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Circulating tumour cell clusters: isolation, biological significance and therapeutic implications

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ABSTRACT

Circulating tumour cells (CTCs) and CTC clusters are considered metastatic precursors due to their ability to seed distant metastasis. However, navigating the bloodstream presents a significant challenge for CTCs, as they must endure fluid shear forces and resist detachment-induced anoikis. Consequently, while a large number of cells from the primary tumour may enter the circulation, only a tiny fraction will result in metastasis. Nevertheless, the metastatic potency dramatically increases when CTCs travel in conjunction with other cell types to form CTC clusters, including neutrophils, myeloid-derived suppressor cells, macrophages, platelets, cancer-associated fibroblasts and red blood cells found in circulation. Such heterotypic CTC clustering events have been identified in a variety of cancer types and may serve as intriguing therapeutic targets and novel biomarkers for liquid biopsy. This review summarises recent advances in microfluidic technologies designed for the isolation of CTC clusters and explores the biological properties of distinct types of CTC clusters within the circulatory system. Investigation of the mechanisms of CTC cluster–blood microenvironment interactions may offer a promising avenue for gaining fresh insights into CTC cluster-mediated metastatic progression and reveal potential opportunities for devising personalised antimetastasis treatments.

INTRODUCTION

Cancer stands as a major leading cause of mortality globally, with distant metastasis accounting for nearly 90% of all cancer-related deaths.[1 2] First identified by Ashworth in the mid-19th century, circulating tumour cells (CTCs) are considered metastatic precursors that leave the primary sites, transit in circulation and eventually give rise to distant metastasis.[3 4] The blood circulation appears to be a harsh microenvironment for the survival of CTCs, as they have to resist the tear of fluid shear forces,[5] reprogramme themselves to avoid the detachment-induced anoikis[6] and evade the surveillance by the immune cells patrolling in the bloodstream.[7] Given these obstacles, it has been shown that only a tiny fraction of CTCs will survive in circulation and eventually establish distant metastasis.[8]

Isolating rare CTCs from billions of surrounding blood cells presents a significant technological challenge. However, recent advances in microfluidic technologies have enabled the successful isolation of viable CTCs from blood circulation, such as antigen-dependent CellSearch systems and antigen-independent microfluidic CTC isolation chips.[9 10] These technologies have made it feasible to perform systematic molecular profiling and ex vivo culturing of CTCs and have shown to be powerful platforms supporting both biological characterisation and clinical application of CTC liquid biopsy. Recent studies have shown that although the viability of single CTCs in circulation is extremely low, their proliferative and metastatic potential may be dramatically enhanced when they travel as CTC clusters or are interconnected with other cell types in the bloodstream, such as neutrophils, myeloid-derived suppressor cells (MDSC), macrophages, platelets, cancer-associated fibroblasts (CAFs) or red cell count (RCC).[11 12]

The clustering of CTCs may protect them from shear forces, anoikis and surveillance by the immune system, and it has been demonstrated that the presence and increased number of CTC clusters in circulation are associated with worse prognosis in a variety of cancer types.[11 12] CTC clusters exhibit a metastatic potential that is 23–50 times greater than single CTCs,[4] and they are reported to be responsible for about 97% of metastases.[13] Moreover, the unique cell type compositions within each individual CTC cluster may significantly influence their metastatic potential.[11 12]

Therefore, the detection and characterisation of CTC clusters are highly relevant for cancer diagnosis and treatment. Either homotypic or heterotypic interactions within CTC clusters could play a critical role in the metastatic progression. Through precise molecular analysis of the composition and dynamics of CTC clusters derived from individual patients, novel therapeutic strategies could be employed to treat metastasis in a patient-specific manner.

Technologies for the isolation of CTC clusters

Although distinct physical or biological properties of CTCs have been used to isolate single CTCs, isolation of CTC clusters is more challenging given their extreme rarity, vast heterogeneities in size and differences in intercellular compositions.[14] CTC cluster isolation methods can be broadly categorised into direct isolation and microfluidic techniques. Direct isolation methods include the use of magnetic beads with specific antibodies attached or simple filtration based on cluster size. Microfluidic techniques, on the other hand, employ a combination of screenings based on various properties of CTC clusters, including physical and biological characteristics. Among the direct isolation methods, Magsweeper is based on immunomagnetic beads and a set of magnetic rods covered with a plastic sheath. By sweeping through the sample well, washing in fresh buffer, releasing and repeating for several rounds, it can collect CTCs with high purity. The purity reaches 100% with a capture efficiency above 80% while the contaminating white cell count (WCC) is fewer than 2×10^4.[15] Another method, EasySep, involves a negative depletion step to remove CD45+cells after RBC lysis. This two-step purification strategy reaches twice the capture efficiency of the EpCAM-positive selection method (58%), and the WBC contamination level is lower than 1%.[16] However, these two methods are unable to remove single CTCs effectively and may damage the CTC clusters during the enrichment steps. The ScreenCell device addresses this issue partially by allowing blood to flow through a single-use, low-cost microporous membrane filter for size-selective isolation. This method has a sensitivity above 90% when the concentration is greater than 5 cells/mL of blood and shows the ability to retain CTC clusters.[17] A similar method, FMSA, uses a flexible structure at the microscale to minimise cell damage, achieving a 90% capture efficiency and 80% viability from 7.5 mL of whole blood.[18]

Microfluidic technologies appear to offer more specificity in capturing CTC clusters while preserving their viability. Researchers have recently developed shifted triangular pillars that could form two openings (Cluster Chip), thus resulting in drag forces from two directions that help the clusters stay stable on the upper side. By applying a defined pressure to the fluid, smaller blood cells may pass quickly through the filter while larger CTC clusters would be retained (figure 1A).[19] At a flow rate of 2.5 mL/hour, the Cluster Chip exhibits a capture efficiency of 99% for MDA-MB-231 clusters consisting of at least four cells, 70% for three-cell clusters and 41% for two-cell clusters, and the integrity of captured clusters is well preserved. However, this method is less effective for capturing small CTC clusters and may cause cellular damage and loss of viable clusters. Moreover, its processing speed is quite slow, taking up to 3 hours for the complete processing of 7.5 mL blood. Another device based on mesh microwells with slanted sidewalls was recently developed (Cluster-Well). The cross-like mesh structure in the microwells may function as a stable supporting platform for CTC clusters. The mesh microwell structure may help CTC clusters resist the shear force and avoid potential damage (figure 1B).[20] At a flow rate of 25 mL/hour, this device captures over 90% of the MDA-MB-231 doublets and almost all the larger clusters. Importantly, the fraction of contaminating WBC and platelets is lower than 0.06% and 0.025%, respectively. Since this method relies on filtration, some CTC clusters could still be damaged.

