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Metastatic potential of tumor-initiating cells in solid tumors

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1. ABSTRACT

The lethality of cancer is mainly caused by its properties of metastasis, drug resistance, and subsequent recurrence. Understanding the mechanisms governing these properties and developing novel strategies to overcome them will greatly improve the survival of cancer patients. Recent findings suggest that tumors are comprised of heterogeneous cell populations, and only a small fraction of these are tumorigenic with the ability to self-renew and produce phenotypically diverse tumor cell populations. Cells in this fraction are called tumor-initiating cells (TICs) or cancer stem cells (CSCs). TICs have been identified from many types of cancer. They share several similarities with normal adult stem cells including sphere-forming ability, self-renewability, and expression of stem cell surface markers and transcription factors. TICs have also been proposed to be responsible for cancer metastasis, however, scarce evidence for their metastatic potential has been provided. In this review article, we have attempted to summarize the studies which have examined the metastatic potential of TICs in solid tumors.

2. INTRODUCTION

Earlier findings from the Dick's laboratory have led to the proposal of a new concept for the origin of cancer; using human acute myeloid leukemia (AML) cells they demonstrated that tumors consists of heterogeneous cellular populations, of which, only a small fraction, called tumor-initiating cells (TICs) or cancer stem cells (CSCs), have the ability to initiate tumors (1, 2). Comparisons between stem cells from normal haematopoietic and leukemic tissues successfully demonstrate their phenotypic similarities including stem cell surface marker profiles and the capabilities of proliferation, differentiation, and self-renewal (2). These results suggest that normal haematopoietic primitive cells are the targets for leukemic transformation and that TICs originate from the transformation of normal stem cells. However, there might be alternative mechanisms that give rise to CSCs; cancer cells could acquire stem cell properties via de-differentiation or by fusion with progenitor cells, although there is no evidence for these mechanisms (3, 4). Thus, the origin of TICs remains unresolved.

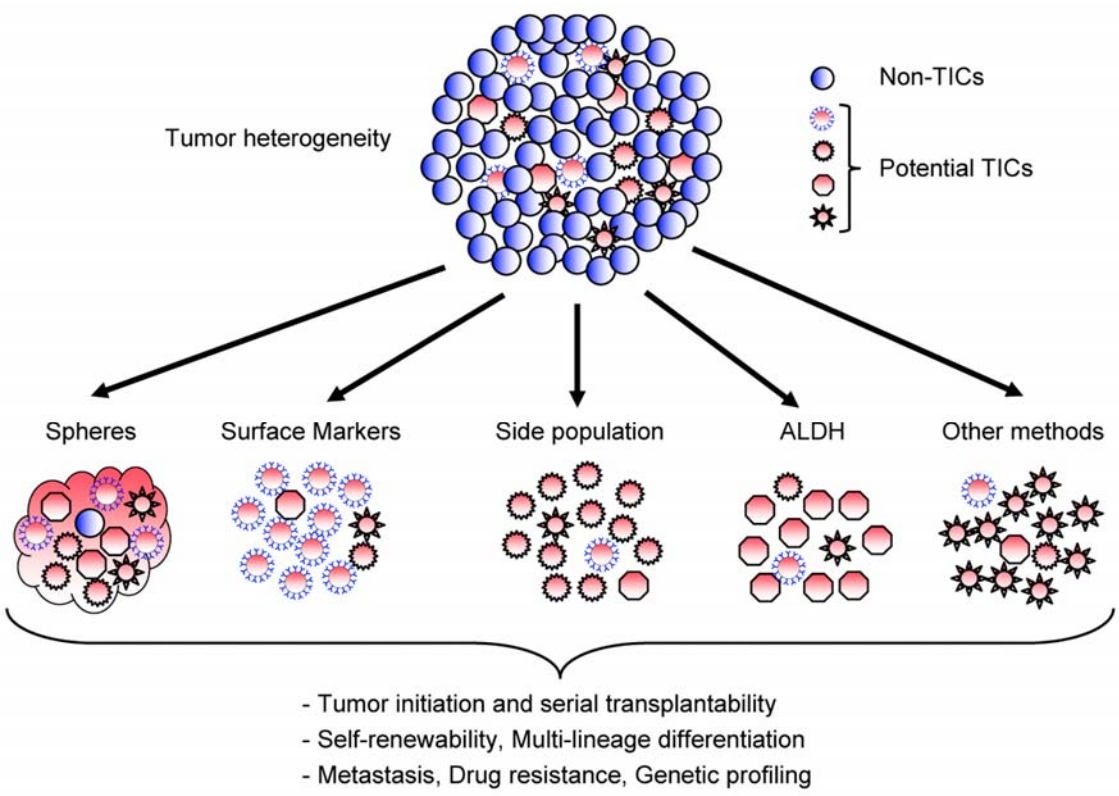

Figure 1. Schematic diagram showing tumor heterogeneity and methods for enrichment of tumor-initiating cells (TICs).

