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Tumor-Initiating Cells: Emerging Biophysical Methods of Isolation

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Abstract The discovery and subsequent isolation of tumor-initiating cells (TICs), a small population of highly tumorigenic and drug-resistant cancer cells also called cancer stem cells (CSCs), have revolutionized our understanding of cancer. TICs are isolated using various methodologies, including selection of surface marker expression, ALDH activity, suspension culture, and chemotherapy/drug resistance. These methods have several drawbacks, including their variability, lack of robustness and scalability, and low specificity. Alternative methods of purification take advantage of biophysical properties of TICs including their adhesion and stiffness. This review will provide a brief overview of TIC biology as well as review the most important methods of TIC isolation with a focus on biophysical methods of TIC purification.

Keywords Cancer stem cells · Tumor-initiating cells · Isolation and purification · Tumorigenicity · Stemness · Adhesion

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Introduction

Increasing numbers of parallels are being drawn between cancer and stem cell research. Until recently, cancer progression was described using mainly the clonal evolution model [1–3] which postulates that cancers evolve by a repeating process of clonal expansion, mutation, and selection. As cancer progresses, different mutations accumulate in clones within the tumor and selective pressure leads to the survival of some clones and the extinction of others in a manner similar to the Darwinian natural selection. Within this model, all cancer cells have the ability to rapidly divide and give rise to a new tumor [3]. A growing body of data supports an alternative view of cancer, dubbed the cancer stem cell (CSC) model. In contrast to the clonal evolution model, the CSC model proposes a hierarchical organization of cells in which a small population of tumor-initiating cells (TICs) is capable of self-renewal into more TICs and "differentiation" into bulk cancer cells. As the name suggests, TICs are defined by their unique ability to initiate new tumors, whereas other cancer cells cannot, but also display distinct marker expression profiles, chemotherapy/drug resistance, and biophysical properties [4–7] (Table 1).

TICs are thought to be responsible for the maintenance, progression, recurrence, and metastasis of cancer [29, 30]. Often, their higher propensity to be drug resistant allows TICs to survive conventional therapies and leads to drug-resistant cancer relapse and metastasis development [31–33]. It is for this reason that targeting TICs in cancer therapy has attracted such excitement from the field [1, 34, 35]. However, TICs are usually rare populations within a tumor and their purification has proven challenging, even after in vitro culture. Efficient isolation and enrichment of TICs would facilitate their study and the development of drugs that selectively target them. This review will give a brief overview of the most

Table 1 Characteristics of tumor-initiating cells (TICs)

Property	Description	Reference
Tumor initiation	TICs have the capacity to form tumors that resemble the tumor of origin in immunodeficient hosts	[8–10]
Drug/stress resistance	An increased resistance to stresses including hypoxia, radiation, chemotherapy, and treatment with other cancer drugs has been observed in TICs. This has been party attributed to an enhanced DNA damage response as well as more effective clearance of cytotoxic agents from the cell	[11–14]
Surface marker expression	Surface marker expression levels are frequently used as tools for TIC purification. The markers vary widely among cancer types	[8, 15–17]
High ALDH activity	ALDH activity is increased in TICs which results in protection from ROS damage and increased survival	[18, 19]
Sphere formation	TICs have an increased ability to grow and form spheroids in suspension culture	[20, 21]
Pluripotent gene activation	The expression of pluripotent genes such as Oct4 and Nanog is increased	[22, 23]
Unique metabolic activity	Higher mitochondrial membrane potential, lower quantity of mtDNA, and lower i ntracellular concentration of ATP and ROS have been observed in TICs	[24]
Changed cell adhesion	The expression of adhesion proteins such as integrins is dysregulated, resulting in a changed cell adhesion profile	[25, 26•]
Decreased cell stiffness	Decreased cell stiffness and increased deformability have been observed in TICs	[27]
Differential Hoechst 33342 staining	The increased activity of the ABC transporter results in differential staining of TICs by Hoechst 33342, allowing for isolation by side population (SP) staining	[28]

important aspects of TICs and will focus on methods of TIC isolation, purification, and enrichment. Several excellent reviews have discussed marker-expression-based approaches for TIC isolation, including surface protein expression- and ALDH-based methods [36–39]. This review will therefore only briefly mention these approaches and focus on the biophysical methods of TIC isolation.

