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Advancements in Cancer Stem Cell Isolation and Characterization

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Abstract
Occurrence of stem cells (CSCs) in cancer is well established in last two decades. These rare cells share several properties including presence of common surface markers, stem cell markers, chemo- and radio- resistance and are highly metastatic in nature; thus, considered as valuable prognostic and therapeutic targets in cancer. However, the studies related to CSCs pave number of issues due to rare cell population and difficulties in their isolation ascribed to common stem cell marker. Various techniques including flow cytometry, laser micro-dissection, fluorescent nanodiamonds and microfluidics are used for the isolation of these rare cells. In this review, we have included the advance strategies adopted for the isolation of CSCs using above mentioned techniques. Furthermore, CSCs are primarily found in the core of the solid tumors and their microenvironment plays an important role in maintenance, self-renewal, division and tumor development. Therefore, *in vivo* tracking and model development become obligatory for functional studies of CSCs. Fluorescence and bioluminescence tagging has been widely used for transplantation assay and lineage tracking experiments to improve our understanding towards CSCs behaviour in their niche. Techniques such as Magnetic resonance imaging (MRI) and Positron emission tomography (PET) have proved useful for tracking of endogenous CSCs which could be helpful in their identification in clinical settings.

Keywords Cancer stem cells · Flow cytometry · Microfluidics · Fluorescent nanodiamonds · Positron emission tomography · MRI

Introduction

Cancer is a result of numerous genetic manipulations which impart immortality, abandoned growth and incessant proliferation to healthy cells [1]. Neoplasm possesses assorted cell population whose conjoint functioning is responsible for their greater survival than normal counterparts. Recent research in oncology have shed light on existence of peculiar population of cells in cancer known as cancer stem cells (CSCs) which were first identified in 1970 in leukaemia's and later in solid tumours. CSCs can form secondary tumours with differentiated cell population similar to that in primary tumour [2, 3]. This minor subset of cells is a major cause of tumour reoccurrence, metastasis and resistance to anti-cancer therapies. Thus, eradication of CSCs is inevitable for complete cure [4]. Therefore, CSCs become important targets for cancer therapy.

Dissecting complete profile of cancer stem cells has been a major concern due to difficulties in isolation of pure population, unavailability of CSC cell lines and reliable characterization methods. Currently, different techniques like tumorsphere assay, colony formation assay and flow cytometry analysis can be used to study CSCs. Most reliable approach used for the isolation and enrichment of CSCs have exploited surface markers and aldehyde dehydrogenase (ALDH) expression, the latter being highly expressed in all CSCs regardless of cancer type [5, 6]. However, still there is no common consent on these approaches as there has been the heterogeneity among CSCs sharing same cell surface markers. Some of the common CSCs markers are CD133, CD44, CD24, ALDH1, oct4, nanog and sox2. Among these, CD44 and CD133 have been extensively used for isolation and characterization. CD44 plays a crucial role in CSCs for communication with the microenvironment, as well as it also help in maintaining stemness [7]. CD133 is a well-known marker of stem/progenitor cells in normal adult tissues, such as kidney, brain, prostate and liver. It has proved as an useful marker for the sorting of CSCs from non-CSCs within tumours [8, 9]. The population of CSCs varies depending on the type of cancer and the biomarker used for their

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identification. In breast cancer, cell population with high CD44 and low CD24 expression was designated as CSCs. Higher expression of CD44 was found to be associated with poor prognosis. Also expression of these markers changes with intrinsic subtypes [10]. Similarly, pluripotency markers such as sox2, oct4, nanog have also been studied in colon cancer, head and neck cancer, breast cancer, lung cancer and cervical cancer, as biomarkers for CSCs. Numerous studies have been done to identify biomarkers for CSCs and it can be comprehended that a single biomarker will not serve the purpose. The expression of biomarker to study CSC population may also vary depending on the cell line [11]. We have summarized the reports of biomarker studied in various cancer types in Table 1. Owing to the heterogeneity present among the CSCs population, the isolation of these cells require methods which should consider different properties of CSCs. MACS and FACS are currently the most reliable techniques used for isolation of CSCs. However these techniques mainly rely on the surface markers whereas the microfluidics and laser capture micro-dissection works on the functionality of CSCs. Although, these methods provide population of CSCs like cells based on markers used, it becomes difficult to study the behaviour of these cells as the cells are teased apart from their original niche. Lineage specific tagging of CSCs like cells has revealed *in vivo* progression and differentiation of these cells. Furthermore, the identification of endogenous CSCs is crucial for the targeting and prognosis of cancer as CSCs are well correlated with relapse of the disease. Here, we have detailed the different techniques used for isolation, tracking of exogenous (labelled) and endogenous CSCs.

Cancer stem cells: Are they similar to circulating tumour cells?

Circulating tumour cells (CTCs) exist in blood stream of cancer patients and are thought to be responsible for invasion and metastasis cascade [60]. These are shaded from primary tumour by process of epithelial to mesenchymal transition (EMT) and possess self-renewal and tumour initiating capacity. Various studies have supported the homogenous nature of CSCs and CTCs (Table 2). Similar to CSCs, population of CTCs is also very low and varies from 1 to 10 cells per ml of blood [80]. CTCs also share various markers with CSCs such as $CD44^{+}/CD24^{-/low}$, $CD133^{+}$, $ALDH1^{+}$, CD326/EpCAM, Bmi and Nestin [66]. Furthermore, expression of various markers also changes according to the stages of cancer, for example in early breast cancer, CTCs from blood have showed low ALDH and cytosolic localization of TWIST (twist related protein 1), while CTCs from metastatic breast cancer demonstrated high ALDH with TWIST translocation to nucleus [71]. High number of CTCs is also well correlated with shorter survival, poor prognosis and reoccurrence in cancer patients [73, 74]. Number of CTCs is found to be increased in metastatic cancers and also after chemotherapy, suggested their chemoresistance and radio-resistance nature [70]. Therefore, liquid biopsies from patients and number of CTCs may serve as prognostic marker for overall survival of cancer patients [81]. Similar to CSCs, CTCs were shown to develop spheres and could resist detachment induced cell death. Unlike, CTCs in blood, CSCs were also found in the core of the solid tumors. Thus, they exist in distinct microenvironments which may cause different metabolic adaptations

Table 1 Biomarkers used for the isolation of CSCs from patient samples

S. no.	Cancer	Markers (Analysed in patient samples)	References
1.	Colon cancer	CD133, CD44, CD166, EpCAM, Oct4, Nanog, Sox2, Tbx3,Tcl1, Esrrb,Dppa4, Bmi, Zfx	[12–14]
2.	Colorectal cancer	$EpCAM^{high}/CD44^{+}$, Lgr5, CD133, CD44v9, CD44, ALDH1	[15–20]
3.	Head and neck	CD44, CD24, Integrin-β1, ALDH1, SOX2, CD133, CD44 $CD271^{+}$, ALDH1A1, Oct4, $ALDH1A1^{+}$, $CD44^{+}$, $ALDH1A1^{+}/CD44^{+}$, $ALDH1A1^{-}/CD44^{-}$	[21–29]
4.	Oral cancer	CD44v6, ABCG2, ALDH1 CD133, ALDH1, CD24, CD44 CD133, CD29, Ki-67	[30–32]
5.	Lung cancer	EpCAM, CD133, ABCG2, ALDH1A1,CD117, BCRP1, ALDH1, Bmi1, CD44, Sox2	[13, 33–42]
6.	Prostate cancer	EpCAM, ALDH1A1, Oct4, Nanog, Sox2, Tbx3, Tcl 1, Esrrb, Dppa4, Bmi, Zfx, ALDH1, EZH2, Sox2, CD44, CD133, ALDH1A1	[13, 14, 43–46]
7.	Breast cancer	EpCAM, CD44, CD24, ALDH1, Bmi1, Sox-2, CD133 Oct-4, Nanog, CASP14, $CD44^{+}CD24^{-}$, $ALDH1^{+}CD44^{+}CD24^{-}$	[10, 13, 47–52]
8.	Uterus cervix squamous cell carcinoma	EpCAM	[13]
9.	Cervical squamous cell cancers	Sox2, Oct4, ALDH1, Msi 1, CD49f, Nanog	[53–58]
10.	Acute myeloid leukemia	TIM3	[59]