Apart from the multiple-opening filtration methods, the deterministic lateral displacement (DLD) technology has been developed for the isolation of CTC clusters. This method consists of two stages. The first stage consists of an array of cylindrical micropillars with row shifts. Because of the designed space between the micropillars, blood cells and single CTCs will go down directly while the large CTC clusters will be isolated by DLD. In the second stage, the pillars are asymmetric, which could cause rotations of small asymmetric clusters, while the symmetric singlets will be continuously separated by going down vertically (figure 1C).[21] This technology relies on fluid mechanics to separate large CTC clusters, small CTC clusters and the rest of the blood cells, thus minimising the mechanical damage caused by the flow. The method achieved capture efficiencies of 90%, 89% and 97% for large clusters, small clusters and single cells, respectively. Remarkably, this two-stage DLD cluster capture chip yielded a 99.9987%–99.9995% reduction of RCC in stage 1 and a 99.9966%–99.9990% reduction of RCC in stage 2. For WCC, the reductions were 96.6893%–98.0501% and 99.5373%–99.8005% in stages 1 and 2, respectively. However, the blood processing speed was relatively low, only about 0.5 mL/hour. One advantage of this device is that there was minimal fragmentation of CTC clusters during its operation, thus enabling the recovery of viable CTC clusters.[21] Compared with the two-dimensional technologies, a three-dimensional (3-D) microfluidic device has recently been developed. This device consists of a 3-D scaffold coated with hydrogel, which could be dissolved rapidly at 37°C, thus allowing efficient and safe release of CTC clusters. Furthermore, the hydrogel within the 3-D scaffold is functionalised with anti-EpCAM monoclonal antibody, which could support the antigen-dependent enrichment of epithelial CTC clusters (figure 1D).[22] Through quantitative experiments, they demonstrated a capture rate of 115.38% for two-cell clusters, 98.33% for three-cell clusters, 93.62% for four-cell clusters, 97.50% for clusters with 5–8 cells, and 68.42% for clusters with more than nine cells.[22] The overview of these isolation methods is summarised in table 1.

