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REVIEW ARTICLE

Cancer Stem Cells: Formidable Allies of Cancer

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Abstract Cancer stem cells (CSC) represent the subpopulation of cells within a tumour showing two fundamental properties of stem cells – self-renewal (the ability to make more of their own kind) and differentiation (the ability to generate diverse cell types present within a tissue). The CSC hypothesis posits that CSCs play an important role in tumour initiation, maintenance and progression. Furthermore, owing to their intrinsic drug resistance, they remain refractory to currently used therapy, thereby contributing to tumour relapse. Thus, targeting or taming CSCs can lead to more effective cancer treatment in the coming decades. In this review, we will discuss about the origin of CSC hypothesis, evidence showing their existence, clinical relevance and translational significance.

Keywords Cancer stem cells · EMT · Drug resistance · Microenvironment · Targeted therapy

Introduction

Cancer is a disease that has plagued humans since centuries with incidences recorded throughout our history. The earliest record of cancer, though the term was not used then, is in an ancient Egyptian text on trauma surgery called Edwin Smith Papyrus. There are eight cases of breast tumours that were described in the text which had been removed surgically (cauterization) with a tool called the fire drill. Evidences for cancer were also found in other ancient manuscripts, amongst fossilized bones and ancient Egyptian mummies.

The coining of the term 'cancer' is credited to Hippocrates (460–370BC), recognized as the father of modern medicine. He had used the Greek term *carcinoma*, which refers to a crab, to describe cancerous growth. It was the Roman physician, Celsus (28–50BC) who translated the Greek term into *cancer*, which is the Latin word for crab.

Efforts to understand the origin of cancer had started since these times. Some of the earliest theories of cancer origin include infectious disease theory, humoral theory, lymph theory, etc. It was in 1863 that Johannes Muller, a German pathologist, demonstrated that cancer is made up of cells. But he wrongly believed that cancer cells did not come from normal cells. His student, Rudolph Virchow (1821–1902) determined that all cells, including cancer cells originate from other cells. This was the turning point after which the study of cancer, oncology, commenced in earnest [1].

Today, we know that, cancer is caused by abnormal cell growth, in most cases giving rise to solid cellular mass called tumour. Abnormal cell growth results from deregulations of pathways that govern cell proliferation/division and cell death/apoptosis of mutated cells. Earlier it was believed that cancer is caused by acquisition of mutation/s in one cell which divides rapidly to establish the tumour ('Concerning the origin of malignant tumour" By Theodor Boveri, 1914) [2] i.e. the tumour consists of clones of the originator cell. However, we now know that there is striking variability amongst the cancer cells within a single tumour with respect to cell size, morphology, proliferation rate, surface marker expression, metastatic proclivity, sensitivity to chemotherapy, etc. There are two warring schools of thought to explain the establishment and maintenance of this "*tumour heterogeneity*": one is the *clonal evolution theory* and the other is *cancer stem cell theory.*

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According to the clonal evolution theory, all cancer cells have the potential to seed new tumours i.e. all cancer cells are tumorigenic. This might be due to their ability to acquire additional mutations that confer new characteristics, owing to genetic instability and rapid proliferation rates. The cancer cells may acquire such variation due to mutations or epigenetic changes. Some of these characteristics can confer rapid proliferative capabilities or a growth advantage, ultimately giving rise to a separate sub-clone within the tumour population. Depending on the extent of the selective advantage, this sub-clone may become the dominant sub-clone within the tumour or it may be one sub-clone amongst many others that co-exist within the tumour. Additionally, it may be that some sub-clones dominate one spatial location within the tumour, depending on its microenvironment [3].

The other theory is the *cancer stem cell theory.* According to this theory, only a subset of tumour cells, called *cancer stem cells (CSCs)* harbour the ability to self-renew indefinitely and to differentiate to give rise to all the cell types that comprise the tumour. The cancer stem cell hypothesis states that only the CSCs are tumorigenic while the bulk of the tumour is not. The tumorigenic CSCs are responsible for driving tumour initiation, maintenance and recurrence, whereas the non-tumorigenic cells comprise the bulk of the tumour but cannot self-renew or initiate tumour formation. Thus, cancer stem cell hypothesis posits that the functional heterogeneity seen in cancer is due to differences in differentiation status, with CSCs at the top of the hierarchy, followed by progenitor cells and bulk of the tumour cells [4, 5]. Hence, nowadays, tumours are seen more as caricatures of "abnormal" organs, sustained by a minority of CSCs [6] (Fig. 1).

Even in the CSC hypothesis, there is controversy whether normal stem cells in the body acquire mutations that give rise to cancer stem cells or whether CSCs arise from dedifferentiation of transformed cells. Thus, the two theories do not state what the originator cell for cancer is. They mention how the tumour becomes heterogeneous, since the earlier perception was that cancer is made up of clones of the originator cell. Additionally, nowadays, studies indicate that both the models have merit and should not be considered mutually exclusive [7, 8].

Discovery of Cancer Stem Cells

Let us first understand the meaning of the term "stem cells". Stem cells are defined by two properties: (1) their ability to perpetuate themselves through self-renewal and (2) to differentiate into progenitor cells via asymmetric division: each stem cell divides to form two daughter cells, one is an undifferentiated stem cell thereby maintaining the pool of stem cells, while the other, is a progenitor cell which is committed to differentiation. The progenitors or transit amplifying cells undergo few rounds of rapid cell division to generate the diverse array of differentiated cells. We will take the example of hematopoietic stem cells (HSCs) that are present in the bone marrow, and are well characterized, to understand this better. The existence of HSCs was first discovered in serial transplantation experiments in mice, which demonstrated the existence of clonogenic precursors in

