the development and testing of new treatments. The heterogeneous nature of CTCs warrants the use of single cell platforms to better inform our understanding of these cancer cells. Current techniques for single cell analyses and techniques for investigating interactions between cancer and immune cells are discussed. In addition, methodologies for growing patient-derived CTCs in vitro or propagating them in vivo to facilitate CTC drug testing are reviewed. We advocate the use of CTCs in appropriate microenvironments to appraise the effectiveness of cancer chemotherapies, immunotherapies, and for the development of new cancer treatments, fundamental to personalizing and improving the clinical management of metastatic breast cancer.

Keywords

Circulating tumor cells (CTCs) · CTC culture · CTC-derived explants (CDX · also called CTC-derived xenografts by some authors) · CTC drug testing · CTC heterogeneity · Immunotherapy · Patient-derived xenografts (PDX) · Single cell analysis (SCA) · Single cell interactions

P. K. Kujur · S. S. Jeffrey (✉)
Department of Surgery, Stanford University School of Medicine, Stanford, CA, USA
e-mail: ssj@stanford.edu

B. C. T. Flores
Department of Surgery, Stanford University School of Medicine, Stanford, CA, USA

International Research Center, A.C. Camargo Cancer Centre, São Paulo, Brazil

N. Ramalingam
New Technologies Group, Fluidigm Corporation, South San Francisco, CA, USA

L. T. D. Chinen
International Research Center, A.C. Camargo Cancer Centre, São Paulo, Brazil

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5.1 Introduction

The mortality rate from breast cancer has declined over time, with improvements in screening, earlier diagnosis, and treatment [1–3]. However, although survival is increasing, the great majority of patients who progress to or are diagnosed *de novo* with metastatic breast cancer (MBC)—which in the U.S. is projected to be over 168,000 cases in 2020—will eventually show resistance to sequential therapies over time and be the major cause of breast cancer death [4–7]. MBC is comprised of a molecularly heterogeneous group of tumors and diverse clinical presentations that influence survival patterns and treatment [7–10]. Thus, the search for better treatments for MBC continues, with continued need for real-world data and appropriate disease-relevant models for preclinical studies [11–14].

5.2 Tumor Heterogeneity

Heterogeneity is ubiquitous in human cancer. As tumors grow, genomic instability and environmental conditions—such as local oxygen concentrations, pH, local nutrients, mechanobiological factors, and immune cell interactions—may favor survival and clonal growth of distinct tumor cell subpopulations. Moreover, during multiple cycles of drug treatment, only cancer cells sensitive to a treatment are ablated, while drug-resistant cancer cell subpopulations survive [15, 16]. Consequently, tumors and their subsequent metastases may consist of individual cells with differing genomic composition, metabolism, physiology, and drug sensitivity. Genetic, epigenetic, protein and biomarker expression levels are commonly used to characterize tumor heterogeneity. Biomarker discordance has been documented between different regions of a primary tumor, between primary and metastatic tumors, and between different metastases [17–26]. Such discordances can limit selection of optimal therapy at any given point in the disease process.

Tumor heterogeneity is broadly classified as intertumoral (tumor by tumor) and intratumoral (differences within a tumor) heterogeneity. Intertumoral heterogeneity is a main barrier for cancer classification, and single-cell analysis plays a limited role in answering questions related to cancer classification. In contradistinction, intratumoral heterogeneity (ITH) is widely recognized as a barrier to overcome drug resistance and achieving effective cancer therapy.

5.3 Tumor Heterogeneity at Single-Cell Resolution

Single cell analysis (SCA) is uniquely powerful in resolving ITH and understanding tumor evolution [27]. ITH is a net effect of heterogeneity of malignant cells and diverse nonmalignant cells, such as immune cells, endothelial cells, and stromal fibroblasts [28]. Collectively, the tumor cells and associated nonmalignant cells comprise the complex tumor microenvironment (TME). To decipher the basic mechanism of drug resistance, metastasis, and immunotherapy response, it is essential to profile the heterogeneity of all cell types and states in the TME. Furthermore, it is essential to understand cancer-immune cell interactions [29] and immune response through various biomolecules such as cytokines [30].

Intratumoral heterogeneity has been widely studied by single-cell DNA [27, 31] and RNA-seq methods [32–35]. SCA analysis using single cell RNA sequencing (scRNA-seq) has been used to study CTCs from patients with advanced breast cancer that lacked human epidermal growth factor receptor 2 (HER2) expression, showing a bimodal distribution of HER2+ and HER2− CTC subpopulations with an increasing fraction of HER2+ CTCs during disease progression; of note was that single cell growth of CTCs showed interconversion of HER2 status [32]. Miyamoto et al. studying gene expression profiles (using mRNA-seq) of 77 CTCs from 13 prostate cancer patients, noted heterogeneity in Wnt signaling pathways that could contribute to outcome of a therapy [33]. Using scRNA-seq, Patel et al. reported that individual tumors of primary glioblastoma contained a spectrum of subtypes and hybrid cellular states showing a

