Stem Cell Reviews and Reports
https://doi.org/10.1007/s12015-025-11003-6

REVIEW

Dysfunctional, Tissue-Resident, Very Small Embryonic-Like Stem Cells (VSELs) Initiate Cancer and Result in its Progression and Metastasis, Independent of Epithelial-Mesenchymal Transition

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Received: 23 June 2025 / Accepted: 6 October 2025

Abstract

It is widely believed that epithelial cells in solid tissues undergo epithelial-mesenchymal transition (EMT) during carcinogenesis. EMT transforms polar and adherent epithelial cells in solid tumors into mesenchymal cells that get mobilized as circulating tumor cells (CTCs) and trigger metastasis. Isolating normal and neoplastic epithelial stem cells and their characterization remains challenging and vague even today. Most deaths in cancer patients are due to metastasis and hence a huge interest exists in understanding and developing tools to prevent and overcome metastasis. EMT during cancer remains clouded by controversies and questions persist as to its precise role. Besides a lack of histological evidence, lineage tracing studies have also failed to provide definitive proof supporting role of EMT in metastasis. Pluripotent, very small embryonic-like stem cells (VSELs) express sex hormone receptors and exist in a quiescent state in all tissues. They are responsible for regular turnover of epithelial cells, maintain lifelong homeostasis and their dysfunctions result in various pathologies including cancer. Developmental exposure to endocrine disrupting chemicals directly impacts VSELs, results in epigenetic changes that transform VSELs into cancer stem cells (CSCs). CSCs enter cell cycle, undergo excessive self-renewal and initiate cancer. CSCs (epigenetically altered and dysfunctional VSELs) are mobilized into circulation and are studied by our group for early prediction of cancer unlike CTCs, in a liquid biopsy, that fail to detect cancer in early stages. In this article, we discuss that besides initiation, CSCs also play a key role in cancer spread. Open questions surrounding EMT are reviewed and discussed in the context of VSELs biology. Existing hallmarks of metastasis-initiating cells produced by EMT are critically examined considering CSCs with a crucial role in cancer initiation, progression, metastasis and recurrence, challenging the existing focus on EMT and CTCs.

Key Highlights

- Epithelial cells in solid tumor undergo epithelial-mesenchymal transition (EMT) during which epithelial cells acquire mesenchymal features, get mobilized as circulating tumor cells resulting in tumor initiation, progression and metastasis.
- However, multiple lineage tracing studies and histological evidence do not support a role of EMT in metastasis.
- Tissue-resident, pluripotent very small embryonic-like stem cells (VSELs) in solid organs are responsible for regular turnover of epithelial cells, participate in regeneration upon chronic injury and their dysfunctions initiate various pathologies including cancer.
- The cancer stem cells (CSCs, epigenetically altered VSELs) get mobilized from the tumor tissue in increased numbers into the peripheral blood. Our group is focussed on studying these stem cells for early prediction of cancer.

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Published online: 05 November 2025

- We suggest that rather than EMT of epithelial cells, it is the CSCs that besides initiation, are also responsible for cancer progression, spread, metastasis and recurrence.
- Like the normally quiescent, and undifferentiated VSELs, CSCs exhibit the properties of chemo- and radio-resistance. Both are immune-privileged, survive hypoxia and being pluripotent, show plasticity, clonogenic potential, and can differentiate into multiple cell types, including cancer cells, mesenchymal stromal cells (MSCs), neuronal and endothelial cells at distant sites.

Keywords Stem cells · VSELs · Cancer stem cells · EMT · Circulating tumor cells · Metastasis

Introduction

Cancer is a leading cause of death globally and most patients who die of cancer die of metastases. During metastasis, cancer cells break away from the primary tumor, travel through the blood and/or lymphatic system, and form new tumors in other organs of the body [1]. However, no consensus exists on the cellular events that lead to metastasis, and oncologists are clueless about preventing it. Once metastasis occurs, cancer by definition is mostly advanced and available options to treat include surgery, hormonal therapy, radiotherapy, chemotherapy, targeted therapy, and immunotherapy. However, most of these treatments are associated with the risk of recurrence due to tumor heterogeneity resulting in therapeutic resistance and eventually death. Metastatic cancer is mostly fatal and despite various advances to extend life span, 5-year cancer survival rates have remained stagnant during the period 2000–2014 [2]. Weinberg [3] concluded that developing anticancer therapies has proved very challenging and the war on cancer is not yet fully prosecuted even after 50 years since it was declared, rather it has just begun.

As a result, there is a huge interest in understanding how metastasis occurs and developing methods to prevent and target it. The epithelial-mesenchymal transition (EMT) program has emerged as a central driver in explaining the mobilization of tumor cells resulting in metastasis. Besides having a role during metastasis, EMT has been widely studied during embryonic development and in pathological conditions like wound healing and inflammation [4, 5]. EMT is a reversible transition process during which epithelial cells undergo simultaneous dedifferentiation (reduce their organ-specific epithelial properties) and transdifferentiation (gain mesenchymal characteristics) into mesenchymal cells. EMT endows cancer cells with increased motility and invasiveness, cancer stem cell generation, and broad resistance to routine chemotherapy not only in solid cancers but also in leukemia [6, 7]. Most solid tumors originate from epithelial cells (carcinomas) and it is widely believed that these polar and adherent epithelial cells undergo EMT to provide a ready source of mesenchymal cells which mobilize as circulating tumor cells (CTCs) and transport to distal sites to cause metastasis by undergoing mesenchymal-epithelial transition (MET). CTCs in peripheral blood exist singly or as small clusters and may be homotypic or heterotypic [8].

Besides promoting invasion by MSCs, EMT also induces stem cell-like properties to initiate primary tumors and accelerate metastasis [4, 9, 10]. One needs to refer to publications in 2009 to understand the role of EMT during cancer metastasis. An editorial by Raghu Kalluri [11] for a Review Series published in The Journal of Clinical Investigation focused on EMT, made it obvious that at that time, scientists were fascinated by MSCs and their presence in adult tissues. Epithelial cells were thought to be highly plastic and able to switch back and forth between epithelia and mesenchyme. Kalluri and Weinberg [12] discussed that all cells in the body are derived from other cells, and that allare derived from a single cell, the fertilized egg. During embryogenesis and organ development, the epithelial cells appear to be plastic and thus can move back and forth between epithelial and mesenchymal states via the processes of EMT and MET. Once fully differentiated, epithelial cells exert tissue-specific function only, while mesenchymal cells play a supporting role. EMT is a concept developed to provide a logical source for MSCs/CTCs in circulation.

Neoplastic cells that emerge due to EMT are the mesenchymal cells that lack cell-cell adhesion, are dysmorphic in shape, and spread to distant organs to complete the metastatic cascade. The CTCs detach from the primary tumor, intravasate into the circulatory and lymphatic systems, move freely on their own, enter the bloodstream and survive, evade immune attack, extravasate at distant capillary beds, invade and proliferate/colonize in distant organs. The Wnt signaling pathway is reportedly activated in many cancers and is the best connection between stem cells and EMT. Activation of β-catenin appears to promote tumor progression and Wnt induces EMT in cell lines via stabilization of Snail and induction of expression of Snail, Slug and Twist1 [13, 14].