Compared to the haematopoietic system, cell surface markers or functional assays for identifying and evaluating normal adult stem cells from other tissues are underdeveloped. Further, cells within solid tumors are less accessible than those from haematologic malignancies. Nonetheless, for the last several years, TICs have been identified from solid tumors of diverse origins, such as breast (5), brain (6), colon (7), pancreas (8), prostate (9), lungs (10, 11), ovaries (12), liver (13), and bone and soft tissues (14-18). In this review, we have considered cells as TICs, only when isolated cells were shown to initiate tumors *in vivo* as compared with other cellular populations or in studies that used already established methods for TIC isolation. Findings from these studies confirm that TICs share many properties with normal adult stem cells including self-renewability, multi-lineage differentiation potential, sphere formation, and the expression of genes related to stem cell maintenance and proliferation (19, 20). Another important property observed in both normal adult stem cells and TICs is the ability to efflux Hoechst 33342 and rhodamine dyes, a property that is used to identify a subpopulation of cells, called the side population (SP) (21, 22). This property is mainly mediated by ATP-binding cassette (ABC) transporters and confers a drug-resistance phenotype. The SP cells in many types of cancer have been shown to be enriched in tumorigenic stem-like cancer cells (23).

TICs have also been proposed to be responsible for cancer metastasis. This is mainly due to the following reasons: 1) TICs are believed to possess an increased ability to survive and grow in a foreign environment (24, 25); 2) if TICs are the only population that can initiate tumors, tumor formation at secondary sites should be initiated by the TICs (26); and, 3) cancer cells use the same molecular machinery for invasion and metastasis as normal stem cells do for homing or mobilization (27-31). Direct evidence for metastatic potential of TICs, however, is just beginning to emerge. In this review, we focus on the recent studies which provide evidence of the metastatic property of TICs with the hope of understanding the mechanisms behind cancer metastasis as well as accelerating the development of novel therapies that target metastatic cancer.

3. METHODS TO ENRICH TUMOR INITIATING CELLS

Based on the hypothesis that TICs might originate from normal adult stem cells and share many properties with them (2), methods to isolate normal adult stem cells have been used to enrich TICs from solid tumors. These include the presence of stem cell surface markers, ABC transporters, aldehyde dehydrogenase (ALDH) activity, and stem cell transcription factors. Additionally, biological properties of stem cells, such as sphere formation and resistance to chemotherapeutic drugs have also been used (Figure 1). Properties of TICs identified using one method may be different from those identified by other methods, because of the possible

heterogeneous nature of TICs. In this section, we summarize the methods for enriching TICs from various types of cancer.

3.1. Stem cell surface markers

TICs in acute myeloid leukemia were enriched with $CD34^+CD38^-$ cells similar to the haematopoietic stem cells. When transplanted into immunocompromised mice, $CD34^+CD38^-$ cells initiated tumors, whereas $CD34^-CD38^+$ cells did not (1, 2). Since then, cell surface markers present in normal adult stem cells such as CD44, CD133, and CD117, in combination with tumor-type specific or lineage markers have been used to enrich TICs from solid tumors. CD44, a receptor for hyaluronic acid (HA), is a transmembrane glycoprotein involved in cell growth, survival, differentiation, cell-cell interaction, and motility (32, 33). CD44 is expressed in both embryonic and adult stem cells (34). TICs from solid tumors were first identified from breast cancer using $CD44^+CD24^-Lineage^-$ (5). In breast cancer cells, CD24 is expressed in more differentiated cells, whereas CD44 is expressed in more progenitor-like cells (35). In pancreatic adenocarcinoma, $CD44^+CD24^+$ epithelial specific antigen $(ESA)^+$ was used to identify the TICs (8). To enrich prostate TICs, $CD44^+alpha2beta1^+$ and $CD44^+CD24^-$ were used (9, 36). CD133 is a glycoprotein also known as Prominin 1 (PROM1), is expressed in haematopoietic stem cells, endothelial progenitor cells, and neuronal/glial stem cells (29, 37). Therefore, CD133 was used to identify TICs from many different types of cancer including brain tumor (6), prostate cancer (38), colon cancer (7), lung cancer (10, 11), and melanoma (39). CD117/c-kit is a 145 kDa transmembrane glycoprotein and is expressed in both haematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). Recently, our group has shown that CD117 in combination with another MSC-specific marker Stro-1 can identify osteosarcoma TICs (40). Thus, TICs can be identified from various types of cancer using stem cell surface markers. However, it remains unclear whether the stem cell surface markers play an active role in the biological properties of TICs in addition to serving as their identification markers. Further studies are required to clarify this issue.

