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Reporter Systems to Study Cancer Stem Cells

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Abstract

Cancer stem cells have been identified in primary tumors, patient derived xenografts, and established cancer cell lines. The development of reporters has enabled investigators to rapidly enrich for these cells and more importantly track these cells in real time. Here we describe the current state of the reporter field and their use and limitations in multiple cancers.

Keywords: Tumor heterogeneity, Cancer stem cells, NANOG, Reporter, Pluripotency transcription factor

1 Introduction

1.1 Tumor Heterogeneity and Cancer Stem Cells

Tumors are composed of heterogeneous populations of cells, which differ in their phenotypic and genetic features [1]. This multifaceted heterogeneity accounts for the differences in tumor biology and treatment sensitivity, not only amongst different individuals with same tumor types, but also between primary cancers and metastases [2]. Originating from this concept of tumor heterogeneity, a distinct subpopulation of self-renewing cells termed cancer stem cells (CSCs) has been shown to be present in both solid and hematopoietic tumors [3–7]. CSC are defined as a cell within a tumor, which has the capacity to give rise to the heterogeneous lineages of cancer cells that constitute the tumor and also undergoes self-renewal to maintain its reservoir [8]. Because of their intrinsic ability to repopulate tumor heterogeneity, CSCs are also known as "tumor-initiating cells" [1]. Additionally, CSCs have been shown to be more resistant to chemotherapy and radiotherapy as compared to non-CSCs [9–11]. Therefore, the residual pool of CSCs after initial cancer therapy has been postulated to be responsible for tumor recurrence [12]. Due to their cardinal roles in tumor initiation, maintenance, progression, and treatment-resistance, various drugs targeting critical CSC pathways are being extensively investigated in clinical trials [13–15].

1.2 Isolation of CSCs by Surface Markers

To date, the isolation and characterization of CSCs have largely been through the differential expression of surface markers between CSCs and non-CSCs [16]. Since the first prospective identification of $CD34^+CD38^-$ CSCs in acute myelogenous leukemia [17], various other markers shared between CSCs and normal human stem cells have been used to isolate self-renewing CSCs in solid tumors. These include CD133, a human hematopoietic progenitor cell marker, which is highly expressed in CSCs of lung [18], colon [19], brain [20], liver [21], ovarian [22], and breast [23] cancers. Moreover, certain adhesion molecules can reliably enrich CSCs within a mixed population of tumor cells. The cell surface glycoprotein CD44 is a well-characterized CSC marker used to isolate CSCs in tumors of breast [5], colon [24], prostate [25], pancreas [26], ovary [27], lung [28], and stomach [29]. Likewise, CD49f (integrin α6) and CD166 were reported to be reliable surface markers for CSC isolation [30, 31]. Other surface markers that were used to isolate CSCs include CD24 [32], CD117 [33], CD138 [34], EpCAM [24], CXCR4 [35], CD66c [36], and CA125 [37].

In order to isolate CSCs from a heterogenous population of tumor cells, two methods are commonly used: fluorescence-activated cell sorting (FACS) and antibodies conjugated to magnetic beads (MACS). The purity of isolation is typically higher with FACS but the survival of the cells is higher with the magnetic beads separation methods [1].

1.3 Reporter Systems to Identify CSCs

Despite the common use of cell surface markers to enrich CSCs in various tumors, this approach has limitations due to the inter-patient heterogeneity and fluctuation of the expression of these markers at different points of cell cycle. The CSC state is dynamic with rapid transitions between CSC to non-CSC states. As sorting/isolation approaches are limited in their ability to track changes in the stem cell state in real time, efforts have been made towards developing reporter systems that can actively monitor certain intracellular markers [38].

The pluripotent stem cell transcription factors NANOG, SOX2, OCT4 are highly expressed in CSCs and have been used to develop reporter systems based on their promoters. The utility of promoter reporter systems allows interrogation of the CSC state in real time [39–41]. Certain CSC-related signaling pathways have also been explored to generate reporters, of which Notch pathway is particularly useful in breast cancer models [42]. Moreover, telomerase reverse-transcriptase (TERT) promoter-driven green fluorescence protein (GFP) reporter was successfully utilized to enrich human osteosarcoma stem cells [38]. The major reporter systems used to isolate CSCs are summarized in Table 1, together with the types of tumors that they have been useful.