Existing Disbelief in EMT

EMT is a dynamic and transient process, and mesenchymal features disappear once the cells undergo MET to facilitate metastasis. Strong evidence is lacking to document both EMT and MET in vivo. Descriptive studies provide evidence in support of EMT, but no direct proof is available showing that EMT occurs at the invasion front of solid tumors. Despite many publications over the last two decades, EMT as a process activated during cancer progression remains shrouded by several mysteries even in 2024 that need to be resolved [15]. EMT has been extensively studied in vitro but its relevance and direct evidence to support it in vivo in human tumors remains controversial and this has been discussed by several groups [15–18]. Williams and co-authors [18] provided three reasons for the existing disbelief in what they term as epithelial-mesenchymal plasticity (EMP) and included both EMT and MET including (i) paucity of robust evidence for EMT remaining transient and reversible in vivo (ii) scarcity of data supporting the occurrence of MET at the metastatic site and (iii) tumor cells can retain complete metastatic capability while retaining their epithelial phenotype.

Moreover, studies on tissue sections only provide a snapshot at a point in time, and it has proved impossible to view the whole process as it occurs. Tarin [19] discussed that evidence suggesting EMT during development and in vitro experiments also remains unconvincing. According to him, EMT described during gastrulation is possibly not EMT in the strict sense because none of the components have been characterized well at this stage. Neither the surface ectoderm nor the mesoderm is fully committed at this stage and still retains plasticity and its ability to interconvert. In adult life mesenchymal and epithelial cells lose plasticity post full development, their fate gets fixed, and interconversion is not likely. During in vitro culture studies, the environment remains highly artificial with no dynamic vascular, endocrine, or neurologic contributions. Importantly, various features of EMT are not convincingly seen during surgical pathological studies on tumor tissues at any stage of neoplasm. One needs to exercise caution while developing a hypothesis regarding mechanisms underlying metastasis based on the premise that a similar process is also seen during early development and in vitro. Detailed ultrastructural studies do not support EMT during wound healing [20, 21]. Attempts have also been made to gather proof to support EMT by lineage tracing studies (Table 1) but the results obtained suggest that EMT is not essential for metastasis but may have a role in inducing chemoresistance.

Weinberg's group [26, 27], the major advocates of EMT, responded to the negative results obtained by Fischer [22] and Zheng [23] groups. As shown in Table 1, both these studies concluded that EMT is not necessary for metastasis but may have a role during recurrence. Later two more studies were published with improved lineage tracing technology again concluding that EMT is not essential for metastasis [24, 25]. In their Perspective, after two decades of research on EMT, Lambert and Weinberg [28] concluded that EMT drives epithelial cells into a range of more mesenchymal phenotypic states and as a result, the link between EMT and stem cells is still not clear and requires further research. EMT programs generate multiple intermediate, partially mesenchymal cell states with differing phenotypes and functions that are highly metastatic. Isolating pure populations of stem cells from normal and cancerous tissues and their characterization remains a challenge. In addition, Mani's group [29, 30] discussed the presence of two types of cancer stem cells – those derived from embryonic/progenitor cells harbored amongst epithelial cells and those produced from differentiated cells by EMT. They discussed that more research is required to understand which stem cells result in metastasis. Ledford [17] discussed 10 years of research in the wrong direction by believing that EMT results in metastasis since pathologists were not convinced about its role. We suggest that even in 2025 after an additional 14 years, the scientific community is still groping in the dark and metastasis remains an imbroglio. Disproving the role of EMT during metastasis could result in a major paradigm shift in the field of cancer biology, and this is precisely the intent of this article.

Our Views on Epithelial Mesenchymal Transition (EMT)

According to us, a differentiated and senescent epithelial cell with a finite lifespan lacks plasticity and can neither accumulate mutations to initiate cancer as suggested by Somatic Mutation Theory nor can it transdifferentiate by undergoing EMT into a mesenchymal cell, let alone produce cancer stem cells (CSCs) to result in cancer initiation and metastasis nor can it be responsible for recurrence since there is no explanation to clarify how CTCs could acquire properties of chemo- and radio-resistance. Earlier, we challenged the concept of paligenosis (dedifferentiation and reprogramming of epithelial cells) resulting in cancer initiation [31]. Cancer is neither a genetic disease nor initiated by paligenosis and rather initiates due to the dysfunction of tissue-resident very small embryonic-like stem cells (VSELs) and somatic mutations occur as a consequence of cancer [32]. Other groups have also questioned SMT due to surrounding paradoxes and consider mutations to occur secondary to the onset of cancer [33, 34]. We discussed various technical shortcomings that have resulted in existing misconceptions leading to

Table 1 Lineage tracing studies have failed to delineate a role of EMT during metastasis

Study Ref	Salient findings of the studies
Fischer et al. 2015 [22]	• Used two oncogene-driven triple-transgenic mouse models, MMTV-PyMT/Rosa26-RFP-GFP/FSPl-Cre (Tri-PyMT) and MMTV-neu/Rosa26-RFP-GFP/FSPl-Cre, (Tri-Neu) by crossing MMTV-PyMT or MMTV-neu mice with Cre-switchable fluorescent reporter (ROSA26-lox-RFP-STOP-lox-GFP) and FSP 1-Cre mice to track EMT during metastasis in vivo. They assumed that EMT process will switch fluorescence expression from RFP to GFP. Thus, RFP and GFP would serve as readouts to monitor cells in epithelial and mesenchymal state, respectively. • Their results showed that carcinoma cells metastasized without activating EMT and the majority of metastatic lesions in the lungs were RFP^{+} • They also inhibited EMT by ectopic expression of miR-200, an inhibitor of Zeb1, a transcription factor crucial for EMT. This also did not affect number of lung metastasis formed.
Zheng et al. 2015 [23]	• Similar results were observed in the MMTV-Neu model or when the Vimentin promoter was used • Tissue-specific knockout of Snail or Twist, two EMT-inducing transcription factors, in spontaneous pancreatic adenocarcinoma mouse models were used to inhibit EMT. But, despite the loss of Snail or Twist, the incidence of metastasis remained similar
Li et al. 2020 [24]	• Genetic tracing of transient gene expression in vivo remains technically challenging. A genetic fate-mapping system for temporally seamless tracing of transient cell fate was developed to study EMT gene activity from the local primary tumor to a distant metastatic site in vivo. • In a spontaneous breast-to-lung metastasis model, primary tumor cells activated vimentin and N-cadherin in situ, but only N-cadherin was activated and functionally required during metastasis. Tumor cells that have ever expressed N-cadherin constituted the majority of metastases in lungs, and functional deletion of N-cad significantly reduced metastasis. • Gene activity of vimentin was not detected during tumor metastasis
Chen et al. 2024 [25]	• Mouse with small cell lung carcinoma (SCLC), a high-grade neuroendocrine carcinoma, with a poor prognosis due to high metastasis was studied • Based on frequent concurrent genetic inactivation of RB1 and TP53 in human SCLC, $Rb1^{L/L}$;$Trp53^{L/L}$;VIM-Tracer mice were generated to trace EMT in SCLC mouse model. Switch from ZsGreen to tdTomato indicates VIM expression and thus permanently records VIM-mediated EMT activity • SCLC initially develops ~ 24 weeks and metastasizes into the liver ~ 40 weeks afterwards. Mouse lungs and livers were collected at 40 weeks and all tumors were green. There was no sign of Vimentin expression during metastasis • Most NCAM + SCLC cells (~ 88.6%) were ZsGreen + tdTomato–. • A few tumors uniformly displayed a tdTomato + pattern suggesting the existence of EMT in primary SCLC, albeit at a low incidence. • ~ 7.9% of SCLC cells were double positive for ZsGreen and tdTomato, indicative of a transitioning state • No association between VIM expression and human SCLC metastasis