Table 2 List of key markers reported in CSC like nature of CTCs

Type of cancer	Sample type	Number of patient	Markers expressed	References
Metastatic breast cancer	Blood	20 7	$CD44^{+}/CD24^{-/low}$ $ALDH^{high}/CD24^{-/low}$	[61]
Metastatic colorectal cancer	Peripheral blood	40	$CD44^{+}$ $CD133^{+}$ $ALDH1^{+}$	[62]
Metastatic breast cancer	Blood	42	$ALDH1^{+}$	[63]
Castration resistant prostate cancer	Blood	11	$CD133^{+}$	[64]
Breast cancer	Blood	1	Microsphere formation Nanog+	[65]
Melanoma	Blood Metastatic lesion	32 6	$CD133^{+}$ Nestin	[66]
Primary breast cancer	Blood	502	$ALDH1^{+}$	[67]
$HER2^{+}$ Metastatic breast cancer	Peripheral blood	28	$CD44^{+}/CD24^{-/low}$ $ALDH^{+}/CD133^{+}$	[68]
Primary breast cancer	Blood	61	$ALDH1^{+}$ $CD44^{+}$ Bmi	[69]
Nonmetastatic breast cancer	Blood	98	$CD133^{+}$	[70]
Early breast cancer Metastatic breast cancer	Blood	80 50	$ALDH1^{low/neg}/TWIST^{cyt/neg}$ $ALDH1^{high}/TWIST^{nuc}$	[71]
Endometrial cancer	Blood	34	$ALDH^{+}$ $CD44^{+}$	[72]
High risk localised prostate cancer	Blood	35	$CD133^{+}$	[73]
Colorectal cancer	Tumour mass Mesentric venous blood	158 135	$CD133^{+}$ ESA^{+}	[74]
Metastatic colorectal cancer	Blood	5	CD44v6	[75]
Colorectal cancer.	Blood	150	CD44v9	[18]
Small cell lung cancer	Biopsy	38	$CD44^{+}$ SOX2	[42]
Oral squamous cell carcinoma	Blood	30	CD44v6 Nanog	[76]
Ovarian cancer	Blood	3	CD44 ALDH1A1 Nanog Oct4	[77]
Gastric cancer	Blood	26	$CD44^{+}$	[78]
Breast, Colon, Prostate, Sarcoma, Multiple myeloma, Glioblastoma, Ovarian squamous cell carcinoma, Melanoma, Unknown origin	Blood	71	OCT3/4 SOX2 NANOG Nestin CD34	[79]

in these cells. However, their low number is a major hurdle for isolation and studies related to CTCs. Most of the isolation methods are based on specific markers present on the surface of CTCs; nevertheless these markers are not exclusive to CTCs and also found on normal epithelial cells and some of the blood cells. Moreover, isolation with label free microfluidic systems has shown differential expression of these markers on CTCs [82, 83]. Expression of these markers varies from type and subtype of cancer [84]. Metastasis of cancer requires epithelial to mesenchymal transition for the detachment from main tumor; however, reverse transition is required for cell attachment and seeding of tumour [82]. Therefore, differential expression of these markers on CTCs could vary depending upon the stage in which cells are present. These cells present high clinical implication for prognosis and diagnosis of cancer. Hence, improved methods for their isolation and characterization are greatly needed. The recent progress in the separation and

advance characterization methods of CSCs has been discussed in following section.

Cell separation platforms for the isolation of cancer stem cells:

As discussed above, there were numbers of markers which have been identified can be used for the isolation of CSCs and CTCs. However, the specificity of these markers towards stem cell population has also been questioned. Here, we have discussed various techniques used for isolation and studies related to cancer stem cells (Fig. 1). Current isolation techniques for CSCs have some advantages over the other but they also possess some technical challenges (Table 3). Combination of methods simultaneously or enrichment with one and then isolation with other may increase the efficiency of isolation and could help us understand the heterogeneity among CSCs. Flow cytometry and magnetic cell separation rely on the specific cell surface antigens and antibodies conjugated to fluorescent molecule or magnetic particles. Hence, these techniques provide highly specific targeted cells.

Flow cytometry

Flow cytometry is the most frequently used platform for the isolation, counting and sorting of CSCs by using specific markers. Method could be implied for multiple markers simultaneously, which increases its applicability and specificity [88]. Conventional flow cytometry does not imply spatial resolution system. The method only identifies the physical binding of proteins with tagged antibody. Thus, detection and isolation does not represent functional value of CSCs. More recently, the new adaptation to flow cytometry has been evolved known as imaging flow cytometry (IFC). IFC has been used to detect CTCs from liquid biopsy samples of hepatocellular carcinoma (HCC) using biomarkers, size, morphology and DNA content. Out of 45 samples, 29% and 18% of CTCs were positive for epithelial markers cytokeratin and EpCAM respectively. While the additional 28% were negative for all markers but vary in cell size and were hyperploids [85]. Various other studies have also reported that stem cells markers were also present in maximum tumor population. However, the functional proteins were only found in few cells which may represent CSCs [105, 106]. Furthermore, mass cytometry where CyTOF mass cytometer, immunocytochemistry and immunohistochemical methods were coupled with high resolution laser ablation, which can detect 100 biomarkers at cellular and sub cellular localizations. Breast cancer samples have been analysed by mass cytometry using tagged antibodies. These high throughput techniques could be powerful tools to understand the heterogeneity among CSCs in solid tumors [107, 108].

ALDEFLOUR assay (STEM-CELL technologies) was used to detect CSCs with high activity of aldehyde dehydrogenase (ALDH). Assay contained BODIPY-aminoacetaldehyde (BAAA) as a substrate for ALDH. BAAA diffused into live cells converted into a negatively charged, fluorescent compound BODIP-aminoacetate (BAA) by the activity of ALDH. BAA accumulated inside the cells which can be detected by flow cytometry. ALDH has been reported to have increased expression in the CSCs and was related to chemoresistance as it is involved in detoxification of number of anti-cancer drugs [109]. Nevertheless, high enzyme activity is also found in haematopoietic stem (HSCs) cells [110] but in some cancers, high activity is not well correlated to CSCs population [111]. Use of other markers along with ALDH expression has shown a more reliable phenotype of CSC which has revealed the heterogeneity among CSC population [112, 113].

Cancer stem cells have been reported to be a slow dividing or quiescent in nature [114]. These properties of CSCs have also been explored for their isolation. Various organic dyes have been employed to analyse slow division of cells e.g. 5-ethynyl-2′-deoxyuridine (EdU) is an analog of thymidine which incorporates into DNA during cell division [115]. EdU showed toxicity to cells as its 5'monophospate inhibits the thymidylate synthase, making it unsuitable for studying live CSCs [116, 117]. Carboxy fluorescein diacetate succinimidyl ester (CFSE) is a membrane permeable dye that binds to lysine and amine containing molecules inside cells. It has been used to track tumor cell population in solid tumors and recently been used for detection of glioblastoma stem cells [98, 118]. PKH26 is a membrane binding dye used for the tracking of stem cells. The membrane binding dye gets segregated with every division and its intensity is inversely proportional to cell division. More recently, fluorescent nanodiamonds (FND) have found promising application in cancer stem cell tracking. FNDs contain nitrogen vacancy (NV^-) centres, when exposed to green orange light they emit bright fluorescence at ~700nm which is distinct from cells autoflourescence. FNDs were taken up by cells through receptor mediated endocytosis, were highly photostable and non-genotoxic for cells [93, 94]. A comparative analysis of all the standard dyes used for quiescent cell detection has shown that FNDs have longest retention time in cells with subsequent cell divisions (Fig. 2a). FNDs were also visible under confocal microscope upto 20 days in mammospheres of AS-B145-1Rcells (Fig. 2b). Furthermore, it has been observed by Miranda-Lorenzo et al that some of the cells show autoflourescence when excited by 488 laser, these cells were analyzed for the expression of stem cell markers. Interestingly, autoflouroscence cells have also demonstrated CSC markers such as oct-4, sox2 and nanog had ability to form spheres and showed *in vivo* tumor forming capacity. The marked reason for their autoflourescence was due to the accumulation of

riboflavin in membrane bound cytoplasmic structures containing ATP-dependent ABCG2 transporters. Nevertheless, suppression of ATP Binding Cassette Subfamily G Member 2 (ABCG2) cleared the autoflourescence but could not eliminate the CSCs gene expression. The exact function of these vesicles remain unknown however these could serve as functional marker for tracking and identification of CSCs.