It is important to distinguish between the cancer cell of origin (CCO) that initiates a tumor and the CSCs/TICs that sustain it, as they may not necessarily be related [40]. The CCO is the original cell that accumulates the first genetic mutations that lead to cancer. While the CCO is involved in the initiation of the primary tumor, CSCs/TICs are involved in the maintenance of this tumor and the initiation of secondary ones [41]. The terms CSC and TIC are often used interchangeably to denote cancer cells that can self-renew to make more of themselves as well as "differentiate" into bulk cancer cells [42]. As mentioned previously, these cells are often referred to as cancer stem cells because of their similarities to somatic stem cells and tumor-initiating cells because they are able to initiate tumors in immunocompromised mice [43].

Controversies and the Evolving CSC Model

The field has been plagued by controversy surrounding the existence and the properties of TICs, with many still doubting the existence of these cells [7, 44]. This debate has been caused in part by the use of the term "cancer stem cells," which suggests that they are derived from somatic stem cells. TICs can develop from normal somatic stem cells as well as progenitors and perhaps even terminally differentiated cells [4, 45–47]. Furthermore, TICs are referred to as cancer stem cells because the definition of a stem cell is a cell that can both self-renew and differentiate, both of which a TIC can do [44, 48]. Nevertheless, to avoid confusion, they will be referred to as TICs in this review.

Other controversies stem from reports that the percentage of TICs within a tumor varies widely, sometimes accounting for a small fraction, whereas other times, the vast majority of cancer cells have the ability to reinitiate tumors [7, 49]. Several studies have suggested that the melanoma TIC frequency varies from around 2 % to greater than 40 % [2, 50, 51]. In addition to demonstrating the vast variability in TIC frequency, these studies challenge the idea that only a small population of cells within a tumor are TICs. Regardless, populations of cells with TIC properties have been identified in a variety of cancers including those of lung [52], ovarian [53], brain [8], breast [9], colon [54], and prostate [55] origins which have the signature TIC characteristics outlined above.

Although useful, the original CSC model for cancer progression has evolved over the years. Current evidence suggests an intermediary progenitor state in between the TICs and the differentiated cancer cells. While TICs are quiescent and self-renewing, progenitors, sometimes called transit-amplifying cells, rapidly proliferate and have a limited self-renewal capability [6, 56]. Notably, the CSC model and the clonal evolution models are not mutually exclusive but rather extremes in a spectrum into which most tumors fall. While there is a hierarchy of cancer cell phenotypes, there is also clonal selection within the TIC population, with different clones evolving in parallel and experiencing selection [6, 57]. Furthermore, it seems that differentiated cancer cells

can dedifferentiate and go back to a TIC state, although how often this happens is not known [1, 57, 58]. A new report also suggests the existence of several TIC states in breast cancer, including mesenchymal quiescent TICs that are CD44^{high}/CD24^{low}, a phenotype associated with TIC phenotype [59], epithelial proliferative TICs that are positive for the TIC marker aldehyde dehydrogenase (ALDH), and a double positive TIC population that is even more tumorigenic [1, 60•].

Relationship Between EMT and TICs

The epithelial-mesenchymal transition (EMT) is an important process during embryogenesis which allows polarized epithelial cells to transdifferentiate into migratory and invasive mesenchymal cells [61, 62]. This process is also activated during cancer progression and is believed to be crucial driver of metastasis, enabling cells to migrate away from the primary tumor to secondary sites [63, 64]. EMT has been correlated with the acquisition of TIC properties [65–67]. Activation of EMT leads to the expression of TIC markers, increased ability to grow in suspension, and higher tumorigenesis in vivo [66]. However, recent studies have challenged the perception that EMT is crucial for cancer progression and TIC phenotype: one study showed that only some TICs undergo EMT, while others retain their epithelial phenotype [60•], while another study showed that EMT inhibition does not affect lung metastasis [68]. The exact relationship between EMT and TICs, whether EMT activation results in TIC phenotype or just promotes it, and whether all TICs undergo EMT remains to be elucidated. What has been shown is that drivers of EMT such as Slug, Snail, and the Wnt pathway are implicated in the acquisition of TIC characteristics [69, 70•]. Conversely, forced expression of pluripotency genes such as Oct4 and Nanog in breast cancer cells leads to the upregulation of Snail, Slug, and mesenchymal markers such as N-cadherin, whereas CD44^{high}/CD24^{low} cancer cells have activated EMT markers and a fibroblast-like morphology [63]. It may be that EMT facilitates TIC phenotype acquisition but is not necessary for it. A more in-depth discussion of this relationship can be found elsewhere [65, 69].