diverse views on how cancer initiates [35]. VSELs exist as a sub-group amongst the epithelial cells and are the possible root cause for cancer initiation as well as metastasis.

An Introduction to Very Small Embryonic-Like Stem Cells

Primordial germ cells (PGCs) are mobilized to all developing organs during early embryonic development rather than only to the gonadal ridge and survive throughout life as VSELs [36–39]. Like PGCs, VSELs are pluripotent, express pluripotent markers including nuclear OCT-4, and can differentiate into 3 germ layers and germ cells in vivo as well as in vitro. The presence of VSELs and their pluripotent characteristics have been reported by more than 60 groups working independently worldwide using both mouse and human tissues including cord blood and Wharton's jelly [36–42]. Normally, VSELs remain quiescent, in G0 stage of cell cycle and undergo occasional asymmetrical cell division to give rise to lineage-committed and multi- or unipotent progenitors which undergo symmetrical division and clonal expansion before differentiating into tissue-specific cell types [43]. This way a close coordination between VSELs and the progenitors ensures tissue homeostasis throughout life. Being quiescent and virtually immortal (Supplement pages 1–5), VSELs are the only cell type present in adult tissues with the properties of chemo- and radio-resistance and account for recurrence after oncotherapy. VSELs survived total body irradiation in mouse bone marrow [44] and chemotherapy in mouse bone marrow [45], ovaries [46], and testes [47, 48]. VSELs survived for >15–20 years in a

quiescent state amongst Sertoli cells and were reported in azoospermic testicular biopsies of adult survivors of childhood cancer [49]. The underlying reason for this is their quiescent nature (Supplement, pages 1–5).

VSELs normally exist in G0 stage of the cell cycle but readily exit dormancy in response to chronic injury to restore homeostasis. Bhartiya's group has delineated this novel property of VSELs while studying the effects of chronic injury to mouse uterus upon inflicting mechanical injury [50] and pancreas by partial pancreatectomy [51, 52]. VSELs increased in numbers on day2 and differentiated into large numbers of progenitors by day5 upon chronic injury in the mouse uterus and pancreas. Evidence has also been published that VSELs differentiate into epithelial cells for their regular turnover [53]. VSELs became controversial in 2013 [54] but after more than a decade of further research, robust protocols are now available to isolate them from any solid tissue [55–57], and many more independent groups have confirmed their presence [58–60]. However, the very existence of VSELs remains disputed, and scepticism persists due to their small size. But seeing is believing! Figures 1 and 2 show their presence in mouse uterine sections amongst epithelial cells. Under normal homeostatic conditions, they are not observed due to their small size and scarce nature. However, they are increased in numbers upon neonatal exposure to diethylstilbesterol

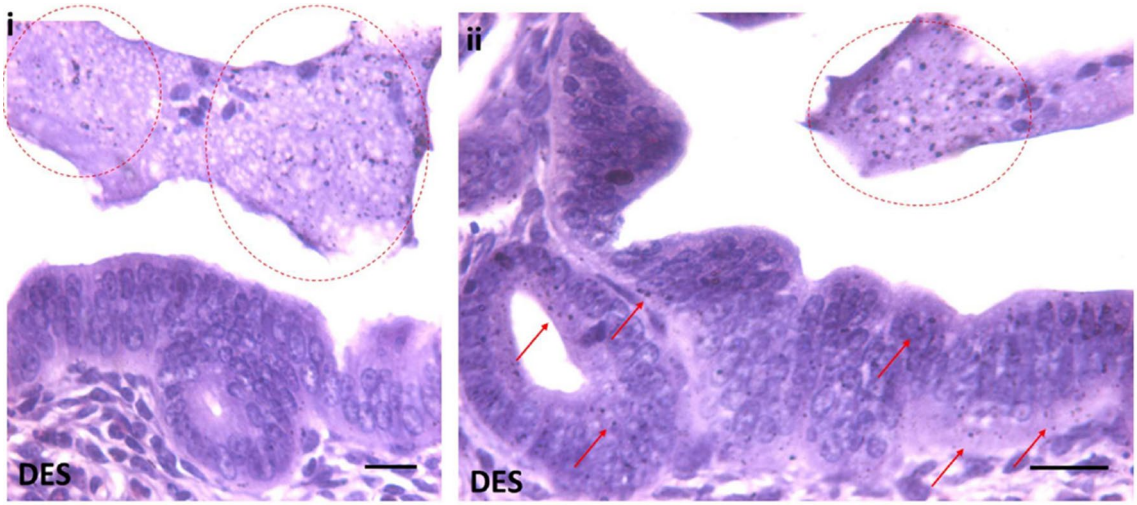

Fig. 1 VSELs in Haematoxylin and Eosin stained endometrial sections of 100-day-old mice upon neonatal exposure to diethylstilbesterol (2 ug/day/pup on postnatal days 1-5). These are small-sized, spherical cells with dark-stained nuclei and can be seen both amongst epithelial cells (red arrows) and get sloughed off in the lumen (red broken circles). They are not seen in normal uterine sections, but the neonatal exposure to diethylstilbesterol results in an increase in their numbers, and as a result, they are visible. This is published data by Singh and Bhartiya [61]. It is these stem cells that result in cancer initiation and spread, rather than the fully mature epithelial cells