Magnetic activated cell sorting (MACS)

MACS is one of the most standard techniques available for cell separation that utilizes functionalized nanoparticles or microparticle conjugated to an antibody corresponding to a specific cell surface antigen [102, 119–121]. Magnetic cell sorting can be done either by high gradient magnetic separation (HGMS) or low gradient magnetic separation (LGMS). HGMS involve low volume columns packed with stainless steel beads or wool which can be magnetized using magnetic field. It provides a uniform magnetic field for the separation of cells labelled with conjugated beads. Once the cells are bound to wool or beads magnetic field is turned off to isolate bound cells [122]. LGMS is utilized for larger volumes; the magnetic gradient is generated by permanent magnets. Relatively larger beads are utilized to encompass the opposite forces such as sedimentation for separation [101]. In both cases positive and negative selection could be implied. In case of positive selection, particles of interest are retained while the supernatant is discarded. In negative selection, non specific particles are retained and particles of interest are collected in fraction [101]. CELLSEARCH® CTC (Janssen Diagnostics Inc. (formerly Veridex LLC) was the first FDA approved system used for isolation of CTCs from blood of patients with breast [123], colorectal and prostate metastatic cancer [124]. It utilizes nanoparticles against CD326 (EpCAM) for the isolation of CTCs using magnetic field. Another system approved by FDA in 2014 was based on magnetic separation was CliniMACS® CD34 Reagent System (MiltenyiBiotec) by using CD34 antibody [125]. Surface markers for lung cancer stem cells (LCSCs) are not well known. However, $CD133^{+}$ LCSCs from lung tissue were sorted by flow cytometry and enriched by MACS have higher potential to form tumors in NOD/SCID mice as compare to negative population. This two step isolation of LCSCs also overcome the requirement for time consuming *in vitro* assays for CSCs enrichment and has established CD133 as LCSCs marker [126]. Further, MACS have been well integrated with microfluidics to achieve

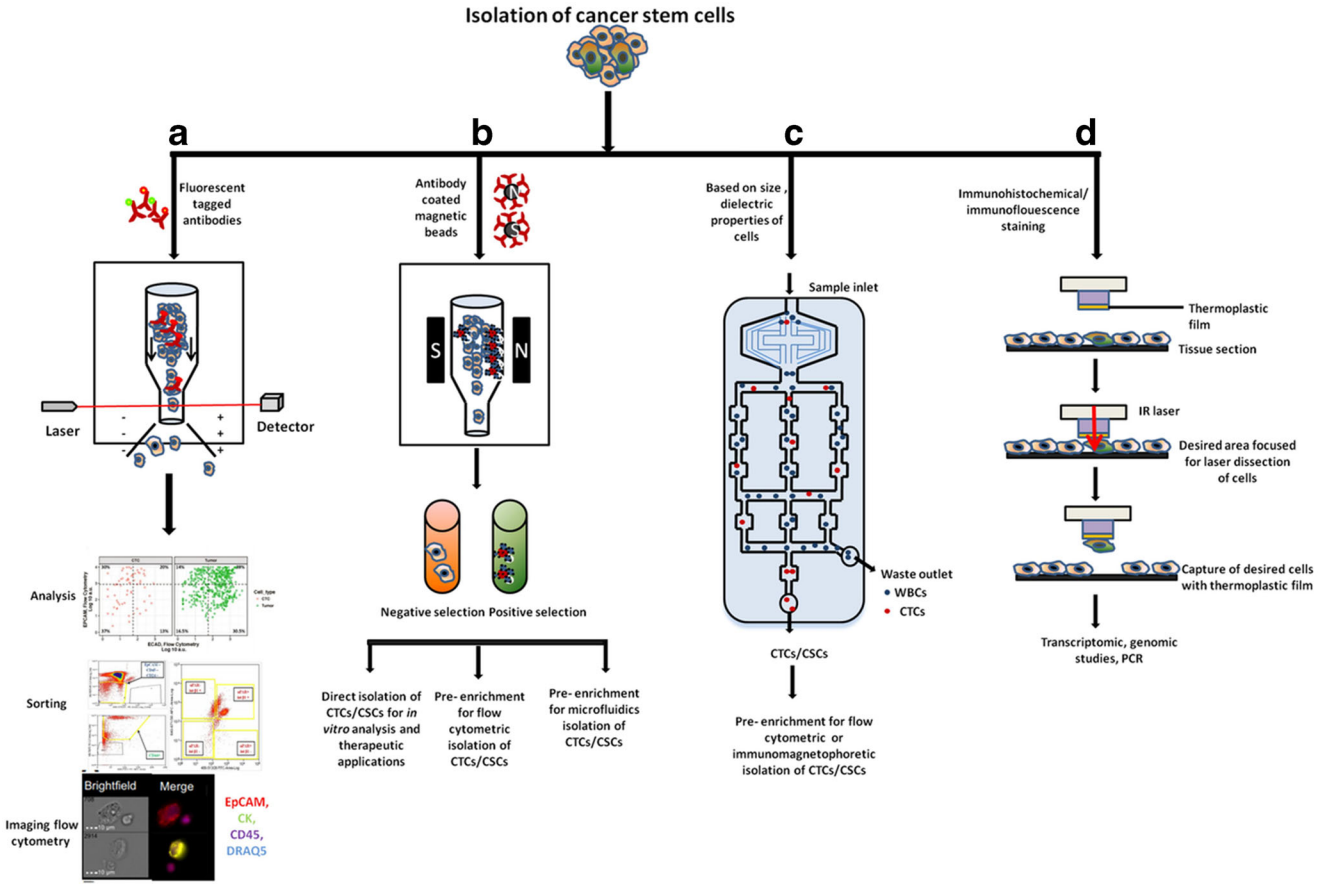

Fig. 1 Representative image of techniques used for isolation of CSCs/CTCs (**a**) Flow cytometry Reproduced from Ref. [85–87] with permission. (**b**) Magnetic activated cell sorting (MACS). (**c**) Microfluidics. (**d**) Laser capture micro-dissection

Table 3 Summary of techniques used for the isolation of cancer stem cell.

S. no.	Technique	Markers used	Specimen type	Advantages	Disadvantages	References
1.	Flow Cytometry	Cancer specific marker	Cell lines and tissue samples	Specific markers for stem cells can be included	Tedious sample preparation, cell loss through stress during sorting, costly	[88]
	Side population analysis	Hoest33342	Cell lines and tissue samples	Easy	Non specificity	[89]
	Attached-cell aldefluor method (ACAM)	ALDFLUOR Assay To enrich CSCs population and grown in adherent conditions	Cell lines oesophagus cancer	Easy to handle after cells are attached in 2D culture	1. Sorting is mandatory to establish 2. Authentication only through ALDH expression 3. Cells may lose their original properties as grown in adherent conditions.	[90]
2.	Laser micro dissection	Microscopic visualization, cells with particular fluorescent markers in tissue	Tissue sample	Provides location of cell in the tissue sample	Cell viability is lost during sample processing Processing of single cell requires highly sensitive methods and instrumentation facility	[91, 92]
3. [91]	Fluorescent nanodiamonds	Slow proliferation	Cell lines, in vivo tracking of transplanted stem cells (lung stem cells)	Endocytosis mediated uptake, Based on functional parameter rather than structural markers, Tracking could be done for 20 days	Genotoxic for embryonic stem cells,	[93–95]
4.	PKH26 dye	For slow proliferating cells	Cell lines and in vivo tracking	Cationic flourchrome with long aliphatic tails incorporates into lipid regions of membrane, staining retained up to weeks to months	Microenvironment contamination (transfer of dye to neighbouring cells mediated by direct cell-cell contacts or diffusible microparticles), incubation time before imaging may significantlychange the labelling intensities.	[96, 97]
3.	CFSC (carboxy fluorescein diacetate succinimidyl ester)	Slow proliferation	In vitro and in vivo tracking	Cell permeable dye, bind to lysine and amino groups inside the cell, homogenous labelling, staining retained up to weeks to months.	Toxicity at higher concentrations.	[98, 99]
4.	Magnetic activated cell sorting	Cancer specific marker	In vitro and ex vivo	Specific markers can be used for isolation (surface markers) using conjugated magnetic beads against surface antigen	Toxicity due to endocytosis of nanoparticle beads, lack use of multiple markers.	[100–102]
5.	Microfluidics	Dean migration and inertial focusing), Filtration, deterministic lateral displacement (DLD) separation based on size of cell (CTCs -10 to 20 μm, RBCs-8 μm, leukocytes-7 to 12 μm)	Cell lines and tissue samples	Label free method, short processing time, highly reproducible methods	Non target cells may be isolated owing to similar physical properties, medium viscosity and cell-cell interactions, clogging may reduce the efficiency	[103, 104]

