



Technological Advancement in Cancer Stem Cell Research **14**

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Abstract

Cancer stem cells (CSCs) display a significant role in cancer research, evidenced from past decade studies. Although, with the passage of time, effective cancer therapy has been developed, still up to now, cancer possesses the second highest mortality worldwide. The only defined characteristic for every therapy failure is the presence of cells with self-renewable capacity known as cancer stem cells in the heterogeneous population of tumor. These CSCs provide a tumor resistance against various therapies like chemotherapy and radiotherapy. Thus, to prolong survival time period of cancer patients, it is prerequisite to eliminate CSC population. Thus, to develop novel effective therapeutics against primary tumors, isolation and characterization of CSCs will provide a novel insight to develop cancer therapeutics. Thus, various in vitro and in vivo approaches have been developed to isolate and target CSCs. In this chapter, we will discuss about how researchers have developed various powerful tools to characterize CSCs to develop better therapeutics to target CSCs and thus cancer and also how technology has sprung up to generate advanced preclinical models of human tumors.

Keywords:

Cancer stem cells (CSCs) · Spheroids · Organoids

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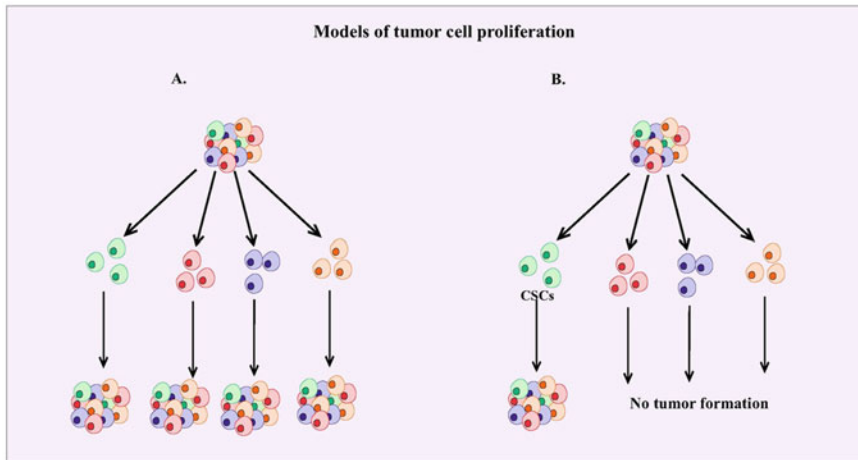


Fig. 14.1 Models for tumor cell proliferation. (a) Stochastic model: this model proposes that every cell has the potential to proliferate and behaves as stem cells. (b) Cancer stem model: according to this model, only subset of cell has the self-renewable capacity which can generate whole tumor. This distinct subpopulation of cells is known as cancer stem cells

14.1 Introduction

Despite advancement in intensive experimental approaches and progress in cancer treatment, cancer still causes the second highest death [1]. A deep insight into the mechanism of development of carcinogenesis has caused a drift toward cancer research and treatment. Previously, much attention has been paid on genetic and biochemical mechanisms that induce drug resistance. Prevailing theories have reported that tumor is not a mass of homogeneous malignant cells albeit, composed of heterogeneous population of cells. It has been well established that during carcinogenesis, treatment failure primarily occurs due to intratumoral heterogeneity. A subpopulation of cells present within a tumor is responsible for the genesis of resistance against chemotherapy and radiotherapy and the roots of tumor relapse. These minor populations of cells are known as cancer stem cells (CSCs) and can repopulate after therapies causing tumor recurrence [2].

Tumor progression has been well explained by two models, the stochastic model (Fig. 14.1a) and cancer stem cell model (Fig. 14.1b). The stochastic model is known as clonal evolution model. According to the stochastic model, all cells of the tumor possess carcinogenic potential with uncontrolled proliferation potency, and therapeutic treatment requires targeting of all tumorous cells [3, 4]. The cancer stem cell model states that tumor is originated from a stem cell with self-renewable capacity, possessing resistance to chemotherapy and radiotherapy [5]. These subpopulations of tumor are known as cancer stem cells (CSCs) due to their ability to drive whole tumor, and these cells cause tumor recurrence [5]. CSCs are involved in tumor initiation, progression, maintenance, development of metastasis, and

reappearance. Thus, specifically targeting and eliminating CSCs population from tumor could be an effective treatment strategy that can pause tumor relapse and can be sustained as a long-lasting treatment [5].