diversity of transcriptional programs, and this heterogeneity played an important role in glioblastoma biology, prognosis, and therapy [34]. Tirosh et al. profiled the multicellular ecosystem of metastatic melanoma by scRNA-seq [35]. In this work, the authors noted that the tumor cells displayed transcriptional heterogeneity associated with the cell cycle, spatial context, and a drug-resistance program, while the nonmalignant immune cells displayed dynamic connection between T cell exhaustion and activation, and heterogeneity was reported across 19 patients. There have been numerous studies analyzing mutation evolution and gene expression profiles of various tumors. However, the cellular processes and function, such as immune response, depend on the expression level of proteins. For most of the genes, there is a poor correlation between mRNA expression level and corresponding proteins [36] or cytokines [37]. Compared to RNA-seq studies, there have been limited studies analyzing single-cell protein expression in the context of heterogeneity. This is primarily because flow cytometry allowed profiling of limited numbers of proteins (< 10). Introduction of cytometry by time of flight (CyTOF®) [38] technology and Imaging Mass Cytometry [39] (IMC) enabled analysis of 32 proteins and protein modifications. CyTOF was used to elucidate ITH in acute myeloid leukemia (AML) [40]. The authors reported that surface phenotypes and regulatory intracellular signaling are decoupled in leukemia. Single cell protein profiling of cancer and immune cells have been applied to other cancer types such as renal cancer [28], acute lymphoblastic leukemia [41], ovarian carcinoma [42], hepatocellular carcinoma [43], and lung adenocarcinoma [44].

To further understand the complexity of ITH, development of technologies that can profile multi-omics such as genome, epigenome (chromatin accessibility, methylation), transcriptome, proteome, and secretome (cytokines) simultaneously per cell would be required. Recently, Gkountela et al. profiled DNA methylation patterns in circulating tumor cells from breast cancer patients and xenograft models [45]. The authors reported that hypomethylation profiles of CTC clusters correlated with poor prognosis in breast cancer, and disruption of CTC clusters reverted the methylation profile and suppressed metastases. There also has been limited work on multi-omics profiling of the TME [46, 47]. Bian et al. profiled somatic copy number alterations, DNA methylation, and transcriptome simultaneously through a single-cell triple omics sequencing (scTrio-seq) technique. The authors demonstrated the feasibility of reconstructing genetic lineages based on epigenetic and transcriptomics signatures [46]. Rodriguez-Meira et al. reported a novel method called TARGET-seq that combines genomic DNA and cDNA genotyping with single-cell RNA-seq [47]. Further development of multi-omics techniques will enable profiling of cytokines and other -omics at single cell resolution.

For patients with multifocal metastatic disease, tissue biopsy may be impractical or risky and, if metastatic biopsy is performed, it is generally not repeated as metastases grow or new metastases develop. Using CTCs from blood draws allows live cells shed from metastases in multiple sites to be interrogated as surrogates of the spectrum of surviving cancer cells in metastatic disease. Using high dimensional single cell transcriptional profiling, we have shown that individual CTCs from patients with primary and metastatic breast cancer are heterogeneous, even within a single blood draw, and distinct from single cells from cancer cell lines used for drug discovery [48]. It is likely that sequential SCA investigations of CTCs through the course of disease may offer insight into more optimally tailored regimens, revealing markers or signaling pathways that may suggest unexpected therapeutic approaches.

5.4 Single Cell Interactions

New platforms to study single-cell RNA-sequencing have enabled the detection of cell-cell interactions, delving more deeply into ligand-receptor (L-R) interactions and its effects on gene expression [49]. Considering the importance of the development of new cancer thera-

pies, research groups are seeking to better understand characteristics that define the interaction between cancer and immune cells. While interactions between cells within the primary tumor and its microenvironment are often studied, the role of immune cells and their interaction with tumor cells during cancer dissemination may be equally if not more important [50]. As precursors of metastasis and when isolated from blood, CTCs may be found to be associated with white blood cells (WBCs), tumor-derived fibroblasts, and/or endothelial cells [51, 52], interactions that may modify cell programs. In another interaction model, a component of innate immunity (macrophages) was described promoting incongruously aggressive pro-tumorigenic behavior when stimulated by an immune checkpoint inhibitor [53].

Transcriptional profiles present in individual malignant and non-malignant cells within a metastatic melanoma tumor were studied using scRNA-seq and t-distributed stochastic neighbor embedding (t-SNE) plots to define different transcriptional states associated with different spatial locations within a tumor and presence of various neighboring immune and non-immune cells [35]. scRNA-seq was used to study ligand-receptor interaction pattern across different immune cell types and tumor cells, particularly chemokine interactions [54]. Chen et al. developed a microchannel plate with three-dimensional (3D) concave microwells for growing liver tumor spheroids and co-culturing them with hepatic stellate cells. Co-culture studies accompanied by drug testing showed recapitulation of epithelial-mesenchymal transition (EMT) and chemoresistance that suggested its use for not only cell-cell interaction studies, but also for drug response testing [55]. We have been using a single cell microfluidic platform that allows single cell selection, cell-cell interactions, drug perturbations, and on-chip preparation for RNA-Seq analyses (Polaris™, Fluidigm Corporation) to investigate single tumor cell-immune cell interactions, focusing on the role of NK cells in antitumoral activity (Fig. 5.1).

Another interesting approach is to evaluate both transcriptome and protein profiles simultaneously following cell-cell interactions. Each cell may be labeled and then measured following an interaction, targeting each cell with appropriate barcoded probes and then incubating both cells together. CITE-seq (cellular indexing of transcriptomes and epitopes by sequencing) [56] and REAP-seq (RNA expression and protein sequencing assay) [57] are similar techniques that use DNA barcodes attached to antibodies, enabling the discovery of multi-omic interaction effects [58]. Different mass spectrometry methodologies may be applied to study single-cells and the biology of cell-cell interactions [59, 60]. A method using high-throughput protein analyses is mass cytometry, allowing measurement of about 40 proteins simultaneously in single cells. In this technique, target cells are labeled with multiplex metal-conjugated antibodies, and the target protein abundance are detected using CyTOF mass spectrometry [61]. Another study using imaging mass cytometry has shown activated signaling pathways spatially distributed among heterogeneous subclones of triple-negative breast cancer and the effect of therapeutics on signaling pathway activation patterns and subclonal communication with other subclones and the TME [62].