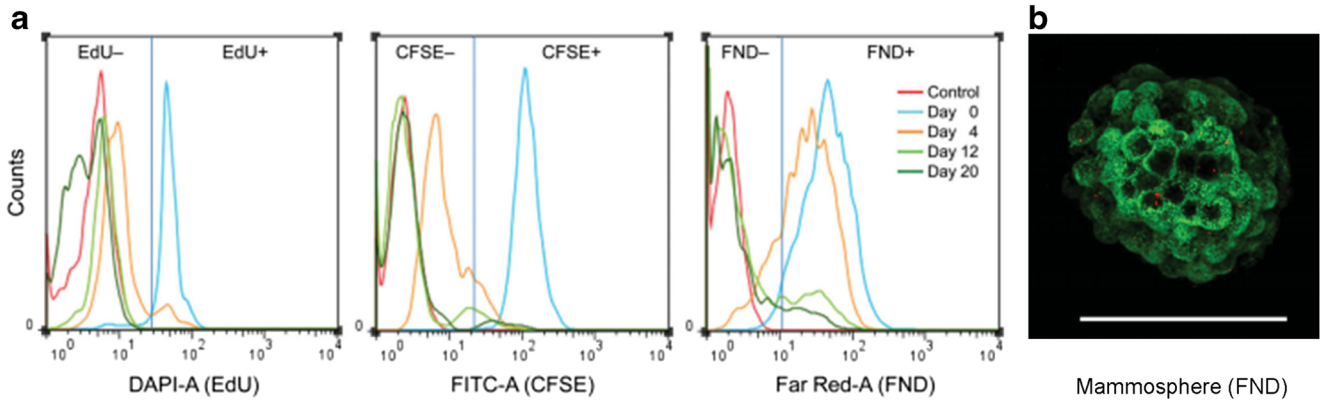

Fig. 2 (**a**) Flow cytometry analysis of dissociated AS-B145-1R mammospheres labelled with EdU, CFSE and FND for quiescent CSCs identification. 82.5%, 12.4% and 9.5% cells were labelled specifically at 4 with FNDs, CFSE and EdU respectively. (**b**) Confocal fluorescence image secondary mammospheres of AS-B145-1R cells labelled with FNDS. Mammospheres are also stained with wheat germ agglutinin (green) and FNDs appeared as red dots in the cells. Ref: [93]

sorting of target cells in a continuous flow manner. More recently, tumor initiating cells were isolated from breast cancer cells line with two bead immunomagnetic separation using $CD24^{-}/CD44^{+}$ markers. Briefly, non-magnetic bead coated antibodies for CD24 were used before adding magnetic bead coated antibody for CD44 then a microfluidic channel was used to trap CD24-/CD44+ cells. This two bead immunomagnetic separation increased the efficiency of cell isolation from 10.3% (before separation), 19.4% (using only anti-CD44-coated magnetic beads) to 41.7%. Negative cell separation approach is an alternative way to enrich CTCs and CSCs without labelling ligands. Negative strategy for the enrichment of CTCs has been utilized to facilitate the recovery of the unlabeled CTCs from whole blood samples from cancer patients [127]. MACS has emerged as a powerful tool for cell separation, however use of MACS has been limited in CSCs isolation when compare to FACS. Because MACS is unable to separate cells based on variable expression of markers and can be only used by utilizing cell surface markers. Some of the other challenges to overcome include detachment of magnetic beads after isolation, separation of multiple markers, etc [100, 102].

Microfluidics

Microfluidics is mostly used for the separation of CTCs from blood samples taking advantage of size variations between CTCs and blood cells. Microfluidics is technology where fluids and particles are transported at microscale. Devices typically contain microporous membranes of a particular size range; through which, fluids can pass and cells can be separated based on their size and deformability [128]. Mainly three basic separation methods were used: 1) Filtration; where cells of a particular size can only pass through porous membranes [129, 130]. 2) Deterministic Lateral displacement; where fluids can passed through microposts and particles below a critical hydrodynamics diameter can passed with the stream but the one with size above than the critical hydrodynamic diameter cannot get streamline and bump into microposts, thus, displaced laterally [103, 131]. 3) Inertial flow based methods utilized the principle where magnitude of lift force required for lifting a particle varies with its diameter, thus microfluidic devices are used to induce inertial forces to separate cells of different sizes [132, 133]. Another microfluidics based approach includes dielectrophoresis (DEP). Dielectrophoresis causes the movement of particles in a non-uniform electric field. Cells become polarized when an asymmetric electric field is given and every cell has a different DEP signature. DEP is independent of the net charge present on the cells and varies with size and surface area, therefore on frequency- dependent dielectric properties of cells. DEP crossover frequency determines whether the cells will be attracted or repelled from high field regions, thus, an electric field frequency that lies between the crossover frequencies of two cell types was chosen for cell separation [134]. The forced equilibrium method of dielectrophoresis field flow fractionation (DEP-FFF) provided higher discrimination efficiency and was applicable for clinical isolation of CTCs. ApoStream™ a microfluidic based platform is commercially available for the isolation of CTCs from clinical blood samples. It has been successfully used for the isolation of CTCs from 7.5 ml blood from lung, prostate, melanoma and breast cancer patients with positive CTC count of 90% from NSLC, 93% from prostate cancer and 100% for breast cancer and melanoma sample [135]. Furthermore, CELLSEARCH® and ApoStream™ were compared to check the presence of CK^{+}/ $CD45^{+}$/ $DAPI^{+}$ CTCs in samples from pancreatic adenocarcinoma ranging between 1-10 CTCs per 7.5ml of blood in 50 % patients. Interestingly, ApoStream™ had also recovered CK^{-}/ $CD45^{-}/DAPI^{+}$ population in 100% of patients and $CA19\text{-}9^{+}$ cells were found in both populations [136, 137]. The DEP devices have efficiently recovered 40% to 95% cancer cells spiked in peripheral blood mononuclear cells (PBMNs) in a timeframe of 30 min. However, the purity of isolated samples

was found to be average due to contamination of dying PBMNs having weak membrane integrity. Therefore, the sample processing for DEP devices becomes crucial to avoid loss of viability [136]. Urine of bladder cancer patients was analysed for the isolation and characterization of rare tumor associated cells using deformability and size based microfluidic system. The study has shown importance of liquid biopsies in bladder cancer to reveal diversity of cancer cells, patient survival and follows up [138]. Optically induced dielectrophoresis based microfluidic system was also used to isolate CTCs from blood samples of head and neck cancer patients. First, immunofluorescence staining was used to observe the target cell microscopically and then OPED based cell manipulation was done to isolate $CD45^-/EpCAM^-$ population. Most of techniques used for CTC isolation rely on $EpCAM^+$ expression; however, the study disseminated the role of $EpCAM^-$ population in CTCs undergone EMT transition. Gene expression analysis have further revealed that same population ($CD44^-/EpCAM^-$) found in healthy donors did not express genes related to EMT, multi-drug resistance and stemness [139]. Similarly, a labyrinth microfluidic device was developed for label free isolation of CTCs from blood of breast and pancreatic cancer patients with a yield of >90% and processing time of 20ml blood in less than 30 min. It has shown heterogeneity among CTCs found in blood with the expression of genes for epithelial cells, mesenchymal cells or cells undergoing EMT [140]. Microfluidic devices have been used for isolation of CTCs and CSCs like cells from patients of pancreatic ductal adenocarcinoma. Whereby, the microfluidic system functionalised by antibodies against EpCAM and CD133 showed two subtypes of CTCs and CSCs ($EpCAM^+$ CK^+ $CD45^-$ $DAPI^+$ $CD133^+$) or ($EpCAM^+$ CK^+ $CD45^-$ $DAPI^+$ $CD133^-$) [141]. Further, gel island chip was also developed to isolate and study heterogeneity within CSCs. Device contain islands made up of ECM gel where cells can be trapped with 34% efficiency while having good media exchange with high cell viability [142]. Microfluidic system is also utilized as platform to recognize specific aptamers against CSCs. These aptamers can act as ligands for the isolation of CSCs [143]. Moreover, microfluidics approach has been combined with various other techniques to improve the efficiency of CTC isolation such as immunomegnatophoresis, immunoflourescence. These methods enhance the specificity by using specific surface markers for CTCs and exclude contamination from leukocytes present in blood. Detailed reviews have been recently published on development of microfluidics for CTC isolation [144, 145].