14.2 Cancer Stem Cell Models

Various tumor biology questions can be answered by studying CSCs. CSCs in a tumor population can be defined as those cells which have potential of self-renewal and are multipotent. Identification and quantification of cancer stem cells like cells can be done by either by in vitro or in vivo assays.

14.3 In Vivo Assays

14.3.1 Xenotransplantation Assay

14.3.1.1 Severe Combined Immunodeficient (SCID) Mice

Immunocompromised mice have been widely used to study CSCs. SCID mice model was first explored for the development of “leukemic stem cells (LSCs)” to study acute myeloid leukemia (AML). During acute myeloid leukemia, cells are restricted with low proliferation capacity, indicating that leukemic clones are maintained by rare population of stem cells [6]. Lapidot et al. [6], used SCID mice model and engrafted different population of cells expressing CD34⁺ CD38⁻, CD34⁺ CD38⁺, and CD34⁻ CD38⁺, resulting in the development of leukemia by cells expressing CD34⁺ CD38⁻ only. It was also observed that 1 in 2.5×10^6 cells had the potential to generate leukemic graft [1, 6]. This study provided an evidence that not all the AML cells had potency for tumor formation, but the limitation of using SCID mice was that the frequency of isolated LSCs was very low.

14.3.1.2 Nonobese Diabetic, Severe Combined Immunodeficient (NOD/SCID) Mice

Human malignant melanoma was studied using NOD/SCID mice model which is more immunocompromised than SCID mice model [7]. Xenotransplantation of human melanoma cells in NOD/SCID mice resulted in identification of only one tumorigenic cell out of million cells [7]. Researchers also observed that most cancers had less than 0.1% of tumorigenic cells when transplanted in NOD/SCID mice [7, 8]. It was questioned whether NOD/SCID assays poorly estimate the frequency of tumor generating cells [8, 9]. Thus, it was a demand to develop an efficient model which could increase the number of detection and isolation of cancer stem cells. To solve this problem NOD/SCID IL2R γ ^{null} model was used [8].

14.3.1.3 NOD/SCID IL2R γ ^{null} Mice

NOD/SCID IL2R γ ^{null} mice is the one which lack the interleukin-2 (IL-2) receptor gamma chain and natural-killer cells and is highly immunocompromised mice model [10]. Quintana et al. showed that transplantation of melanoma cells into the

NOD/SCID IL2R γ^{null} mice resulted in detection of increased number of tumorigenic cells [8]. Using this model, researchers were able to generate new tumor *in vivo* from 25% of melanoma cells. This was quite high in comparison to tumor-initiating potential of NOD/SCID mice which was 1 in 1,090,000; in NOD/SCID IL2R γ^{null} , it was 1 in 9 melanoma cells which had tumor-generating capacity [10].

This model then became the choice of many researchers. For example, AML cells were xenotransplanted in NOD/SCID IL2R γ^{null} mice, and it resulted in the presence of long-term engrafting, self-renewing LSCs in very few 10^3 bone marrow hCD34 $^+$ hCD38 $^-$ cells, but not in hCD34 $^+$ hCD38 $^+$ or hCD34 $^-$ cells [11]. Ishizawa et al. did comparative study between NOD/SCID and NOD/SCID IL2R γ^{null} mice for human pancreatic, lung carcinoma, and head and neck cancer [12]. For all tumor under investigation, about tenfold elevation was detected for tumorigenic cells in NOD/SCID IL2R γ^{null} mice [12].

14.3.1.4 Limitation of Xenotransplantation Assays

There are many technical errors in *in vivo* detection of cancer stem cells by using xenotransplantation assay and limiting dilution analysis. These errors occur due to murine microenvironment and inadequate immune response at the transplanted site and also sex of recipient mouse strain. These factors compelled scientists to develop a more accurate approach to study cancer stem cells and led to use of genetically engineered mouse models (GEMM).