Epigenetic alterations that may occur after in vivo interactions can be profiled by Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) [63]. This method identifies gene regulatory elements through transposition of sequencing adaptors in regions where the chromatin is accessible. Further extension of this method led to ATAC-see, which enables profiling of spatial organization of the accessible genome. ATAC-see utilizes optimized Tn5 transposases with fluorescent adaptors to profile open chromatin structure in single cells fixed on a substrate [64].

One of the approaches of monitoring in vivo cell-cell interaction is a strategy that uses ligand-induced intramembrane proteolysis. This has been shown with glial cells and neurons in transgenic Drosophila, based on the Notch-Delta interaction mechanism which controls cell fate during fly development through cell-cell interactions [65]. However, this strategy can only be used to investigate cell-cell interaction for Notch-

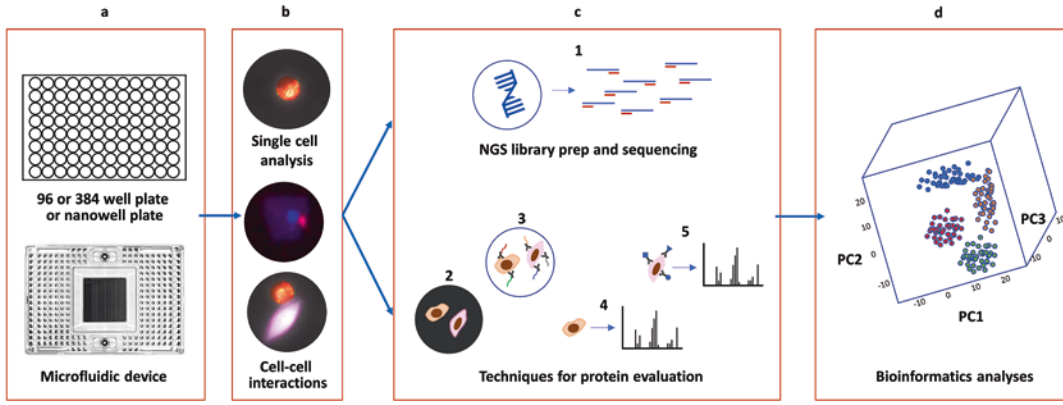

Fig. 5.1 Single cell analysis and cell-cell interactions analysis. Single cell analysis or single cell interactions between individual tumor cells (including CTCs) and immune cells may be assayed to evaluate anti-tumoral activity, differential gene expression, or evaluation of protein and/or metabolic markers. (a and b) microfluidic devices or small chamber well plates may be used to perform single cell analysis or cell-cell incubations; (c1) cells are lysed, mRNA is reverse transcribed, cDNA is pre-amplified, and the library is prepared and sequenced; other techniques may be used to study protein expression: immunofluorescence (c2), DNA-barcoded antibodies (c3), mass spectrometry (c4), or mass cytometry (c5); (d) bioinformatic analyses are then performed, such as by principal component (PC) analysis and multiple other techniques. *NGS* = next generation sequencing

Delta signaling pathways. Another important interaction to investigate is the effect of myeloid-derived suppressor cells (MDSCs) on cells of the immune system with downstream consequences on tumor growth and spread. MDSCs target T cells, which then suppresses the immune system. Some proteins involved in MDSC immune suppression mechanisms include arginase (ARG1), inducible nitric oxide synthase (iNOS), transforming growth factor beta (TGFβ), interleukin 10 (IL10), cyclooxygenase-2 (COX-2), indoleamine 2,3-dioxygenase (IDO) sequestration of cysteine, and decrease of L-selectin expression by T cells [66]. There is also evidence to suggest that MDSCs interact with the innate immune system and modulate the activity of macrophages, dendritic cells (DCs) and natural killer (NK) cells [67]. Recent in vivo studies in breast cancer patients have shown that polymorphonuclear-MDSCs (PMN-MDSCs) interact with CTCs in heterotypic clusters; these PMN-MDSCs induce pro-survival responses in CTCs, and in xenograft models, these interactions enhance metastasis formation [68]. Tumor-educated platelets (TEPs) interact with tumor cells to influence tumor growth and dissemination [69]. This interaction affects both the expression of genes in tumor cells and the RNA (coding and non-coding) profile of blood platelets [70]. Still under investigation are the mechanisms involved in platelet education and how different platelet subpopulations change in cancer patients. TEP RNA biomarkers may contribute to the liquid biopsy field through easier disease monitoring or even earlier detection [71]. This interaction may also serve as a treatment target, as discussed in a later section.

5.5 Reason for Propagating CTCs

The clinical relevance of available preclinical models is frequently debated due to problems such as lack of genetic heterogeneity, transcriptomic drift, cross-contamination, and whether they are indeed representative of the patient being treated [72]. Thus, the development of appropriate and clinically-relevant cancer models is critical. CTCs are critical effectors of cancer metastasis, but their numbers are limited. Moreover, the process of growth and expansion of CTCs in culture still remains challenging owing to their rarity and low viability [73]. Variability in morphological, molecular, and functional aspects due to genetic heterogeneity

further adds to this conundrum [74]. Thus, strategies for the expansion of CTCs may shed light on the molecular signature and biology, including metastatic homing mechanisms, of the parent tumor or tumors. Due to their rarity, the propagation of CTCs holds promise for establishing patient-specific preclinical models for accurate genetic and phenotypic evaluation and for testing preclinical efficacy of various drugs or drug combinations [75]. Notable progress has been made regarding the isolation and in vitro propagation of CTCs from the peripheral blood of cancer patients. Recent studies have shown the possibility of developing 2D and 3D (tumor spheroid or organoid) cultures that closely relate to the CTCs from which they were derived.