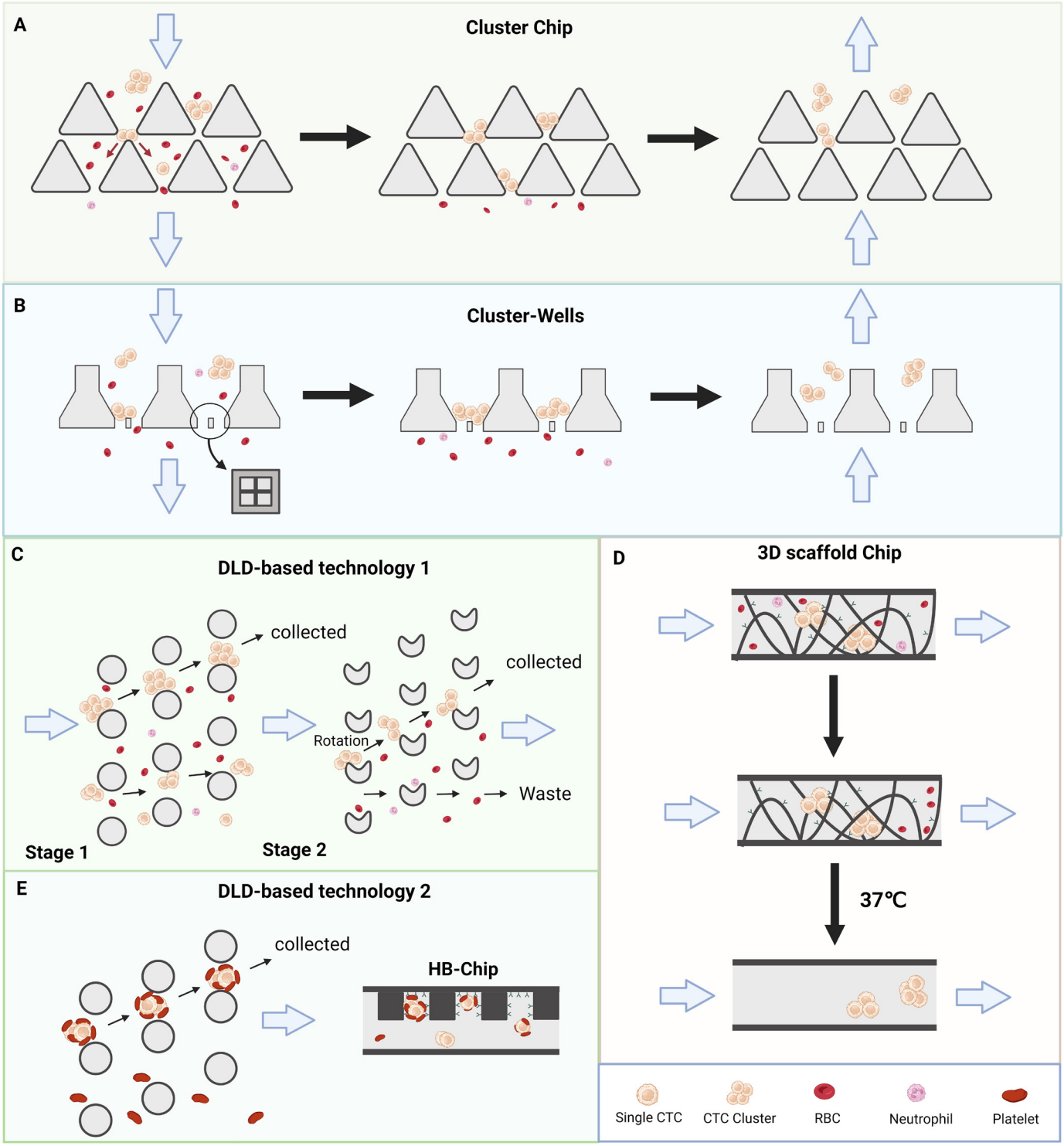

Figure 1 Overview of representative microfluidic technologies that are used for circulating tumour cell (CTC) cluster isolation. (A) Filtration method with shifted triangular pillars, forming two openings to restrain the CTC clusters stably. (B) Filtration method with mesh microwell, the cross-like mesh structure in the microwells can help to hold the CTC clusters. (C) DLD-based method with two stages. Stages 1 and 2 are used to isolate large and small clusters, respectively, according to the difference between the hydrodynamic characteristics of clusters and other cells. (D) Three-dimensional (3-D) structured filtration method. The scaffold is coated with a thermosensitive hydrogel which will dissolve at 37°C and attached with corresponding antibodies, resulting a safe release of CTC clusters. (E) A combination method of DLD and affinity (HB-Chip) was used to isolate CTC-platelet clusters or other types of heterotypic CTC clusters. (Created with BioRender.com). DLD, deterministic lateral displacement; HB, herringbone; RBC, red blood cell.

Table 1 Comparision of different CTC and/or CTC cluster isolation strategies

Technology	Key feature	Strengths	Weaknesses	Capture efficiency	Contamination level
Magsweeper[15]	Use of magnetic beads and rods for enrichment	High purity	Not specific for CTC clusters	>80%; background WCC <2×10⁴	Negligibly low
EasySep[16]	Negative enrichment by depleting CD45+cells	Convenient	Not specific for CTC clusters; low capture rates rate	>58%	<1% WCC
ScreenCell[17]	Size-based microporous membrane filter	single-use, low cost	Potential cluster damage; contain single CTCs	>90% (including single CTCs)	/
FMSA[18]	Size-based microporous membrane filter	Reduced CTC damages	Contain single CTCs	~90%	/
Cluster Chip [19]	Shifted triangular pillars	High efficiency for large CTC clusters; preserves cell viability	Less effective for small CTC clusters; slow process time	Up to 99% for large CTC clusters	/
Cluster-Well[20]	Mesh microwells with slanted sidewalls	Resists shear force; fast process time	Potential CTC cluster damage	>90% doublets and large CTC clusters	<0.06% WCC
DLD[21]	Two-stage cylindrical micropillars	Minimises mechanical damage; high purity	Low throughput	Up to 97% for single CTCs; ~90% for CTC clusters	Negligibly low
3-D Microfluidic Device[22]	Hydrogel-coated scaffold	Antigen-dependent enrichment; efficient release	Less effective for large CTC clusters	>90% for clusters with 8 cells or less; ~68% for CTC clusters with 9 cells or more	About 1000 WBC remaining per millilitre of blood

CTC, circulating tumour cell; WCC, white cell count.

Remarkably, some researchers have recently developed a CTC cluster isolation device that specifically targets platelets-containing CTC clusters (figure 1E).[23] This technology first used the DLD device, followed by the enrichment using an antibody-coated herringbone CTC chip (HB-Chip) strategy. By coating the HB-Chip with CD41 antibody, the researchers achieved an efficient capture of CTC-platelet clusters (figure 1E). This method reliably detected CTC-platelet clusters in 66% of lung cancer, 60% of breast cancer and 83% of melanoma patient samples, respectively. However, this method yields relatively low purity, with the number of contaminating leukocytes up to 10^5/mL.[23]

Thus, microfluidic devices with distinct design principles have been successfully developed to isolate viable CTC clusters from the blood microenvironment, allowing subsequent molecular and functional characterisations of these rare metastatic precursors at single-cell resolutions.

Homotypic CTC clusters

CD44-positive homotypic CTC clusters were shown to maintain lipid raft integrity on cell membranes, enhance stemness and survival, and suppress anoikis.[24 25] Mechanistically, the overexpression of γ-secretase within CTC clusters induced CD44 cleavage and generated the CD44 intracellular domain (CD44 ICD), which could subsequently activate the Rac1-Pak2 pathway to promote the survival of CTCs (figure 2A, ①).[24] This implied that therapeutically targeting γ-secretase, CD44 ICD or the Rac-Pak2 axis may be effective in disrupting homotypic CTC clusters. Apart from this, when the CTC clusters travel in the bloodstream, the outer layer of cells could protect the inner CTCs from shear force (figure 2A, ②).[5] Remarkably, CTC clusters were shown to be able to reshape themselves into a chain-like structure (figure 2A, ③), which may facilitate their traverse through the capillary-sized vessels.[26] These clusters appeared to have a lower turnover rate in circulation as compared with singlets, thus potentially facilitating more efficient metastatic extravasation.[26]

Seminal work by Aceto *et al* has shown that Plakoglobin plays a pivotal role in the generation of CTC clusters, contributing to both adherens junctions and desmosomes formation (figure 2C).[4] It was found that the intercellular adhesion molecule (ICAM) could regulate the assembly of CTC clusters by inducing CTC aggregates.[27] Using single-cell epigenomic profiling technologies, it was elegantly demonstrated that the hypomethylation of