3.2. Side population (SP)/ABC transporters

The ABC transporters, which are expressed in a wide variety of stem cells and are associated with cellular drug resistance, are molecular determinants of SP cells (23). SP cells can be identified as a cellular fraction that effluxes the fluorescent dyes Hoechst 33342 and rhodamine 123 (41). Specifically, ABCG2 effluxes Hoechst 33342, while ABCB1 and ABCB5 efflux rhodamine 123 (42, 43). Since the SP is used to identify stem-like cells in various normal tissues (44), this population along with ABC transporters have also been used to identify TICs from various types of tumors including gastric cancer (45-47), hepatocellular carcinoma (48, 49), nasopharyngeal carcinoma (50), lung cancer (51), esophageal carcinoma (52), glioma/glioblastoma (53, 54), pancreatic cancer (55, 56), and breast cancer (57, 58). SP cells have also been used to isolate TICs from bone and soft tissue sarcomas, such as malignant fibrous histiocytoma, rhabdomyosarcoma, and osteosarcoma (16). Additionally, ABCB5 was used to identify melanoma TICs (59). However, cells that are non-SP or negative for ABC transporters can occasionally initiate tumors in some types of cancer (46, 60, 61), suggesting that these markers are not universal for all tumor types and that TICs cannot exclusively be enriched by these methods.

3.3. Aldehyde dehydrogenase (ALDH)

The enzyme aldehyde dehydrogenase (ALDH) is responsible for the oxidation of intracellular aldehydes. Since ALDH is highly expressed in haematopoietic stem/progenitor cells (62, 63) and also in primitive cells from other lineages including neuronal and mammary epithelial cells (64), high levels of ALDH activity measured by the ALDEFLUOR system have been used as a novel approach for the identification of stem/progenitor cells. Cells with high ALDH activity become brightly fluorescent and can be identified and enumerated using a standard flow cytometer. Since cells exclusively having an intact cellular membrane can retain the ALDEFLUOR reaction product, only viable $ALDH^{+/hi}$ cells are identified and the isolated cells are readily available for both *in vitro* and *in vivo* studies. $ALDH^{+/hi}$ cells have been isolated from several types of cancer and show high tumorigenic stem cell-like properties including breast cancer (64-68), hepatocellular carcinoma (69), colorectal cancer (70), osteosarcoma (71), and pancreatic cancer (72). It should be noted that several studies show little correlation between ALDH expression and the $CD44^+CD24^-$ phenotype in breast cancer cells (64, 65, 68). Thus, it remains unclear if TICs identified by ALDH activity possess similar stem cell-like properties to those identified by other methods and whether ALDH activity can be used as a universal method for all types of cancer.

3.4. Sphere formation

Sphere formation is the ability of progenitor cells to propagate in the absence of serum and in an anchorage-independent manner (73). This assay was first introduced by Reynolds and Weiss to identify normal neural stem cells in a defined sphere-specific medium whereby striatal embryonic progenitors could be isolated and maintained (74, 75). By culturing tumor cells in a sphere-specific condition, formed spheres have been shown to be enriched in TICs. These studies include glioblastoma (76), ovarian cancer (77), melanoma (78), cervical cancer (79), and osteosarcoma (40, 80). However, sphere formation requires prolonged culture times, amounting to about two weeks, which could give rise to non-TICs after cell division. Another caveat of the sphere formation assay is that the observed spheres could be just aggregated cells, unless performed using a single cell clonogenic assay. For these reasons, this method is mainly used to test the *in vitro* tumorigenic potential and self-renewability of the TICs isolated by other methods.