Organoid cultures are grown in a 3D environment and are emerging as a novel preclinical model to understand the structure and function of the organ sample from which they originate. When tumor tissues are isolated from a patient and cultured, these tumor organoids are able to partially mimic the complexity of the original tumor [76]. When tumor tissues are cocultured with immune cells and tumor-associated stromal cells, the patient's tumor tissue phenotype may be sustained, allowing therapeutic responses to different drugs to be effectively studied [77]. This ex vivo model has become a crucial tool in the emerging field of personalized medicine. Sachs et al. successfully prepared more than 100 mammary epithelial tumor organoid lines from primary and metastatic breast cancer patients. These organoids typically mimicked the micro-anatomy of the original tumor, including the hormone receptor and HER2 status of the original tumor. Importantly, therapeutic response of organoid cultures to tamoxifen, when determinable, showed a match between the in vitro response and the therapeutic response of the patients from whom the organoids were derived, as would be expected for an in vitro surrogate of a patient's breast cancer; similarly, comparing drug response in xenograft models generated in mice implanted with organoids grown from patient tumors, the in vivo response of the mice to drugs blocking the HER2 signaling pathway generally matched the in vitro response of the organoid culture [78]. In a separate study designed to systematically assess T cell-mediated tumor recognition, tumor organoid cultures positive for major histocompatibility complex (MHC) class 1 from non-small-cell lung cancers and mismatch repair-deficient colorectal cancers were co-cultured with peripheral blood lymphocytes from the same patients to assess the activation of T cell response against tumor cells. They indeed showed that tumor-reactive T cells were induced by co-culture, and that further co-culture of tumor organoids with these autologous tumor-reactive T cell populations caused apoptosis and reduced survival of the tumor organoids [79]. Patient-derived 3D organoid lines from patients with advanced prostate cancer were successfully developed from bone and soft tissue metastases, a pleural effusion, and, in one case out of 17 blood samples with CTC counts greater than 100 in 8 ml of blood, a CTC organoid line from a patient with castration resistant prostate cancer (CRPC); the organoid lines and subcutaneous xenografts made from the organoid lines recapitulated the histopathological and molecular features of the original samples, reflecting the diversity of genomic, transcriptomic, and protein expression features found in CRPC, and showed expected results during drug testing [80].

Growing such cultures ex vivo facilitates intervention by chemotherapeutic drugs and also its interactions with immune cells, which can be monitored and studied in real time. These cultures can further be readily integrated into in vivo studies, either by orthotopically or subcutaneously injecting them into immunocompromised mice to establish a CTC-derived mouse xenograft [81]. In a different approach, CTCs isolated by negative enrichment from the peripheral blood of patients can be tumorigenic after direct implantation into mice to establish CTC-derived explants (CTX) [82]. These ex vivo models exploit the potentially invasive nature of CTCs and serve as emerging preclinical models for patients with invasive cancers. Ex vivo expansion of CTCs by culturing of CTCs in vitro, both short-term and long-term, and in vivo growth of CTCs from patient blood samples are exciting approaches for investigations into the biology and treatment of breast cancer.

5.6 In vitro Expansion

5.6.1 Short-Term Culture

CTCs from patients have been propagated in vitro by multiple groups for various types of cancer [83–93]. Short-term cultures of CTCs vary from a few days to a few months depending on the type of experiments. These short-term cultures have been mostly utilized for karyotyping, immunohistochemical analysis, cytomorphological analysis, genomic profiling, gene expression profiling and proteomic profiling. Short-term culture may be more closely related to the malignant cells of the tumor as longer term tumor growth may accumulate genetic or phenotypic changes through prolonged passaging. Short-term ex vivo expansion of CTCs from breast cancer patients has also been established. In one study, CTCs from six patients were cultured for 16–18 days, and contained heterogeneous populations of cells, with epithelial cell adhesion molecule (EpCAM)-positivity of cultures from each patient ranging from 35% to 86%. The cultures were then analyzed using a panel of genetic mutations and compared with those of the primary tumor; the similarity of mutation profiles also demonstrated the suitability of the CTC cultures as in vitro surrogates for breast cancer molecular studies [94]. Another group isolated CTCs using a size-based filtration membrane and cultured them briefly for 3–5 days for use in downstream molecular analyses and monitoring patient response to different therapeutic regimes in different types and stages of breast cancer [95]. Using the same size-based and antigen-independent membrane filter technique, CTCs from 167 breast cancer patients were either analyzed immediately or cultured in vitro by placing the filter in a 6-well cultivation plate for a minimum of 14 days, facilitating immunocytochemical as well as downstream molecular analyses by qPCR. In some patients, expression status of HER2 and estrogen receptor (ER) in CTCs differed from that of matched primary tumors, and over time in multiple different blood samples, *HER2* status change of CTCs was bidirectional, with only unidirectional change in ER status (*ESR*+ to *ESR*−) [96].