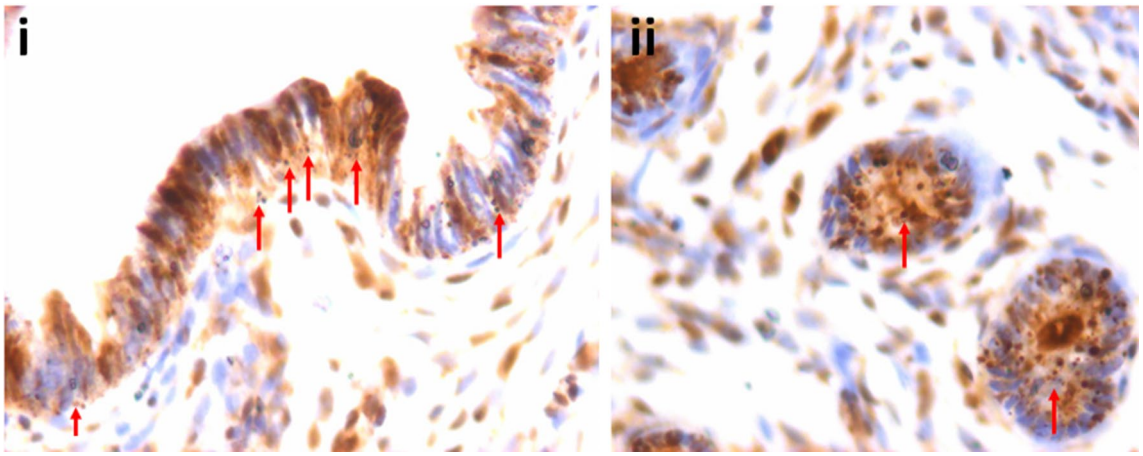

Fig. 2 Pluripotent VSELs express nuclear OCT-4 and are stimulated by follicle-stimulating hormone (FSH), exist as a sub-population amongst the epithelial cells lining both the lumen and the glands in mouse endometrial sections. The mice were bilaterally ovariectomized and later treated with FSH (5 IU/day for 7 days). This data is published earlier [63]

(Fig. 1) [61]. Figure 2 shows that they survive in an atrophied uterus upon bilateral ovariectomy, express nuclear OCT-4, and respond directly to follicle-stimulating hormone treatment [even in the absence of ovaries, FSH primarily acts on ovarian granulosa cells in females, [62]. This data should convince the reader about the existence of VSELs in adult tissues.

Despite being pluripotent, VSELs do not exhibit a few of the hallmark features described for pluripotent stem cells that exist in vitro [63]. VSELs do not readily divide and expand in vitro (but can differentiate into all three lineages and germ cells), neither form teratoma nor integrate into a developing embryo. This is because VSELs possess a unique property of quiescence and because they remain epigenetically locked under homeostatic conditions [64]. If they were to behave like embryonic or induced pluripotent stem cells, tumors would erupt spontaneously all the time in the human body.

Being small in size and non-cyclic, VSELs inadvertently get discarded while processing cells for various studies including flow cytometry and single-cell RNAseq and also remain elusive during lineage-tracing studies [35]. VSELs in mouse endometrium [61, 65], testes [66, 67] and ovaries [68] were reported to initiate various pathologies including cancer in adult life due to neonatal exposure to endocrine insults. Similar data about the presence and expansion of VSELs is available for human ovarian cancer [69]. VSELs in bone marrow are suggested to have a role in both hematopoiesis and leukemogenesis [39]. VSELs undergo epigenetic changes and transform into cancer stem cells (CSCs) in response to various extrinsic and/or intrinsic insults (like increased exposure to endocrine disrupting chemicals and agents that result in inflammation in the body) resulting in cancer initiation [70]. These CSCs are possibly the root cause for cancer initiation, progression and recurrence. They get mobilized into circulation and are being studied by our group for early detection of cancer [71]. We posit that rather than EMT and CTCs having a role in metastasis, CSCs (epigenetically altered VSELs) are possibly the much sought-after metastasis-initiating cells (MICs) responsible for metastasis.

Early Prediction of Cancer Based on Stem Cells in Liquid Biopsy Rather than CTCs

Our group has developed a pan-cancer test for prediction of early stages of cancer based on studying causative, pluripotent markers expression including OCT-4 in stem cells enriched from the peripheral blood [71]. These markers are specific to VSELs and CSCs (greatly increased in cancer) and are supposedly better candidates for early prediction of cancer compared to studying CTCs in circulation, which are unable to detect early stages. In a 1000 sample proof-of-concept study, it was possible to successfully detect multiple types of cancers based on studying OCT-4 A levels in peripheral blood [72]. VSELs are developmentally linked to primordial germ cells that do not express Major Histocompatibility Complex (MHC) molecules, a key component of the immune system. Consequently, undifferentiated VSELs, CSCs (epigenetically altered VSELs) and early progenitors are immune-privileged and express low levels of MHC class1 molecules [73]. They are not attacked by immune mechanisms; can survive hypoxia; and conceptually implant in distant organs to result in metastasis or survive and later cause relapse/recurrence. Cellular events like dedifferentiation of epithelial cells, followed by their transition into mesenchymal cells during EMT and mobilization as the CTCs (with intricate mechanisms to avoid immune rejection) seem dispensable for metastasis. Recent publications have discussed the shortfall of using CTCs and ctDNA for early prediction of cancer [74–79]. We suggest that CSCs (epigenetically altered VSELs) are the elusive metastasis initiating cells (MICs).

Re-Examining the Hallmarks of Metastasis-Initiating Cells (MICs) in the Absence of EMT

A set of hallmarks have been published for MICs including cellular plasticity, clonogenic potential, metabolic reprogramming, ability to survive hypoxia, enter and exit dormancy, resist regular apoptosis and anoikis, ability to evade immune attack, and ability to build or take advantage of a supportive stromal niche [80–82]. Most importantly, MICs should have the property of chemo- and radio-resistance. Table 2 compiles various cell types found in circulation and their characteristics. MSCs or hybrid-EMT cells produced as an outcome of EMT or even epithelial cells do not possess most of these properties. Thus, the relevance of EMT during metastasis becomes ambiguous and needs to be re-examined. CTCs exist in circulation as single cells or as small clusters (either homotypic or heterotypic) along with cancer associated fibroblasts (CAF). CAFs are central to tumor microenvironmentt in primary and metastatic tumors, influence behaviour of cancer cells and are involved in cancer progression [83]. Tumor-associated macrophages (TAMs) within tumors, and their precursors, resident macrophages and monocytes, help create an immunosuppressive tumor microenvironment by producing cytokines, chemokines, growth factors, and triggering the inhibitory immune checkpoint proteins in T cells [84].

PGCCs in solid cancers arise in response to stress and are considered to have a more crucial role to play in

Table 2 Metastasis-related properties exhibited by various cell types in circulation doubtful (?), no (X), yes (Y)

	Epithelial Cells	MSCs CAFs	CTCs, CTC clusters, CTCs interaction with hemato-poietic cells TAMs, CAML etc.	VSELs, CSCs PGCCs, CHIP
Cellular plasticity	?	?	?	Y
Ability to self-renew	X	?	X	Y
Expression of stem cell markers	X	X	X	Y
Clonogenic potential	X	X	X	Y
Metabolic reprogramming	X	X	?	Y
Ability to survive hypoxia	X	X	?	Y
Resist apoptosis and anoikis	X	X	?	Y
Immune evasion	X	X	?	Y
Enter and exit dormant state	X	X	X	Y
Ability to interact with stromal niche	X	?	?	Y

metastasis than CTCs [85–88]. CHIP (clonal haematopoiesis of indefinite potential) is characterized by the expansion of hematopoietic cells harbouring leukemia-associated somatic mutations in otherwise healthy people and occurs in at least 10% of adults over 70 [89, 90]. People with CHIP have increased rates of hematologic malignancy, increased risk of cardiovascular disease, and worse all-cause mortality compared with those without CHIP.