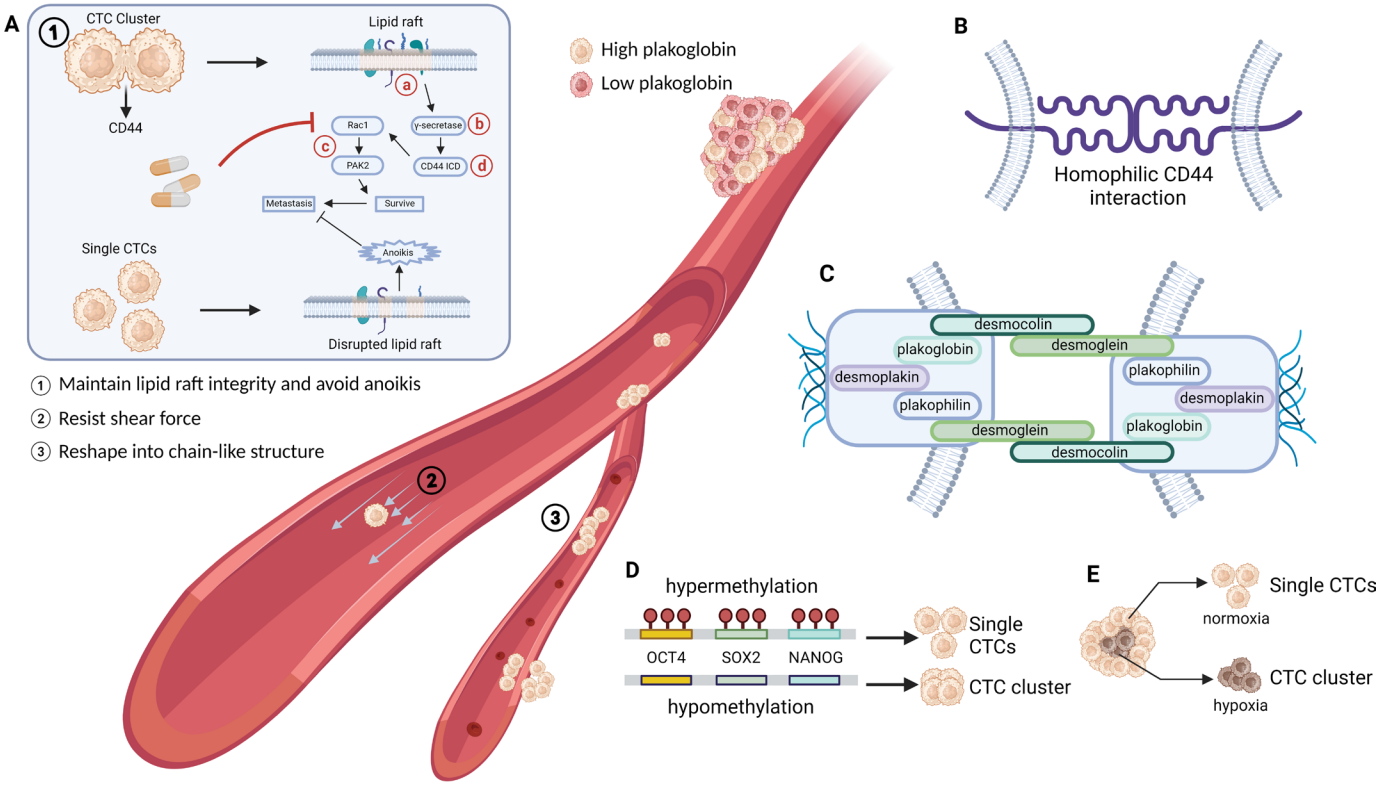

Figure 2 The formation and effect of homotypic circulating tumour cell (CTC) clusters. (A) ① The clustering of CTCs could avoid anoikis by maintaining lipid raft integrity. (a–d) Potential therapeutic interference of the lipid raft formation or the downstream key elements in the pathway may lead to anoikis. ② Clusters may help the CTC cells to resist shear force effectively; ③ CTC clusters could reshape into chain-like morphology, facilitating traversal through capillary-sized vessels; (B) CTC clusters can be induced by CD44, among which homophilic CD44 interaction is the most common type. (C) Plakoglobin is essential in the process of adherens junctions and desmosomes formation, thus regulating the formation of CTC clusters. (D) Hypomethylation of binding sites for OCT4, NANOG and SOX2 could lead to the formation of CTC clusters. (E) Hypoxia environment could help with the formation of CTC clusters. (Created with BioRender.com).

binding sites for key transcription factors (TFs) involving stemness and proliferation is specifically enriched in CTC clusters. TFs including OCT4, NANOG, SOX2 and SIN3A appeared to be involved in the regulatory network, a feature reminiscent of those observed in the embryonic stem cells (figure 2D).[28] Interestingly, Donato *et al* have discovered that hypoxia is a significant factor in promoting the generation of CTC clusters, largely due to its ability to trigger the upregulation of cell-cell junctional components. They found that while conventional antiangiogenic therapy could reduce the primary tumour burden, the number of CTC clusters and metastasis was significantly increased. In contrast, the proangiogenic therapy led to increased primary tumour size but significantly reduced metastatic burden in breast cancer mouse models.[29] Thus, the hypoxia signalling pathway actively regulates CTC cluster generation (figure 2E).

Heterotypic CTC clusters

CTC-neutrophil clusters

While analysing the regulatory mechanisms involved in homotypic CTC clustering is critical for a better understanding of CTC survival and dissemination, the mechanistic investigation of heterotypic CTC clusters may provide novel insights into the interactions between CTC and the blood microenvironment. CTC-WC clustering has been consistently observed in various cancer types and is associated with poor prognosis.[11 30] Szczerba *et al* demonstrated that neutrophils are the most frequently encountered cell type in conjunction with CTCs in both mouse models and breast cancer patients (figure 3A).[31] The neutrophils within the clusters were found to support the proliferation and cell cycle progression of CTCs. Reciprocally, CTCs from the clusters could possibly stimulate neutrophil activation in the cluster.[31] Interestingly, Wculek and Malanchi discovered that neutrophils could promote metastasis by secreting neutrophil-derived leukotrienes, which appeared to selectively expand a subpopulation of cancer cells with high tumourigenic potential.[32] The genetic knockout of vascular cell adhesion molecule 1 (VCAM1) successfully disrupted the formation of CTC-neutrophil clusters, suggesting the functional importance of VCAM1 in mediating CTCs-neutrophil cluster formation.[31] Another receptor-ligand pair, the integrin subunit alpha M (MAC-1)/ICAM1, was identified to play an important role in CTC-neutrophil interaction and facilitate cancer cell adhesion and metastasis.[33]