Pizon et al. isolated a variable fraction of circulating cells from breast cancer patients based on EpCAM expression and grew those with high-CTC counts as tumor spheroids, culturing them up to 28 days. CTCs isolated from different patients were heterogeneous and when examined individually, showed variable expression of nanog and vimentin; the ability to grow as tumor spheroids appeared to correlate with tumor aggressiveness [97]. A similar study used a functionally-based approach to isolate breast cancer CTCs by enriching for an invasive subpopulation of CTCs using collagen adhesion matrix (CAM) assay and, using gene expression analysis, identified variable CTC populations with epithelial lineage, tumor progenitor cells with stem/invasive cell properties, and mixed epithelial/progenitor phenotypes; CAM-enriched CTCs were also capable of growing in culture on the CAM scaffold for up to 33 days [98]. A related work used fluorescence-activated cell sorting (FACS) to isolate CTC subsets from the blood of breast cancer patients with and without brain metastases and cultured them as 3D CTC spheroids for up to 30–40 days. Prior to culture, EpCAM-negative CTC subpopulations were selected for CD44+/CD24− cells, related to stemness, and then selected for urokinase plasminogen activator receptor (uPAR) and integrin beta-1 (int β 1) positivity, related to breast cancer dormancy. These distinct molecular attributes allowed the CTCs to form spheroids and grow in culture for use in identifying patients at risk for forming brain metastases [99]. Khoo et al. also were able to establish in vitro cultures of CTCs for 2–8 weeks from breast cancer patient blood using special laser-ablated microwells. Cultivated cells were stained with multiple markers to determine cell composition over time, including markers for leukocytes (CD45 and CD18), hematopoietic precursors (CD34), monocytes (CD14 and CD16), megakaryocytes (thrombospondin-1), and endothelial cells (CD31 and von Willebrand factor, VWF). After 2 weeks, cultures consisted mainly of three cell types: 1) CTCs that expressed cytokeratin but not CD45 (CK+/CD45-), 2) macrophages that expressed migratory inhibitory factor (MIF) and CD68, and 3) NK

cells that expressed CD56. The CD45 negative cells generally were either small cells (≤25 microns with a high nuclear/cytoplasmic ratio) corresponding to CTCs and large cells (>25 microns with a low nuclear to cytoplasmic ratio) comprised of macrophages and NK-like cells. The fraction of stem-like cells in CTC cultures was also increased by cultivation under hypoxic conditions (1% oxygen). The CTCs grown for 2 weeks contained heterogeneous groups of cells expressing both epithelial (pan-CK) and mesenchymal (vimentin) markers, confirmed by RNA fluorescence in situ hybridization using nine epithelial genes and four mesenchymal genes (*PTX3, SERPINE2, VIM,* and *FASCIN*). Moreover, the formation of CTC clusters was inversely correlated with treatment duration and the persistence of CTC cluster formation appeared predictive of lack of response to anti-cancer therapy [100] .

5.6.2 Long-Term Culture

We define long-term culture of CTCs as cultures that can be maintained for greater than 6 months. Culturing of CTCs isolated from peripheral blood of breast cancer patients is challenging, with few reports of long-term culture. The first successful attempt at cultivating CTCs as a continuous culture was done by Zhang et al. in 2013. They established three CTC lines (CTC-1, CTC-2, and CTC-3) from metastatic breast cancer patients. CTCs captured by FACS using the molecular pattern EpCAM-/ALDH1+/CD45- were able to grow continuously and form cell lines, while CTCs selected using EpCAM+/ALDH1+/CD45- were not able to survive in culture for more than 2 weeks. Intracardiac or tail vein injection of these three cell lines into nude mice produced brain and lung metastases for CTC-1 only, whereas the other two CTC lines formed only lung metastases. Further, after expansion of these three EpCAM-negative cell lines, selection by FACS for CTC subpopulations that expressed HER2, epidermal growth factor receptor (EGFR), heparanase (HPSE), and Notch 1 (EpCAM-/EGFR+/HPSE+/Notch1+, known as brain metastasis selected markers, BMSM) produced cells capable of homing to and forming brain metastases. All three new CTC cell lines had brain metastatic potential and were capable of generating brain and lung metastases in nude mice [101].

Subsequently, other laboratories were able to successfully culture CTCs long-term. In one study, six CTC cell lines were derived from patients with metastatic ER-positive breast cancer whose disease was progressing on therapy (BRx-07, BRx-33, BRx-42, BRx-50, BRx-61, and BRx-68). These oligoclonal CTC cultures were cultivated from microfluidically-captured CTCs and grown as tumor spheres in serum-free media supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (FGF) under hypoxic conditions (4% oxygen), with continuous growth in vitro for over 6 months. However, unlike the other five ER-positive CTC lines, BRx-07 did not retain its original ER expression in vitro. Of these six cell lines, three (BRx-07, BRx-61, and BRx-68) were able to develop tumors in NOD *scid* gamma (NSG) mice, depicting in vivo tumorigenicity, and with BRx-07 regaining ER expression in vivo [81]. The first report of a CTC-derived cell line established from colon cancer was by Cayrefourcq et al. and was named CTC-MCC-41. After examining blood from 71 patients with metastatic colorectal cancer (CRC), 50 patients were identified whose blood sample had at least one detectable CTC, and three had greater than 100 CTCs. Two of these high CTC number blood samples produced CTC cultures that propagated for at least 2 months when initially incubated under hypoxic conditions (2% oxygen); however, only one developed into a permanent CTC cell line, derived from a patient with rapidly progressive metastatic CRC that was unresponsive to multiple therapies, with that CTC line still growing under normoxic conditions for more than 16 months at time of publication. CTCs were isolated by negative selection and grown as tumor spheres in non-adherent conditions. This CTC line was also expanded as a CTC-derived xenograft following subcutaneous injection into SCID mice. Interestingly, the CTC cell line expressed