As evident from Table 2, various hallmarks of MICs are possessed by VSELs that transform into CSCs in response to various insults to initiate cancer. Rather than EMT and associated MSCs, and CTCs; CSCs that transition from tissue-resident VSELs along with polyploid giant cancer cells (PGCCs) have a crucial role during metastasis. We recently discussed that PGCCs appear due to clonal expansion of VSELs/CSCs that survive chemo- and radiotherapy [91]. Clonal expansion of stem cells is a normal feature of stem cells that get augmented in tumor tissues and CSCs survive oncotherapy and undergo clonal expansion and form PGCCs. Discussed below are the various hallmarks of MICs in the context of VSELs/CSCs.

Hallmarks of MICs are described in published literature focused on CTCs in circulation that are generated by EMT and are thought to mobilize and cause metastasis [92]. But these hallmarks need to be carefully reassessed keeping in mind that CSCs (epigenetically altered VSELs) that exist as a distinct sub-population and with distinct cellular features and properties (including the ability to form PGCCs), amongst the epithelial cells, get mobilized to initiate metastasis.

Molecular Hallmarks of MICs

Cellular Plasticity

It is suggested that epithelial cells have plasticity and undergo EMT to produce MSCs which can mobilize to distant places as CTCs to initiate metastasis. EMT also results in cells with stem-like properties. But these cells are difficult to characterize and lack clarity [29, 30]. Rather than this widely held view in the field of cancer biology, we posit that being pluripotent, VSELs exhibit plasticity and the ability to differentiate into the three germ layers as reported in mice as well as humans. Ratajczak's group was the first to report on the plasticity of VSELs. Being pluripotent, VSELs from mouse bone marrow differentiate into the 3 germ layers and germ cells in vitro [39, 93]. CD45- VSELs, enriched from GFP + 5-FU treated mice bone marrow transition into CD45 + cells within 14 days in vitro [40]. Pluripotent VSELs with nuclear OCT-4 co-exist with lineage-restricted and tissue-committed HSCs expressing cytoplasmic OCT-4 [94]. A similar ability to differentiate into the 3 germ layers has also been shown using VSELs isolated from human cord blood [41, 42]. Ratajczak's group [36] recently compiled various studies that demonstrated the differentiation potential of VSELs into cardiac cells, bone cells, MSCs, blood cells, liver alveolar epithelium, and gametes in vivo. We have reported that VSELs participate in and regenerate mouse pancreas in vivo after partial pancreatectomy [51, 52]. Thus, VSELs can differentiate into cells of all 3 lineages in vitro and regenerate multiple tissues in vivo (Supplement pages 6–8).

Expression of Stem Cell Markers

Dedifferentiation and reprogramming of epithelial cells into stem cells expressing OCT-4 and other markers occur neither in vivo in solid cancers nor in vitro to produce induced pluripotent stem cells. We have discussed this in detail in our earlier publication [32]. No study has yet been published where a 'pure' population of somatic cells was subjected to reprogramming in vitro; the starting cell population always remains heterogeneous. A study published in PNAS journal reported that MUSE cells alone in fibroblast culture can reprogram in vitro providing support to the view that fully

differentiated cells do not dedifferentiate to form pluripotent embryonic colonies [95, 96].

On the other hand, VSELs are developmentally linked to primordial germ cells (PGCs) [36]. During early development, PGCs migrate to the gonads and differentiate into germ cells. But it has been discussed that the PGCSs indeed mobilize to all developing tissues in the body and survive in small numbers throughout life [36, 37, 97]. VSELs play a crucial role in maintaining lifelong homeostasis by balancing between proliferation and differentiation. However, VSELs are directly impacted by endocrine-disrupting chemicals, undergo epigenetic changes and transform into CSCs. It needs to be appreciated that VSELs exist in very few numbers in normal tissues, but the CSCs enter cell cycle and increase in numbers (Supplement pages 9–18). We reported marked increase in VSELs numbers upon exposure to estradiol and diethylstilbestrol based on flow cytometry and qRT-PCR studies in the mouse uterus and testis [67, 98]. Both VSELs and CSCs express transcription factors OCT-4, SOX-2, NANOG, REX1 essential for maintaining a pluripotent state, and these markers are also reported in various cancers in humans [99, 100]. Transcriptome profile of VSELs in mouse bone marrow and in women with borderline ovarian cancer is reported [69]. A recent single-cell RNAseq study on VSELs sorted from the human cord blood reported their transcriptional signatures and showed that imprinted genes regulate their germ lineage origin [101].

Like PGCs, VSELs quiescence is maintained by certain parentally imprinted genes. Under normal conditions, VSELs show bilateral erasure of parental imprinting at Igf2-H19 locus [102]. Every time a pluripotent VSEL undergoes asymmetrical cell division (ACD) [32, 91], besides inactivation of majority of chromatin in the progenitor which is now tissue-committed, somatic imprints are reestablished. VSELs express sex hormone receptors and are directly impacted by neonatal exposure to endocrine disrupting chemicals [61, 65–67]. This results in altered expression of various epigenetic marks and the imprinting gets altered. Imprinted gene loci *Igf2-H19 and Dlk-Meg3* are dysregulated and exhibit modified methylation patterns. Increased expression of growth-promoting genes *Igf2* and *Dlk1*, while the reduced expression of growth inhibitory genes *H19* and *Meg-3* suggested that the stem/progenitor cells come out from their quiescent stage and undergo aberrant self-renewal, proliferation blocked differentiation [61, 65–67, 98]. As a result, although CSCs express similar pluripotent transcripts like VSELs, they are distinct, have altered expression of sex hormone receptors (estrogen dominance and progesterone resistance), epigenetic state and genomic imprinting. This explains why CSCs show global hypomethylation, increase in numbers, express Ki67 and specific markers like CD166 along with reduced tumor suppressor genes and genes related to the methylation machinery (Supplement pages 9–18).