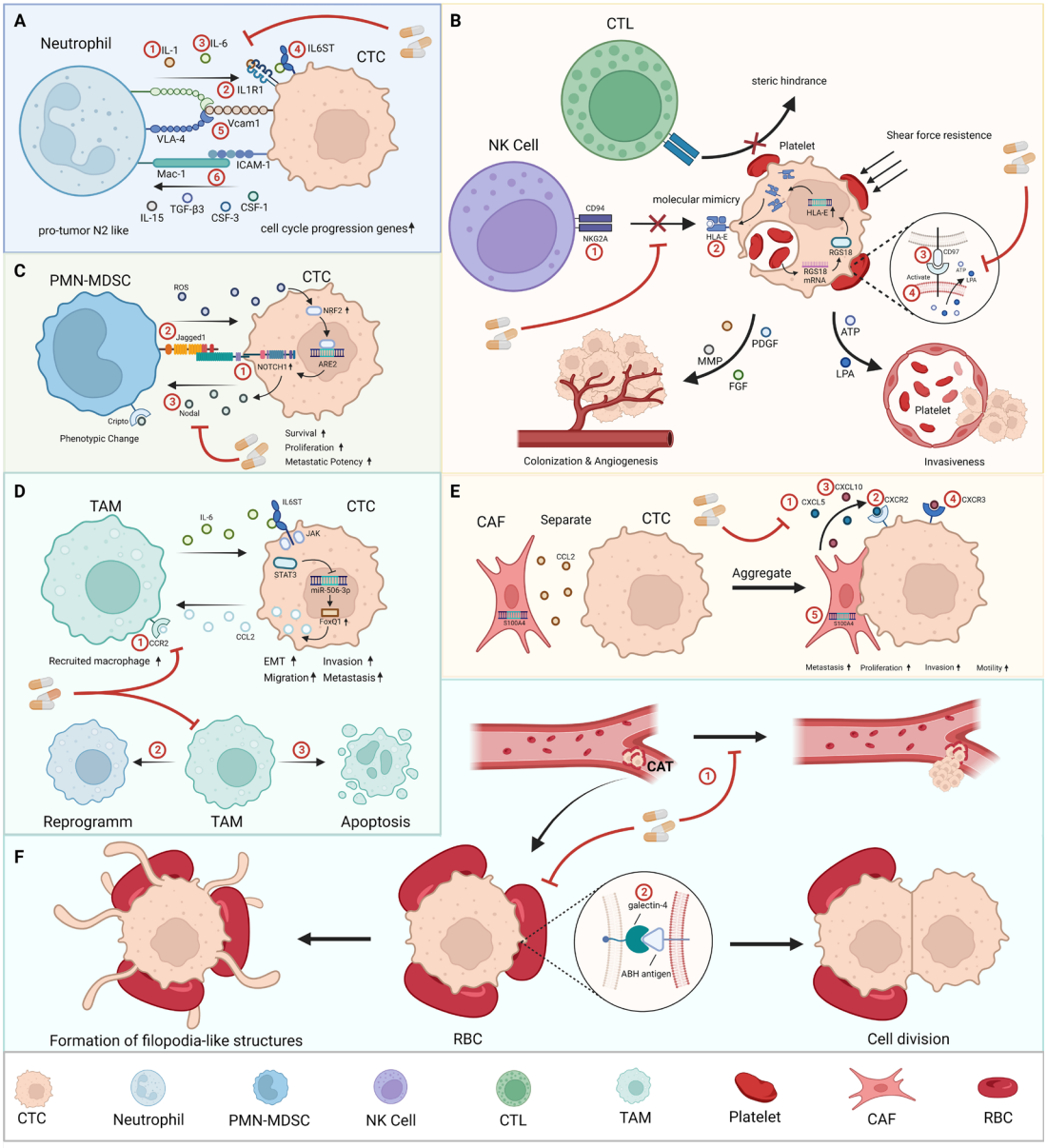

Figure 3 The formation, effect and possible therapeutic targets of distinct heterotypic circulating tumour cell (CTC) clusters. (A) CTC-neutrophil cluster: Neutrophils in the clusters can secrete cytokines such as IL1β and IL6 to stimulate the expression of genes involved in cell cycle progression and proliferation. Possible therapeutic strategies and targets to block CTC-neutrophil interactions: ① IL-1; ②IL1R1; ③ IL-6; ④ IL-6R; ⑤ ICAM-1; ⑥Vcam1. (B) CTC-platelets cluster: Platelets in the cluster could help CTCs resist shear force and avoid immune surveillance from Nature Killer cells (NK) and Cytotoxic T Lymphocytes (CTLs). Possible therapeutic strategies and targets to disrupt CTC-platelets interactions: ① NKG2A; ② HLA-E; ③ CD97; ④Platelets membrane-based to drug delivery. (C) CTC-MDSC cluster: The MDSCs in the cluster are mainly PMN-MDSC, which could regulate gene expression of CTCs through the Notch1-Jagged1 pathway. Possible therapeutic strategies and targets to inhibit CTC-MDSC interactions: ①–② Notch1-Jagged1 axis; ③ Nodal. (D) CTC/TAM interactions: TAM could stimulate the epithelial-to-mesenchymal transition (EMT) programme and enhance CTC-mediated metastasis through the modulation of the JAK2/STAT3/miR-506-3 p/FoxQ1 axis. Possible therapeutic strategies and targets: ① CCR2; ② Drugs lead to M1 polarisation of TAM; ③ TAM depletion by small molecule inhibitors. (E) CTC-CAF clusters: CAF could secrete specific cytokines when CAFs were in physical contact with the cancer cells, which might be a contributing factor to the survival and proliferation of cancer cells. Besides, CAF cells within the cluster may regulate ECM and facilitate metastatic colonisation. Possible therapeutic strategies and targets to inhibit CTC-CAF interactions: ① CXCL5; ② CCR2; ③ CXCL10; ④ CXCR3; ⑤ S100A4. (F) CTC-RBC cluster. RBCs in the cluster may increase the adhesion affinity to the endothelial wall and facilitate successful extravasation. Furthermore, it could also help CTCs to divide by providing alternative mechanical attachment signals. Possible therapeutic strategies: ① CAT dissociation; ② galectin-4. (Created with BioRender.com). CAF, cancer-associated fibroblast; NK, natural killer; PMN-MDSC, polymorphonuclear myeloid-derived suppressor cell; RBC, red blood cell; TAM, tumour-associated macrophage.