3.5. Other methods

A novel approach to isolate TICs is based on the previous observation of enhanced Oct-4 expression in osteosarcoma-derived spheres (81). Levings *et al.* (18) generated a transgenic human osteosarcoma cell line stably

Table 1. Studies demonstrating metastatic potential of TICs in solid tumors

Cancer	Materials	Methods to enrich TICs	Metastasis assays		Results	Ref
			In vitro	*In vivo*		
Breast	Human cell lines	$ALDH^+$	Invasion		HER2 overexpression increased $ALDH^+$ population. $ALDH^+$ cells were more tumorigenic and invasive compared with $ALDH^-$ cells.	66
	Human cell lines	$ALDH^+$	Invasion	Intracardiac	$ALDH^+$ breast cancer cells showed higher tumorigenic and metastatic potential than $ALDH^-$ cells. CXCR1/IL-8RA increased $ALDH^+$ population and invasion of the cells.	67
	Human cell lines, primary xenograft	$ALDH^+$	Invasion	Intracardiac	$ALDH^+$ inflammatory breast cancer cells showed high tumorigenic and metastatic potential, whereas $ALDH^-$ cells failed to initiate tumors.	68
	Human cell lines	$ALDH^{hi}CD44^+CD24^-$ $ALDH^{hi}CD44^+CD133^+$	Migration Invasion Adhesion	Tail vein Orthotopic	$ALDH^{hi}CD44^+CD24^-$ and $ALDH^{hi}CD44^+CD133^+$ cells developed larger tumors and more metastases than $ALDH^{low}CD44^{low/-}CD24^+$ and $ALDH^{low}CD44^{low/-}CD133^-$ cells, respectively.	65
Pancreatic	Human cell lines	$CD133^+/CXCR4^{-\text{ or }+}$	Invasion	Orthotopic	Both $CD133^+CXCR4^-$ and $CD133^+CXCR4^+$ cells efficiently formed tumors but only $CD133^+CXCR4^+$ developed liver metastases.	85
	Human cell lines	Side population	Invasion	Intrasplenic	Side population (SP) cells showed superior potential of the epithelial to mesenchymal transition (EMT) and metastasis to main population (MP) cells.	55
	Human cell lines	$ALDH^+$ $CD44^+CD24^+$ $ALDH^+CD44^+CD24^+$	Migration		$ALDH^+$, $CD44^+CD24^+$, and $ALDH^+CD44^+CD24^+$ cells developed tumors more efficiently and showed higher migratory potential compared with unsorted cells.	72
Lung	Human cell lines	Drug surviving cells (DSCs)	Migration Invasion	Tail vein	DSCs formed spheres, tumors, and metastases more efficiently than the parental H460 cells.	82
Gastric	Human cell lines	Side population	Adhesion	Intraperi-toneal	SP cells formed tumors more efficiently and were more adhesive than unsorted cells. Upon intraperitoneal injections, only SP cells showed peritoneal metastasis.	45
Prostate	Human cell lines	$CD44^+$	Invasion		$CD44^+$ cells were more invasive than $CD44^-$ cells. Genomic profiles of $CD44^+CD24^-$ cells and Matrigel-invasive cells were similar.	87
Osteosarcoma	Human and mouse cell lines	$CD117^+Stro\text{-}1^+$	Invasion	Orthotopic	$CD117^+Stro\text{-}1^+$ (DP) cells were enriched in spheres and drug surviving cells. DP cells showed higher potential of tumor initiation, metastasis, and drug resistance with enrichment of $CXCR4^+$ and $ABCG2^+$ cells than $CD117^-$ $Stro\text{-}1^-$ cells.	40

expressing an *Oct-4* promoter-driven green fluorescent protein (GFP). Cells expressing Oct-4/GFP showed high tumor-initiating potential. Rhabdomyosarcoma TICs were identified by the expression of fibroblast growth factor receptor 3 (FGFR3) (17). The FGFR3-positive cells showed an elevated expression of progenitor-related genes, such as *CD34, Pax3, Oct-4, Nanog,* and *Sox2*, and initiated tumors more efficiently than the FGFR3-negative cells. Based on the idea that TICs are responsible for tumor regeneration after chemotherapy, they can be enriched and maintained following the treatment of particular chemotherapeutic drugs. Levina *et al.* (82) treated a lung cancer cell line with several chemotherapeutic drugs and demonstrated that the drug surviving cells (DSCs) were enriched in TICs having high metastatic potential.