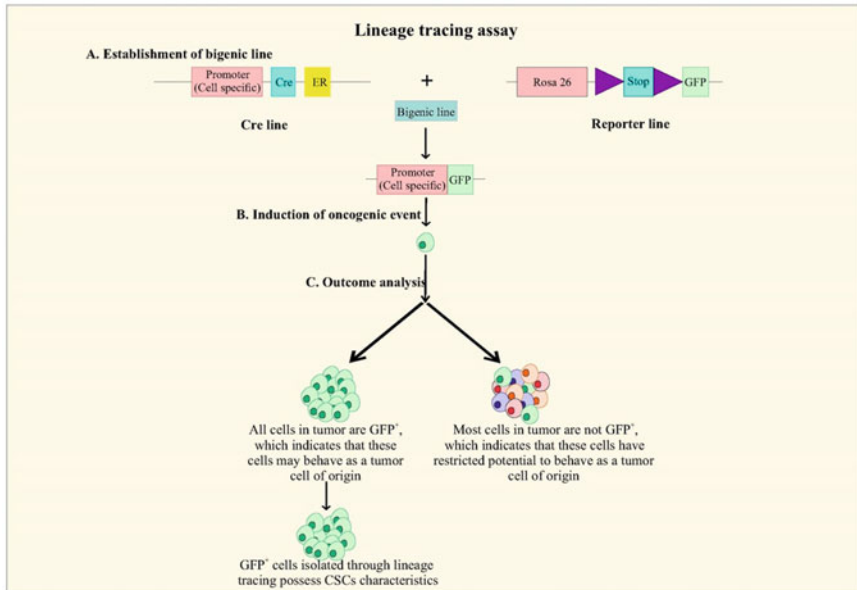


Fig. 14.2 Lineage tracing assay. Schematic representation of lineage tracing assay. The first step includes establishment of bigenic line. The second step involves induction of oncogenic event which is followed by third step, where analysis of outcome is done

14.3.1.5 Lineage Tracing Assay

In lineage tracing assay (Fig. 14.2), different cell-specific promoters are used to label different cells which enables tracking of single cells [13]. Various steps are involved in lineage tracing assay. The initial step is to generate bigenic mouse line (Fig. 14.2a). The bigenic mouse line is generated by crossing an inducible Cre (expressing Cre recombinase) with a reporter line (expressing reporter) which helps in labeling of cells [13]. The second step involves either introduction of oncogenes or deletion of tumor suppressor genes by crossing bigenic mouse generated with third conventional Tg line overexpressing either oncogenes such as Myc, Tcf, and Ras or deleted tumor suppressor genes such as p53, PTEN, and Rb (Fig. 14.2b) [13]. In spite of expressing oncogenes using Tg line, chemical carcinogens can also be used to induce oncogenic event. The most widely used carcinogen is DMBA (7,12-dimethylbenz[a]anthracene). In the final steps of tumor formation, tracing of labeled cells is done. If all the cells are reporter positive, it suggests that these cells have tumor-repopulating capacity (Fig. 14.2c). Thus, purification of these cells is done to perform serial transplantation and then CSCs are isolated. But, if majority of cells are reporter negative, then it suggests that cells do not possess CSC properties. Lineage tracing assay has gained momentum, and various studies have been performed using this assay which employs the use of genetically engineered mouse models (GEMM) [14].

For example, Chen et al. performed a lineage tracing study for glioblastoma multiforme (GBM). This study showed that dormant subset of endogenous glioma cells is responsible for tumor maintenance and recurrence of GBM after chemotherapy [15]. They used a *Nestin-ΔTK-IRES-GFP* (*Nes-ΔTK-GFP*) transgene that labels both adult NSCs (neural stem cells) and endogenous glioma tumor cells. This *Nes-ΔTK-GFP* was crossed with *Mut7* line which is a glioma-prone mouse line [16]. This *Mut7* mouse line is generated by deleting three tumor suppressor genes, i.e., *PTEN*, *p53*, and *Nf1* [15]. The resultant *Mut7* mice developed glioblastoma with deleted *PTEN*, *p53*, and *NF1* tumor suppressor genes [15]. These *Mut7*;*Nes-ΔTK-GFP* tumor cells also expressed Sox2 and had two population of cells. One subset of cells expressed $GFP^+/Sox2^+/ki-67^-$ and $GFP^-/ki-67^+$. Treatment with temozolomide eliminated actively dividing $GFP^-/ki-67^+$ tumor cells, and a fraction of quiescent cells responsible for tumor recurrence $GFP^+/Sox2^+/ki-67^-$ was left. GFP^+ cells could be targeted by ganciclovir; thus, ganciclovir administration significantly decreased tumor growth with prolonged survival and co-administration of temozolomide- and ganciclovir-retarded tumor growth [15]. This lineage tracing study demonstrated that dormant endogenous glioblastoma cells $GFP^+/Sox2^+/ki-67^-$ responsible for tumor recurrence possess CSC properties and are responsible for long-term tumor growth [14].

