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Culture of Circulating Tumor Cells - Holy Grail and Big Challenge

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Abstract

Circulating tumor cells (CTCs), the most important representation of "liquid biopsy", provides a minimally invasive approach to tumor tissue, and has been a hot topic in cancer research for years. CTCs bear great potential to provide a surrogate for traditional biopsy, and the culture of CTCs is essential to investigate the biological features of CTCs and their roles in cancer metastasis as well as to provide the opportunity for *in vitro* therapeutic sensitivity tests to guild treatment selection. However, it is challenging to cultivate CTCs due to many factors. 1. They exist in very low numbers in peripheral blood. 2. A large proportion of CTCs may not be viable, and we have not developed reliable analysis tools to distinguish viable from non-viable CTCs. 3. The biological features of CTCs, in particular the favorable environment for their growth, are unclear. Many cell culture methodologies have been tested for CTC propagation and several successful examples have demonstrated the possibility to capture viable CTCs for cultivation, thus inaugurating a new era of CTC research. Long-term culture of CTCs bears great promise as a preclinical model for human cancer, whereas short-term culture is more efficient and gives reasonable molecular information for disease progression status and potential therapeutic responses and targets. In this review, we give a brief summary of CTC isolation and culture strategies, and present published data on CTC culture, either for short-term or long-term, in several major cancer entities. The major challenges in propagation of CTCs have been identified and potential research directions to overcome these difficulties have been proposed.

Keywords

Circulating tumor cell, Isolation, Viability, Propagation, Challenges

Introduction

Circulating tumor cells (CTCs) are cancer cells shed from any tumor sites into the bloodstream and has been proposed to play an important role in cancer metastasis [1]. CTCs are a rare population of cell in the circulation blood at a ratio of one CTC to 10^6-10^7 nucleated blood cells [2]. Though the concept of CTC was firstly described more than a hundred years ago [3], only recently have people started to use them in cancer clinical diagnosis and prognosis, owing to technology development. The initial study of CTCs has mainly focused on enumeration, which has been proven to be useful for diagnosis and prognosis in several cancer types including breast [4-6], colon [7], prostate [8-10] and lung [11] cancers. The automated CTC isolation and counting system, CellSearch has been approved by FDA for detecting and enumerating tumor cells and is widely used in CTC studies [6,12-14]. However, as it is an antibody-based immune magnetic capture and automated staining methodology, cells lose viability during fixation and thus cannot be cultured after isolation. The clinical utility of CTCs for early cancer detection, diagnosis, prognosis, prediction, stratification and pharmaco dynamics have been explored [15,16], but the limited amount of CTCs poses a hurdle to comprehensive studies especially functional studies. Thus, propagation of CTCs is a requisite for in-depth functional study. In addition, expanding CTCs has the potential of providing patient-specific tumor models for individualized therapeutic prediction/evaluation of cancers [17]. Significant advancement of technologies including a number of isolation devices and culture conditions has been made over the past few years on CTC propagation. CTC cultures, whether short-term or long-term, *ex vivo* or *in vivo*, have shown great value in cancer genetic studies and individualized medicine developments. Recent achievements on CTC expansion have also led to a better understanding of the cancer biology. However, the propagation of CTCs is still technically challenging and only a few out of the many CTC culture attempts are successful. In this review, we will summarize published cases of CTC expansion (both short-term and long-term) in several major cancer entities, identify the major challenges in CTC culture, and propose the potential strategies to overcome these difficulties.

Viable CTC isolation and culture strategies

Viable CTC isolation strategies: An enrichment step for CTCs is indispensable before putting cells into culture. Existing techniques of viable CTC isolation can be divided into three types: antibody-based approaches, physical property-based approaches and function-based approaches [18].

Cell surface proteins are often used as targets in antibody-based CTC isolation methods. Due to the absence of strictly tumor-specific antigens, epithelial-specific proteins such as EpCAM have been employed for the positive selection of CTCs and antibodies against leukocyte-specific surface antigens, such as CD45, have been used to deplete leukocytes from the blood sample for negative selection [19]. Either immune-magnetic separation [20,21] or flow cytometry [22] can be used to enrich viable CTCs. Immuno-affinity based micro fluidic platforms have also been developed. The CTC-chip, which