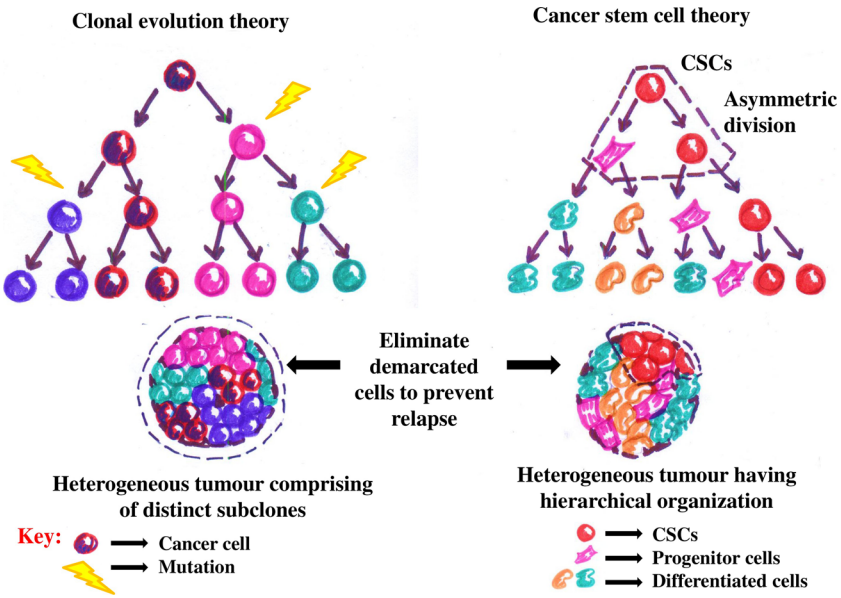

Fig. 1 **a Clonal evolution model:** During proliferation of a cancer cell, it might spontaneously acquire mutation/s giving rise to a distinct sub-clone within the tumour. Many such varied sub-clones constitute the tumour mass. Each of these cells possesses the ability to seed new tumours and hence, all of them must be eliminated for effective therapy. **b Cancer stem cell hypothesis:** Cancer stem cells (CSCs) are at the top of the hierarchical organization of tumours, which divide asymmetrically to produce two daughter cells: one CSC itself and the other is a progenitor cell. The progenitor cell, in turn, gives rise to more differentiated cells in the tumour, which form the tumour bulk. Thus, tumours show heterogeneity with respect to differentiation status. CSCs alone have the capacity to seed new tumours, and hence, elimination of this fraction is critical for preventing tumour relapse

the bone marrow that are capable of long term expansion and multipotent myelo-erythoid differentiation. These constitute a small population, representing as little as 0.5 % of the total bone marrow, and are of three types: long term self-renewing HSCs, short-term self-renewing HSCs and multipotent progenitors without any detectable self-renewing capacity [9, 10]. They form a hierarchy with the long-term renewing HSCs forming the short term renewing HSCs, which in turn give rise to the multipotent progenitor. The multipotent progenitors differentiate irreversibly to form specific myelo-erythoid lineage. The long-term self-renewing HSCs are quiescent in nature. As the quiescent long term self-renewing HSCs differentiate to ultimately form the progenitors, they progressively lose their self-renewal capacity and become mitotically active. Thus, HSCs maintain homeostasis in blood, that is, they divide to maintain the repertoire of blood cells which undergo rapid turnover in the body [11, 12]. Similarly, other organ mass and tissue architecture is maintained by tissue-specific stem cells. Thus, normal stem cells within the body function to replace the cells lost by wear and tear, or become activated when the organ suffers physical damage to replenish the damaged cells.

Since cancer is believed to be caused by the acquisition of multiple genetic mutations in a single target cell, sometimes over a period of several years, normal stem cells, which are the only long-lived cells in many tissues, may be the cell-of-origin of cancer. This idea is quickly gaining favour and is called "the stem cell hypothesis of cancer origin". Cancer stem cells (CSCs) are so named because they exhibit the same properties as normal stem cells, particularly, self-renewal and ability to generate other cell types. Like normal stem cells maintain the various cell types within an organ, CSCs are a minor population within the tumour with the ability to differentiate and give rise to the heterogeneous progeny of cells found in the bulk tumour.

The earliest hints about the existence of cancer stem cells came from the studies of Bruce and van der Gaag [13] and Clarkson [14] wherein they observed that only 1–4 % of patient-derived lymphoma cells can form colonies in vitro or can initiate formation of macroscopic colonies in the spleen of irradiated mice. Another study identified rare, slow cycling cells within leukemic cell lines that also showed resistance to anti-proliferative agents [15]. However, clear evidence about the existence of CSCs is generally credited to the study by Bonnet and Dick in 1997. They showed that only the $CD34^+$ $CD38^-$ cells from acute myeloid leukemia (AML) patients could initiate hematopoietic malignancies in NOD/SCID mice and possessed the ability to self-renew and differentiate [16]. The existence of such tumor-initiating cells or CSCs in solid tumours came from the study of Al-hajj et al. in 2003 in the context of breast cancers. They observed that only a small fraction of breast cancer cells formed tumours in immune-compromised mice. This fraction could be distinguished by $CD44^+$ $CD24^{-/low}$ phenotype, and as little as 200 cells of the phenotype $CD44^+$ $CD24^{-/low}$ were able to form tumours in mice whereas more than 10,000 cells with alternate phenotype failed to do so (Fig. 2a). This fraction could be serially transplanted in NOD/SCID mice, that is, these cells had long term repopulation capacity. After each serial transplantation, the $CD44^+$ $CD24^{-/low}$ cells gave rise to cells representing the entire heterogeneity of the initial tumour, that is, the cells differentiated to give both tumorigenic as well as phenotypically diverse mixed population of non-tumorigenic cells (Fig. 2b) [17].

Following this study in breast cancer, many papers have established the existence of CSCs in other cancer types including brain [18], melanoma [19], ovarian [20], prostate [21, 22], head and neck [23], pancreas [24, 25], sarcoma [26], colon [27], and lung [28]. In all the above studies, CSCs are primarily characterized based on their ability to seed new tumours in immune-deficient host mice; hence, the term '*tumour initiating cells*' (TICs) is also used to refer to them.

Apart from their ability to seed new tumours, CSCs are also known to possess enhanced drug resistance capacity [29] that allows them to escape chemotherapy and cause relapse. In addition to cancer recurrence, CSCs are also associated with epithelial-mesenchymal transition (EMT) [30, 31] which helps in the invasion and metastasis cascade. Hence, it is important to understand CSC biology and the various signalling pathways that regulate them, which in turn will help in identifying novel targets that can be exploited for their elimination.