Relationship Between CTCs and TICs

Circulating tumor cells (CTCs) are a population of cancerous cells that have been shed into the vascular or lymphatic systems [71, 72]. A significant amount of research has been done on CTCs since they can be used as a tool for cancer prognosis and other clinical applications [72–74]. Although they have been reported to be more aggressive than other cancer cells [75, 76], it is important to note that not all CTCs are TICs but rather a fraction of them are [71, 74, 77, 78]. The CTCs that do not have the plasticity characteristic of the TIC phenotype are not capable of forming secondary tumors [74], suggesting that a cell needs to have properties of both CTCs and TICs in order to successfully metastasize [71]. A significant number of systems for CTC detection and isolation have been developed as discussed elsewhere [71, 74]. However, many of them rely on specific CTC markers, such as EpCAM, which may not be expressed in all CTCs [1, 71]. Therefore, there is still a significant need for the development of robust platforms to purify TICs.

Methods of TIC Purification

Many different methods of TIC purification have been developed to exploit unique attributes in these cells. Common methods of enrichment include surface marker-based purification and isolation based on TIC intrinsic functional markers, such as ALDH expression, reactive oxygen species (ROS) levels, flow cytometric side population (SP) analysis, and mitochondrial membrane potential differences. Many of these purification platforms rely on probes such as antibodies and separation technologies such as flow cytometry and magnetic beads. Although popular, these methods have several drawbacks including high price, non-specificity, inability to scale-up, and lack of robustness, which have led to the recent development of biophysical methods of TIC purification based on differences in adhesion, stiffness, and niche/scaffold affinity. The methods of TIC enrichment are summarized on Fig. 1.

Marker-Based Purification

Surface Markers

TICs have been identified in many types of solid tumors based on their expression of surface markers (Table 2). Various surface markers continue to be identified; however, no universal marker exists. Instead, TIC surface markers appear to be tissue specific and may vary among different tumors requiring extensive validation. Moreover, even well-validated markers such as CD133 seem to fail to specifically identify TICs in certain applications [101, 102]. In spite of their limitations, surface markers are widely used for TIC purification, with some groups developing non-antibody-based aptameter probes [103, 104]. Many of the developed markers are conjugated with fluorescent labels and used in combination with techniques such as fluorescence-activated cell sorting (FACS) [105] and magnetic-activated cell sorting (MACS) [33] for isolation. More information regarding surface marker-based purification can be found in recent reviews [15, 16].

Intrinsic Functional Markers

ALDH ALDHs are a family of enzymes that play a role in the metabolism of aldehydes [106]. Studies dating more than a