CTC-platelet clusters

Platelets are frequently observed to envelop CTC clusters, and the aggregates thus formed were shown to be significantly associated with adverse prognosis (figure 3B).[34] Platelets may support metastasis in multiple ways. By shielding CTCs from shear force-induced stress, platelets could facilitate CTC cluster survival and metastasis formation.[35] Furthermore, it was found that platelets could help CTC clusters escape immune surveillance by establishing a physical barrier and inducing steric hindrance between CTCs and NK cells.[7] In another study, it was shown that platelets could be 'eaten' by CTCs, and by doing so, it provides platelet-derived RGS18 to the host CTCs. The accumulation of RGS18 in CTCs is capable of blocking NK-mediated cytotoxicity by engaging the immune checkpoint HLA-E:CD94-NKG2A axis by promoting the expression of HLA-E.[36] Remarkably, platelets were also capable of directly transferring platelet-derived major histocompatibility complex class I into CTCs, forming a molecular mimicry that resulted in attenuated antitumour reactivity of NK cells by disrupting the self-recognition.[37] Furthermore, studies have demonstrated that platelets can secrete TGFβ and induce epithelial-to-mesenchymal transition (EMT) to facilitate the extravasation of CTC in mouse models of lung metastasis.[38] In another study, it was shown that platelets facilitate the dissemination of CTC clusters by secreting TGFβ, which dissociates these clusters into individual CTCs in vitro.[39]

It has been shown that platelets could be attracted to the sites of tissue injury to prevent excessive bleeding and promote wound healing by secreting various cytokines.[40] Intriguingly, it was revealed that platelets could be recruited by tumour cells via P-selectin expressed on the surface of the platelets.[41] This might allow tumour cells to hijack the physiological wound-healing process for their own benefit by acquiring a variety of pro-angiogenic factors including platelet-derived growth factor, vascular endothelial growth factor, matrix metalloproteases and fibroblast growth factor to promote tumour invasion.[41 42] Furthermore, platelets could be activated and release granules via interaction with CD97 on the tumour cell surface. Those granules contain critical mediators regulating cancer invasion, including ATP and Lysophosphatidic Acid (LPA). ATP serves as a potent disruptor of the endothelial junctions and hence may facilitate CTC intravasation and extravasation while LPA could promote tumour invasiveness and metastasis via the proximal CD97-LPAR heterodimer signalling.[43] Platelets have also been implicated in promoting the distant colonisation of CTCs. Studies have shown that the platelet adhesion receptor complex, such as integrin αIIbβ3 and P-selectin, play a significant role in metastatic outgrowth.[44 45]

CTC-MDSC/macrophage interactions

MDSCs represent a heterogeneous population of cells originating from bone marrow. In patients with solid tumours, an increased number of MDSCs showed a significant correlation with poor prognosis.[46] MDSCs are mainly composed of two subsets: polymorphonuclear MDSC (PMN-MDSC) and monocytic MDSC (M-MDSC). PMN-MDSCs appeared to be significantly expanded in patients with cancer as compared with those with infections and/or inflammation.[47] The number of MDSCs has been reported to correlate with CTCs in patients with metastatic breast cancer and elevated MDSC levels were associated with poor survival.[48] The direct evidence of CTC-MDSC interactions is demonstrated by Sprouse *et al*, who have confirmed the existence of CTC-MDSC clusters in breast and melanoma patients. They showed that the protumourigenic differentiation of MDSCs is induced in the clusters through the paracrine Nodal signalling activation, which could lead to increased ROS production and Notch1 receptor expression in CTC, thus resulting in enhanced metastatic potency (figure 3C).[49]

Macrophages could be broadly categorised into M1 and M2 subtypes, and tumour-associated macrophages (TAMs) frequently exhibit an M2 polarisation.[50] TAMs play a pivotal role in modulating tumour angiogenesis, metastasis and immune suppression, and their abundance is correlated with poor prognoses.[51 52] Researchers have found that isolated CTC clusters tethered to the macrophages, primarily the M2-like subtype,[53] suggest the existence of CTC-macrophage clusters, but the underlying mechanisms require further investigations (figure 3D). CTCs have been demonstrated to induce the differentiation of monocytes into TAMs in small-cell lung cancer (SCLC),[54] and reciprocally, TAMs have been shown to stimulate the EMT of CTCs, thereby enhancing metastatic colonisation through modulation of the JAK2/STAT3/miR-506-3p/FoxQ1 axis.[55]

CTC-CAF clusters

The presence of CAFs has been linked to clinical metastasis and poor prognosis in a variety of cancers.[56] CAFs have been found to co-travel with CTCs, thereby augmenting the efficiency of metastasis (figure 3E).[57] The CTC-CAF clusters may possess a higher metastatic potential compared with homotypic CTC clusters, and CD44 appeared to be involved in this process.[58] Through co-culture of CAFs and tumour cells, Hurtado *et al* discovered that CXCL10/IP-10 was only detectable when CAFs were in physical contact with the tumour cells, and a significantly elevated level of CCL5/RANTES was observed in the culture medium under this condition. This implies that CAFs may engage in intercellular communications with tumour cells through direct physical contact.[59] In addition, Grum-Schwensen *et al* showed that tumours growing in mice lacking the S100A4 gene, which led to deficient fibroblast migration, failed to metastasise. Remarkably, metastatic potency was restored when S100A4-expressing fibroblasts were supplied.[60] The direct cell-cell contact between CAFs and CTCs not only could offer a crucial survival cue but also may enable the protection of CTC clusters from shear force and anoikis.[61] Furthermore, it is well documented that CAFs could regulate the structural dynamics of the extracellular matrix (ECM), either

by secreting various collagen-modifying enzymes such as PLOD2[62] and LOX,[63] or by exerting contractile force through stretching and pulling.[64] The remodelling of the ECM by CAFs may allow efficient colonisation of CTC clusters, thus promoting metastasis.

CTC-RBC clusters

The role of RBC in CTC biology is relatively underexplored (figure 3F). It has been shown that various cancer patients with higher pretreatment RBC distribution width have a worse prognosis.[65–67] The CTC-RBC cluster may enable the CTC to roll steadily along the vessel wall at a low flow rate, potentially enhancing their adhesion affinity to the endothelial wall and facilitating extravasation.[68] Consistent with this observation, Helwa *et al* reported that tumour cells with high galectin-4 expression could bind significantly more RBCs, and these RBCs may serve as surrogate mechanical attachment surfaces for tumour cells to adhere and proliferate.[69]

Potential therapeutic strategies targeting CTC clusters

CTC clusters not only exhibit a greater metastatic potential than single CTCs but also are able to shape the blood microenvironment through the formation of heterotypic clusters with diverse blood cell types. Through physical interactions or the paracrine secretion of a wide array of cytokines, CTC clusters could effectively modulate the vasculature and immune microenvironment, facilitating successful extravasation and metastatic colonisation. Recent advances in single-cell sequencing and CTC ex vivo-culturing methods have begun to shed light on the molecular mechanisms of CTC cluster formation and present potential therapeutic targets for novel antimetastasis therapies.