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captures epithelial cancer cells circulating in the blood using anti-EpCAM coated on microposts, is the first successful immuno-affinity based micro fluidic platform [23]. Since then, many other antibody-based platforms have been developed to achieve better purity and faster isolation speed, [24] for example, CTC-iChip, an micro fluidic CTC capture platform with two immune magnetic sorting modes to isolate CTCs. The positive selection mode (^{pos}CTC-iChip) identifies CTCs and sorts on the basis of their expression of EpCAM, and a negative selection mode (^{neg}CTC-iChip) depletes leukocytes by immune magnetically targeting both the common leukocyte antigen CD45 and the granulocyte marker CD15 [25]. As immuno beads linked to the cells may interfere with cell viability and proliferation, special beads that can be separated from cells after isolation have been developed for CTC culture [26]. The overt drawback of positive selection is the low recovery rate, as the selected antigen may not cover all CTCs, such as losing epithelial-mesenchymal transformed cells when EpCAM antibody is used. Thus, a negative selection, which depletes leukocytes, may circumvent the problem of losing some subtypes of CTCs. There are several commercially available products for negative selection. RosetteSep® antibody cocktail, which crosslinks unwanted cells in human whole blood to multiple red blood cells forming immune rosettes, has been used to enrich viable CTCs and helps to achieve long-term cultures of CTCs [21,27]. Although a negative selection will cause a CTC purity problem, this is not critical for CTC culture as leukocytes are eliminated during culture.

Physical property-based techniques are large part in the field of CTC isolation. "Ficoll," a synthetic low-viscosity, high-molecular-weight hydrophilic sucrose, and epichlorohydrine polymer and sodium diatrizoate, have been extensively used for depleting red blood cells through density gradient centrifugation [28]. It is often used before further purification steps to deplete lymphocytes. Recently, a large number of size-based devices have been developed for CTC isolation. They are designed based on the hypothesis that tumor cells are larger than blood cells [29-31], though not all CTCs are guaranteed to have a bigger size [32]. Epithelial immunospot (EPISPOT) assay, which captures viable CTCs on the membrane, enables short-term CTC culture. Captured cells on the membrane can be cultured for 24h or longer and then stained for targeted proteins [33]. MetaCell, another membrane-based technique for CTC capture, has been used for CTC isolation and short-term culture in several cancer types [34-37]. Many other micro fluidic devices use microchips to enrich CTCs based on the cell size difference. Parsortix, a cell size and deformability based CTC separation technology, has been tested to preserve nearly 100% cell viability after cell isolation using cancer cells spiked into healthy donors' blood, and the isolated cells proliferated into colonies [38]. Sarioglu, *et al.* reported a Cluster-chip which is designed to capture CTC clusters through specialized bifurcating traps under low–shear stress conditions topreserve their integrity [39]. By staining with the proliferation marker Ki67, the authors showed that the captured CTCs were actively proliferating cells. However, they did not attempt to culture the cells [39]. Huang, *et al.* reported micro fluidic platform which isolated CTCs based on both their size difference and electric properties [40]. This system has been tested using cell lines and the viability of isolated cancer cells has been evaluated to be as high as 94 ± 2% and 95 ± 3%, for the PC-3 and OEC-M1 cells respectively, and proliferated in a conventional monolayer format [40].

Another strategy to enrich viable CTCs is to capture a specific portion of CTCs based on their function. A method, which is based on tumor cells' ability to attach and ingest collagen adhesion matrix, has been described as collagen adhesion matrix (CAM) assay [41]. To enrich tumor cells, blood samples were simply transferred into a CAM-coated tube and incubated for several hours. Unattached cells were then washed off and adherent cells were collected [41,42]. Similarly Wang, *et al.* developed an invasion assay to detect CTCs which could invade into Matrigel [43]. These function-based methods do well in preserving the viability of CTCs, thus enabling further expansion of CTCs. However, not all cancer cells in the circulatory system are viable, so this method is specifically developed to capture viable CTCs. The non-viable CTCs, which may still be valuable for diagnosis/prognosis purpose and cancer molecular analysis, will be excluded.

CTC culture conditions: Various conditions have been applied to CTC expansion. There are three main propagation strategies: two-dimensional (2D) culture, three-dimensional (3D) culture and xenotransplantation.