Functional Hallmarks of MICs

Ability To self-renew

Under normal conditions, epithelial cells in solid tissues undergo regular turnover and are continuously replaced. Once they differentiate and become functionally mature, these cells lose the ability to self-renew and have a limited lifespan. As mentioned earlier, VSELs survive as a sub-population amongst the epithelial cells and function in a subtle manner (undergo ACD, give rise to tissue-specific progenitors that further differentiate into tissue-specific cell types) to renew the epithelial cells in solid organs. These stem cells are immortal, normally exist in G0 stage of the cell cycle and undergo ACD that helps maintain their genetic integrity. ACD results in the formation of cells of two distinct sizes and fates, including a smaller one to self-renew and the other is a progenitor cell that further expands by undergoing symmetrical divisions and clonal expansion before initiating differentiation [33, 92]. The progenitor that arises by ACD becomes lineage-restricted and fate-committed. ACD is a dynamic event that involves chromatin remodelling and extensive epigenetic changes that result in the speciation of the progenitors. However, this process gets altered due to extrinsic/intrinsic insults that transition VSELs into CSCs. CSCs can undergo excessive self-renewal; however, further differentiation is compromised (Supplement pages 9–18).

Clonogenic Potential

VSELs undergo ACD to give rise to progenitors that in turn undergo symmetrical divisions and clonal expansion (rapid endoreduplication with incomplete cytokinesis) before initiating differentiation into tissue-specific cell types [43]. Clonal expansion of tissue-specific progenitors is a feature of normal tissues but remains underreported as clonally expanded cells quickly proceed to differentiate into tissue-specific cell types. This process of clonal expansion gets amplified in cancer tissues (since further differentiation remains blocked) and this is why PGCCs are being reported by several groups. CSCs undergo clonal expansion to form PGCCs in solid tissues, and leukemic stem cells give rise to CHIP in blood which are increasingly observed with advanced age (suggesting compromised further differentiation) and indicate increased risk of developing leukemia. This has been discussed in detail elsewhere [91].

Metabolic Reprogramming

Cancer is often described as a metabolic disease [33]. It is a well-known fact for almost 100 years that cancer cells depend on glycolysis for their energy requirements even in the presence of oxygen, 'Warburg effect' compared to adjacent normal tissues, which depend on OXPHOS [103]. Targeting CSC metabolism is considered a promising therapeutic strategy [104]. Positron emission tomography (PET) using an analog of glucose [^{18}F-2-fluoro-2-deoxy-D-glucose (FDG)] along with computed tomography (CT) is the gold standard test used globally to determine the presence, progression, metastasis, and recurrence of multiple types of cancers by taking advantage of the 'Warburg effect' [105]. Dysfunctional mitochondria are reported in multiple types of cancers by various groups and have been reviewed [33]. Scientists have tried to explain this peculiar behaviour of cancer cells based on the metabolic reprogramming of differentiated cells as they undergo paligenosis (dedifferentiation and reprogramming) into cancer cells [106]. But the increased dependence on glycolysis could be simply because, like the inner cell mass cells from which embryonic stem cells are obtained in vitro and PGCs, have a paucity of functional mitochondria and have few, spherical mitochondria with poorly developed cristae [36] and depend on glycolysis. During normal differentiation, mitochondria increase in numbers, become elliptical, and develop well-formed cristae where OXPHOS can occur. However, CSCs and cancer cells (with blocked differentiation) retain few mitochondria with poorly formed cristae. This could explain increased dependence on glycolysis by cancer cells.

Ability To Survive Hypoxia

Solid tumors outgrow their vasculature, resulting in hypoxia. Over-expression of HIF1α has been reported in many types of malignancies, including lung, prostate, breast, colon and rectum carcinoma, and in both regional and distant metastases [107]. HIF-1 regulates cellular oxygen homeostasis and plays a key role in hypoxic conditions during tumor angiogenesis, invasion, and metastasis. HIF-1 is a heterodimeric transcription factor that consists of α and β subunits. The β subunit is constitutively expressed, while the oxygen levels regulate HIF-1α expression. As a transcription factor, HIF-1α regulates the expression of dozens of genes that maintain homeostasis as oxygen concentrations change. Circulating tumor cells (CTCs) are implicated as precursors and harbingers of metastasis. Shedding of CTCs as a result of EMT or the physical shedding of tumor epithelial cells results in the dissemination of cancer cells to distant regions to cause metastasis. A recent study discussed that hypoxia induces CTC-platelet cluster formation that promotes breast cancer metastasis [108]. CTC clusters are reported to be 23–50 times more metastatic than single CTCs [109].

VSELs can survive hypoxia due to their quiescent nature, high nucleo-cytoplasmic ratio, and minimal cytoplasmic organelles, and lower metabolic rate. Zhang et al. [110] have reported expression of HIF-2α in VSEL-like MSCs, and thus, it is not clear if undifferentiated VSELs express HIF, but it is expressed at the onset of differentiation. They show minimal metabolic activity and survive hypoxia. VSELs depend on glycolysis for their energy requirements and are virtually immortal in nature (Supplement pages 1–5). They exist in the peripheral blood of normal subjects and are increased in numbers in patients with cancer. We utilize this property of VSELs for early prediction of cancer [71]. Being immortal and pluripotent, VSELs are most likely candidates for metastasis compared to CTCs or tumor epithelial cells, which lack plasticity and have a half-life of few hours [111].

VSELs survived chemo- and radiotherapy and remained dormant for more than 2 decades. This was reported by Kurkure et al. [49], who showed the presence of VSELs in otherwise non-functional and azoospermic testis of cancer survivors of childhood cancer. These VSELs in azoospermic testis could restore spermatogenesis and fertility when exposed to a healthy niche, e.g., by transplanting mesenchymal stromal cells. On similar grounds, the epigenetically altered CSCs that escape oncotherapy and can survive long term - could start multiplying again when exposed to a conducive niche, resulting in recurrence. But this remains a hypothesis, and more work needs to be undertaken to provide evidence in support of this concept.

Resist Apoptosis and Anoikis

Apoptosis and anoikis are different forms of programmed cell death and the ability of cancer cells to evade or resist them is considered a significant hallmark of cancer [92]. Apoptosis could eliminate abnormal cells with mutations and DNA damage but it is normally suppressed, allowing the growth of cancer.

Anoikis is induced upon cell detachment from the extracellular matrix. Anchorage-independent growth and EMT, two features associated with anoikis resistance are vital steps during cancer progression and metastatic colonization. The ability of cancer cells to resist apoptosis and anoikis are areas of interest to develop therapeutics. Several recent articles challenge this hallmark of resisting or evading apoptosis [112–114]. Rather, increased apoptosis and mitosis are found associated with cancer aggressiveness and poor prognosis [115]. Also, overexpression of BCL2 (anti-apoptotic gene) is linked with better survival of cancer patients [116]. Cancer cells do not live longer than normal cells but have

a shorter life span. No clarity exists on how tumor tissue repopulates itself after chemo- and radiotherapy, resulting in relapse or recurrence. Patients who relapse are rarely cured and often have only a short-term progression-free survival. Effective elimination of cancer cells by apoptosis has been a mainstay and goal of clinical cancer therapy for over 3 decades. However, apoptotic cells can promote tumor repopulation through, e.g., the Phoenix Rising pathway that is orchestrated by executioner caspases [117]. 'Phoenix Rising' explains how apoptotic cells promote the proliferation of adjacent cells, resulting in recovery after oncotherapy. Li et al. [118] showed that caspases released from apoptotic cells release PGE2 that stimulates stem cell proliferation and tissue regeneration. This process of Phoenix Rising has been studied in solid tumors in vitro [119, 120]. It is emerging that such processes are mediated by EMT forced by therapy-induced damage, which increases the migratory capability of cancer cells, promoting metastases. Microscopic observations have identified giant cells termed PGCCs with nuclear abnormalities amongst the dying cells upon chemotherapy.