4. METASTATIC PROPERTY OF TICs IN SOLID TUMORS

Metastasis is the ability of cells to detach from a primary tumor, migrate into lymphatic or blood vessels, disseminate and survive in the lymphatic or blood systems, and initiate new tumors at secondary sites. Most solid cancers develop metastases, which are directly responsible for the majority of cancer-related deaths. Although developing therapies to target cancer metastasis is essential, a lack of complete understanding of the underlying mechanisms remains a major hurdle. Recent studies suggest that the molecular machinery for cancer invasion and metastasis is similar to that involved in the activation, mobilization, and homing of normal stem cells (27-31). Since non-TICs do not have the ability to initiate tumors at secondary sites and because TICs share several molecular and biological properties with normal stem cells, TICs have been proposed to be responsible for metastasis (26, 83). Unfortunately, till date, only a small number of studies have investigated the invasive and metastatic properties of TICs. In this section, we summarize the recent studies that demonstrate the metastatic potential of TICs in solid tumors (Table 1).

4.1. Breast cancer

Since the first report of breast cancer TICs using $CD44^+CD24^-Lineage^-$ (5), several other studies have also identified breast cancer TICs using different methods (57, 58, 64). Many of them, however, lack investigation on the metastatic property of TICs. The first report suggesting the high metastatic nature of TICs was made by Balic *et al.* (84). They demonstrated significant enrichment of breast cancer cells, having stem cell-like phenotypes within the bone marrow micrometastases. Later studies providing evidence for the metastatic property of breast cancer TICs include those from Wicha's group (66). They demonstrated that HER2 overexpression increased the $ALDH^+$ population in several human breast cancer cell lines and that $ALDH^+$ cells showed higher abilities of tumor formation and invasion compared with $ALDH^-$ cells (Table 1). Furthermore, the same group used *in vitro* assays and

intracardiac injections to show that $ALDH^+$ TICs from human breast cancer cell lines or a xenograft possessed higher metastatic potential (67, 68). Interestingly, CXCR1/IL-8RA signaling increased the $ALDH^+$ population and self-renewability (67). ALDH1 expression was also associated with the development of early metastasis, as well as poor clinical outcome in inflammatory breast cancer patients (68).

Croker *et al.* (65) identified a subpopulation of human breast cancer TICs having high metastatic potential by combining two methods for TIC identification, namely ALDH activity and the presence of stem cell surface markers CD44 or CD133 (Table 1). $ALDH^{hi}CD44^+CD24^-$ and $ALDH^{hi}CD44^+CD133^+$ TICs showed greater abilities of colony formation on soft agar as well as tumor formation following orthotopic injections. Furthermore, $ALDH^{hi}CD44^+CD24^-$ and $ALDH^{hi}CD44^+CD133^+$ cells displayed enhanced metastasis compared with $ALDH^{low}CD44^{low/-}CD24^+$ and $ALDH^{low}CD44^{low/-}CD133^-$ cells. Most notably, they examined the metastatic behavior of these cells by performing *in vitro* cell adhesion, migration, and invasion assays, as well as *in vivo* tail vein and orthotopic (mammary fat pad) injections. This is one of the few studies that determined the metastatic potential of TICs using orthotopic injections.

4.2. Pancreatic cancer

TICs in pancreatic cancer were first reported by Simeone's group in 2007 using $CD44^+CD24^+ESA^+$ (8), however, this study did not investigate the metastatic ability of these cells. Hermann *et al.* (85) used CD133 as a marker for the isolation of human pancreatic adenocarcinoma TICs. They showed that $CD133^+$ cells were tumorigenic and highly resistant to the chemotherapeutic drug gemcitabine when compared with the $CD133^-$ cells. Immunohistochemistry using human pancreatic tumors revealed a distinct subpopulation of $CD133^+CXCR4^+$ cells at the invasive front of tumors. They further performed orthotopic injections using a $CD133^+CXCR4^+$ group (equal number of $CD133^+CXCR4^+$ and $CD133^+CXCR4^-$ cells) and a $CD133^+CXCR4^-$ group. Although both groups efficiently formed tumors, only the $CD133^+CXCR4^+$ group showed liver metastasis, while no metastasis was observed in $CD133^+CXCR4^-$ cells. This *in vivo* result was supported by *in vitro* invasion assays where the CXCR4 neutralizing antibody inhibited the high invasive potential of $CD133^+$ pancreatic TICs (Table 1). Thus, these findings provide direct *in vivo* evidence for the heterogeneity of $CD133^+$ TICs and high metastatic property of $CD133^+CXCR4^+$ TICs.