The standard two-dimensional cell culture condition has been widely used for CTC culture, especially for short-term culture [35,44]. In some cases, CTCs were expanded in 2D conditions for over two weeks [45]. A 2D culture system is easy to set up and has been extensively used for the culture of cell lines as well as primary cell cultures. However, for primary cancer culture, specific medium or feeder cells have to be used and the success rate is low for long-term cultures, apart from for a few very skillful laboratories. It is claimed that non-adherent conditions are important for long-term expansion of CTCs, as CTCs would senesce after a few cell divisions in adherent monolayer culture [26]. To circumvent this problem, 3D conditions have been applied for long-term expansion of CTCs. A wide-range of techniques have been developed that are referred to as 3D culture, organotypic culture or organoid culture [46]. To form 3D conditions, extracellular matrix (ECM) is often added. Several commercial ECMs are available, for instance, Matrigel [20] and agarose [47]. There are also some scaffold-free systems that maintain cells in suspension, for example, ultra-low attachment culture dish can force cells to stay in unattached conditions and form spheroids [48]. So far there is no standard 3D format for CTC culture as CTCs of different cancer types behave differently. Yu, *et al.* achieved long-term culture of CTCs from breast cancer patients using ultra-low attachment culture dishes, while the other five conditions involving Matrigel and feeder cells failed [26]. Some publications have highlighted that hypoxia is critical in CTC culture [26,49]. However, CTCs have also been successfully cultured without hypoxic conditions [20]. Since we know very little of the biological features of CTCs and their favorable culture conditions, many CTC culture attempts are trial and error, based on the cell culture knowledge of other cells and assumptions of CTC features. It is still not clear if hypoxic conditions are essential or favorable for CTCs from certain types of cancers.

Xenotransplantation is another strategy to propagate CTC and is totally different from 2D and 3D culture systems. By directly injecting CTCs into immune-compromised mice, researchers have obtained CTC-derived tumors in lung cancer [21]. The advantage of xenotransplantation is that CTCs can expand and prove to be tumorigenic which are of great value in cancer research especially for cancer metastasis study. However, there are also limitations. It has been reported that some CTC-derived cell lines are not tumor-generating in mice [26], indicating that this method may fail in certain cases when the CTCs have the potential to be expanded *in vitro*. Moreover, CTCs without tumorigenic ability can be lost in the process of xenotransplantation, thus it may not represent the heterogonous populations of CTCs existing in the blood samples.

The main cancer types with successful CTC culture

Short-term culture has been achieved in multiple cancer types, even from early stage cancers in certain instances, whereas most long-term cultures have been established from advanced metastatic cases, where a large number of CTCs can be isolated. Short-term culture of CTCs *in vitro* further enriches tumor cells after the isolation procedure, as white blood cells are eliminated during culture and CTCs can be further identified by cytomorphological tests. Moreover, short-term culture to some extent better preserves the original characteristics of CTCs. Thus, short-term culture of CTCs can assist single cell analysis and gene expression analysis on CTCs. Long-term culture, whether *ex vivo* expansion or generating xenografts *in vivo*, holds great promise for developing personalized cancer medicine, as drug screening can be performed on cultured CTCs and CTC-bearing animals provide *in vivo* models for testing therapeutic efficacy. The long-term culture will also generate sufficient cells viable for many

cell biological studies to understand mechanisms of the development of cancer metastasis and therapeutic resistance.

Breast cancer:

Short-term culture: In breast cancer, CK19-releasing cells (RCs) in bone marrow, detected by EPISPOT assay, were correlated to metastases [33] and CK19-RCs from peripheral blood of metastatic breast cancer patients can be a potential predictor of overall survival [50]. In 2015 Khoo, *et al.* proposed a method of short-term culture of breast CTCs and indicated that the CTC cluster formation ability may predict treatment response [44]. Generally, nucleated cells from patient blood were seeded in laser-ablated micro wells under hypoxia condition after red blood cell lysis. After two weeks of culture, the proliferative cells formed multilayered clusters, whereas the non-proliferative cells generated a noticeable amount of cell debris. The cultured cells were heterogeneous and exhibited a population of macrophage and natural killer-like cells in addition to CTCs. Moreover, the cultured cells could be transferred to Geltrex® or ultra-low adhesive dishes and grown as spheroids withstanding multiple passages. Although people placed great expectations on establishing cell lines from CTCs, the authors argued that their efficient short-term culture system was more advantageous, since prolonged culture and multiple passages often led to phenotypes that were no longer representative of the original tumor in terms of the cell's epigenetic and gene expression [44].