Against this backdrop, VSELs being virtually immortal and quiescent in nature (Supplement, pages 1–5), do not undergo apoptosis or anoikis or autophagy to survive oncotherapy. Data is published in mouse models and humans that VSELs survive radio- as well as chemotherapy because of their quiescent nature [44–49]. CSCs that exist as a small sub-population amongst cancer cells, will survive oncotherapy (although >90% of tumor cells die), and cause recurrence.

Immune Evasion

One of the primary roles of the immune system is to detect and kill cells that have acquired cancerous mutations. Natural killer (NK) cells, cytotoxic T cells, and other immune cells recognize and eliminate these aberrant cells. Cancer metastasis depends on the ability of cancer cells to evade attack from the immune cells [121, 122]. Cancer cells avoid detection and attack by the immune system by using various mechanisms including (i) by decreasing the expression of surface proteins like MHC that identify them as abnormal, (ii) by suppressing immune cells through the release of cytokines and recruitment of regulatory T-cells. Cancer cells attract Tregs that suppress other immune cells that might attack the cancer (iii) By activating immune checkpoint pathways like PD-L1, which binds PD-1 on T cells, putting the brake on T cell killing (iv) by hijacking immunosuppressive cells like tumor-associated macrophages (TAM) to create tolerance. Myeloid cells, including macrophages, are usually first responders of the immune system. However, cancer can reprogram these cells to help it instead. These corrupted cells, known as tumor-associated macrophages (TAMs), can suppress other immune cells and even help the cancer to grow and spread (vi) by masking tumor antigens through shedding decoys, forming physical barriers against immune cell infiltration (vii) by enhancing resistance pathways to withstand immune assault. (viii) tumors can release factors (e.g., TGF-β, IL-10, CXCLs) that suppress immune responses. The sum of these evasive methods creates an immunosuppressive tumor niche that enables immune escape [123–125]. The immune landscape in the tumor microenvironment (TME) is composed of macrophages, neutrophils, natural killer (NK) cells, dendritic cells (DCs), bone marrow-derived suppressor cells (MSDCs), and tumor-infiltrating lymphocytes (TILs). However, the precise mechanism of EMT-induced immunosuppression is not very clear. Weinberg's group have suggested that the mesenchymal cells produced due to EMT possess immunosuppressive properties [11, 12]. However, epithelial and mesenchymal carcinomas significantly differ in their susceptibility to immune attack [126]. Epithelial carcinomas are more susceptible to immune responses and respond well to immunotherapy, while mesenchymal carcinomas are more resistant and are refractory to therapies like immune checkpoint blockade due to their immunosuppressive microenvironment. This difference is largely due to EMT which mesenchymal tumors undergo to evade the immune system. Also, Snail1 is involved in tumor immunosuppression by inducing chemokines and immunosuppressive cells into the tumor microenvironment (TME) [127].

VSELs play a crucial role during regular turnover of cells in multiple tissues, maintaining lifelong homeostasis, and regeneration in response to chronic injuries in healthy tissues. They are normally quiescent, long-lived, virtually immortal and have mechanisms in place to protect themselves from adverse autoimmune responses and chronic inflammation. It is known that early embryos, undifferentiated embryonic, and adult stem cells (mesenchymal and neural stem cells) are very likely immune-privileged [73, 128]. Like VSELs, CSCs also express OCT-4 and other transcription factors specific to pluripotent stem cells. Being pluripotent, primitive and undifferentiated VSELs/CSCs are most likely immune privileged and are expected to show reduced expression of MHC I and II antigens and thus can evade immunological attacks and be responsible for metastasis in all types of cancers.

Enter and Exit Dormant State

Cancer relapse, metastasis and recurrence, following successful treatment, is a major threat and responsible for majority of deaths amongst cancer patients. During metastatic tumor dormancy, disseminated cancer cells remain in

a viable, yet not proliferating state for a prolonged period. Dormant cancer cells are characterized by their entry into cell cycle arrest and survival in a quiescence state to adapt to their new microenvironment through the acquisition of mutations and epigenetic modifications, rendering them resistant to anti-cancer treatment and immune surveillance [129–131]. Under favorable conditions, disseminated dormant tumor cells 're-awake', resume their proliferation and thus colonize distant sites [132]. A temporary mitotic arrest in G_0-G_1 stage of cell cycle occurs through a complex interplay of intrinsic and extrinsic mechanisms. Intrinsic mechanisms include genetic alterations, autophagy, intracellular signaling pathways (ERK/p38 balance), and epigenetic changes. Extrinsic factors influencing dormancy are the extracellular matrix (ECM), hypoxia, and immune surveillance [133].

Under normal conditions, VSELs exist in G0 stage of cell cycle in a 'reversible' quiescent state. Data is available to show that VSELs enter the cell cycle in response to chronic injury and once homeostasis is achieved, they return to G0 stage. This sets VSELs apart from the differentiated epithelial cells which lack plasticity, exist in a state of irreversible quiescence, and have a limited life span. VSELs also enter the cell cycle in response to extrinsic/intrinsic insults, undergo epigenetic changes, and transform into CSCs to initiate cancer (Supplement pages 9–18). Similar mechanisms also possibly exist at distant sites during metastasis and recurrence.

The question that needs to be addressed is why the sites of cancer initiation, progression, metastasis, and recurrence show dependence on glycolysis with increased glucose uptake and are detected by PET-CT? This is strong and direct evidence supporting the expansion of a novel population of stem cells at primary and metastatic sites and at times of recurrence that depend on glycolysis. As discussed above, these cells are the VSELs/CSCs with paucity of mitochondria.

Ability to Interact with the Stromal Niche

A favourable microenvironment, or niche, is crucial for metastatic progression. Distant tissues are normally hostile environments for the cancer cells that get mobilized from the primary tissue. During metastatic colonization, tumor cells must establish a favourable microenvironment or niche that will sustain their growth at the metastatic sites. However, both the cellular and molecular details of this process remain poorly understood. Most of the mobilized cells die by anoikis/apoptosis, hypoxia, energy resources, or by death signals from the incompatible stromal and immune cells of the host tissue. MICs have evolved multiple mechanisms to turn a potentially hostile environment in a secondary organ into a supportive niche. This is achieved by releasing systemic growth and survival signals from the primary tumor, competing for existing normal stem cell niches, and engaging and converting the stromal cells to thwart death signals and immune attack [134, 135]. A main threat to MICs is the immune cells present at the new organ sites. EMT-associated transcription factors and markers like TGF-β support metastasis [136].