Laser Capture Microdissection

Laser capture microdissection (LCM) enables the precisely controlled lasers to cut an area containing the cell of interest and then eject it out to a sample collection tube. The method is mostly used to dissect cells from tissue samples. In case of tumors the localization of cells may create heterogeneous or rare cell population, where microenvironment of cell plays an important role [91, 146]. Therefore, LCM is the tool to study CSCs in their microenvironment, which is lost during processing of samples when isolated using other cell isolation methods. Formalin fixed- paraffin embedded sample are used for LCM and target cells are identified either by visual identification, staining, immunohistochemical or immunoflourescence staining [147]. More recently ALDH positive (CSCs population) and ALDH negative cells were isolated from colon cancer patient (stage III) sample to analyse differential protein expression between two groups [148]. LCM has also been used to isolate xenograft models of head and neck cancer for studying cancer stem cell signalling [92]. Furthermore, live cells from HeLa cell line were also isolated and clonally expanded using laser dissection using gravity transfer [149]. LCM stands out among other cell isolation methods due to identification of cells through anatomical visualisation. Some of the limitations of LCM such as imaging and isolation of only fixed cells have been resolved owing to advances in technology while others such as tedious sample preparation, loss of integrity of sample for further downstream processing such as transcriptomic or proteomics still needs a critical handling.

Current Techniques to Study and Trace Cancer Stem Cells (*in vivo*):

There are number of evidences for the occurrence of a small population of cancer cells which is resistant to chemotherapy, radio therapy and is responsible for reoccurrence of tumors regarded as putative stem cell like population. Isolation of CSCs from the tumors (patient samples or *in vivo* tumor models) or cell lines has immensely developed our understanding towards the molecular mechanisms involved in self renewal, metastatic metastasis and drug resistance. However, knowledge of CSCs behaviour in their niche is required and thus, tracking of CSCs become inevitable for their identification and therapeutic targeting in *in vivo* conditions. Next, the question arises whether one want to study the fate of exogenous CSCs isolated from humans *in vivo*, that could be done by ex vivo labelling of cells or one desires to know about the endogenous population of CSCs present in humans by injecting highly specific and sensitive probes. Here, we will try to elucidate different models and techniques for studying exogenous and endogenous CSCs (Fig. 3).

Techniques for Studying Exogenous CSCs

Fluorescent imaging (FI) and bioluminescence imaging (BI) are the most commonly used techniques to study *in vivo* behaviour of CSCs. FI is the best modality to study

differentiation and plasticity of CSCs as it provides high resolution at single cell level. Multiple fluorescent proteins can be used to tag CSCs for simultaneous tracking [151] (Fig. 4a). BI is another major modality used for *in vivo* CSCs tracking where cells were tagged with luciferase promoters and luciferin is injected for tracking purposes [150]. BI have eliminated the disadvantage of autoflourescence and demonstrated low background activity. FI is more oftenly used for *ex vivo* sample analysis, for *in vivo* imaging it requires surgical procedures while BI provides a non invasive method to trace CSCs. However, BI suffers from low resolution and requires minimum 2500 cells with luciferase reporter for detection [153, 154] (Fig. 4b). Another important optical modalities used for *in vivo* tracking are quantum dots (QDs). QDs are semiconductor nanocrystals that can emit light of a tuneable wavelength and are photostable. QDs have been used in number of studies for tracking stem cell population and cancer cell imaging *in vivo* [155–157]. Recently, rhodamine functionalized QDs were used to detect Fe^{3+} in CSCs of prostate cancer models with a resolution of 0.02μm [158]. Antibody conjugated QDs have also been used for studying endogenous haematopoietic stem cell population at single cell level. QDs were especially useful for imaging with near infrared fluorescence (NIRF) which provides highest penetration among the optical methods with least surgical manipulations [155]. Cellular barcoding is a technique where cells are labelled with a unique nucleotide sequence called barcodes; these barcodes are delivered into cells with the help of vectors such as plasmids or lentiviruses. Barcodes are detected by nucleic acid extraction and amplification by PCR [159]. Recently, cellular barcoding has been used to identify proliferative hierarchy of glioblastoma cancer stem cells. There is a population of slow cycling cells which give rise to highly proliferative progenitor cells and further to a non proliferative population. Also, there is a population which was drug resistant and expanded in response to chemotherapy [160].

Other commonly used techniques for *in vivo* CSCs detection include positron emission tomography (PET), computed tomography (CT), and magnetic resonance imaging (MRI). PET and MRI have been used for the detection of both endogenous and exogenous CSCs and will be discussed later. For the detection of exogenous CSCs, it requires them to be labelled with specific probes and then transplanted into mice

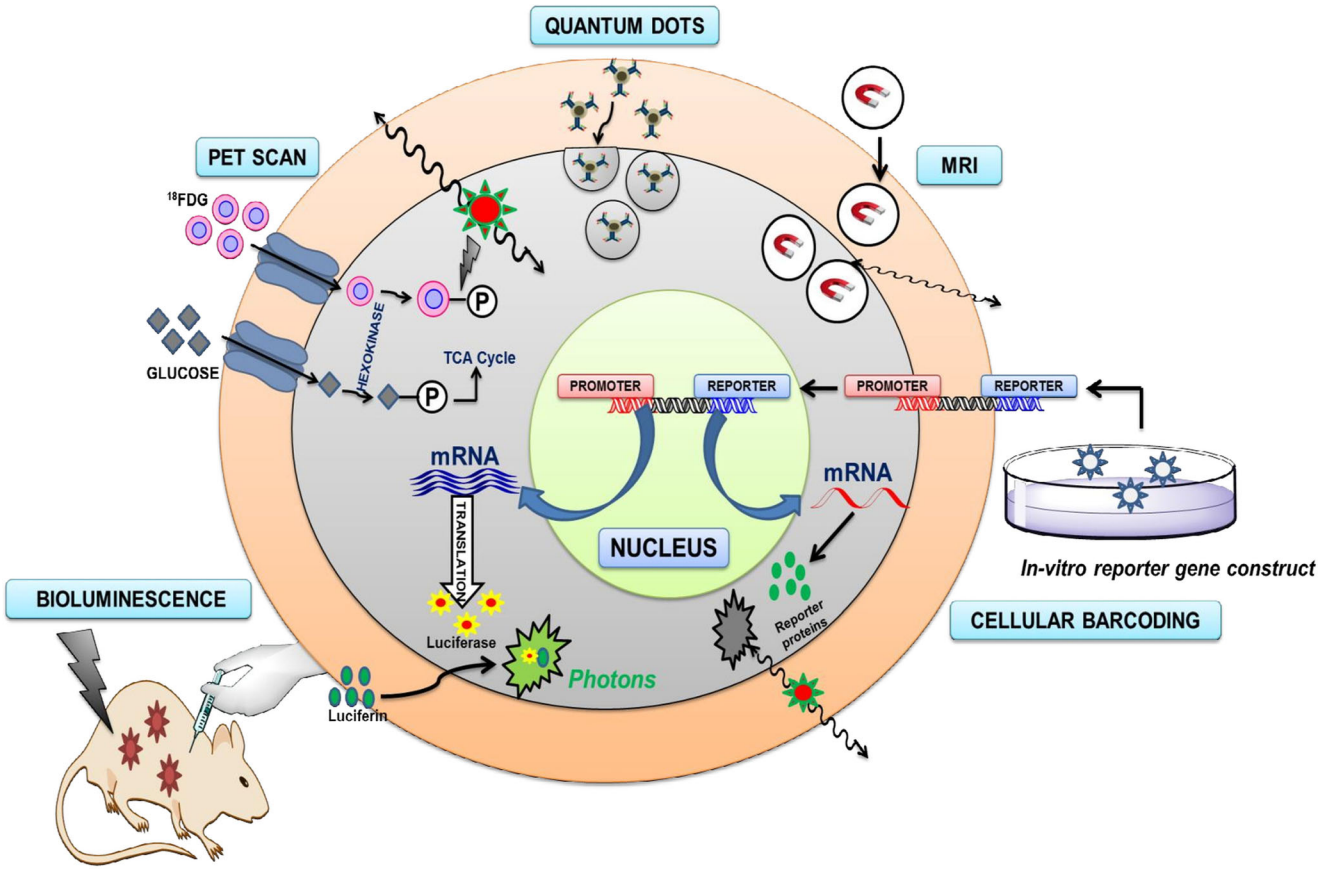

Fig. 3 Conceptual basis of exogenous and endogenous labeling techniques for cancer stem cells imaging: Stem cells can be tracked through endogenous technique that include PET with ^{18}FDG, MRI with SPIONs, super paramagnetic iron oxide coated nanoparticles. Exogenous technique involve reporter gene imaging where stem cells can be stably transduced with reporter genes, bioluminescence using luciferase reporter for detection and quantum dots nanocrystals conjugated with antibody (Redrawn with permission from [150]