CSC Biomarkers and Techniques Used to Identify and Isolate Them

Since CSCs represent a small fraction of the bulk tumour mass, we need to identify and isolate them for further studies. This can be achieved using fluorescence-activated cell sorting (FACS) based on the distinct and specific cell surface that are expressed by CSCs that sets them apart from other tumour cells and normal stem cells. As mentioned previously, $CD34^+$ $CD38^-$ phenotype distinguishes leukemic stem cells (LSCs) from non-tumorigenic ones [16]. Recent studies have determined more selective marker phenotype $CD34^+$ $CD38^-$ $HLA\text{-}DR^-$ $CD71^-$ $CD90^-$ $CD117^-$ $CD123^+$ for LSCs that distinguishes them from normal HSCs [32]. Similarly, ESA^+ $CD44^+$ $CD24^{-/low}$ phenotype identifies and allows isolation of Breast cancer stem cells (BCSCs) [17]. Other studies have identified CD133 along with specific other markers to identify CSCs in liver [33], brain [18], lung [28], colon [27, 34], pancreas [25] and prostate cancer [21]. Although this technique allows easy isolation of CSCs, it faces many limitations. A specific cell surface marker phenotype may not be known for all cancer types. Additionally, there is a possibility that the marker phenotype is ambiguous and even certain non-CSC also expresses the same. FACS itself is a harsh technique that the cells are subjected to before we study them further. However, for now, this is the best technique available to identify and isolate CSCs.

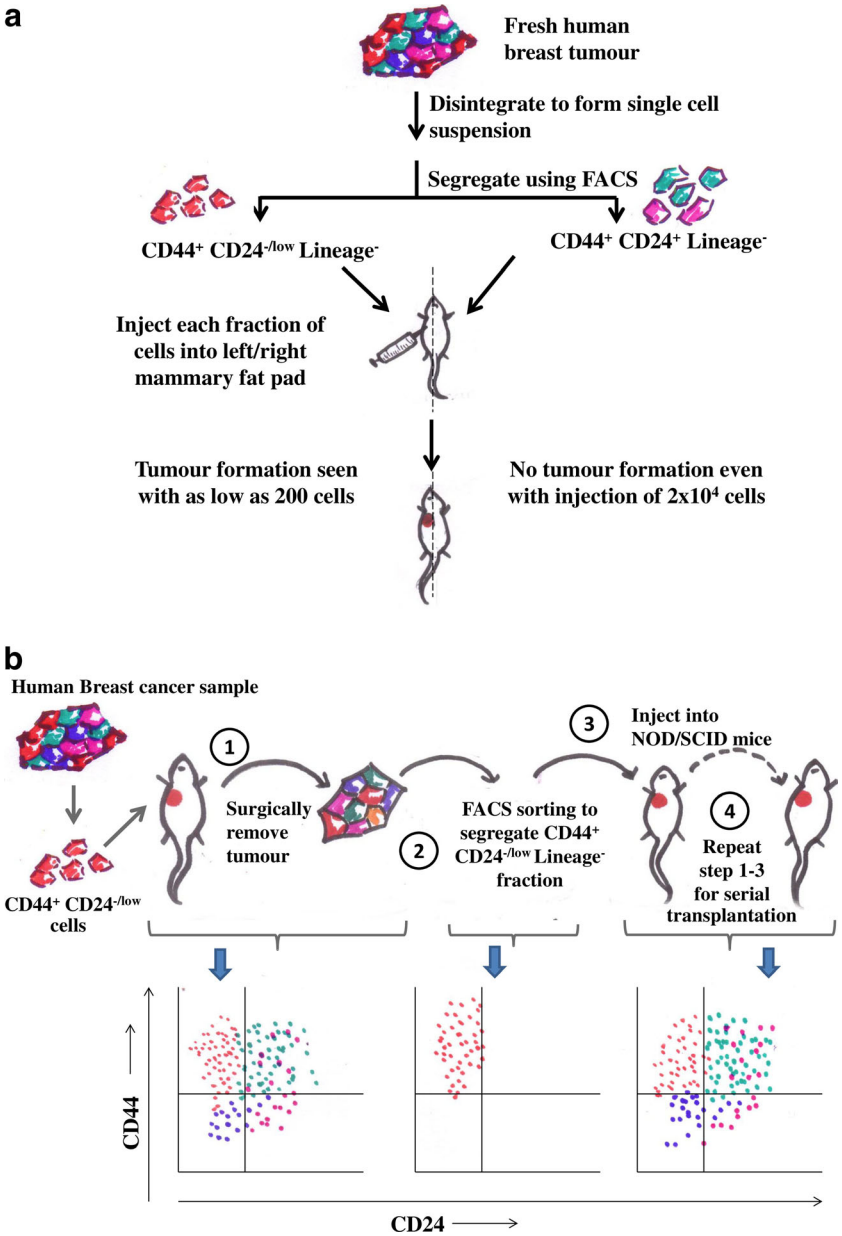

Fig. 2 Properties of cancer stem cells (using breast cancer as a model): **a** A subset of cells within primary tumour, characterized by $CD44^+$ $CD24^{-/low}$ marker phenotype, exhibiting enhanced tumour initiation ability: Fresh human breast tumour sample is minced, disintegrated into single cell suspension using collagenase and trypsin treatment and subjected to FACS sorting using CD44 and CD24 surface markers. Varying number of cells from the two fractions so obtained, $CD44^+$ $CD24^{-/low}$ and $CD44^+$ $CD24^+$, are injected into the left and right 2nd mouse mammary fat pad of NOD/SCID mice. Palpable tumour formation is monitored. For the $CD44^+$ $CD24^{-/low}$ fraction, as few as 200 cells gave rise to tumour of >1 cm diameter. For the $CD44^+$ $CD24^+$, no palpable tumour was detected even with injection of 20,000 cells. **b** CSCs differentiate to give rise to all the cell types found in the primary tumour and exhibit self-renewal and long-term repopulation capacity: Patient tumour passaged in mice, as described above, shows a heterogeneous expression of CD44 and CD24 markers. Enriched breast cancer stem cells (BCSCs) fraction ($CD44^+$ $CD24^{-/low}$) can be isolated from this passaged tumour and re-injected into another NOD/SCID mouse. The tumour so formed will have regained the original heterogeneity of the primary tumour as seen by the FACS profile, including the $CD44^+$ $CD24^{-/low}$ fraction. Repeating the above steps, the tumour can be passaged multiple rounds in NOD/SCID mice, indicating they have long-term repopulation capacity

In addition to cell surface marker expression, functional characteristics of cancer stem cells are employed for their identification and isolation. Tumorsphere formation assay, more commonly called "sphere formation assay" is a common technique used to identify and enrich for cancer stem cells from a given primary tumour sample or from cell lines. It exploits the ability of cancer stem cells to grow under conditions of low-attachment/suspension with minimal growth factors as 3D spheres of tumour cells. This in turn correlates with their tumour-initiation potential in vivo. This technique was first developed to maintain neural stem cells in an undifferentiated state as neurospheres [35, 36]. It

was later on adapted by Dontu et al. to cultivate primary mammary epithelial stem/progenitor cells in an undifferentiated state [37], while Yuan et al. used it for the first time to correlate with glioblastoma cancer stem cells [38] It is now used as a routine assay to detect presence of stem cells in various cancers [39, 40]. It overcomes the limitation of having to rely on a specific cell surface marker phenotype to identify cancer stem cells. Additionally, it allows us to quantify the number of CSCs within a sample by counting the number of spheres formed from single cell suspension grown in low-attachment condition.