stem cell-like markers and an intermediate epithelial-mesenchymal phenotype that may have added to its ex vivo growth advantage. Further, *KRAS* and *BRAF* mutational status and CK20 expression were preserved between the CTC cell line, the original patient tumor tissue, and xenograft tumor tissue, indicating suitability for a personalized medicine approach for testing future drug therapies [102]. In addition to CTC-MCC-41 described above, eight more permanent CTC lines were generated from sequential blood draws from the same patient during progression of that patient's metastatic CRC, and thereby facilitating further study of clonal selection in metastatic cancer. Notably, this newer paper describes that these remain the only CTC cell lines derived from this one patient after testing blood samples from 168 patients with metastatic CRC [103]. As also described, Gao et al. were able to make an organoid line from CTCs isolated from a patient with castration-resistant prostate cancer. The organoid line, MSK-PCa5, was established from a patient who had a CTC count of >100 cells per 8ml of blood. These CTCs were cultured as organoids in Matrigel (BD Biosciences) with reduced growth factors. Further, it formed tumor when it was injected as a subcutaneous xenograft in a SCID mouse [80]. In another report, a CTC-derived cell line (CTC-3) was established and characterized from the blood sample of a patient with metastatic ER-positive breast cancer. These cells had high nuclear/cytoplasmic ratio and were able to form spheroids [104].

Our collaborative group has previously shown that some of the drivers for the immortalization of cells include *hTERT* expression, telomerase activity, downregulation of genes associated with TGFβ signaling, and overexpression of oxidoreductase genes [105, 106]. Studies have shown that additional genetic and phenotypic changes are acquired when stable cell lines are generated from the patient-derived samples [72, 107]. However, studies have shown that continuous cultures from CTCs retains the important genetic features of the patient's tumor [80, 102].

5.7 In vivo Expansion

In addition to the short-term and long-term in vitro culture of CTCs, in vivo platforms have also been used for their expansion. Breast cancer is a highly heterogeneous disease both intertumorally and intratumorally, as previously discussed, and there can be significant clonal diversity within a patient's tumor. Inconsistency between xenograft studies from a diverse array of cell lines and individual patients' tumors may be bridged by patient-derived xenograft (PDX) models. Such models are generated by the implantation of freshly resected cancerous tissue from a patient's tumor either subcutaneously or orthotopically into an immune-deficient mouse. Conceptually, PDX models maintain the complex tumor heterogeneity by preserving the crucial molecular properties of the original tumor and by providing associated TME when implanted as tumor fragments (although mouse stroma will eventually replace human stroma by the second passage in PDX models [108]). PDX models also provide a renewable source of original patient tumor for interrogation with diverse targeted therapies and new drug development, in contrast to the clinical setting. However, because the tumor growth time of some PDX models (often 2–8 months or more), this may or may not be of benefit the specific patient from which it was derived and instead benefit future patients with molecularly similar tumors. Zhang et al. showed the response of mTOR inhibitors in a panel of seven triple-negative breast cancer (TNBC) patient-derived orthotopic xenograft (PDOX) models, representing four different molecular subtypes of TNBC, and with all histologically and genomically matching original patient tumors [109]. In breast cancer PDX research, a consortium of academic researchers worldwide has curated over 500 stably transplantable breast cancer PDX models and their information, representing three major clinical subtypes of breast cancer, estrogen receptor positive (ER+), HER2+, and TNBC [110]. PDX models (also called patient-derived tumor xenografts, PDTX) and

short-term culture of cells from PDTX models (PDTX-derived tumor cells, PDTCs) are both platforms that offer clinically relevant options to guide the testing and development of drug therapies for individualized breast cancer management [111]. PDX models provide a lot of promise in the field of precision medicine but also require protocol standardization for tissue collection, tracking and handling, and the propagation of the primary tumor from patient to mouse as well as further growth of xenografted tissue in 3D culture [112]. PDX models can also be a useful source of CTCs for in vitro interrogations. Our lab and others have shown that PDX models of breast cancer are able to shed CTCs and metastasize to distant organs; these CTCs can be then used for downstream molecular investigations using immunofluorescence, flow cytometry, real-time quantitative reverse transcription-PCR (qRT-PCR), and single cell gene expression analyses [113–118].

Tumor samples used to generate PDX models are derived from patients at the time of surgical excision or needle biopsy of a tumor. Both approaches limit the use of PDX models to track temporal changes that tumors undergo following treatment and during disease progression. However, blood samples (i.e., liquid biopsy) offer an easy and minimally invasive approach for obtaining patient tumor material serially and in real-time. CTCs isolated from the peripheral blood of patients may be grown in immunocompromised mice to generate CTC-derived explants (CDXs) [119]. CDX models recapitulate the molecular characteristics and heterogeneity of patient tumors to shed light on metastatic biology and, importantly, for use as preclinical models for drug testing and drug development.

The first successful attempt to create CDX model was done by Hodgkinson et al. from patients with metastatic small-cell lung cancer (SCLC), which unlike most solid tumors, shed hundreds to thousands of CTCs. They implanted negatively enriched CTCs from both chemosensitive and chemorefractory SCLC patients; regardless of the therapeutic responsiveness, CDX models were successfully generated when CTC numbers were greater than 400 per 7.5 ml of blood. Genomic analyses between the isolated CTCs and tumor from the CDX models showed similar molecular signatures. Interestingly, the CDX models perfectly recapitulated the original patient's response to platinum and etopside treatment, proving the possibility of predictive tailored therapy on patients [82]. Since then, many studies have reported successful attempts to propagate CTCs in vivo through CDX models in lung cancer [120–122], melanoma [123], and breast cancer [124, 125]. CDX (also used to denote 'CTC-derived xenografts' by other authors) tumor cells have also been subsequently propagated in vitro in short-term culture (up to 5 weeks) and have shown similar drug sensitivities, thereby facilitating in vitro drug screening [126].