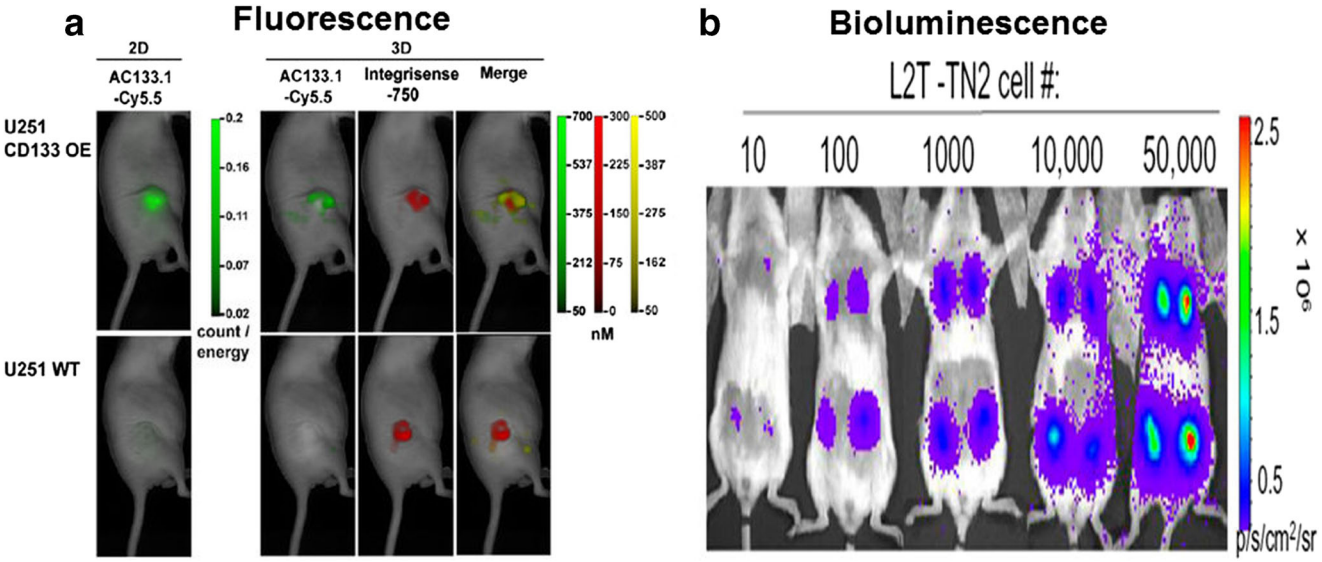

Fig. 4 Tracking of exogenous CSCs: (**a**) In-vivo Fluorescent imaging of U251 glioma cells expression of CD133. 5×10^6 U251 wild-type, CD133-overexpressing U251, cells were injected per animal with 75 μg AC133.1-Cy5.5 and 2nmol of IntegriSense 750. Fluorescent images correspond to day 7[135]. (**b**) Bioluminescence imaging using firefly luciferase expression with ubiquitin promoter of L2T-TN1 tumor cells (10–50,000) implanted in mammary fat pads of NOD/SCID mice [152]

models whereby two common approaches are used; transplantation assays and lineage tracing assays.

The Transplantation Assay

In transplantation assay, cells with presumed markers can be sorted using flow cytometery followed by limiting dilution assay and transplanted into immunodeficient mice to access the ability of tumor cell population to recapitulate the tumor. The disadvantage of this assay was that preparation method for single cell suspension may interfere with the nature of cells due to changes in metabolism or the niche in which they were present earlier. Another major disadvantage of these studies is lack of immune-competent microenvironment. However, it is also reported that CSCs lack expression of MHCs and thus could escape immune surveillance. As CSCs possesses tumorigenic potential human xenograft models are easier route to establish their role in oncogenesis [3]. Human CSC xenografts were obtained in similar manner as cancer xenografts by injecting isolated CSCs subcutaneously or orthotopically into immunocompromised mice such as severely compromised immunodeficient mice (SCID) or athymic nude mice [161]. Development of such models had been reported for the identification of cancer stem cell population for the first time in solid tumour [162]. Patient derived cancer stem cell xenograft models of brain, colon, pancreatic and lung cancer provided concrete evidence about tumour initiating ability of CSCs [163–167]. Discovery of CSCs led to the development of therapies for their eradiation. The CSCs xenografts are better tools to determine therapeutic utility of such therapies. Wakimoto,H. et al, have determined the effect of oncolytic herpes simplex virus (oHSV) vectors on glioblastoma stem cell (GBM-SC) model. GBM-SC were sensitive to oHSV oncolysis and the small population which was not lysed remain non proliferative, thus could have translational value as a therapeutic in GBM patients [168]. Liu et al, studied role of breast CSCs in metastasis by tracking labelled BCSCs *in vivo*, thus providing advanced orthotopic BCSC xenograft combined with non invasive imaging [152]. The bone metastasis was studied in mice as well as zebrafish model of osteosarcoma stem cell models respectively. CSCs (ALDH ^{high}) and non CSCs (ALDH ^{low}) isolated from prostate cancer cell line PC-3M-Pro4 were transplanted into 2 days post fertilized embryos through duct of cuvier and lineage tracing experiment were done to check the distribution of cells. Cancer cells when metastasise to bone marrow CSCs number was found to be increased, these cells compete with hematopoietic stem cells for hematopoietic niche suggesting an important role of niche in stem cell plasticity [169]. 3AB-OS pluripotent cancer stem cells- with and without matrigel were transplanted to athymic mice. Tumors developed showed multileniage commitment when injected with matrigel, particularly mesenchymal leneage and also developed vasculature and muscle fibres mimicing tumours in clinical settings [170].

Although xenografting is the main avenue while studying CSCs. The presence of stem cell niche unlike other cellular niche, excruciating experimental conditions during model development and barring impact of immune system on cancer stem cells are major limitations of these models. Vaidyanath et al, gave an approach of ligand based isolation of CSCs to overcome above mentioned limitation of xenograft models. The hyaluronic acid was used as specific ligand for the

isolation of $CD44^+$glioblastoma and established glioblastoma CSC xenograft [3]. Besides this, constructing genetically engineered CSC mouse model as well as developing human CSC xenograft within humanized mice might serve as forthcoming ways to give advanced panels for cancer stem cell study [171].

The Lineage Tracing Assay

In lineage tracing studies, cells are labelled using cell specific promoters which allow the tracking of single cell derived clones in animals. Lineage tracing assays were earlier used to study stem cells and their lineage differentiations. It has been suggested that CSCs serve as the cell of origin for tumor cells. The studies related to skin, intestinal and brain tumors have supported the concept of CSCs as the cells of origin for tumors [172–174]. These studies revealed that some of the cells in tumors remain quiescent and can divide asymmetrically to give rise to the bulk of the tumor cells. Recent studies in colon cancer have employed patient derived organoids from colorectal cancer. Introduction of LGR5 (which is a marker of adult intestinal stem cells) cassette into their genome using $CRISP^{103}R$/Cas9 gene editing technology were further studied to check their behaviour and plasticity *in vivo* (Fig. 5a) [176, 177]. Another study on mammary tumors observed that some cells initially serve as cells of origin, eventually disappear and new population of stem cells arise which gives rise to tumor (Fig. 5b) [175]. Although these studies have improved our understanding of how CSCs could lead to the development of tumours and its plasticity. Major drawback of this study that the CSCs with lineage tracing utilize common stem cell markers which may also be present on other surrounding cell lineage. It is also difficult to have these studies in complex tissue tumors like mammary glands as they have different architecture during puberty, adult age and during pregnancy. Other parameters which may also change the interpretation of results includes time of induction of labelling, enzymatic digestion during mount preparations and efficiency of labelling [178].