A functional evidence for the presence of stem cells in intestinal adenomas was provided by the study done by Schepers et al. [17]. In this study, they used multicolor Cre reporter R26R-Confetti mouse strain. They crossed $Lgr5^{EGFP-Ires-CreERT2}/Apc^{fl/fl}$ mice with the R26R-Confetti strain, and tamoxifen injection resulted in generation of $Lgr5-GFP^{hi}$ and $Lgr5-GFP^{low}$. Gene expression and clonogenic potential analysis showed that $Lgr5-GFP^{hi}$ had multipotent stem cell characteristics,

and retracing of these cells showed that these cells were obtained from single adenoma stem cells [17].

Another lineage tracing study done by Driessens et al. utilized a chemical two-stage carcinogenesis model to generate skin papillomas [18]. The bigenic mouse strain K14CreER/Rosa-YFP was obtained by crossing K14-driven CreER line with the Rosa26-YFP reporter line. By injection of tamoxifen, K14-expressing keratinocytes will be labeled as YFP⁺ cells [18]. Administration of both DMBA and tamoxifen resulted in generation of YFP⁺ cells and had majority of cells with limited proliferation capacity, while a fraction had stem cell-like characteristics. Confocal analysis of clones showed that papillomas were sustained by small population of tumor cells having characteristics like of stem cells [18].

14.3.1.6 Limitations of Lineage Tracing Assay

Lineage tracing can be performed utilizing mouse model only, and various fundamental differences exist in human and mice cells/organs. For example, mouse prostate is divided into 4 lobes that do not exist in humans, and also, mouse cells do not express PSA which is an important molecule of human prostate gland. Another difference is that mouse cells express high telomerase activity, which indicates that mouse cells may never undergo true terminal differentiation. Thus, results obtained using mouse models may not directly reflect human system [14].

14.3.2 In Vitro Assays

14.3.2.1 Side Population Assay

Side population assay has been used for the isolation and characterization of cancer stem cells (Fig. 14.3) [13]. SP assay was developed by Goodell and mulligan [19, 20]. It was observed by the researchers that a distinct population existed in murine bone marrow cells which were poorly stained for Hoechst 33342. These cells occupied a distinct position in flow cytometry dot plot, hence named as side populations [21]. The exclusion of Hoechst stain by side population is a specific property of CSCs. Interestingly, the efflux of Hoechst stain was due to ATP-binding cassette (ABC) transporter. ABC transporter uses ATP to efflux out many small endogenous molecules like peptides, cholesterol, and bile acids. These transporters help in detoxification of cells and also contribute to cancer stem cell-like properties to CSCs. ABC transporters induce chemoresistance in CSCs as chemotherapeutic drugs are also substrates for these pumps and efflux of drugs occurs by ABC transporters [21].

14.3.2.2 Retention of PKH26 and PKH6 Dye

It has been reported that CSCs proliferate slowly and remain quiescent. These CSCs when divided result in two daughter cells; one possesses stemness (remains quiescent) and other proliferates. PKH26 and PKH6 are two lipophilic dyes [21]. In this assay (Fig. 14.4), the cell membranes are labeled with these dyes. After division both daughter cells receive equal portion of these dyes [21]. The CSCs which are