Homotypic CTC cluster

As summarised in table 2, CD44 ICD could be a promising target for the elimination of CTC homotypic clusters. One strategy is to block the proteolytic cleavage of CD44 by using antibodies[70] or alter the conformation of CD44 using Angstrom6, a small molecule that binds to CD44.[71] Inhibition of CD44 has demonstrated effectiveness in various in vitro experiments.[70 72] An anti-CD44 antibody, RG7356, has progressed to a phase I clinical trial, exhibiting tumour uptake and modest efficacy.[73 74] However, these clinical trials primarily targeted solid tumours. Given that disrupting CD44 may impact the formation of CTC clusters, further research is necessary to determine whether anti-CD44 therapies can effectively reduce cancer metastasis. Another potential target is the lipid raft. Nystatin, an antifungal agent that binds to cholesterol and disrupts lipid rafts, might be an effective candidate.[75] Alternatively, the γ-secretase inhibitors such as MRK003[76] and Nirogacestat,[77] could be applied to block the generation of CD44 ICD. MRK003 has shown promising preclinical activity,[78] and Nirogacestat has proceeded to a phase III clinical trial for desmoid tumours, where it showed statistically and clinically significant improvements in progression-free survival and overall response rate.[79] Furthermore, KP372-1, a specific Akt inhibitor, could be used to inhibit the downstream signalling pathway of CD44 ICD, namely the Rac1-Pak2 axis.[80] A more direct way to impair the formation of homotypic CTC clusters is to target ICAM1, which mediates cell-cell adhesion. A-205804, a small molecule that inhibits ICAM1, might be used to test its effect on CTC cluster survival and metastasis.[81]

CTC-neutrophil cluster

To disrupt the CTC-neutrophil clusters, one potential approach is to interfere with crosstalk between CTC and neutrophils by targeting IL1β, IL6 or their receptors. Zhou *et al* demonstrated that suppressing IL-1 signalling with the IL1β-specific blocking antibody Canakinumab, or the IL1R1 antagonist Anakinra, could effectively eradicate breast cancer-induced bone metastasis.[82] Canakinumab has shown effects in reducing lung cancer incidence in a clinical trial,[83 84] and a phase I clinical trial involving Anakinra for rectal cancer has been initiated.[85] Moreover, it has been revealed that IL6 inhibitors, such as siltuximab (anti-IL6 mAb) and tocilizumab (an anti-IL6R mAb), are effective in multiple clinical cancer treatments, including myeloma, metastatic renal cell carcinoma and prostate cancer.[86–89] Alternatively, Mac-1/ICAM1 inhibitors A-205804[81] and Vcam1/VLA-4 antagonist natalizumab may be applied to disrupt the formation of CTC-neutrophil clusters.[90]

CTC-platelet cluster

Targeting the HLA-E:CD94-NKG2A axis is a possible strategy to disrupt CTC-platelet clusters.[36] The NKG2A inhibitor monalizumab has been put into various clinical trials for its potential in treating different cancers.[91–93] However, NKG2A blockade may interfere with the normal process of NK cell education, thus targeting HLA-E might be a better option.[94] Monoclonal anti-HLA-E mAbs such as TFL-033 have been developed and were shown to restore NK cell and CD8+anticancer cell cytotoxicity.[95] Moreover, inhibiting CD97 to prevent the activation of platelets by CTCs might be a choice, but CD97-specific inhibitors are yet to be developed.

CTC-CAF cluster

CXCL5, CXCL10 and their receptors are potential therapeutic targets for CTC-CAF clusters. Although an anti-CXCL5 monoclonal antibody has been developed, its efficacy needs further validation.[96] By contrast, SB225002, an antagonist of CXCR2, has been shown to impair lung cancer progression and enhance the sensitivity to cisplatin treatment.[97] Similarly, butyrate may be an effective inhibitor of CXCL10 that requires further validation.[98 99] AMG487, a CXCR3 antagonist, has been demonstrated to prevent lung metastasis in a mouse model.[100] Therapeutically targeting S100A4 could be a potential strategy, and the S100A4-specific inhibitors cantharidin and its analogue norcantharidin might be

Table 2 Potential CTC cluster-specific drug targets and their clinical development

Type	Target	Possible drugs	Possible mechanisms	Clinical trial	Characteristics
CTC-CTC cluster	CD44 ICD	Angstrom6[71]	An 8 amino-acid peptide that interferes with the conformation of CD44[71]	Phase 2[114]	Delayed progression; no severe adverse effect[114]
		RG7356	An anti-CD44 humanised antibody	Phase 1 (NCT01358903)[73]	Limited efficacy; mild side effect[73]
	Lipid raft	Nystatin[75 115]	An antifungal inhibitor disrupting lipid raft formation[75 115]	To be determined	To be determined
	γ-secretase	MRK003[76]	A γ-secretase inhibitor[76]	Preclinical[78]	Induce apoptosis[78]
		Nirogacestat[77]	Similar to MRK003[77]	Phase 3 (NCT03785964)[79]	Better ORR; mild side effect[79]
	Rac1-Pak2 axis	KP372-1[80 116]	A cell permeable Akt inhibitor[80 116]	To be determined	To be determined
	ICAM	A-205804[81]	A potent and selective inhibitor of E-selectin and ICAM1[82]	To be determined	To be determined
CTC-neutrophil cluster	IL1β	Canakinumab[82]	A human monoclonal antibody targeting IL1β[82]	Phase 3 (NCT01327846)[83]	Reduced incidence and mortality; higher sepsis rate[83]
	IL1R1	Anakinra[82]	A IL1R1 antagonist[82]	Phase 1 (NCT04942626)[85]	Enhanced survival; no severe adverse effect[85]
	IL6	Siltuximab[86 87]	A human monoclonal antibody targeting IL6[86 87]	Phase 2[88]	Limited efficacy; mild side effect[88]
	IL6R	Tocilizumab[86 87]	A human monoclonal antibody targeting IL6R[86 87]	Phase 2 (NCT03999749)[89]	Under evaluation[89]
	Mac-1/ICAM1	A-205804[81]	A selective inhibitor of E-selectin and ICAM-1 expression[81]	To be determined	To be determined
	Vcam1/VLA-4	Natalizumab[90]	A human monoclonal antibody that blocks α4 integrin from forming VLA-4[90]	To be determined	To be determined