Cell line development from stem cells has sharply increased in past two decades. Currently there are number of stem cell lines commercially available from both embryonic and adult stem cell origins which are used for research worldwide [179–182]. However despite of many challenges related to isolation and study of cancer stem cells, the cell line development from CSCs is lagging far behind. There are very few cell lines available for CSCs studies, Peter et. al., have developed three glioma stem cell lines G144, G166 and G179 from human patients in adherent conditions and characterized them for CSCs like properties. Study implies the usage of these cell lines in screening of compounds that target CSCs and also ascertain that adherent cultures are better than their sphere counterparts [183]. Hundreds of these cells when injected into NOD-SCID mice were sufficient to generate aggressive tumor mass.

In vitro generation of CSCs like cells has been studied from transformation of primary fibroblast cells. Primary fibroblast cells were immortalized using hTERT and transformed with H-RasV12 and SV40 LT and ST antigens. These transfected fibroblast cells were studied for the formation of CSCs like character. Total of 1% population from the transformed cells have shown the expression of SSEA-1 which was not detected in immortalized cells. $SSEA\text{-}1^+$cell population was able to differentiate into adipocytes and osteoblasts. It have also showed symmetric and asymmetric division which gave rise either to both $SSEA^+$ or one $SSEA^+$ and one $SSEA^-$ daughter cell. $SSEA^-$ cells were also able to generate $SSEA^+$ cells spontaneously after 5 days of culturing under same condition. The study have demonstrated proof of evidence that normal somatic cell can give rise to CSCs like cells and can maintain hierarchy and heterogeneity of tumors [184].

Patient derived tumor xenograft (PDX) lines showed stable genomic, proteomic and histology as that of tumor of origin. These PDX models from breast cancer were studied for the presence of CTCs. Study aims if these PDX lines can be used as a renewable source to study CTCs, which is hindered by their low number in patient samples. These results correlate between the presence of CTCs and lung metastatic potential to study in these preclinical models. The study also suggested that CTCs and BM-DTCs found in BC PDX-bearing mice could serve as a valuable and unique preclinical model for investigating the role of CTCs in tumor metastases [185].

Techniques to Study Endogenous CSCs:

High Resolution MRI

MRI has been used for investigation of both exogenous and endogenous stem cell population. Various studies have been conducted to detected single stem cells (exogenous) in tumors of small animals using MRI [186] [187]. To achieve a high resolution and identify a single cell of interest, a probe with significant positive or negative contrast is required. Recently, different probes used for increasing resolution of MRI have been reviewed by Monnica Carril [188]. Conti et al, has explored the L-ferritin receptors which are over expressed in CSCs as compare to differentiated cells. Gd-HPDO3A, a commercially available contrasting agent was used to load Apoferritin cavity along with curcumin (Gd-APO-Curcumin) for the detection of CSCs *in vivo,* using MRI. Although, the results were not encouraging owing to low sensitivity of MRI to detect small population of CSCs; nonetheless, study opens a way to use addiction of CSCs for designing PET based sensitive contrasting agents to identify CSCs [189]. Fe_3O_4 mediated MRI has been used to track Fe_3O_4@PPr@HA nanoparticles loaded with notch inhibitor

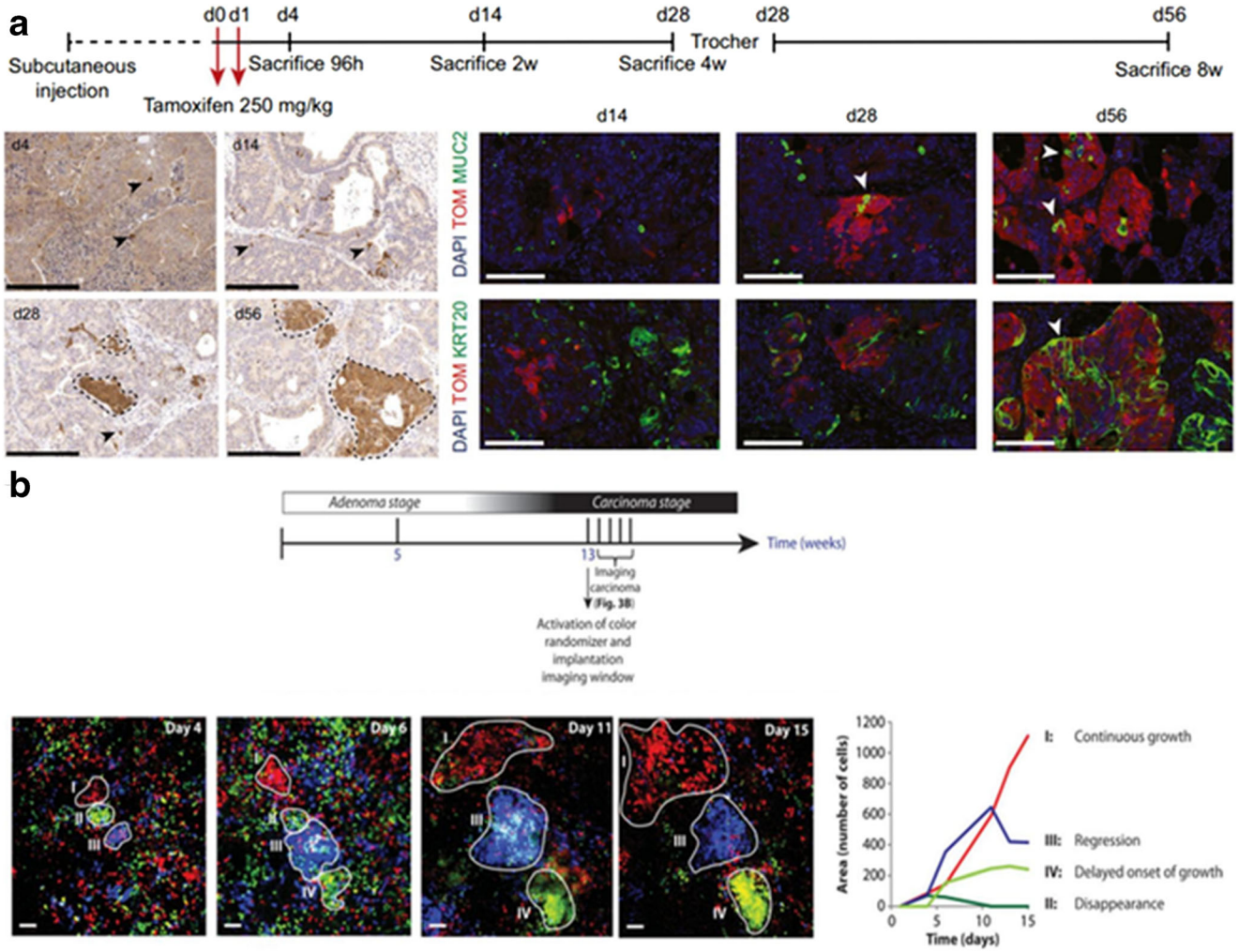

Fig. 5 (**a**) Lineage tracing assay of patient derived organoids from colorectal cancer. Cells are introduced with LGR5 cassette for in vivo tracing. Representative immunohistochemistry using anti-tomato antibodies after tamoxifin induction. Arrowheads point to single and two cell clones. Dashed lines delimit large clones. Scale bars indicate 250 lm. Expression domains of TOM and differentiation markers MUC2 and KRT20 at different time points. White arrowheads indicate double-positive cells. Scale bars indicate 100 lm. Ref: [176]. (**b**) Lineage tracing in mammary carcinoma: The four confetti colours (cyan fluorescent protein, green fluorescent protein, yellow fluorescent protein, and red fluorescent protein) are randomly expressed in mice bearing genetic mammary tumors. After induction of confetti randomizer, different growth patterns of the clones were observed (indicated by I-IV). Representing that stem cell is plastic and can be acquired or lost with time. Ref: [175]

to successfully target CSCs. Hyaluronic acid have greatly enhanced the accumulation of Fe_3O_4@PPr@HA nanoparticles at tumor site owing to CD44 presence [190]. In a study, single-walled carbon nanotube (SWCNT) probes conjugated with CD44 antibody was traced with MRI, SPECT and NIR, to demonstrate their efficient targeting of bCSCs [191]. Extracellular domain of fibronectin (EDB-FN)-Specific peptide (APT_{EDB}) superparamagnetic iron oxide nanoparticles (SPIONs) loaded with doxorubicin were used for tracking and targeting bCSCs more specifically than non specific SPIONs and drug alone [192] (Fig. 6a). Lactate /pyruvate (Lac/Pyr) ratios in glioblastoma orthotropic models generated from cancer cells, U251 and cancer stem like cells, NSC11 lines were studied using 13C- MRI. The study has demonstrated that Lac/pyr was higher in tumor tissues as compare to normal brain tissue. However, Lac/pyr ratio was higher in U251 than in NSC11 tumor tissue [194].