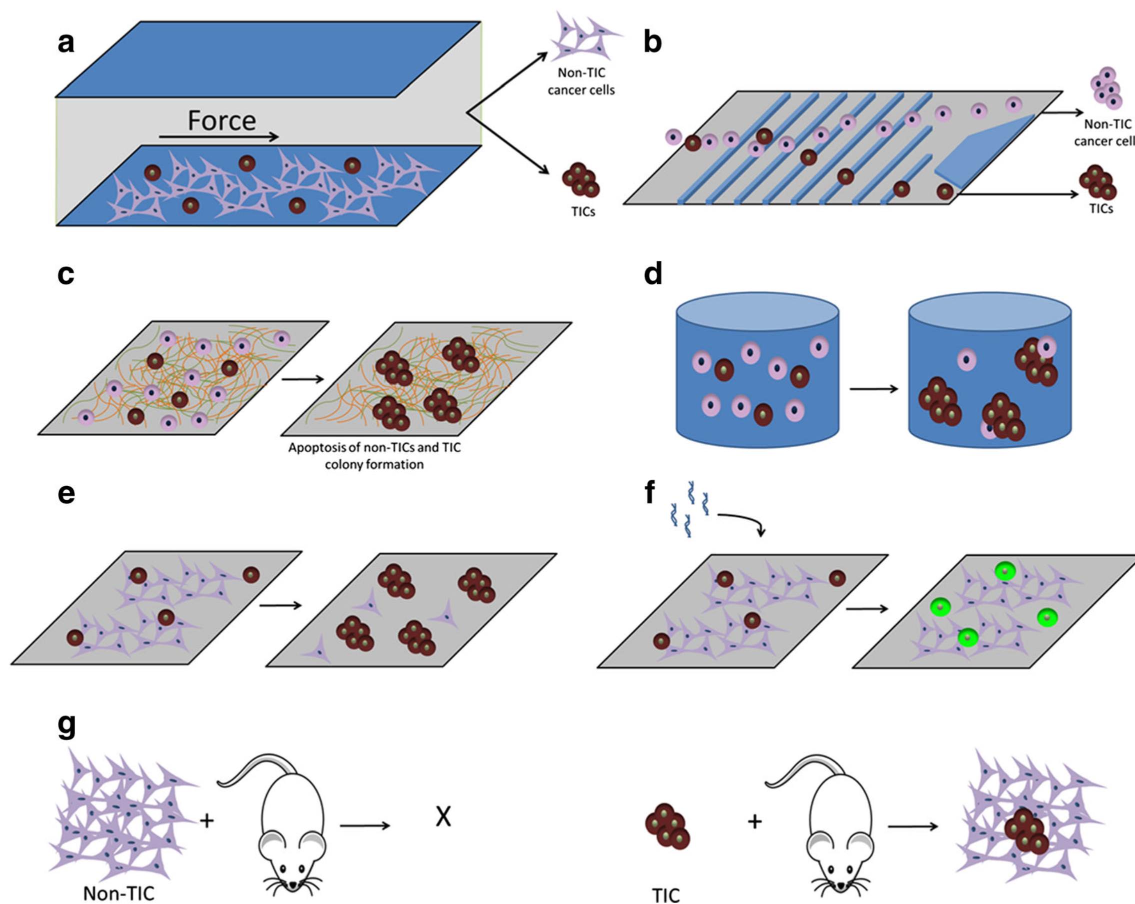

Fig. 1 Methods of TIC purification, isolation, and enrichment. **a** Differential adhesion: Cells can be separated from surfaces based on their adhesion strength by applying defined amounts of force. **b** Differential stiffness: Cells are flowed through a microfluidic channel with diagonal ridges that interact with flowing cells. More deformable cells travel perpendicular to the ridges while stiffer cells attempt to travel parallel to them. **c** Niche mimicking/biomaterials: Cells are introduced into a biomaterial or plated on a coated surface that mimics some property of the TIC niche which leads to enrichment of TICs and/or apoptosis of non-TICs. **d** Suspension culture: Cells are grown in suspension culture. TICs have an enhanced ability to grow in these conditions resulting in their enrichment. **e** Drug resistance: Culture is treated with a drug, which results in the apoptosis of non-TIC cancer cells. **f** Reporter-based selection: A reporter line is made with a fluorescent protein under the control of a TIC marker (e.g., pOct4-GFP). **g** In vivo tumorigenesis: Cells are introduced into an immunocompromised mouse. Only TICs have the ability to form tumors at low doses

Table 2 Common tumor-initiating cell surface markers

Tumor type	Markers	Reference
Breast	$CD44^+/CD24^{low}$, $CD133^+$, $CD44^+/CD176^+$, ESA^+ ($EpCam^+$), $CD24^+/CD29^+$, $CD24^+/CD49f^+$	[9, 79–81]
Colorectal	$EpCAM^{high}/CD44^+$, $CD133^+$	[82, 83]
Liver	$CD90^+$, $CD44^+/CD176^+$, $CD133^+$, $CD13^+$	[80, 84–86]
Pancreatic	$CD44^+CD24^+ESA^+$, $CD133^+$ $CXCR4^+$	[33, 87]
Ovarian	CD133+, $CD44^+CD117^+$, $CD24^+$	[88–90]
Prostate	$CD44^+/\alpha_2\beta_1{}^{hi}/CD133^+$	[55]
Bladder	$CD44^+CK5^+CK20^-$	[91]
Lung	$CD176^+$, $CD133^+$, $CD44^+$	[80, 92, 93]
Brain	$CD133^+$, $SSEA\text{-}1^+$	[8, 94]
Melanoma	$CD20^+$, $CD166^+$, $CD133^+$, $ABCB5^+$	[51, 95–97]
Gastric	$CD44^+$, $CD133^+$	[17, 98]
Osteosarcoma	$CD133^+$, $CD117^+$, $Stro\text{-}1^+$	[99, 100]