Yet another functional assay that allows CSC isolation in the absence of cell surface markers came from the study of Cheung et al. who first discovered that increased aldehyde dehydrogenase (ALDH) activity can be used to isolate leukemic stem cells [41]. Subsequent studies have suggested that increased ALDH activity might be a universal marker since it identifies CSCs in liver [42], colon [43], lung [44], melanoma [45], head and neck [46], prostate [47], bladder [48], thyroid [49], glioblastoma [50] and osteosarcoma [51]. The aldefluor assay is used to measure ALDH activity and also allow FACS sorting of $ALDH^{+/high}$ cells. However, it gives a cumulative reading for all the isoforms of ALDH within the cell. It might be that different cancers have a specific ALDH isoform that may have the prognostic ability.

While the following functional assay utilizes the exclusion of vital dyes like Hoechst 33342 stain by CSC owing to overexpression of adenosine triphosphate-binding cassette proteins called ABC transporters. The CSC population that excludes the dye appear as a separate fraction in FACS and is called the *side population*. It can be isolated for further studies. One limitation of this assay is that even certain non-CSCs can express ABC transporters [52, 53].

Other assays that are used to determine the presence of CSCs and to quantify them are the serial transplantation assay (described in Fig. 2b) and the limited dilution assay [54], respectively. At least three or more of the above mentioned assays are generally used to demonstrate the presence of cancer stem cells.

Controversy About the Existence of Cancer Stem Cells

Cancer stem cells are defined functionally. Hence, till today, all CSCs studies have included sorting cells from cancer biopsies based on their cell surface markers to other strategies and injecting them into mice to score for tumour formation. Thus, the ability to initiate tumours in immuno-compromised mice is the major criterion for identifying CSCs. However, these assays raised many disputes: foremost being that human cells are transplanted into mice which have completely different microenvironment that might affect the behaviour of these cells. Furthermore, before transplantation the cells are subjected to vigorous isolation techniques that can affect them. Hence, the proportion of cells that can initiate tumour might be grossly underestimated. These issues raised the concern that cancer stem cells, though an attractive concept, might actually be just an experimental artefact.

These controversies have to a large extent been silenced by three pioneering studies using mouse models that have demonstrated the existence of CSCs and their role in tumour maintenance and recurrence. All the three studies have exploited lineage tracing, a technique that allows permanent in vivo fluorescence marking of specific cells and their progeny.

Using glioblastoma mouse model, Chen et al. provided compelling evidence about the existence of CSCs and their ability to cause relapse [55]. They brought the transgene expressing a genetic marker that labelled (GFP^{+}) adult neural stem cells, but not their differentiated progeny in the background of glioblastoma-prone (driver mutation is inactivation of p53, PTEN and Nf1 tumour suppressors) mice. All the gliomas developed in these mice contained a subset of GFP expressing cells, which also co-expressed stem cell marker Sox2, but did not express ki67 alluding to their quiescent nature. This indicated the presence of CSCs in gliomas. Next, they elegantly demonstrated that these normally quiescent CSCs regained proliferative ability after chemotherapeutic treatment that eliminated most of the dividing cells to cause cancer relapse. Using endogenous lineage tracing, they found that the relapsed tumours contained quiescent CSCs as well as actively dividing differentiated cells that had been derived from the CSCs. They also showed that if GFP labelled cells were specifically killed followed by conventional chemotherapy which kills the bulk cells, then the tumours regressed and there was no relapse [55]. This study is a proof of principle that targeting CSCs along with the bulk tumour cells is the "only" effective way of eradicating cancer. Thus, this paper proved that only CSCs have the capacity to sustain tumour growth and are responsible for recurrence after therapy.

Schepers et al. used the intestinal adenoma (a pre-malignant precursor lesion; driver mutation is APC null mutation)-prone mouse model and Driessens et al. used a benign papilloma mouse model to drive home an important point: tumours are maintained by dedicated cancer stem cells, and tumour growth is analogous to normal tissue renewal and architecture [56, 57]. Schepers et al. brought a loxP cassette containing four fluorescent labels in the background of adenoma-prone mouse which conditionally express Cre recombinase and in which intestinal stem cells are labelled with GFP. On treating with tamoxifen, Cre was expressed which edited the LoxP cassette such that one fluorescent label is expressed. This single colour will label the cell as well as its progeny with that colour. They found that adenomas are labelled by a single colour, indicating that tumours are formed from a single intestinal cell (already having driver mutation) and have 5–10 % of GFP labelled stem cells, called cancer stem cells. Second dose of tamoxifen allowed some cells within the single colour adenomas to change colour by "flipping"

within the loxP cassette. This is called lineage retracing. Early after retracing, there was co-expression of new colour and GFP, indicating that colour change has tracked CSC. When this was followed for many days after retracing, progeny of newly coloured stem cells was seen to populate the adenoma with the architecture resembling normal intestinal growth, indicating that tumours are maintained by CSCs still retaining a semblance of the normal tissue architecture [56]. Similarly, Driessens et al. labelled individual tumour cells, without labelling stem cells specifically, in the papillomas formed in the carcinogen followed by mitogen applied mice. They found two subsets of cells: one subset, which included the majority, divided only a few times whereas the other subset persisted a long time, giving rise to progeny that occupied a significant part of the tumour. This suggested that the former subset included the differentiated/progenitor cells, while the later might be stem cells which maintain the tumour growth. Additionally, the growth characteristic of the tumour was reminiscent of the normal skin stratified architecture [57]. Taken together, these three mouse models, employing skillful imaging and lineage tracking studies, proved beyond doubt the existence of CSC and their role in tumour initiation and relapse.