Positron Emission Tomography (PET)

PET is a highly sensitive, non-invasive technique that plays an important role in diagnosis of cancer. It quantitatively detects high energy γ-rays emitted from intravenously injection of radiopharmaceutical agent into patient (e.g., ^{11}C, ^{13}N, ^{15}O, ^{18}F, and ^{68}Ga). It has achieved highly stable resolution of about 1-2mm with an independent depth [195]. Identification of CSCs requires high resolution (upto 200μm) which can be achieved only in small animal imaging PET scanners [196]. Various probes have been utilized for PET mediated cancer scanning among which 2-[18F]-fluoro-2-deoxy-D-glucose (18F-FDG) is

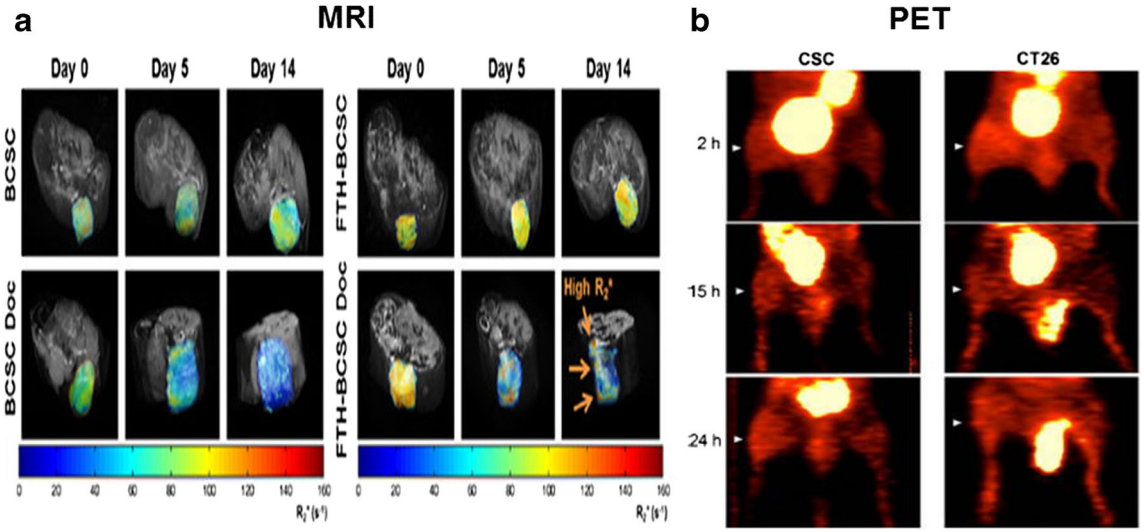

Fig. 6 Tracking of endogenous CSCs (**a**) MRI images of BCSC, BCSC Doc, FTH-BCSC and FTH-BCSC Doc tumor. 1×10^6 BCSCs or FTH-BCSCs were injected into the mammary fat pads of NOD/SCID mice. Distribution of R2* values showing pre-treatment and docetaxel treated BCSC and FTH-BCSC at day 0, day 5 and day 14. Studies were performed using ferritin based MRI with a high spatial resolution [187]. (**b**) In-vivo PET images of tumor cells in the hind limb of ischemic mice. Transplantation of tumor cells was performed 24hrs after hind limb ischemia. 3×10^6 CSCs and 3×10^6 parental CT26 infected with Ad.EGFP.NIS were implanted into the ischemic mice PET images demonstrate I-124 uptake with NIS expressing CSCs and CT26 was performed in all the eight animals at 2, 15 and 24hr [193]

most widely used in clinical and preclinical setup. FDG is actively transported in cancer cells which were detected through PET scan [197, 198]. Keratin 19 (K19) and GLUT1 expression is reported to be increased in Hepatocellular(HCC) CSCs. Study conducted on 98 patients who underwent ^{18}F-FDG-PET scan revealed higher uptake of ^{18}F-FDG in K19$^+$ cells than in K19$^-$ cells, thus, could be used to detect K19$^+$ CSCs population in HCC [199]. Similarly, thyroid CSCs related markers such as CD133, CD44, oct4 and nanog were correlated with higher FDG uptake in thyroid cancer patients [200]. I-124 PET imaging has been used to identify sodium/iodine symporter (NIS) -enhanced green fluorescent protein (EGFP) transfected CSCs *in vivo* with high resolution [193] (Fig. 6b). Of note, a recent study in ovarian cancer patients showed that cells with low FDG uptake may have CSC properties. Sato et al. also isolated CSCs from cell lines and demonstrated the lower expression of L-type amino acid transporter 1(LAT1) and glucose transporter1 (GLUT1) in CSCs than in non–CSCs which may account for decreased FDG uptake and altered glutaminolysis in Ovarian cancer CSCs in patients [201]. Most common reporter gene used for tracing exogenous stem cells *in vivo* via PET scan is herpes simplex virus type-1 thymidine kinase (HSP-tk) which phosphorylates the18F-fluoropenciclovir probe and retained in cytosol of cells for detection [196, 202].

All these modalities used for *in vivo* imaging have their own limitations and advantages. Also the techniques for *in vivo* tracing of CSCs require high resolution as the number is very less. Modalities used for in vivo tracking of CSCs are summarized in Table 4. Although the imaging of exogenous stem cells has been achieved upto single cell levels in small animals but tracking and detection of endogenous CSCs is still correlative. The major drawback for identifying endogenous CSCs is that it share common markers of stem cells and metabolic discrepancies of CSCs and differentiated cancer cells

Table 4 Comparison of imaging modality for CSC study

S.no.	Molecular imaging	Resolution	Detection limit: Cells	Model	Imaging agent	Cancer stem cell Biomarker	Clinical use	References
1.	Fluorescence	2–3 mm	~10^6	Glioblastoma	Cy5.5	CD133	Preclinical	[203]
2.	Bioluminescence	5–20 mm depending on depth of signal	~10^3	Breast cancer	Firefly luciferase (Luc)	CD44+	Preclinical	[152]
3.	Quantum dots	0.02μm	~10^3	Prostate cancer	Rhodamine-Functionalized Graphene	Fe^{+3}	Clinical	[158]
4.	PET	~1 mm; 4–6 mm	~10^4	Glioblastoma	^{64}Cu-NOTA	CD133	Clinical	[204]
5.	MRI	25–500 μm; 0.5–5 mm	~10^4	Breast cancer	Ferritin heavy chain (FTH)	$CD44^+/CD24^-$	Clinical	[187]

are not finely demarcated. However, multimodal imaging could enhance the clarity to visualize CSCs, where probes are designed to visualize cells in two or more imaging systems. Examples of dual imaging modalities include PET/FI, PET/CT, PET/BLI and MRI/ FI etc [153, 205].

Conclusion

Stem cells in cancer present a valuable avenue in tumor research. These cells could be used as good prognostic markers and are the main targets for anticancer therapies. However, their, scanty number and presence of common stem cells markers for CSCs present a major challenge for the isolation and studies related to drug targeting. Various techniques have been used for the isolation of these rare cells exploiting the differences in expressed markers, size, deformability and quiescent nature. The flow cytometry and MACS platforms for the isolation of CSCs can provide both preparative and analytical scale purification but recent progresses in development of microfluidics platform could provide a better method to isolate CSCs for their analysis. Moreover, the laser capture micro-dissection is very useful in analysing CSCs at single cell level. However, every technique has its advantages and disadvantages for the isolation of CSCs. The growing interest in the studying CSCs have pushed the establishment of *in vitro* and *in vivo* models such as cell lines, PDX models and 3D cell culture models to enhance our current knowledge. Various imaging techniques such as MRI and PET have shown its potential in studying endogenous CSCs in various cancer types. Need of the hour demands a model which could mimic the heterogeneity of cancers which varies at individual levels and using the information for personalized medicine.

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Compliance with Ethical Standards

Conflict of Interest Authors declare that they have no conflict of interest.

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