Other studies have used long-term cultured CTCs from the breast cancer patients to form xenografts [81, 101]. But there are few studies that have directly isolated CTCs from breast cancer patients to make CDX models because CTC numbers are generally low (single digit to double digit range), even in metastatic breast cancer. Baccelli et al. developed CDX models from a metastasis-initiating cell population among CTCs isolated from primary human luminal breast cancer patients. These CTC subpopulations, which were EpCAM+CD44+CD47+MET+, were injected into the femurs of NSG mice, a bone marrow compartment that potentially represents a privileged hematopoietic stem cell niche, and then were able to grow and disseminate, forming multiple lung, liver, and bone metastases. However, it was observed that only when over 1100 CTCs were transplanted into the femur did successful xenografts occur [127]. In another study, the CDX models were developed in NOD/SCID mice where CTCs positive for M30 and HER2 were isolated from metastatic breast cancer patients. It was interesting to observe that only approximately 200 and 400 CTCs were injected into the mice that successfully formed metastases in spleen and bone marrow. Further, they were able to detect CTCs in the mouse peripheral blood [128]. In a recent study, Pereira-Veiga et al. successfully made CDX models from CTCs isolated from a patient with

metastatic TNBC whose CTC count was 969 CTCs/7.5 ml blood and included 74 CTC clusters of 2–7 cells. A nude mouse was injected subcutaneously with negatively enriched CTCs, and after tumor growth, the xenograft tumor was split: a portion was implanted subcutaneously in a Scid Beige mouse and another portion was disaggregated and cultivated in vitro for 2 weeks and then injected orthotopically into the mammary fat pad of another Scid Beige mouse and tumor growth was monitored. CTCs from mouse blood were also detected. Gene Ontology (GO) analysis on CDX tumors and matched patient tumor and lymph node metastases indicated WNT signaling pathway and genes associated with cell cycle were crucial to TNBC tumor progression [124]. Vishnoi et al. successfully created a TNBC CDX model that specifically formed liver metastases. They used a negative depletion strategy to isolate a CD45-/CD34-/CD105-/CD90-/CD73- cell population from TNBC patients that were enriched for CTCs. When these CTCs were injected by an intracardiac route into NSG mice, about 66% of them developed liver metastases. They then sequentially propagated the metastatic liver tumor for four generations using the cells from the liver tumor to determine a TNBC liver metastasis gene signature as well as identify six candidate drug target genes for the development of new therapeutics [125]. CDX models can be further used as the sources of CTCs or patient's tumor cells, which again can be interrogated for metastatic research studies. In addition, human cell line-derived tumor xenograft models [129] and syngeneic and transgenic mouse models [130, 131] using established cancer cells lines have been previously used to isolate, culture, and propagate CTCs, offering insights into the relation between hypoxia and CTCs and other biological insights.

All the above studies point toward continued sources of patient CTCs or tumor cells expanded ex vivo that can be used for novel therapeutic targeting along with the multi-omics analyses that can provide a large array of data for biomarker and drug screening in cancer and for use in investigating metastatic biology, as depicted in Fig. 5.2. These ex vivo preclinical tumor models preserve the original molecular characteristics of the parent tumor tissue or CTCs and should prove useful for advancing personalized medicine.

5.8 Drug Testing Using CTC Models

Breast cancer is still the leading cause of cancer death in women worldwide and the second leading cause after lung cancer in North America, Northern Europe, and Australia/New Zealand [132]. There are diverse molecular phenotypes of breast cancer based on gene expression profiling, corresponding pathology biomarkers, and integrative cluster groupings based on genetic fingerprinting and genomic copy number drivers [133, 134]. However, intratumoral heterogeneity and spatiotemporal heterogeneity among different metastases, including frequent acquisition of driver mutations in distant metastases not identified in the primary tumor, will impact the effectiveness of therapeutic drugs that may only target tumor subclones with specific genetic aberrations, inferring a need for new therapeutic strategies for treating metastatic breast cancer [135–137].

CTCs and CTC-derived preclinical models offer solutions for studying tumor heterogeneity and molecular changes over time, thus helping guide, develop, and test new therapeutic strategies against breast cancer. The characterization and monitoring of CTCs may offer insight into the molecular landscapes of a patient's tumor in real-time and help monitor tumor growth and therapeutic response [138]. Many prospective studies contribute to the efficacy of chemotherapy in breast cancer by monitoring the CTCs from blood biopsies [139–142].

CTCs may themselves be utilized as therapeutic targets. Novel methods of targeting CTCs, such as by incorporating synthetic microparticles containing apoptosis-inducing substances into CTC microemboli, thereby using them as a "Trojan Horse" for delivering therapy,