Continued

Table 2 Continued

Type	Target	Possible drugs	Possible mechanisms	Clinical trial	Characteristics
CTC-platelet cluster	NKG2A	Monalizumab[36 91 92]	A human monoclonal antibody targeting NKG2A[36 91 92]	Phase 2[93]	Disease stabilisation; no severe adverse effect[93]
	HLA-E	TFL-033[94 95]	A monospecific anti-HLA-E antibody[94 95]	To be determined	To be determined
CTC-CAF cluster	CXCR2	SB225002[97]	A selective inhibitor of CXCR2[97]	To be determined	To be determined
	CXCL10	Butyrate[98]	A short-chain fatty acid that can suppress the expression of CXCL10[98]	Phase 2 (ACTRN12612000804886)[99]	Reduced polyp number and size; no severe adverse effect[99]
	CXCR3	AMG487[100]	A small molecule that acts as an antagonist for CXCR3[100]	To be determined	To be determined
	S100A4	Cantharidin[101]	A transcriptional inhibitor of S100A4[101]	Phase 2[102]	Improved clinical benefit with better quality of life; no severe adverse effect[102]
		Norcantharidin[101]	Similar to cantharidin[101]	Phase 1 (NCT04673396, no publication yet)	Under evaluation
CTC-MDSC cluster	Notch1	Crenigacestat[117]	A small molecule that prevents the release of NOTCH intracellular domain (NICD)[117]	Phase 1 (NCT02836600)[118]	Limited efficacy; no severe adverse effect[118]
		CB-103[104 119]	A pan-NOTCH inhibitor that directly targets the NOTCH transcriptional activation[104 119]	Phase 1 (NCT03422679)[119]	Limited effects; no severe adverse effect[119]
		siRNA[120]	Knockdown Notch1 directly[120]	To be determined	To be determined
CTC/Macrophage interaction	TAM reprogramming	ADH-503[109]	A small-molecule agonist target CD11b[109]	Preclinical[109]	Enhanced immune response[109]
	TAM depletion	Clodrolip[110]	Clodronate encapsulated in liposomes that can deplete TAM[110]	To be determined	To be determined
	TAM recruitment	BMS-813160[111]	A small molecule inhibitor that can block CCR2[111]	Phase 1b/2 (NCT03184870)[121]	Under evaluation[121]

Continued

Table 2 Continued

Type	Target	Possible drugs	Possible mechanisms	Clinical trial	Characteristics
CTC-RBC cluster	CAT	LMWH[112]	A coagulation inhibitor that can prevent the formation of CAT[112]	Phase 4 (NCT00942968)[113]	Reduced recurrence rate; manageable bleeding risk[113]

CAT, cancer-associated thrombosis; CD44 ICD, CD44 intracellular domain; CTC, circulating tumour cell; CTL, cytotoxic T lymphocyte; LMWH, low-molecular-weight heparin; ORR, overall response rate; RCC, red cell count; TAM, tumour-associated macrophage.

promising drug candidates.[101] A clinical study investigating the combination of Cantharidin Sodium treatment with chemotherapy in gastric cancer patients has been conducted and the results indicated a statistically significant improvement in the quality of life before and after treatment.[102]

CTC-MDSC cluster and CTC/macrophage interaction

Therapeutic blockade of the Notch signalling might be effective in disrupting CTC-MDSC clusters and suppressing metastasis.[49] Various clinical trials have been conducted on selective Notch1 inhibitors like crenigacestat and CB-103 which were shown to be effective in treating various cancer types, such as adenoid cystic carcinoma,[103] clear-cell renal cell carcinoma[104] and breast cancer.[105] Antibodies against Jagged1 could also be an effective means for Notch1 inhibition.[106] Another strategy to eliminate the CTC-MDSC cluster is to block the Nodal-Cripto axis,[49] which was shown to be effective in treating melanoma using preclinical PDX mouse models.[107] Multiple clinical trials have been carried out to test inhibitors for depleting or reprogramming TAM for cancer treatment.[108] The application of ADH-503 could lead to the repolarisation of TAM and thereby enhance the dendritic cell responses in pancreatic cancer,[109] and the application of clodronate encapsulated in liposomes (clodrolip) has led to significant depletion of TAM, yielding potent inhibition of tumour growth.[110] Clinical trials have been carried out for BMS-813160, a small molecule inhibitor of CCR2/5, to evaluate its efficacy in treating several solid tumours including hepatocellular carcinoma, non-SCLC, renal cell carcinoma and pancreatic ductal adenocarcinoma by combining it with nivolumab and/or the tumour vaccine GVAX.[111]

CTC-RBC cluster

Clinical studies and drug development targeting CTC-RBC clusters remain unexplored. Research findings suggest Galectin-4 might be a promising target.[69] Since CTC-RBC clusters are implicated in the formation of cancer-associated thrombosis (CAT), low-molecular-weight heparin (LMWH) might be useful in reducing CAT and suppressing cancer metastasis.[112] Although multiple clinical trials have been conducted using drug candidates targeting LMWH, the clinical benefits remain controversial.[112 113]

In summary, the mechanistic and functional characterisations of homotypic and heterotypic CTC clusters may unveil innovative therapeutic avenues for effectively targeting these potent metastatic precursors (table 2). These findings warrant additional experimental and clinical validations to advance our understanding and treatment of metastasis.

CONCLUSION

The haematogenous spread of CTCs is characterised by its inefficiency, given the fact that CTCs often face formidable obstacles in the bloodstream. Consequently, the aggregation of CTCs into clusters, including the homotypic and heterotypic CTC clusters, has shown to be a potent means to enhance metastatic potency. The advancement of microfluidic isolation technologies presents powerful tools for the efficient isolation and ex vivo culturing of CTC clusters, facilitating subsequent molecular and functional assessments. The CTC clusters exhibit remarkable heterogeneities as they interact with various blood cell types during the transition in circulation, including neutrophils, CAFs, MDSCs and RBCs. While each heterogeneous cluster may serve a distinct function, collectively, they promote CTC growth, invasion and metastatic colonisation. Exploring the mechanistic aspects of CTC cluster–blood microenvironment interactions may hold great promise in deciphering novel mechanisms of cancer metastasis and uncovering novel strategies for tailored antimetastasis treatments.

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