Long-term culture: It has been demonstrated that primary human luminal breast cancer CTCs contain some metastasis-initiating cells by Baccelli, *et al.* in 2013 [51]. In the study, blood samples from 110 progressive metastatic breast cancer patients were transplanted into the femoral medullar cavity of immuno compromised mice. CTCs from only three patients generated multiple bone, lung and liver metastases in mice and parallel CellSearch showed more than a thousand CTCs were injected into each mice [51]. Rossi, *et al.* injected CTCs from two breast cancer patients into NOD/SCID mice subcutaneously and they detected human cells in the murine peripheral blood, bone marrow and spleen, though no tumor developed [52]. In 2013 Zhang, *et al.* reported success of developing CTC cell lines from three brain metastatic breast cancer patients [53]. $EpCAM^+/ALDH1^+/CD45^-$ or $EpCAM^-/ALDH1^+/CD45^-$ circulating cell subsets were obtained from three patients by FACS and cultured *ex vivo* in stem cell culture medium for the first week, followed by epithelial cell culture medium. Although different cell sets could form colonies for the first few days, only the EpCAM-population survived over 14 days. Brain and lung metastases were observed in nude mice engrafted with the cultured CTCs, demonstrating their metastatic capability [53]. In 2014 Yu, *et al.* reported another success in long-term *ex vivo* culture of CTCs, where CTCs from six of 35 luminal subtype breast cancer patients were propagated and established into cell lines [26]. CTCs were isolated by CTC-iChip and cultured under non-adherent culture conditions. Genome sequencing of the CTC lines revealed same mutation status in the PIK3CA gene between CTC lines and primary tumors. Three out of five CTC lines tested for tumorigenicity generated tumor within animals. Drug sensitivity screening on these cell lines showed that certain drug sensitivity features of the CTC cell lines were concordant with clinical responses and revealed potential new therapeutic targets [26]. This report gives strong evidence that expansion of CTCs is a practicable approach for drug response tests for individualized medicines and reveals the great value of CTC cultures.

Lung cancer:

Short-term culture: It is remarkable that CTCs from 14 of 19 early stage non-small-cell lung cancer (NSCLC) patients have been isolated and cultured for a short time using an *in situ* capture and culture methodology [54]. CTCs were co-cultured with fibroblasts for up to 14 days, after 7-days of culture on the chip, and were able to form spheroids in three-dimension cultures. Nine of 15 patients' CTCs examined had mutations in TP53 gene while five had matched TP53 mutations in primary tumors and *ex vivo* expanded CTCs. Next generation sequencing of 124 selected cancer-related genes further revealed that concordant mutations (APC, ERBB4, CASP8) existed in tumor and CTCs in addition to TP53 [54].

Long-term culture: In small-cell lung cancer (SCLC), researchers demonstrated that CTCs from patients with either chemo sensitive or chemo refractory SCLC are tumorigenic in immuno compromised mice [21]. CTCs, enriched using RosetteSep® from six SCLC patients (three chemo sensitive and three chemo refractory), were injected into immune-compromised mice and CTCs from four patients (two chemo sensitive and two chemo refractory) generated tumors. Applying drugs to mice bearing CTC-derived explants showed that the explants mirrored the donor patient's response to platinum and etoposide chemotherapy. In this report, only blood samples with > 400 $EpCAM^+$ cytokeratin(CK)$^+$ CTCs per 7.5 ml blood (evaluated by Cell Search platform) gave rise to CTC-derived explants (CDXs). The number of CDXs is still limited and this method needs to be tested in a larger cohort [21]. Most recently, the same group reported a case of NSCLC patient-derived xenografts from mesenchymal CTCs [27]. CTCs were enriched using RosetteSep® and injected into immuno compromised mice. Parallel Cell Search analysis of the patient's blood detected zero $EpCAM^+CK^+$ CTCs. Multi parameter immuno fluorescence on the filtered blood sample revealed > 150 CTCs ($CD45^-/CD144^-$) cells/ml comprising epithelial $CK^+/vimentin^-$ (23%), mesenchymal $CK^-/vimentin^+$ (30%) and mixed phenotype $CK^+/vimentin^+$ (47%) CTCs. These data highlight the importance of non-epitope-dependent technologies for CTC enrichment [27].

Prostate cancer:

Short-term culture: MetaCell has been introduced to isolate CTCs from 3 prostate cancer patients and CTCs were cultured *in vitro* for over 14 days [34]. Some cells were able to escape the membrane and grow on the plate bottom. Cultured cells from one patient showed mesenchymal features when counterstained by May-Grünwald-Giemsa (MGG) staining. In another report using MetaCell, CTCs were detected in 28 of 55 patients with localized prostate cancer and cells with a proliferative capacity were identified from 18 out of the 28 CTC positive patients [55].