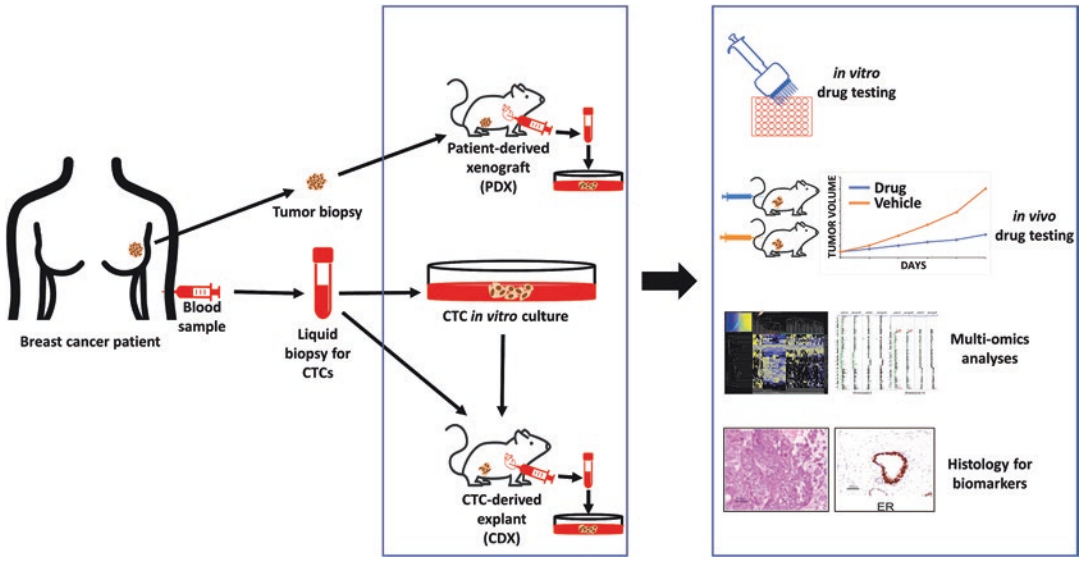

Fig. 5.2 Ex vivo propagation of CTCs from patients and patient-derived models. Tumor fragments or cells from a patient's primary breast cancer or metastasis may be directly implanted or inoculated into the mammary fat pad of immunocompromised mice to generate a patient-derived xenograft (PDX) model; human CTCs isolated from PDX mouse blood may then be propagated by in vitro cell culture. CTCs from a patient blood sample may also be directly propagated by in vitro culture and then inoculated into immunocompromised mice; alternatively, CTCs from a patient may be isolated by positive or negative selection and propagated in mice as CTC-derived explants (CDX, also called CTC-derived xenografts). These ex vivo models may ideally be used for drug testing to predict therapeutic responses of patients or to perform multi-omic, immunohistological, and immunohistochemical analyses for elucidating metastatic biology and identifying new targets for drug discovery

have been reported to decrease lung metastases in a mouse model [143]; interruption of CTC/platelet interactions is another strategy under investigation [144].

CTCs may also be used to identify drug sensitivities of breast tumors [145]. Yu et al. derived CTC cell lines, growing for greater than 6 months, isolated from the blood of patients with metastatic ER-positive breast cancer who were off treatment or progressing on therapy. Cell lines were generated by 3D cultures (tumor spheres) under hypoxic conditions in 6/36 patients. CTC lines and CDX models generated from some CTC cultures were tested for response to an array of anticancer drugs that included inhibitors of PI3K, CDK4/6, IGFR, ER, mTOR, HSP90, FGFR, PARP, and some first-line chemotherapeutic drugs like paclitaxel, capecitabine, and doxorubicin. In this proof of concept study, these drugs alone or in combination targeted CTC-derived cell lines both with mutated oncogenic drivers like *PIK3CA*, *FGFR2, TP53, ESR1,* and *BRCA2* or non-mutated targets like HSP90 and *IGFR*. Some of the drug sensitivity and resistance results were concordant with available clinical histories of the patients, and combination treatments that targeted two pathways were more effective than single drug treatment in some cell lines and CDX mouse models tested with specific oncogenic driver mutations [81]. Another study described the development and testing of a special microfluidic platform designed for growing non-enriched CTCs in short-term culture (within 2 weeks) and then performing on-chip drug screening, finding that co-culture with immune cells promoted cluster formation and CTC expansion. Importantly, the ability to form clusters was inversely correlated with drug concentration and in vitro drug sensitivity, suggesting its use as a CTC drug-screening assay [146, 147].

5.9 Future Perspectives

Patient-derived CTCs can now be cultured ex vivo as short-term cultures or long-term cultures, and can be available for expansion or re-transplanted into immunocompromised murine models as per the requirement of the experiments. While some researchers have been able to establish continuous cell lines with patient CTCs, success rates remain relatively low and these ex vivo culture methods still require further refinement and optimization for regular use in laboratory protocols or clinical applications. As the TME encompasses multiple cell types, biochemical signals, extracellular matrix, varied oxygen levels, and also mechanical stress and tensions that drives towards metastasis, creating biomimetic organ microenvironments or strategies involving the metastatic niche could provide cues for enhancing CTC growth ex vivo [148, 149, 150]. Mimicking these microenvironment by 3D cultures using biomaterials, bio-scaffolds, cytokines, immune cells, and tissue-specific cells on microfluidic platforms could be utilized to create a physiologically relevant cancer model [149]. Such 3D cultures could be manipulated and studied to elucidate the dynamics of TME interaction with CTCs during metastasis formation and growth and also used for developing and testing therapeutic approaches against metastatic breast cancer [148]. Such co-clinical approaches are expected to be used widely in therapeutic development where assessments of CTCs and CDX models can be directly correlated with patients' treatment and clinical outcomes [119, 151]. This allows the evaluation of real-time response to different therapies through disease evolution. However, these strategies have been plagued by the pertinent problems of cultivating and expanding CTCs both in vitro and in vivo. CTC cell lines representing diverse tumor types may be characterized, authenticated, and collected in a CTC biobank, as is the case for the many PDX biobanks used for preclinical investigations. These CTCs biobanks may prove to be powerful resource for multi-omics and therapeutics research. Considering the current poor prognosis of metastatic breast cancer, these CTC-derived preclinical models for basic and preclinical research offer great hope for the identification of novel biomarker signatures, therapeutic drug development and testing, and enhancing our understanding of drug resistance in cancer, so that the promise of precision medicine and improved clinical outcomes for patients with metastatic breast cancer may be achieved.

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