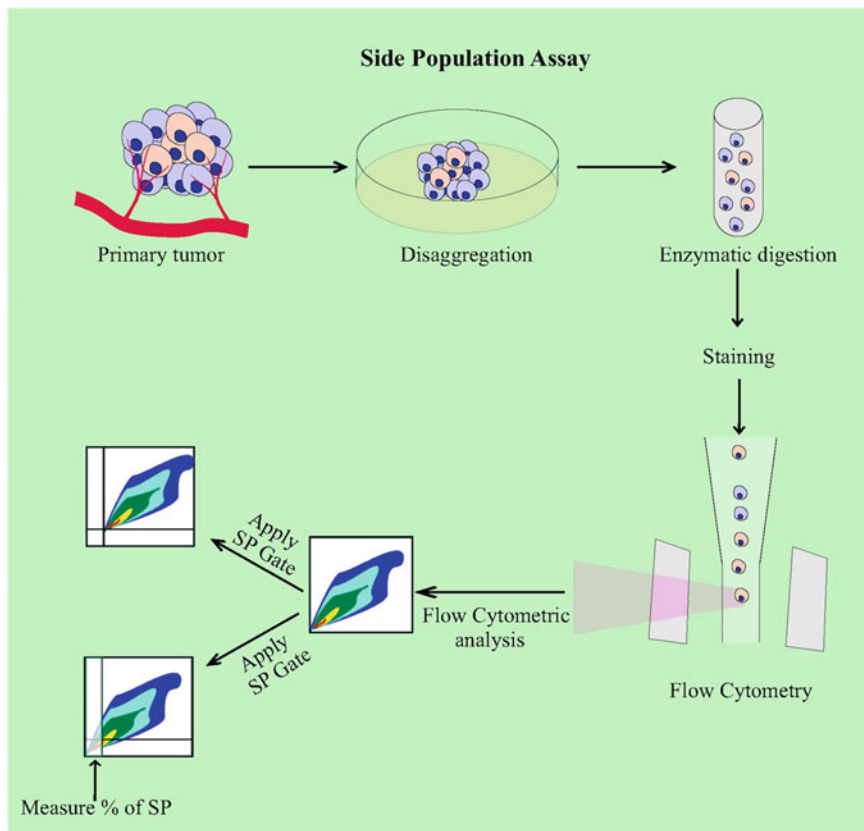


Fig. 14.3 Side population assay. The side population assay measures percent side population of cells after flow cytometric analysis. The steps include single-cell isolation of cells by disaggregation and enzymatic digestion of cells. These cells are then stained with Hoechst 33342, and then cells are subjected to flow cytometric analysis. The cells with CSC characteristics possess more ABC transporters and efflux dye out of the cells, and these cells are obtained on side on flow cytometric plot

quiescent retain dye for longer duration as compared to non-stem cells. This method has been used to isolate CSCs from breast cancer [22].

14.3.3 ALDEFLUOR Assay

Aldehyde dehydrogenases (ALDHs) belongs to the family of enzymes that catalyzes the oxidation of endogenous and exogenous aldehyde substrates to corresponding carboxylic acids [23]. These enzymes are known for their detoxification properties as these eliminate aldehydes synthesized either by physiological metabolic products or by cytotoxic drugs like chemotherapeutic agents. This detoxification property

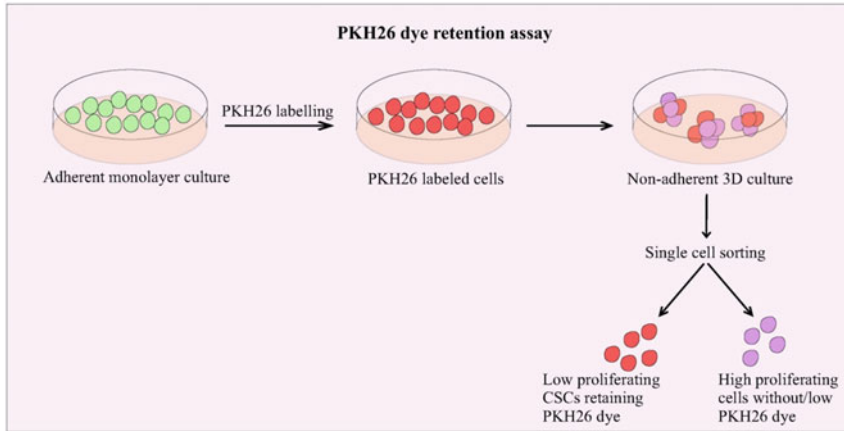


Fig. 14.4 PKH26 dye retention assay. Monolayer culture is first treated with PKH26 and then subjected to non-adherent 3D culture. After single-cell sorting, CSCs are identified as those which retained dye for long due to low proliferating potential

attributes them as a marker of cancer stem cells as they confer chemoresistance in cancer cells [24]. Study done by Hilton et al. firstly revealed that high ALDH activity is responsible for chemoresistance in leukemia stem cells against cyclophosphamide (an alkylating agent) [25]. Increased ALDH activity has been reported in lung, colon, and breast cancer stem cells [21]. *ALDEFLUOR* assay is done to identify cancer stem cells (Fig. 14.5). In this assay, CSCs with high ALDH activity become highly fluorescent and can be detected by using flow cytometer and can be isolated by using cell sorting. *ALDEFLUOR* assay works on the principle of conversion of BODIPY-aminoacetaldehyde (BAAA) substrate to a fluorescent BODIPY-aminoacetate (BAA) product [24]. Thus, this assay isolates CSCs on the basis of intrinsic functional property of CSCs.

14.3.3.1 Two-Dimensional Model

Two-dimensional cultured tumor cell lines have been extensively used to study cancer progression. Various signaling pathways have been studied using 2D cultured tumor cell lines. But, with the advancement in technology to study tumor progression, it has been reported that 2D cultured tumor cell lines provide contradictory results due to culture conditions and number of cell passages [26]. Although research using 2D cultured tumor cell lines is inexpensive, these cannot mimic three-dimensional characteristics of solid tumor models and also tumor microenvironment. Thus, researchers have developed three-dimensional tumor models that may resemble solid tumor characteristics so that more accurate therapeutics can be developed to improve survival of cancer patients (Table 14.1).