decade have described a correlation between high levels of ALDH and stemness and succeeded in isolating hematopoietic stem cells based on ALDH activity [107–109]. More recently, high ALDH levels have been associated with other stem cell types and with TICs [18, 19, 110, 111]. Importantly, overexpression of some members of the ALDH family has been shown to be predictive of poor clinical outcome [112, 113]. This might be in part due to the role ALDH plays in drug resistance development, as these enzymes have been shown to help protect cells from ROS damage [114]. It seems that ALDH overexpression is a characteristic that TICs from different tissues share [111], making ALDH detection kits extremely useful for TIC isolation. Nevertheless, ALDH-based isolation is not capable of detecting all TICs, as even $ALDH^{low}$ fractions of cancer cells can lead to the formation of tumors in vivo [115]. In fact, recent studies suggest that tumors may have a subpopulation of TICs that are not characterized by the $ALDH^{high}$ phenotype [60•]. These developments underscore substantial limitations with ALDH-based purification of TICs.

Other Functional Markers Other functional markers for TICs include ROS levels [116], SP analysis by Hoechst 33342 staining [117], and differences in mitochondrial membrane potential [24]. Of these three methods, the SP exclusion is the most widely used. Although easy and simple to perform, SP exclusion can be culture condition dependent, since the staining conditions influence the number of positive cells and have low specificity [118]. More information on these methods can be found in excellent reviews [37, 119].

Biophysical Methods of Purification

Adhesion-Based Purification

The interaction between cells and their extracellular environment is of critical importance to normal development and function [120, 121]. Integrin receptors mediate this interaction by mechanically coupling to an ECM ligand, associating with the actin cytoskeleton and clustering together [122]. These interactions may strengthen to give rise to focal adhesions, which function as structural links between the cell's cytoskeleton and the surrounding ECM [123]. Abnormal integrin function can lead to a variety of diseases, including cancer [124]. Other proteins involved in cell adhesion and focal adhesion complex formation such as focal adhesion kinase (FAK) [125, 126] are also dysregulated in cancer cells and, along with integrins, contribute to disease progression and metastasis. Integrins in particular are often upregulated in many types of cancer, and the levels of several integrin subtypes are prognostic of disease severity [124]. In addition to having important roles in cancer cell survival, migration, and invasion, integrins have been shown to cooperate with oncogenes to increase tumorigenesis [124].

Given that integrins and other important cell adhesion proteins are upregulated in cancer, it follows that cancer cells might bind to the ECM with a different amount of force than normal cells. A study by Kwon et al. supports this premise, as they were able to separate breast cancer cells from normal mammary cells based on adhesion forces. MCF7 breast cancer cells and MCF10A human breast epithelial cells were introduced into microfluidic channels, allowed to attach to gelatin-coated surface, and differentially detached using shear fluid forces [127]. A limitation of this study, however, is that only cell lines are used which might not recapitulate the behavior of primary cancer cells.

Cell adhesion proteins are also important for normal development and embryogenesis [128]. Integrins are essential for stem cell homing to their niche during embryogenesis and development [129]. Several studies have shown the feasibility of separating stem cells based on their adhesion levels and integrin expression profiles [130, 131, 132•]. For example, our group recently demonstrated different levels of integrin expression in pluripotent stem cells (PSCs) and their differentiated progeny [132•]. These differences resulted in differential ECM adhesion strengths that could be exploited to isolate PSCs from a mixture of differentiated and partially reprogrammed cells. Furthermore, these results suggest that upon differentiation, integrin expression levels change resulting in varying adhesion strengths to ECM proteins.

Given the vital role that adhesion proteins play in normal stem cell function, it is expected that they also play important roles in TICs. In fact, many adhesion proteins have been shown to promote TIC stemness. FAK ablation results in a depletion of the TIC population [133]; integrin $\alpha v\beta 3$ regulates expression of TIC marker CD44 [134] and is necessary to drive stemness and EGFR inhibitor resistance for epithelial cancers [135]; and integrin $\alpha 6\beta 1$ knockdown results in loss of stemness in TICs [136, 137]. In addition to simply being overexpressed in TICs, adhesion proteins potentiate TIC function and enable tumor propagation and drug resistance [138]. The close relationship between integrins and TICs is reviewed elsewhere [138].