Long-term culture: It has been previously reported that CTCs from prostate cancer patients could survive in mice and have been detected in mice spleen and bone marrow, but they were not able to develop into a tumor [52], indicating that prostate cancer CTCs may have a relatively lower tumor-forming potential. Only a limited number of cell lines available in Biobanks also indicate that prostate cancer is very difficult to expand in cell culture [56]. In 2014 Gao, *et al.* reported success in long-term cultures of CTCs from a prostate cancer patient using 3D organoid system [20]. Organoid culture system has been used for primary culture of different cancer tissues [46]. This report demonstrated that organoid system could be used to generate cell lines from CTCs. This CTC-derived organoid was grafted into mice and both the organoid and graft histologically resembled the primary cancer. 67% of point mutations found in the CTC-derived organoid line were identified in the lymph node metastasis while the additional mutations can be acquired during culture or cancer progression [20].

Colon cancer:

Short-term culture: A study using both EPISPOT and CellSearch to isolate CTCs from peripheral and mesenteric blood of colorectal cancer patients was published in 2013 [57]. The enumeration of CK19-releasing cells by the CK19-Epispot assay revealed CTCs in 27 of 41 (65.9%) and 41 of 74 (55.4%) (P = 0.04) patients, in mesenteric and peripheral blood respectively, whereas CellSearch detected CTCs in 19 of 34 (55.9%) and 20 of 69 (29.0%) (P = 0.0046) patients. They detected significantly more CTCs in mesenteric blood compared with the peripheral blood, which supports the hypothesis that the liver is a filter for CTCs [58]. Moreover, the EPISPOT assay showed, for the first time, that CTCs released by the primary colorectal cancer into the mesenteric blood are viable [57].

Table 1: Culture conditions and isolation methods used for CTC long-term culture.

Cancer type	Culture conditions	Isolation methods
Breast cancer	2D culture condition [53]	Ficoll + FACS [53]
	Non adherent culture condition [26]	CTC-iChip [26]
Lung cancer	Xenotransplantation [21,27]	RosetteSep® + Ficoll + xenotransplantation [21,27]
Prostate cancer	organoid culture condition [20]	RosetteSep® + Ficoll [20]
Colon cancer	Non adherent culture condition [49]	RosetteSep® [49]
Gastric cancer	collagen-based 3D culture condition [60]	Magnetic micro beads [60]
	Xenotransplantation [61]	Lymphoprep® + magnetic micro beads + xenotransplantation [61]

Long-term culture: It is well known that the frequency of CTCs is lower in peripheral blood of colon cancer patients as compared to breast or prostate cancer patients, making it even more difficult to find and grow CTCs in colon cancer [56]. A group tried isolating CTCs from patients with colorectal carcinoma and cultured the cells in different conditions. The CTCs were able to adhere to the culture plate. However, they could not proliferate or propagate [22]. So far only one cell line has been established from human circulating colon cancer cells, which also can generate xenografts *in vivo*. All primary tumor, lymph node metastasis, the CTC-derived cell line and the xenograft of this patient bear the same KRAS and BRAF mutations [49]. Another CTC culture from a patient, who had a CTC count of > 300, was maintained for 2 months, but gradually died off after 2 months [49].

Gastric cancer:

Short-term culture: CTCs from gastric cancer patients were captured by MetaCell and successfully cultured *in vitro* for over 14 days [45]. CTCs were detected in seven patients out of ten from the resection group (70%) and six out of 12 from the non-resectable group (50%). Cells were cultured on the membrane *in vitro* and could overgrow the membrane and attach to the plate bottom [45]. Although they reported that the mRNA transcripts of CKs and other possible epithelial markers were more abundant in the enriched CTC-fractions than in the whole blood, little detail was given in the article.