SP cells isolated from human pancreatic cancer cell lines also showed higher liver metastases following intrasplenic injections, compared to the main population (MP) cells (55). Interestingly, SP cells showed superior potential of TGF-β-induced epithelial to mesenchymal transition (EMT) and invasion in relation to MP cells (Table 1).

ALDH activity was also used to identify pancreatic TICs (72). $ALDH^+$, $CD44^+CD24^+$, and $ALDH^+CD44^+CD24^+$ cells initiated subcutaneous tumors more efficiently than unsorted cells. These enriched cells showed higher migratory potential with altered expression of EMT-associated genes, including reduced *CDH1 (E-cadherin)* expression and increased *SNAI2 (SLUG)* expression (Table 1). Interestingly, the high migratory potential and reduced *CDH1 (E-cadherin)* expression were more obvious in $ALDH^+$ and $ALDH^+CD44^+CD24^+$ cells than those in $CD44^+CD24^+$ cells, suggesting that $ALDH^+$ and $CD44^+CD24^+$ cells are biologically distinct. It should also be noted that patients with $ALDH^+$ primary tumors had worse survival rates than patients having $ALDH^-$ primary tumors. Immunohistochemical staining further revealed that $ALDH^+$ cells were observed more frequently in metastatic lesions compared to those in matched primary tumors. These results suggest the crucial role of ALDH activity in the progression of pancreatic cancer.

4.3. Lung cancer

Lung TICs were identified using CD133 as a marker (10, 11). Although these studies showed the tumor-initiating property of lung $CD133^+$ TICs, their metastatic property was not investigated. Levina *et al.* (82) enriched lung TICs by treating a human lung cancer cell line H460 with doxorubicin, cisplatin, or etoposide. DSCs expressed high levels of several embryonic stem cell markers and showed an ability to self-renew and initiate tumors when compared with the parental cells, suggesting that the DSCs functioned as TICs. Morphological analyses revealed that the DSCs displayed a greater migratory phenotype, such as lamellipodia extensions and actin spikes at the leading edge, compared with the parental cells. Consistent with these observations, DSCs also showed high migratory and invasive potential by *in vitro* assays using IL-8 as an attractant. In tail vein injections, DSCs displayed more metastatic nodules than the parental cells (Table 1). Interestingly, DSCs expressed higher levels of human VEGFR2, FGFR2, CXCR1, 2, and 4 receptors than the parental cells. Further, the DSC-derived tumors stimulated murine stroma to produce elevated levels of angiogenic and growth factors. These upregulations in the cytokine network could serve as the basis for the enhanced tumorigenic and metastatic potential of the TICs (82).

4.4. Gastric cancer

Takahashi *et al.* (86) first demonstrated that $CD44^+$ cells in gastric cancer possessed TIC properties such as high sphere-forming and tumor-initiating abilities. Additionally, these cells were resistant to chemotherapy and γ-irradiation. However, the metastatic potential of the isolated $CD44^+$ cells was not studied. TICs from gastric carcinoma were also isolated using the Hoechst 33342 dye exclusion method (45). The SP cells were shown to initiate tumors more efficiently and possessed higher ability of peritoneal metastasis following intraperitoneal injections, as compared with unsorted cells (Table 1). Consistently, the SP cells showed higher expression of adhesion molecules α2-, α5-, β3-, and β5-integrins than unsorted cells (45).

4.5. Prostate cancer

Prostate cancer TICs were identified using $CD44^+\alpha2\beta1^+$ and $CD44^+CD24^-$ markers by Patrawala *et al.* (9) and Hurt *et al.* (36) respectively, yet these studies lacked exploration of their metastatic potential. Klarmann *et al.* (87) recently demonstrated a higher invasive potential of $CD44^+$ prostate cancer TICs compared to that of $CD44^-$ cells. $CD44^+CD24^-$ cells showed similar RNA expression profile to that of the Matrigel-invasive cells, including expression of EMT-related genes and those associated with stem cell maintenance, proliferation, and differentiation (Table 1).

The crucial role of CD44 in prostate cancer metastasis is also supported by the study of Eaton *et al.* (88), where CD44 expression, but not the expression of CD133 or $\alpha2\beta1$ integrin, was observed more frequently in metastases than in primary tumors.