CSC Hypothesis Brought About a Change in our Perception of Chemotherapy

We have already mentioned the salient features of the two existing theories about tumour heterogeneity: clonal evolution theory and cancer stem cell theory. These two theories dictate a fundamental difference to our approach towards cancer treatment. According to the clonal evolution theory, all cancer cells have the potential to initiate a tumour and to cause recurrence. Consistent with this, traditional cancer therapies have sought to destroy as many cancer cells as possible. However, according to the cancer stem cell hypothesis, only CSCs are capable of tumour initiation and recurrence. Thus, an effective chemotherapy must additionally aim to target this small subset of cancer cells. To achieve this, we need to better understand the biology of CSCs.

The Role of CSCs in Cancer Maintenance and Progression

1. **CSCs and EMT:** Epithelial-mesenchymal transition (EMT) refers to the complex series of events that allow epithelial cancer cells to shed their epithelial characteristics like cell-cell and cell-matrix adhesion, apico-basal polarity and lack of motility and to gain mesnchymal characteristics like motility, migratory and invasive capabilities [58]. It is believed, with sufficient evidence, that cancer cells induce EMT and its reciprocal mesnchymal-epithelial transition (MET) program to complete the steps involved in invasion-metastasis cascade [59–61]. As mentioned earlier, only CSCs or TICs can seed new tumours. Hence, intuitively, we realize that for efficient metastasis, cancer cells that undergo EMT should acquire stemness characteristics or CSCs must activate the EMT program to form micro- and macro-metastases. Consistent with this view, many studies have reported that activation of EMT program is associated with acquisition of stemness in many malignancies including breast [30, 31], colorectal [62], pancreatic [63] and hepatocellular carcinoma [64].

   EMT is a complex process that needs integration of various signalling cascades. This is achieved either through major EMT signalling pathways like Wnt, Notch and Hedgehog or through some pleiotropic transcription factors called EMT-TFs that achieve activation of various pathways simultaneously (reviewed in detail in [65, 66]). Further, since EMT is a reversible process with both EMT and MET states, we are left to wonder whether acquisition of stemness is also reversible. This will be opposite to the existing cancer stem cell hypothesis that posits that CSCs are at the top of the hierarchical organization of tumour that divide asymmetrically to give rise to CSC and progenitor cells. The progenitor cells further differentiate to form the bulk of the tumor. Thus, consistent with the ample evidence above, since EMT and stemness are linked, we can presume that stemness is also a reversible trait i.e. non-CSCs can give rise to CSCs. There is some recent evidence that supports this notion [67, 68]. If supplemented with additional evidence regarding the same, particularly in vivo, then CSC hypothesis may very well be looking at an amendment soon.
2. **CSCs and drug resistance:** Failure of chemotherapy is majorly attributed to drug resistance. Cancer cells can acquire drug resistance via multiple mechanisms including mutations, epigenetic changes, over-expression of drug target, inactivation of drug or elimination of drug from the cell. Drug resistance can be either de novo or acquired. De novo drug resistance alludes to the fact that some cancer cells within a tumor already have mechanisms in place to escape drug treatment even prior to encountering the drug. Such cells are refractory to the first bout of chemotherapy itself. On the other hand, if on exposure to drug, cancer cells activate mechanisms that allow them to survive drug treatment, it is referred to as '*acquired drug resistance*'. The proportion of such drug-resistant cells within a tumour may vary.

   One major mechanism of drug resistance in cancer is the over-expression of ABC transporters. ABC transporters can actively efflux out various substrates including chemotherapeutic drugs. Several classes of ABC transporters are known, each efflux different repertoire of

substrates with different affinities. Hence, they generally confer "*multi-drug resistance*". Recent studies have shown that CSCs inherently express high levels of specific ABC drug transporters, which might explain their inherent drug resistance [69]. ABCB1, ABCG2 and ABCC1 are the three major multidrug resistance genes that are up-regulated in CSCs. High level of ABCG2 is expressed by hematopoietic stem cells, but not by committed progenitor and mature blood cells [70]. This drug effluxing ability of stem cells, on account of ABC transporter expression, is used to identify and isolate them using "*side population*" assay. Most cells accumulate Hoechst 33342 or rhodamine 123 used in this assay, but CSCs efflux the dyes out and appear as a negatively stained (low fluorescence) subpopulation separate from the majority of the cells on a density dot plot. Hence, these cells are called *dull cells or side population (SP) cells*, as described earlier. Normal stem cells in various tissues, as well as cancers like breast, lung and glioblastoma show this side population (SP) phenotype. This SP phenotype indicates the presence of inherently drug resistance resistant cancer stem cells [71, 72]. Interestingly, highly invasive cancers are also more drug resistant, suggesting an association between tumor aggressiveness and chemo resistance. Consistent with this, we observed that cells that undergo EMT, in addition to acquiring stemness, also acquire drug resistance. Our work further revealed that the very transcription factors involved in mediating an EMT are also responsible for up-regulating ABC transporters [73], thus explaining the age long association of tumor aggressiveness and drug resistance. Taken together, these observations suggest that cancer stemness, EMT and drug resistance are all phenomenon that may be molecularly linked with other.

Expression of ABC transporters might not be the only mechanism for drug resistance in CSCs. Expression of high levels of anti-apoptotic proteins, and DNA repair capacity found in both normal and neoplastic stem cells are equally contributing factors [74–77]. Moreover, CSCs in certain cancer are shown to be slow-cycling i.e. quiescent in nature [78–80]. There quiescent nature also allows them to escape chemotherapeutic strategies aimed at rapidly dividing cells. Additionally, apart from these inherent properties of CSCs, epigenetic and genetic changes can contribute to acquired drug resistance followed by drug insult [81].

CSCs and Microenvironment

Tumour microenvironment (TME) comprises of mesenchymal stem cells (MSCs), endothelial cells, fibroblasts, adipocytes and immune-modulatory cells. The role of TME in regulating tumour growth is now being realized. The earliest report that suggested that TME can affect tumour development was provided by Mintz and Illmensee in 1975. They injected embryonal carcinoma cells subcutaneously in mice, where they formed teratocarcinomas. However, when the same cells were injected into a blastocyst, they ended up forming chimeric normal mice instead of tumours. The cells injected in the blastocyst also had the same tumorigenic genetic makeup, but the blastocyst microenvironment somehow prevented tumour formation [82]. This experiment was the first that suggested that cells can integrate signals coming from the microenvironment to modulate their behaviour.