Abnormal expression and function of the cell-ECM adhesion apparatus in TICs have allowed groups to isolate them based on their adhesion dynamics. Bansal et al. isolated prostate TICs from other cancer cells by allowing them to bind to collagen-coated surfaces for a short period of time. They found that TICs bound faster, and by washing away other cells, cancer cells with stem-like properties could be enriched [25]. Zhang et al. applied the same idea in a more controlled manner to isolate breast cancer TICs. They used a microfluidic platform to slowly flow cells through a channel coated with selected ECM proteins. Since the TICs interact more rapidly with the ECM, they became trapped in the channel, while other cells flowed through [26•].

It is important to note that adhesion strength separation can be done by exploiting differences in the rates at which cells bind to the substrate or differences in the amount of force required to detach adherent cells. When exploiting differences in binding rate, the cells are slowly rolled over a coated surface and some will bind faster that others allowing for separation. On the other hand, when exploiting differences in detachment force, cells are allowed to reach some level of adhesion strength with the ECM and then forces are applied to separate them. Although this might not seem like a major difference, the two assays measure different cell properties which may vary independently: the first, how fast cells can form adhesion complexes and the second, how strongly these adhesion complexes bind to the ECM. Thus far, only differences in binding speed have been exploited to isolate TICs. Separation based on detachment force might prove more useful since it is generally more scalable and robust.

Stiffness-Based Purification

Cell elasticity (i.e., stiffness) is another important mechanical property of cells, and it is modulated by the cytoskeleton which is comprised of actin filaments, microtubules, and intermediate filaments [139]. Cells are capable of regulating their elasticity by regulating the composition and organization of their cytoskeleton in response to internal and external cues [139, 140].

Cell elasticity has been shown to be increased in cancer cells from a variety of tissues including those from breast, pancreatic, and ovarian origin [139, 141, 142]. Other studies have shown that the trend of decreased stiffness for cancer cells might not hold for cells from different tissues. In contrast to other cancer cells, brain cancer cells appear to be less mechanically compliant than non-cancerous cells [143]. In spite of this, the differences in cell stiffness between cancer cells and benign cells present the opportunity for stiffness-based separation of cancer cells. Several microfluidic platforms have been developed for this application. The platforms use diagonal ridges [144] or triangular posts [145] to force cells to deform as the flow through the channel. The cells deform to varying degrees depending on their elastic properties and travel differentially through the channels to different outlets.

Cell elasticity has been correlated to the metastatic potential of cells, with cells that are more elastic having higher invasive and migratory behaviors [142, 146–148]. It is speculated that the lower stiffness allows cells to squeeze through and migrate to other parts of the body. The activation of the EMT program in tumor cells would also make them more elastic, since the loss of adhesion and stiffness are hallmarks of EMT [149]. Interestingly, both the activation of the EMT program as well as metastatic potential correlate closely with TIC phenotype [150], suggesting some of the highly metastatic elastic cells studied above might have been TICs. Babahosseini et al. used atomic force microscopy (AFM) to directly show that an enriched TIC population is at least 45 % softer than other cancer cells [27]. Isolation of TICs should thus become feasible by using one of the stiffness fractioning microfluidic platforms mentioned earlier. One study suggests the feasibility of this approach. Highly elastic breast cancer cells were isolated and shown to be enriched for the $CD44^{high}/CD24^{low}$ TIC phenotype. They were also tested for mammosphere formation, an ability unique to TICs [151•]. Although encouraging, the study has severe limitation, since it does not include any primary tissue nor does it test the cells for tumor formation capacity in vivo.

Niche/Biomaterial-Interaction-Based Purification

Similar to somatic stem cells, TICs have a niche which regulates their differentiation and self-renewal and protects them from the host's immune system [152–154]. Under certain conditions, TICs will home to normal stem cell niches and hijack them [155, 156]. A variety of methods for TIC enrichment have been developed which take advantage of their preferential adhesion or homing into specific scaffolds or topographies. A study by Tan et al. demonstrated that breast TICs preferentially bind to a specific nanotopography, characterized by thinner grating. A significant enrichment was seen in the $CD44^{high}/CD24^{low}/ESA^{+}$ phenotype when MCF7 cells were cultured in a nanopatterned surface with thin gratings as opposed to unpatterned surfaces and other patterned designs [157]. However, only a slight difference was seen in the $CD44^{high}/CD24^{low}$ population. It is difficult to determine which TIC phenotype was enriched for since no functional testing was done on the cells and only low levels of ESA expression are associated with TICs, whereas ESA^{high} is seen in luminal breast cancer cells [158]. More testing is therefore needed to assess the efficacy of nanopatterned surfaces for TIC enrichment.