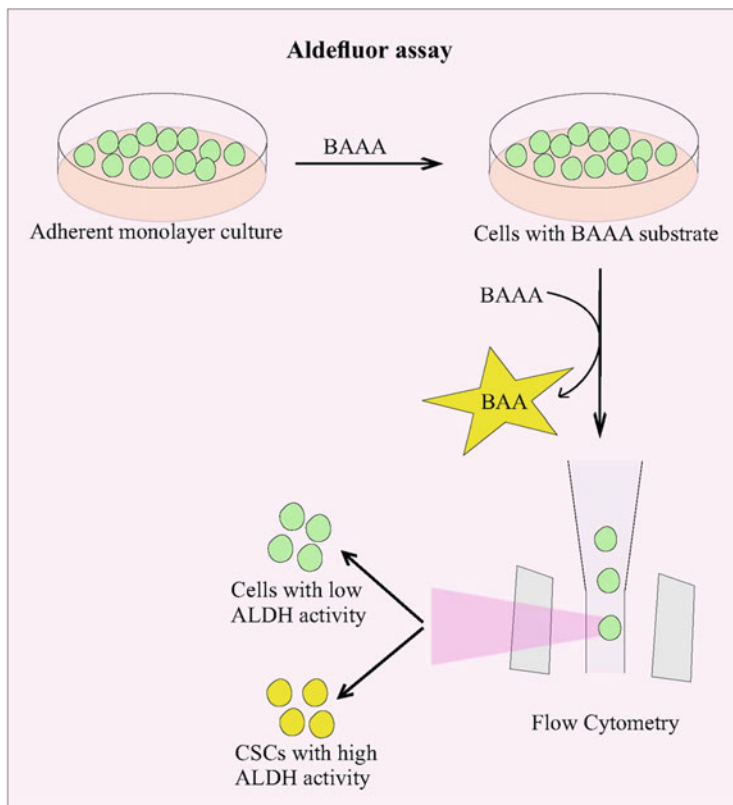


Fig. 14.5 ALDEFLUOR assay. This assay involves treatment of cultured cells with BODIPY-aminoacetaldehyde (BAAA) substrate, and CSCs are identified and isolated on the basis of high ALDH activity which converts BAAA to highly fluorescent BAA

14.3.3.2 Three-Dimensional Models

Three-dimensional tumor cultures are the recent advancement of technology to specifically generate and isolate CSCs. The main purpose of 3D models is to study the effect of tumor microenvironment on the gene expression analysis, pathogenesis, and effective drug testing to overcome chemoresistance. The two important 3D tumor models discussed in this chapter are *tumor spheroids* and *tumor organoids*.

14.3.4 Sphere Formation Assay

14.3.4.1 Tumor Spheroids

Tumor spheroids are spherical aggregates of tumor cells with self-renewable capacity and are generated by sphere formation assay (Fig. 14.6). Sphere formation assay also known as non-adherent 3D culture was firstly described as an approach to study adult stem cells [32]. The principle of this assay is that cancer stem cells in non-adherent conditions proliferate to form a sphere and non-stem cells will go for

Table 14.1 Illustration of key differences between advantages and disadvantages of two-dimensional cell lines and three-dimensional spheroids and organoids

Advantages/ disadvantages	2D cell line	Spheroids	Organoids
Advantages	<ul style="list-style-type: none"> • Cost-effective [2, 26] • Genetic manipulation is easy [2] • It allows high-throughput screening of drugs in short duration [2, 27] 	<ul style="list-style-type: none"> • Provides 3D environment [28] • Allows growth of cancer stem cells [28] • Highly reproducible [29] 	<ul style="list-style-type: none"> • In vivo-type complexity and architecture • 3D structures and resembles mini-organ to the tissue of origin [2, 30] • Patient-derived organoids enable the development of personalized medication [31]. Variable [29]
Disadvantages	<ul style="list-style-type: none"> • Lack of heterogeneity [2, 27] • Do not correspond to tumor microenvironment [27] 	<ul style="list-style-type: none"> • Expansion of CSCs occurs after serial passages, thus, not efficient for investigation of drug activity [31] 	<ul style="list-style-type: none"> • Organoids cannot mimic exact hypoxic gradient occurring in tumor microenvironment [2]

anoikis. Sphere formation assay is a powerful tool that allows to access stem cell-like characteristics residing in tumor and cancerous cells. Cancer stem cells possess the ability to generate 3D (three-dimensional) spheres in vitro when grown in serum-free non-adherent culture conditions [33]. Various 3D in vitro sphere formation assays have been developed to obtain cancer stem cells. This assay requires the growth of cells in an artificial medium resembling stem cell-like conditions which include media supplemented with epidermal growth factor (EGF), low-density condition to avoid aggregation, progesterone, heparin, insulin, and hydrocortisone [34]. Sphere formation assay is widely used as it helps to detect CSCs, and also, self-renewal and differentiation can be studied at single-cell level [32].