There is co-operative communication between TME and tumour cells, the cumulative signalling affected by this will determine the overall tumour development. Tumour cells can cause epigenetic changes in the non-tumorigenic cells of the tumour microenvironment, which in turn, releases factors that cause epigenetic changes in the tumour cells [83, 84]. Cytokines (specifically IL-6 and IL-8), TGFβ, IGF, PDGF, Wnt, Hedgehog ligands, Notch ligands, and MMPs are some factors released by the cells in the TME that regulate tumour progression, invasion and metastasis [85–87].

Targeting Cancer Stem Cells Conventional chemotherapy targets rapidly dividing cells. It leads to tumour regression/shrinkage. However, CSCs, both by virtue of their inherent drug resistance and quiescence, are able to escape such cancer treatment regime, leading to tumour recurrence and metastasis. Hence, in addition to targeting the bulk cancer cells, it is imperative to eliminate the CSCs. Since normal and malignant stem cells share similarities with respect to cell surface proteins, signalling pathways, microRNA and cell quiescence, a major hurdle that needs to be overcome is to find drugs that specifically target only malignant stem cells. Another factor to bear in mind is that while treatment with CSC-specific drugs alone may eliminate the chances of tumour relapse and metastasis, however, it would not cause tumour regression, and cancer symptoms associated with tumour burden will persist. For this reason, CSC-targeted therapy must be given together with conventional chemotherapy.

Studying cancer stem cell biology allows us to identify novel drug targets that can be exploited to eliminate CSCs specifically, without affecting the repertoire of normal stem cells within the body. For example, PTEN dependence can distinguish normal hematopoietic stem cells (HSCs) from leukemic stem cells (LSCs) [88]. Deletion of PTEN tumour suppressor causes rapid proliferation of HSCs leading to leukaemogenesis. However, proliferation of HSCs is limited and ultimately they become depleted via a cell autonomous mechanism. On the other hand, LSCs could maintain themselves without PTEN. Thus, treatment with rapamycin depleted LSCs whereas it rescued the function of PTEN-

deficient HSCs [88]. Similarly, parthenolide (sesquiterpene lactone isolated from the plant feverfew) induced apoptosis of leukemic stem cells (LSCs) in vitro and also inhibited leukemia engraftment in NOD/SCID mice after 16 h treatment in vitro [89]. However, they do not affect normal HSC, thereby keeping the process of normal blood cell formation intact. Work from our lab has identified that extracts of *Tinospora cordifolia* plant, used in many Ayurvedic preparations, is very effective against the drug resistant and stemness enriched side population cells of a variety of epithelial cancers [90]. Similarly, many other phytochemicals targeting CSCs have been identified and have reached stage II of clinical trials [91].

Other potential avenues that are being explored for targeting cancer stem cells include the following:

Targeting CSC-specific cell surface markers: As mentioned previously, CSCs can be identified by a unique cell surface marker profile. This can be also be exploited to target CSCs for their elimination. Several recent studies support the utilization of monoclonal antibody targeting CD44 for treating acute myeloid leukemia [92, 93], CD24 for treating colon and pancreatic cancer [94], and CD133 for treating hepatocellular and gastric cancer [95]. Additionally, these cell surface markers can be utilized to increase uptake of conventional drugs by CSCs. Such cell-surface marker based nano-capsule targeted drug delivery systems are being developed. For example, development of ultrasmall hyaluronic acid (HA) paclitaxel nano-conjugates allow elimination of breast cancer brain metastases via CD44 receptor mediated endocytosis, which eludes p-glycoprotein mediated efflux on the surface of cancer cells. Moreover, free paclitaxel delivery is restricted in ~90 % of brain metastasis, whereas the small size of the nano-conjugates allows them to passively diffuse across the leaky blood-tumour barrier [96].

Targeting self-renewal pathways: As mentioned already in the preceding section, various signalling pathways involved in drug resistance, EMT, microenvironment and others impinge on CSC stemness regulating pathways. Some of these can be exploited to target CSCs. For example, p53 tumour suppressor has been shown to have a role in asymmetric stem cell division. p53 null mammary cells showed higher proportion of symmetric division. However, treatment with Nutlin3, a small molecule inhibitor of MDM2-mediated p53 degradation which restores p53 function, reinstated asymmetric division [97]. Various studies have shown that PTEN/PI3K/Akt/Wnt signalling axis plays a vital role in maintenance of normal as well as malignant stem cell homeostasis [98–100], [88], [101–104]. Similarly, hedgehog signalling has been shown to play a role in regulating normal and malignant stem cells [105–107]. Notch signalling is known to regulate CSC maintenance [108, 109]. Additionally, specific Notch receptors are shown to have differing roles in CSC biology. For example, Notch4 is upregulated in breast CSCs whereas Notch1 is downregulated. Inhibition of Notch4 signalling reduced breast CSCs and inhibited tumour initiation [110]. Indeed work from our lab has shown how monoclonal antibodies targeting Notch can deplete breast cancer stem cells in animal models [111]. Targeting Notch signalling can inhibit tumour-initiating cells as well as Notch-mediated cell proliferation, motility and survival [112, 113]. Several antibodies targeting Notch signalling as well as γ-secretase inhibitors are currently in stage II of clinical trials [114]. Other signalling pathways that are known to regulate cancer stem cells include JAK/STAT, Wnt, hedgehog, Bcl-2, NF-κB and others [115–119]. These various signalling molecules can be targeted by small molecule inhibitors to eliminate CSC homeostasis.

miRNAs to target CSCs: Since the discovery of microRNAs some 20 years ago, they have been shown to play diverse important functions within the cell. microRNAs (miRNAs) are ~20–25 nt long endogenous non-coding RNAs that form a hairpin 2° structure. After transcription, miRNAs are processed and loaded onto RNA-induced silencing complex (RISC) which comprises of Argonaute proteins. 7–8 nt long seed sequence within the RISC loaded miRNA strand is complementary to the 3′UTR of one/many mRNAs and can bind via Watson-Crick pairing. This binding causes either translational repression and/or mRNA destabilization or cleavage [120] (Fig. 3).