Another strategy involves culture within three-dimensional scaffolds to enrich for TIC populations. Different scaffolds have been used in this context including chitosan-alginate scaffolds for glioblastoma [159] and hepatocellular carcinoma [160] TIC enrichment and collagen scaffolds for breast [161] and liver [162] TIC enrichment. In these examples, cancer cells infiltrate the scaffold and are maintained within them. The scaffold modulates cancer cell behaviors, resulting in an enrichment of cells with TIC markers, more resistance to chemotherapy/cancer drugs, and higher tumorigenesis in vivo. Since this approach results in both enrichment of TICs and mimicking of in vivo environment of cancer cells, it might prove to be a useful tool for modeling in vivo behavior and screening new therapies.

Other groups have gone a step further and attempted to model the TIC niche in order to isolate this population of cancer cells [162, 163]. Polyelectrolyte multilayers (PEM)

nanofilms were used to fabricate microenvironments that simulated hepatocellular carcinoma TIC niches. Cells were then seeded on top of the PEM layer. After being cultured for 7 days, 70 % of the cells were positive for CD44/CD133 TIC markers. This enrichment was due to cell death of non-TICs, presumably because the PEM/hyaluronic-acid-based environment mimics a TIC-niche topographical cue which is not conductive for their survival and resulted in a population that was more resistant to chemotherapy agents. Further in vivo testing of the enriched populations is critical to validate this enrichment strategy.

An exciting development is a platform for in vivo capture of early metastatic cells or CTCs [164•]. Poly(lactide-co-glycolide) scaffolds were implanted in immunodeficient NSG mice that had undergone tumor inoculation 7 days beforehand with a highly metastatic variant of the MDA-MB-231 breast cancer cell lines. Cells not only homed to the scaffold but the tumor burden on common metastatic sites was decreased fivefold, suggesting that cancer cells and TICs were being trapped in the scaffold and prevented from migrating elsewhere in the mouse. A light-scattering technique could then be used to detect the cells in the scaffold in a label-free manner. Although this approach does not directly recruit TICs, one could envision modifying the scaffold to facilitate the homing of TICs. Therapeutically, this strategy could serve to deplete the remaining TIC population after initial treatment and reduce the probability of cancer metastasis and relapse.

All these approaches appear to enrich the TIC population by providing topological or adhesive cues that either decrease the survival of non-TICs or increase the amount of cells that remain in the TIC state. Label-free, reproducibility, and relative ease of use are major advantages of these systems. Furthermore, most of them mimic elements of the in vivo environment of cancer cells, which can be useful for other applications such as disease modeling. Nevertheless, a major disadvantage might be the broad applicability of these platforms to different types of cancer. If they indeed work by mimicking the TIC niche, those niches might vary among cancers of different tissues of origin.

In summary, biophysical methods for TIC isolation and enrichment offer several advantages over marker-based approaches. The label-free nature of biophysical methods leads to more objectivity as there is no bias towards a marker, less manipulation of the cells, and potentially lower costs. Furthermore, some of these methods are more easily scalable since they can be performed in large groups of cells instead of requiring each cell to be passed through a flow cytometer one at a time. Nevertheless, the biophysical properties of cells, including adhesion and stiffness, change when EMT is activated. If there indeed are both epithelial and mesenchymal TIC states [1, 60•], then these approaches might not be able to isolate them both at the same time.

Other Methods of Purification

Suspension Culture

It has become increasingly common to use suspension culture to isolate TICs. The spheres that form in these conditions express many of the TIC surface markers, have higher ALDH expression, and are drug resistant [20, 21, 165]. Although widely used, this system has several drawbacks. First, whereas the spheres do have some true TICs, many of the cells are differentiated progeny or progenitor cells, which are also able to form spheres, and contain a necrotic core [37, 166, 167]. There is also a large variation in the percentage of TICs within a sphere, partly caused by the variation in methodology that is employed, which may cause conflicting results and make cross comparison difficult among labs [166, 168, 169]. Some groups have attempted to encapsulate the spheres in a liquid core hydrogel, but the TIC enrichment remains fairly low at 25–30 % [170]. Finally, suspension culture might not be able to detect quiescent TICs [168]. Some groups have even reported better results at isolating pure TIC cell lines by using adherent culture with defined media [167]. While useful in some situations, suspension culture has major drawbacks and attention should be paid to the methodology that is used.