4.6. Osteosarcoma

Although several groups suggested the presence of TICs in osteosarcomas (81, 89-91), the tumor-initiating potential of osteosarcoma TICs was not demonstrated until recently. Wu *et al.* (15) used a dye-excluding SP for enriching TICs from cultured cells of one human osteosarcoma, two malignant fibrous histiocytoma, and one synovial sarcoma. Interestingly, SP cells excluding the Hoechst 33342 dye initiated serially transplantable tumors following subcutaneous injections, whereas SP cells excluding the rhodamine 123 dye did not form tumors, suggesting the involvement of a specific fraction of the SP cells in the TIC properties.

Based on the previous observation that an embryonal transcriptional regulator Oct-4 was expressed in spheres from osteosarcoma cells (81), Levings *et al.* (18) generated a transgenic cell line where cells from an osteosarcoma biopsy (OS521) were stably transfected with a plasmid containing the human *Oct-4* promoter-driven *GFP* reporter gene. The Oct-4/GFP(+) cells were over 100-fold more tumorigenic by subcutaneous injections than the GFP-depleted cells. Interestingly, serial transplantation of the Oct-4/GFP(+) cells into immunocompromised mice resulted in acquired metastatic ability, suggesting a selective adaptation analogous to the process of tumor progression (18).

Wang *et al.* (71) used ALDH activity to enrich TICs from a human osteosarcoma cell line OS99-1. Cells having a high activity of ALDH ($ALDH^{br}$) showed more efficient tumor initiation by subcutaneous injections and higher levels of expression of the stem cell-associated genes *Oct-3/4a*, *Nanog*, and *Sox-2* than cells possessing low ALDH activity ($ALDH^{lo}$). Although various methods were used to demonstrate the presence of TICs in osteosarcoma, none of them demonstrated orthotopic tumor formation or provided direct evidence for their high metastatic potential.

Recently, our group (40) showed *in vitro* as well as *in vivo* evidence of the high metastatic potential of osteosarcoma TICs (Table 1). Using murine primary and established cell lines, we demonstrated that mesenchymal stem cell markers CD117 and Stro-1, but not markers such as CD44, CD105, and CD49b, were preferentially expressed in spheres and doxorubicin-resistant cells. As low as 200 $CD117^+Stro\text{-}1^+$ (double positive, DP) cells from several mouse and human osteosarcoma cell lines efficiently formed serially transplantable tumors following subcutaneous injections, whereas $CD117^-Stro\text{-}1^-$ (double negative, DN) cells rarely initiated tumors. Consistently, orthotopic injections of 200 and 2,000 DP cells from a primary mouse cell line revealed that DP cells possessed significantly higher ability of tumor initiation than DN cells. Importantly, we found multiple metastatic nodules in the lungs and liver of the majority of mice orthotopically injected with DP cells. When DN cells were injected with ten times or higher numbers (20,000 and 200,000) than DP cells, DN cells did form tumors. However, it took a much longer time period than the DP cells. When average numbers of metastatic nodules were compared, DN-derived tumors developed a significantly less number of metastatic nodules than DP-derived tumors (6 vs. 29). Results of *in vitro* invasion assays revealed that DP cells possessed a two-fold higher invasive potential than DN cells, supporting our *in vivo* observations. We further compared the expression patterns of stem cell markers CD117, Stro-1, ABCG2, and CXCR4 between DP-derived primary tumors and their corresponding lung metastases by immunohistochemistry. Although we detected cells positive for all four markers in both primary and metastatic tumors, the intensely stained cells were more frequent at the metastatic sites when compared to those at their primary sites. These results support previous findings that a high expression of TIC markers positively correlates with cancer metastasis (68, 84, 92). Moreover, we demonstrated that DP cells were enriched with cells expressing CXCR4 and ABCG2 in both murine and human model systems. CXCR4 enrichment in DP cells was over 80% and 20% in mice and human cell lines respectively, whereas that in unsorted and DN cells was less than 5%. Regarding ABCG2, over 60% and 80% of DP cells contained $ABCG2^+$ cells in mice and human cell lines, respectively, whereas only a few unsorted and DN cells were positive for ABCG2. Consistent with enrichment of $ABCG2^+$ population in DP cells, these cells also showed a higher drug-resistant property than DN cells. Thus, CD117 and Stro-1 identify osteosarcoma TICs associated with metastasis and drug resistance.