Since a single miRNA can target various mRNAs at a time, it provides a lucrative regulation system for the cell to modulate various signalling events in a coordinated fashion. The first study correlating miRNAs with CSCs was carried out by Yu et al., who found that several miRNAs were down-regulated in breast CSCs, including let-7, miR200a/b/c, miR103, miR107, miR128 and miR20b. Of these, let-7 was consistently and significantly down-regulated, and ectopic expression of let-7 in breast CSCs abrogated their self-renewal capacity as seen by reduced mammosphere formation in primary breast cancer and cell lines and also supressed tumour initiation in NOD/SCID mice. This suggests that let-7 supresses CSC self-renewal [121]. Shimono et al. identified 37 miRNAs that were differentially expressed in breast CSCs; all the five members of the miR-200 family were found to be down-regulated in human breast CSCs. However, the same was also observed for normal human and murine mammary stem/progenitor cells. miR200c strongly supressed the ability

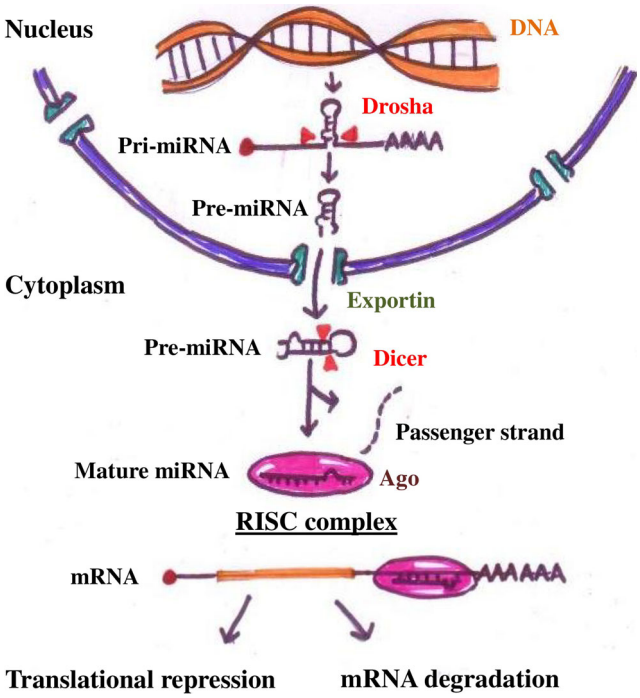

Fig. 3 miRNA biogenesis pathway: The primary transcript of miRNA, called pri-miRNA is transcribed by RNA polymerase II, following which it is cleaved by Drosha. The precursor (pre)-miRNA so formed is exported into the cytoplasm where it is process by Dicer. The passenger strand is degraded when the mature miRNA strand is loaded onto Argonaute proteins to form the RNA-induced silencing complex (RISC). The mature miRNA within the RISC complex mostly binds to the 3′UTR of mRNAs to cause either mRNA degradation or translational repression

of normal mammary stem cells to form mammary ducts and breast CSCs to initiate tumours in vivo [122]. Recently, the mechanism of action of the various differentially modulated miRNAs in CSCs has also been elucidated. For example, Yu et al. showed that ectopic expression of miR30 in breast CSCs inhibits their self-renewal by reducing Ubc9 (ubiquitin-conjugating enzyme 9) and inducing apoptosis through silencing ITGB (integrin B3) [123]. Bmi-1, Sox2 and Klf4 are important transcription factors regulating stem cell biology. 3′ UTR of Bmi1 has binding sites for miR200, miR203, miR183. miR200 also repressed the pluripotency factors Sox2 and Klf4 [124].

Thus, miRNAs provide a lucrative target for cancer. Depending on their role as pro-tumorigenic or anti-tumorigenic, they can be down-regulated or over-expressed, respectively. Down-regulation is achieved using anti-miRNA oligonucleotides (AMOs) or their modifications [125–127], miRNA sponges [128] or miR-masking, whereas overexpression can be achieved using liposomal delivery systems [129] and synthetic miRNA mimics [130].

Targeting Multidrug Resistance One major reason that allows cancer stem cells to escape chemotherapeutic intervention is their inherent drug resistance. Amongst the various possible mechanisms that lead to drug resistance, over-expression of ABC transporters by CSCs is the most common. Hence, ABC transporters may serve as yet another novel target for making CSCs more sensitive to drug treatment. For example, the first ABC transporter inhibitor to be identified is ABCB1 (P-gp) inhibitor named *verapamil*, which is often used as control in side population assay to block efflux of Hoechst 33342 dye [131]. Other novel ABC transporter inhibitors that have been developed include methylene blue [132], MS-209 [133], VX-710 [134], tariquidar and others [135, 136]. However, first generation ABC transporter inhibitors (verapamil, cyclosporine) failed in clinical trials on account of their low binding efficacy of the drug to its target, requiring the drug to be used in high doses that cause cytotoxicity [137]. Now, third generation ABC transporter inhibitor (tariquidar, zosuquidar), some of which are currently in stage II of clinical trials, have been designed that are less toxic than first generation drugs [138] and more specific than second generation drugs (dexverapamil, dexniguldipine) [139]. Another strategy is to down-regulate the protein expression levels of these transporters. For example, hedgehog signalling regulates the expression of ABCB1 and ABCG2. Treatment with cyclopamine (a small molecule inhibitor of hedgehog cascade) leads to downregulation of the two ABC proteins. Similarly, chemoresistance is partially reversed by targeted ABCB1 and ABCG2 using siRNAs [140].

Differentiation Therapy CSCs forms hierarchical organization within tumours, with CSCs at the apex having unlimited self-renewal potential, followed by their progenitors which slowly lose their self-renewing capacity. Hence, inducing differentiation of CSCs offers another specific strategy to target CSCs (Fig. 4). Again here, targeting only malignant stem cells, without affecting normal stem cells, is a major criterion.