Drug Selection

In cancers that do not go into remission or which relapse after what appears to have been successful treatment, TICs have been shown to be chemoresistant [69, 171–173] as well as resistant to other cancer drugs [11, 174] and even radiotherapy [12]. Moreover, treatment with drugs such as tamoxifen [11], temozolomide [175], gemcitabine [176], trastuzumab [177], and staurosporine [178] results in the enrichment of cancer cells with TIC properties [13]. A growing body of literature suggests that this drug resistance allows TICs to survive conventional cancer treatments and leads to relapse and metastasis [69, 172]. The exact mechanism of drug resistance is not known, but it probably involves a combination of ABC transporter expression, increased ALDH activity, and enhanced DNA damage response, quiescence, and activation of other key signaling pathways [173]. The EMT process has also been associated with drug resistance acquisition [69, 179]. Several groups have taken advantage of this property to enrich TIC populations by treating the whole population of cells with chemotherapy or other cancer drugs [180–182]. This leads to the death of normal cancer cells and enrichment of the TIC population. However, there have been reports of cancer drug treatments changing the gene expression profiles of cancer cells [177, 178]. The changes in expression profiles could be due to changes in the relative amounts of TICs to non-TICs as enrichment proceeds, but it could also be due to the process altering the cells themselves. If the cells are changing their

gene expression in response to drug selection, this would present a major limitation since it would mean this method could not be used to isolate naïve TICs that have not been altered by drug selection.

Reporter Genes

Pluripotency genes such as Oct4 and Nanog are expressed in TICs [22, 23]. This has led to the development of TIC reporter systems in which cancer cells expressing Oct4 [183] or Nanog [184] express fluorescent proteins such as GFP. Interestingly, some of the procedures for reporter line derivation have caused the cells to get stuck in a TIC-like state in which the cells express TIC surface markers, have higher tumorigenesis and cancer drug resistance, and cannot exit the TIC state [183]. Although these reporter lines are useful in vitro systems, developing these lines is time intensive and genetically modifies the cells, which may impact other cells functions and behaviors. Furthermore, this technique is not applicable when attempting to purify or enrich cells from primary human tumors.

In Vivo Tumorigenesis

By definition, TICs are characterized by their ability to form de novo tumors that resemble the primary tumor following transplantation [5, 8, 9]. A gold standard for the characterization and identification of TIC populations is their ability to form tumors in serial immunodeficient hosts. In contrast to TICs, differentiated cancer cells cannot give rise to a tumor and progenitor cells are not able to form tumors in serial hosts. Although it has not been shown that a single TIC can give rise to an entire tumor, significantly lower numbers of TICs are required to do so relative to bulk cancer cells. This limitation, however, might result from our technical inability to get completely pure populations of TICs and not a functional inability of single TICs to give rise to tumors. Although a gold standard, protocols and animal models for in vivo tumorigenic evaluation of TICs vary in the literature and a consensus has not yet been reached [7].

Conclusions and Future Directions

TICs are a unique population of cells within tumors capable of establishing new tumors which drive cancer relapse and metastasis. While usually rare, TICs are resistant to chemotherapy and hypoxic environment. In vitro, they are detected by their expression of defined surface markers, ALDH activity, growth in suspension culture, drug resistance, and tumorigenesis in immunocompromised mice. Efficiently isolating TICs will be essential in order to diffuse the debate surrounding the existence and importance of TICs as well as to better understand their biology and develop new drugs that selectively target them.

Much effort has gone into identifying appropriate surface markers for TICs, which vary widely according to tissue of origin. It is important that other methods, such as the ones described in the review, are studied as well in order to develop more reproducible and robust methods for TIC purification.

In summary, the development of the CSC model has revolutionized the cancer research field. Although the model continues to evolve, an impressive amount of data supports the existence of TICs in many cancers. Isolating them will be of pivotal importance to effectively treat and eradicate cancer.

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Compliance with Ethical Standards

Conflict of Interest Efraín A. Cermeño has a pending patent on Adhesion-Based Separation of Cancer Cell Sub Populations for Cancer Diagnostics.

Andrés J. García is an inventor in the patent portfolio associated with the uSHEAR technology licensed to CellectCell, and he is a co-founder and member of the Board of CellectCell, Inc.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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