Long-term culture: Reported long-term culture of gastric cancer CTCs mainly focused on $CD44^+/CD54^+$ proposed gastric cancer stem cells. In a 2012 publication focused on proposed $CD44^+$ $CD54^+$ gastric cancer stem cells, Chen, et al. reported that they successfully cultured CTCs into tumor spheres using isolated $CD44^+$ cells through negative depletion of $CD45^+$ and endothelial marker positive cells followed by positive selection using anti-CD44 antibody in six of seven gastric cancer patients [59]. Moreover, they claimed that injection of the $CD44^+$ $CD54^+$ cells, sorted from the spheres, generated tumor in mice, which histologically resembled original human tumor tissues. It is generally known that a large number of CTCs are required from a sample to make a successful long-term culture case [21,27,49,51]. It is not yet clear if all the viable CTCs are cancer stem cells, but this report indicated that gastric cancer should release a large amount of CTCs so that there are sufficient numbers of CTCs with such a $CD44^+CD54^+$ stem cell feature to make the successful sphere growth. However, no any further gastric cancer CTC studies using this so-claimed efficient CTC culture system have been reported in the following four years since this paper was published. There is only one additional case of successful gastric cancer CTC culture reported since then, in which CD44 highly expressed cells had great tumorigenicity, whereas the $CD44^{-/low}$ fraction was not tumorigenic [60]. In this study, all $CD45^+$ and $CD45^-$ fractions of peripheral blood mononuclear cells from patients with bone marrow metastasis were used to inject immuno deficient mice. While one tumor was generated in mice from $CD45^-$ fraction of peripheral blood mononuclear cells, eight tumor-like-structures were generated from non authorized CTC containing cells expressing CD45 and B-cell markers [60].

Pancreatic cancer: Using MetaCell technology, Bobek, *et al.* [37] maintained CTCs from pancreatic cancer patients viable for a period of minimum 14 days on the membrane. They also observed that some cells invaded through the membrane and grew on the bottom of the 6-well plate, which indicated the plasticity of captured cancer cells. Another group cultured pancreatic CTCs isolated using a microchip [61]. They observed adherent cells on the culture dish and kept them alive over 4 months. However, these cells did not proliferate. To date, there is no report of long-term culture of pancreatic CTC. The long-term culture conditions and isolation methods for CTCs are summarized in table 1.

The challenges and prospective of CTC culture

By virtue of isolation device development and culture condition modification, propagation of CTCs has finally been achieved in a number of human cancers. Series of analyses on cultured CTCs have implicated the great value of CTCs for understanding cancer genetics and the molecular mechanisms of drug response, as well as predicting therapeutic sensitivity. Further investigations using cultured CTCs hold great promise for understanding cancer metastasis mechanisms and developing precision cancer medicine. However, many challenges exist for CTC culture and there are still a very limited number of cases of successful long-term cultures of CTCs. Consequently, the functional analyses of CTCs are currently still at an early proof-of-concept stage [18]. The obvious challenge is that CTCs exist at very low numbers in peripheral blood. In cell culture, cells help each other to survive, so it is usually a challenge to culture single or few cells alone. This explains why the current successful long-term CTC cultures are from cases with high number of CTCs [21,27,49,51]. The second challenge is that we have not developed reliable analysis tools to distinguish viable from non-viable CTCs. A large proportion of CTCs may not be viable so that in certain cases, in particular the cases without metastasis, our attempts in CTC culture may start without any viable CTCs. Stem cell concepts have been used in the gastric cancer long-term CTC cultures [59,60]. However, CTCs with such stem cell features cannot be isolated for CTC culture yet [59], and it is not clear if only CTCs with stem cell features can be cultured for a long-term.

The most important challenge in CTC culture is our limited knowledge of the biological features of CTCs, in particular the favorable environment for their growth. With scarce data, it is still unclear of the features associated with the culture ability of CTCs. Currently, the success of long-term CTC expansion largely relies on a large number of CTCs from each patient, where there is a high chance of certain CTCs capable of proliferation in a given culture condition due to the heterogeneity of CTCs with various molecular and biological properties [62]. Our current attempts at CTC culture are mainly based on our knowledge or established systems of cell culture from cancer tissues. We know that different cancer types required different culture conditions, determined not only by the features of the type of cancer cells but also the micro-environment the cancer cells located. In the circulation, CTCs from any type of cancer are facing the same environment, which is different from their solid tissue location. Single cell culture for pure cell colony generation can be achieved for many established cancer cell lines. Specific culture conditions may be able to be developed for single viable CTC cultures, if we have a good understand of the biological features of viable CTCs. This will also make the success of CTC cultures independent of cases with a large number of CTCs.

These challenges make it difficult, at this stage, to define a CTC culture method to be generally suitable for all cancers or even just for one type of cancer. Based on the above challenges, future research directions in CTC culture should be focused on improving the understanding of the biological features of CTCs, in particular

the features associated with the culture ability of CTCs. With this improved knowledge, we may identify the viable CTCs and culture them in the most suitable culture conditions, which allows single or few cells to proliferate and grow into cell lines. Once this is achieved, we can then begin to establish robust procedures for CTC cultures from different cancer cell types and in patients, not only with metastatic cancer and large amount of CTCs, but also before metastasis developed, as long as viable CTCs exist.

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