Functions of both VSELs and CSCs are controlled by their niche under normal conditions and in cancer. Niche directly controls the balance between the proliferation and differentiation of stem cells under homeostatic conditions. VSELs can survive in a non-supportive niche for years and can also survive inflammation. VSELs were detected in azoospermic testes of survivors of childhood cancers after lying dormant for >20 years [49]. These VSELs that survive in otherwise azoospermic testis in cancer survivors, can restore spermatogenesis when a supportive niche is provided by way of transplanting mesenchymal stromal cells. The niche at the site of the primary tumor also gets affected by various insults and supports excessive proliferation of CSCs and early cancer cells (with tissue-specific signatures) that cause tumor growth and undergo increased mobilization to distant sites. CSCs (epigenetically altered VSELs) survive hypoxia, and immune attack and depend on glycolysis for their energy requirement. They can extravasate into any organ, lie dormant for years, and undergo clonal expansion into metastatic tumors when exposed to a conducive microenvironment.

Discussion and Conclusions

We have reviewed published studies by multiple groups that challenge the process of EMT resulting in MICs that travel to distant places to cause metastasis, can survive for decades, and then recur as a fatal disease. It is widely believed that MICs exist in the peripheral blood as CTCs with a short half-life either singly or in clusters. Varied descriptions exist to explain how the MICs survive hypoxia and immune attack followed by MET in a secondary organ in an avascular environment and either shortly or after extended periods of dormancy undergo clonal expansion and grow out as a metastatic tumor. The concept of the presence of CSCs exists, but how they arise and their characteristics remain vague. Seminal studies have delineated CSCs in cancer initiation, but their origin remains disputed, whether they form during EMT by dedifferentiation of mature cells or arise due to the dysfunctions of stem cells that reside in normal tissues.

Cancer is a more than 200-year-old disease but still remains a black box. Epithelial cells are believed to

dedifferentiate and reprogram into a cancer cell by paligenosis to initiate cancer [107]. We explained the underlying misconceptions behind this concept of cancer initiation [35]. Similarly, it is widely believed that metastasis is associated with cellular events like EMT, fusion of CTCs with platelets to survive hypoxia and evade immune attack, pro-survival properties through phoenix rising, failed apoptosis, and/or anastasis (return from the brink of death) by which MICs show therapeutic resistance and ability to survive. There is no clarity on whether apoptosis is suppressed or upregulated in a tumor. How the MICs enter and exit a dormant state followed by clonal expansion, resulting in recurrence. Whether cellular events like autophagy inhibit the growth of cancer cells and push them into a dormant state explaining recurrence after decades? We undertook a careful review of the literature on EMT leading to metastasis and current understanding of various hallmarks of MICs. But the feeling of futility at the end of the exercise was depressing since the result of the hundreds of thousands of man-years of work on understanding metastasis which results in >90% of cancer-related deaths appear minimal as was suggested earlier [137].

Kaelin Jr [138] commented that lack of knowledge is the true bottleneck to clinical translation and evidently why the war against cancer has not yet been won. Weinburg recently commented that the war against cancer has just begun in 2024 [3]. Diverse opinions and concepts propounded by various groups to explain cancer initiation, metastasis, and recurrence are simply mansions of straw that need to be replaced by a house of bricks. This complete lack of understanding is the reason for >90% deaths due to metastasis. Foulds, a British cancer biologist in 1970 [139], stated that cancer research will reach an outstanding landmark the day we can define cancer in biological terms.

Ratajczak's and Bhartiya's groups [44–48] have reported that VSELs (being quiescent) survive radio- and chemotherapy in mouse models. Kurkure et al. [49] detected VSELs in azoospermic testes of cancer survivors even after >15–20 years of cancer therapy. These publications suggest that VSELs alone have the potential to survive chemo- and radiotherapy in cancer patients, cause recurrence, and satisfy various hallmarks of MICs as discussed above. Being immune-privileged, VSELs will not be affected by immunotherapy and are responsible for immune resistance. Why should a big, fully differentiated cell with abundant organelles, including large numbers of mitochondria, undergo autophagy to get rid of them to shift to glycolysis and enter dormancy? VSELs harbor minimal organelles, depend on glycolysis, survive oncotherapy and thus are ideal candidates to explain metastasis and recurrence.

The question is how to instigate a paradigm shift in metastasis research so that personalized medicine is applied to target MICs rather than solely to the primary tumor. How do we shift the focus from treating metastasis to preventing metastasis when we currently do not have any drugs that specifically target disseminated or circulating tumor cells (DTCs or CTCs)? How can CTCs and DTCs be depleted from circulation to prevent cancer spread? The bigger question that surfaces is whether we should focus on VSELs/CSCs, PGCCS, and CHIP or on EMT, MSCs, DTCs and CTCs. Addressing these challenges requires bold thinking and methodologies to study rare cell populations.

It is hoped that the learnings of this article will provide an altogether different perspective to the process of metastasis during cancer independent of EMT. A fundamental shift in our understanding of cancer biology is crucial for developing effective therapies. A lot more work needs to be done on VSELs in clinical samples and brainstorming is required to address the limitations of current knowledge, challenge existing paradigms, and understand the basic biology of VSELs to provide a framework of cancer initiation, progression, and metastasis.

Abbreviations

CAF	Cancer Associated Fibroblasts
CAML	Cancer-Associated Macrophage-like Cells
CHIP	Clonal Hematopoiesis of Indefinite Potential
CSCs	Cancer Stem Cells
CTCs	Circulating Tumor Cells
DTCs	Disseminated Tumor Cells
EMT	Epithelial-Mesenchymal Transition
MICs	Metastasis Initiating Cells
MSCs	Mesenchymal Stromal Cells
PGCCs	Polyploid Giant Cancer Cells
TAM	Tumor-Associated Macrophages
VSELs	Very Small Embryonic-Like Stem Cells

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12015-025-11003-6.

Acknowledgements We acknowledge the earlier work done by Bhartiya's group at ICMR-NIRRCH, Mumbai. Also, detailed research undertaken by various groups globally on different aspects of VSELs biology. We acknowledge work done by large number of scientists in the field whose work we may not have cited out of ignorance.

Author Contributions Author contributions: DB was involved in the conceptualization of the manuscript, wrote the manuscript, and revised the final manuscript. NJ was involved in critical review of the manuscript. AT and AT actively participated in discussions. All authors read and approved the final version of the manuscript.

Funding Information No separate funds were required to prepare this article as it is based on published literature.

Data Availability No datasets were generated or analysed during the current study.

Code Availability (software application or custom code): Not applicable.

Declarations

Competing interests The authors declare no competing interests. DB and NJ are employees of a startup company named Epigeneres Biotech Pvt. Ltd. (EBPL), Mumbai. AnT and AsT are owners of Epigeneres Biotech Pvt. Ltd. AT is also affiliated with 23Ikigai Pte Ltd., Singapore.

Clinical Trial Details It is not applicable.

Consent to Publication Yes.

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