5. PROSPECTIVES

TICs have been identified using diverse techniques in various types of cancer. Different methods have also been used for the characterization of TICs. Since TICs are defined experimentally by their abilities of *in vivo* tumor initiation and self-renewability, investigators need to have a consensus regarding the methods to isolate and evaluate TICs according to specific tumor types (4).

Considering the possible heterogeneous nature of TICs (65, 72, 93-95), the use of different TIC-isolation methods might result in enriching TICs with different properties, regarding drug resistance and metastasis. The biological significance of stem cell markers for enriching TICs remains unclear. The methods to evaluate TICs

should also be carefully chosen. Different types of immunocompromised mice (NOD/SCID vs. NOD/SCID IL2rγ$^{-/-}$) or use of Matrigel during the TIC injections resulted in dramatically different outcomes regarding the efficacy of tumor initiation (93). NOD/SCID IL2rγ$^{-/-}$ mice lack T, B, and NK cells and are most appropriate for xenografting human tumors. However, the development of mouse models of cancer enables us to test the tumor-initiating ability of TICs in syngeneic or congenic systems having intact immune environment and without any concerns of tumor rejection (96, 97). Additionally, since the tumor microenvironment varies depending on the tissue of origin, the injection of tumor cells into their orthotopic sites is ideal to test the tumor-initiating ability and metastatic potential of TICs. Although *in vivo* assays such as tumor-initiating ability and serial transplantability are the gold standard for evaluating the TICs, these assays with a small number of cells are time-consuming. Developing simpler and more reliable assays would be helpful for the progress of this field.

Several recent studies suggest the functional interaction between TICs and their specialized microenvironment called a niche (98). Tumor cells are actively involved in the creation of their own future premetastatic niche for distant metastasis by secreting cytokines, metalloproteinases, and growth factors or by recruiting bone marrow derived cells (BMDC). The premetastatic niche provides a suitable environment to recruit the tumor cells (20, 29, 99, 100). Calabrese *et al.* (101) showed that tumor endothelial cells increased the number of TICs in brain tumors, whereas their depletion reduced the TIC numbers, suggesting that niche plays a crucial role in the formation and maintenance of TICs. Further studies are required to gain a better understanding of the interaction between TICs and their niche.

Epithelial to mesenchymal transition (EMT) is an important mechanism for metastasis of epithelial tumor cells. Recent studies suggest that EMT endows normal and transformed mammary epithelial cells with stem cell-like properties, including the ability to self-renew and to initiate tumors (102). A recent report by Kabashima *et al.* (55) demonstrated that SP cells from a pancreatic cancer cell line were more responsive to TGF-β-mediated EMT than main population (MP) cells. These observations showing a functional link between TICs and EMT may suggest that the EMT process is involved in the generation of TICs, acquisition of metastatic potential, and formation of metastatic nodules at secondary sites (103).

In addition to the stem cell marker profiles and biological properties, TICs also share expression pattern of genes related to stem cell maintenance, proliferation, and differentiation with those of normal stem cells (19, 20). Comparative analyses of DNA and RNA profiling of TICs with that of normal adult stem cells or between different tumor types will help dissect their properties at molecular levels, thereby accelerating TIC-targeted therapy.

To develop a novel therapy targeting TICs, altering their properties would be crucial. This can be executed by inhibiting their high tumorigenic, drug-resistant, and metastatic properties, as well as manipulating their cell cycle phase, differentiation status, and niche environment. Further, finding strategies that discriminate TICs or their niche from normal cells will accelerate the development of novel drug delivery systems to specifically target TICs.

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Abbreviations: TIC: tumor-initiating cell, CSC: cancer stem cell, ABC: ATP-binding cassette, HSC: haematopoietic stem cell, MSC: mesenchymal stem cell, AML: human acute myeloid leukemia, SP: side population, MP: main population, GFP: green fluorescent protein, MMP: metalloproteinase, BMDC: bone marrow derived cells, ALDH: aldehyde dehydrogenase, EMT: epithelial to mesenchymal transition, DSC: drug surviving cell, DP: double positive, DN: double negative, NOD/SCID: non-obese diabetic/severe combined immunodeficiency, REF: references

Key Words: Tumor-initiating cells, Cancer stem cells, Metastasis, Solid tumors, Osteosarcoma, CXCR4, ABCG2, Side population, CD117, Stro-1, Review

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