14.4 Critical Parameter Consideration

14.4.1 Cell Density and Clonal Formation

Cell density is the most crucial parameter as it directly affects clonality. A central focus of sphere formation assay is that each sphere is obtained from single cell and therefore must be clonal. Different research groups have proposed different cell densities for seeding. High-density seeding is not favored because interpretation of results becomes very difficult due to sphere fusion. Spheres have the potency for aggregation due to both intrinsic and experiment-induced locomotion. It must be ensured that the sphere is formed due to proliferation not due to aggregation [32]. Thus, seeding at 0.2–20 cells per microliter is recommended [35–37].

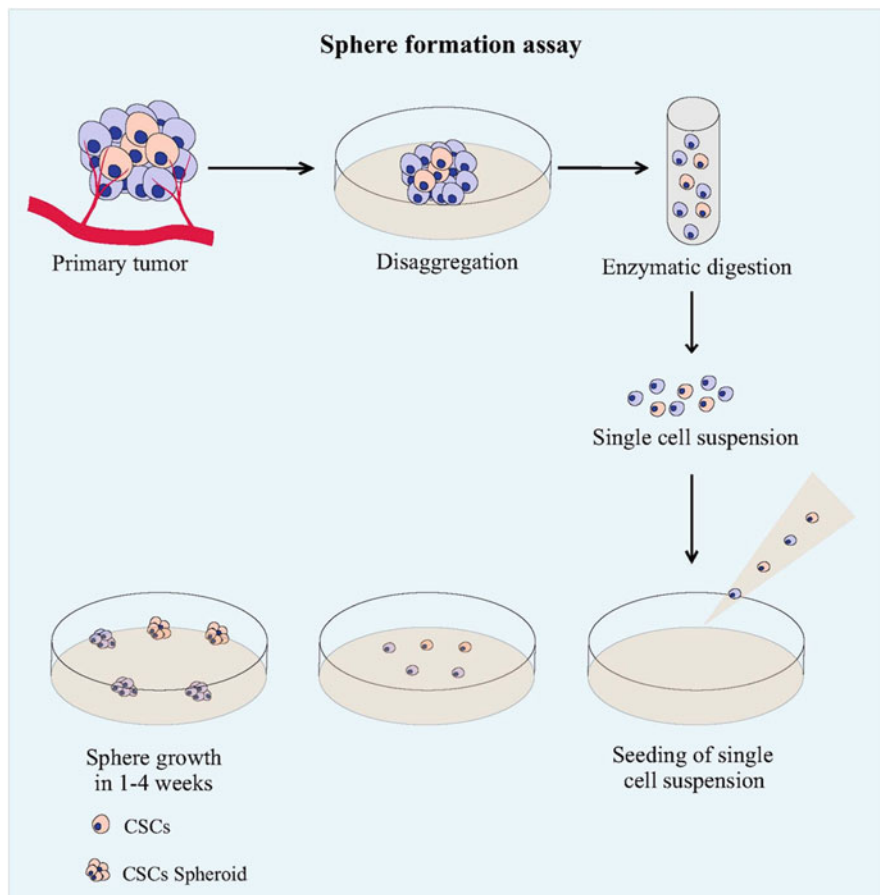


Fig. 14.6 Sphere formation assay. In this assay, firstly single-cell suspension is formed from primary tumor by disaggregation and enzymatic digestion. Then seeding of single cells is done and sphere formation occurs in 1–4 weeks

14.4.2 Mitogen Tolerance

It has been reported that spheres are cultured at very high level of EGF of about 20 ng/ml. This high concentration of EGF may alter differentiation potential of cultured cells [32].

14.4.3 Overestimation of Stem Cell Frequency

Sphere formation assay can overestimate frequency of generated stem cells because neural stem cell purification by FACS has shown that both stem cells and transit amplifying cells have potential to give rise to neurospheres [38]. Similar observation of false readout was observed by culturing mammary cells which formed mammospheres [39].