Differentiation therapy currently practiced in clinical oncology uses all-trans-retinoic acid (ATRA) and arsenic trioxide along with chemotherapy to treat acute promyelocytic leukemia (APL), one subset of acute myeloid leukemia (AML) [141]. ATRA along with arsenic trioxide causes leukemic cell terminal differentiation by degradation of retinoic acid receptor alpha (RARα) oncoprotein [142, 143]. These terminally differentiated cells undergo apoptotic cell death. Ninety percent of the patients subjected to this combination of cytotoxic chemotherapy in conjunction with differentiation therapy show remission [144]. Recently, a high-throughput screening for small molecules that specifically induce differentiation of cancer stem cells has been developed which exploits loss of polarity in cancer stem cells undergoing EMT [145]. Salinomycin, a highly selective potassium ionophore, selectively affects cancer stem cells was picked up by the screen. The exact mechanism by which differentiation targets CSCs in not known, however, in some cases, it causes apoptosis of

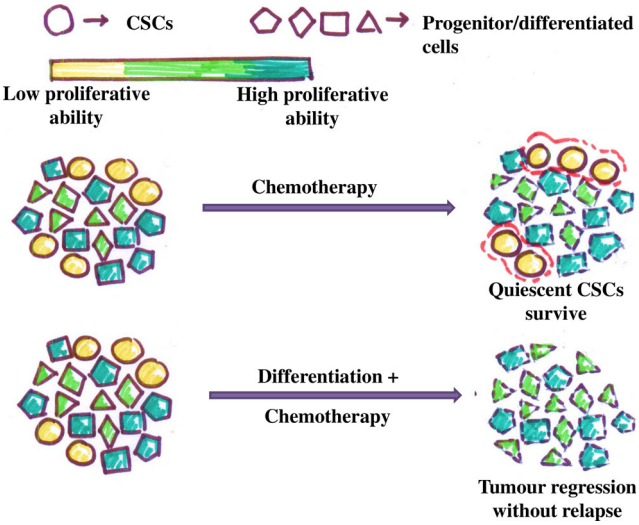

Fig. 4 Differentiation therapy to target CSCs: Cancer stem cells within the tumour escape chemotherapy due to their quiescence and inherent drug resistance. Terminal differentiation of cancer stem cells, along with chemotherapy causes complete tumour regression and prevents relapse

CSCs. Salinomycin appeared to induce terminal epithelial differentiation along with cell cycle arrest both in vitro and in vivo. Tumours derived from Ras-transformed HMLE cells formed less efficiently in mice if the cells were pre-treated with salinomycin. Further, salinomycin has been shown to induce expression of E-cadherin, a cell adhesion protein, indicating that salinomycin might eliminate CSCs by inducing their differentiation. Similarly, other differentiating agents like All-trans-retinoic acid (ATRA), tricostatin A and vorinostat are shown to reduce BCSC fraction.

Discussion

Proposal of cancer stem cell theory and the recent evidences revealing the existence of cancer stem cells within the tumour's native environment has changed our perception of cancer therapy. The conclusive evidence that CSCs are responsible for tumour relapses following therapy has highlighted the importance of eliminating this population from within the tumours for effective relapse-free treatment. Towards this end, various novel targets have been identified that can be exploited for targeting cancer stem cells. Many of these synthetic drugs, phytochemicals and monoclonal antibodies are in clinical trials.

One major point of contention is that therapies targeting cancer stem cells should not affect normal stem cells. Hence, studies that concentrate on signalling pathways in both normal as well as cancer stem cells are of utmost importance as they allow us to remove common targets between the two from the list. We can then focus our attention on the pathways that are distinct between normal and cancer stem cells for designing therapeutics. Additionally, this will ensure that the new therapies coming up would not affect normal tissue homeostasis and regeneration. Thus, understanding normal tissue stem cell biology is also important for us to design effective cancer treatment.

Moreover, there is emerging literature stating that clonal evolution model and cancer stem cell model are not mutually exclusive. This suggests that cancer stem cells can themselves acquire multiple mutations that can be acted upon by selective forces. Thus, within the small cohort of cancer stem cells within the tumour itself there can be clonal diversity. Hence, undertaking clonality studies is also one major avenue that ought to be explored.

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Glossary

Cancer stem cells (CSCs)	Are cancer cells that possess the abilities analogous to normal stem cells, specifically the ability to self-renew and to differentiate to give rise to the heterogeneous population within the tumour.
Cre recombinase and LoxP site	Cre-Lox technique is used to generate knock-in, knock-out or flipping of segments within genes. Cre recombinase is expressed conditionally within the cells where genetic modification is desired and it recognizes loxP sites placed around the desired gene to cause recombination. The orientation of the loxP site determines the outcome of the recombination event.
Differentiation	Is a cellular process by which cells commit to a particular fate, i.e. they commit to form a particular cell type within the organ having a distinct set of functions.
Epigenetics	Refers to the heritable changes in gene expression that does not involve changes to the DNA sequence; a change in phenotype without a change in the genotype. These changes generally occur at the level of chromatin organization.

Epithelial-mesenchymal transition (EMT)	Is a complex process by which epithelial cells lose their epithelial properties like cell-cell, cell-matrix adhesion, apico-basal polarity, etc. and acquire mesenchymal characteristics like migratory and invasive capabilities. The reverse process is called ***mesenchymal-epithelial transition (MET)*** by virtue of which mesenchymal cells acquire epithelial properties.
Fluorescence-activated cell sorting (FACS)	Is a flow cytometry technique that allows heterogeneous populations to be segregated based on the expression of cell surface markers that have been fluorescently labelled.
Invasion and metastasis cascade	Is a multi-step process by which epithelial cancer cells invade into the local surrounding, enter the vasculature, lodge at distant sites where they give rise to secondary/ metastatic tumours.
Mammosphere	3D spheroid structures formed when mammary tumour cells are subjected to suspension condition on long term culture (7–10 days).
Mutation	Is defined as a change in the nucleotide sequence of DNA, the genetic material in most organisms. Heritable mutations are called germline mutations whereas others which affect only the organism in which they occur are called somatic mutations.
NOD/SCID mouse	Non-obese diabetic SCID mouse; an immunodeficient mouse strain that lacks B, T lymphocytes as well as NK cells.
SCID mouse	Severe combined immunodeficiency mouse; an immunodeficient mouse strain that lacks B and T lymphocytes.
Self-renewal	Is the ability by which one stem cell can give rise to more cells of the same cell type.
Stem cells	Are undifferentiated cells within tissues that possess the ability to self-renew and to differentiate into other cell type of that tissue. They maintain tissue homeostasis.
Symmetric division	Produces two daughter cells having the same cellular fate. ***Asymmetric division*** produces two daughter cells with different cellular fates.
Tumorigenicity	Defines the ability of cells to seed/ initiate new tumours. It is generally tested by subcutaneously/ orthotopically injecting cells into immunocompromised mice and checking for tumour formation.

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