14.4.4 Tumor Organoids

Tumor organoids are 3D constructs resembling avascular tumor generated from fresh biopsy samples [26]. The process of tumor organoid formation includes mechanical or enzymatic processing of tumor samples and embedding in extracellular matrix such as collagen or Matrigels and ECM substitutes [26, 40]. The various steps used to generate tumor organoid has been shown in Fig. 14.7. Organoid technology recently has been used extensively, and various cancer organoids such as stomach cancer organoid, intestinal cancer organoid, liver cancer organoid,

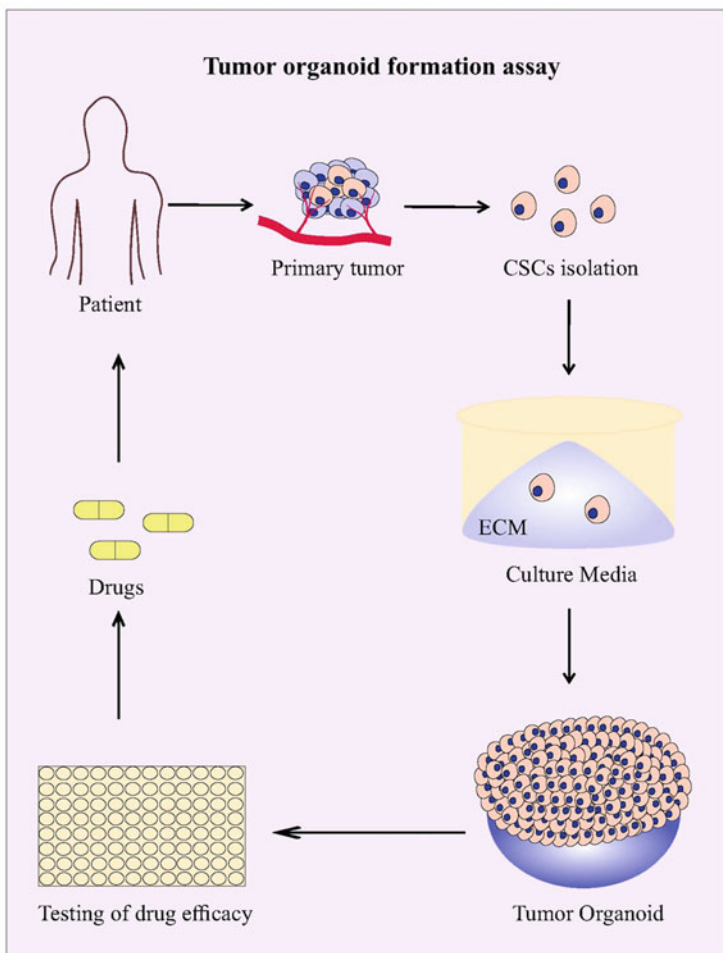


Fig. 14.7 Tumor organoid formation assay. Primary tissue from the patient is disaggregated to obtain CSCs. These cells are then cultured in three-dimensional media to generate tumor organoid, and these organoids can be used to test required drug efficacy and to develop personalized medicines

pancreatic cancer organoid, breast cancer organoid, bladder cancer organoid, and prostate cancer organoid have been synthesized [41–51].

14.5 Conclusion and Further Directions

Plethora of evidences is in agreement with current theory of cancer stem cells. It has been well established that CSCs play a vital role in tumor initiation and maintenance of tumor progression. These CSCs facilitate tumor metastasis to distant site other than the site of origin. Thus, these CSCs have the potential for tumor regeneration and recurrence; hence, these are potential therapeutic targets, and elimination of these CSCs will protect tumor recurrence. Various technological advancements have been made with the primary aim to develop effective drug treatment. Traditional 2D culture cell lines have been in long use to develop cancer treatment and have contributed significantly in cancer research. But these 2D culture cell lines fail to match accuracy to the condition of tumor development in the presence of the immune system; stromal interactions of CSCs and also lack of heterogeneity make them of least choice. To overcome these limitations, 3D tumor models are in fashion for cancer research. Tumor spheroids and tumor organoids both are widely used, but tumor organoids have revolutionized cancer research and have been proven as best models as they recapitulate whole tumor like in vivo. These tumor organoid technologies have provided a way for cancer researchers toward the development of effective drug testing and facilitation of personalized therapy.

Thus, organoid technology upholds potential for new possibilities for personalized medication which will be the best nonsurgical treatment not available today.

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