Table 1
Reporter systems validated for CSC enrichment

Reporter gene	Gene function	Cancers in which the reporter has been validated
NANOG	Transcription factor, expressed in embryonic stem cells maintaining pluripotency, oncogene	Breast [41], prostate [40], nasopharynx [51], liver [43], ovary [52]
SOX2	Transcription factor, expressed in embryonic stem cells maintaining pluripotency, oncogene	Breast [54], glioma [56], skin [53], cervix [55]
OCT4	Transcription factor, expressed in embryonic stem cells maintaining pluripotency, oncogene	Liver [58], melanoma [57], sarcoma [59]
SOX2-OCT4	Transcription factors, expressed in embryonic stem cells maintaining pluripotency, oncogene	Breast [39]
NOTCH	Maintenance of stem cell state by inhibiting differentiation, oncogene	Breast [42], lung [64]
Telomerase reverse transcriptase (TERT)	Catalytic subunit of the enzyme, telomerase	Osteosarcoma [38]
stem-SH2-domain-containing 5′-inositol phosphatase (s-SHIP)	Regulation of growth factor receptor mediated signaling	Prostate [66]
LGR5	Wnt signaling pathway	Colon [68]
Alpha-fetoprotein (AFP)	Fetal plasma protein	Cholangiocarcinoma [67]

In this chapter, various reporter systems used to enrich CSCs from a heterogenous population of tumor cells are discussed with a special focus on NANOG-GFP reporter system that we have successfully employed to study CSCs in breast and ovarian cancers.

2 Materials

1. PureLink HiPure Plasmid DNA Purification Kit for Maxiprep
2. HEK293T/17 cells at low passage number (ideally <10) (**Note 1**)
3. 2× BBS solution
   (a) For 250 mL 2×BBS solution, prepare:

2.665 g	BES (Sigma, B6266)
4.091 g	NaCl (Chemika Fluka, 71380)
100.52661 mg	$NA_2HPO_4 \times 7H_2O$

Add distilled water up to 250 mL. Dissolve and titrate to pH 6.95 with 1 M NaOH. Efficiency of transfection is highly dependent on pH. Filter, sterilize, and store 50 mL aliquots at 4 °C

4. 0.25 M $CaCl_2$ solution
   (a) For 250 mL 0.25 M $CaCl_2$ solution, prepare:

9.8175 g	$CaCl_2$ (Sigma C 7902)

Add distilled water up to 250 mL. Dissolve, filter, sterilize, and store 50 mL aliquots at 4 °C (**Note 2**)

5. DNA packaging vectors
   (a) For two vectors system, prepare:

pMD2G	4 μg
psPAX	7 μg

These vectors are compatible with non-inducible pCDH, pLKO, pGIPZ vectors (**Note 3**)

6. PEG-it virus precipitation solution (System Biosciences, LV810A-1)
7. Polybrene (**Note 4**)
8. Puromycin

3 Methods

3.1 NANOG Promoter-Driven GFP Reporter

3.1.1 NANOG Is a Pluripotency Gene Involved in Oncogenesis

NANOG is a transcription factor expressed in embryonic stem cells, and together with SOX-2 and OCT-4, the so called "core triad," maintains the pluripotent state [43]. Several studies have demonstrated that NANOG mRNA and protein are expressed in various cancers and its expression is positively correlated with poor clinical outcome [43, 44]. Therefore, it has been suggested as a prognostic biomarker. Based on a search of the cBioPortal database, the NANOG gene is frequently amplified in cancers of breast (17.2 %), peripheral nerve sheath (13.3 %), ovary (8.4 %), lung (6.7 %), and brain (4.9 %) (Fig. 1) [45]. NANOG expression also correlates with poor differentiation and vascular invasion in primary tumor samples [43].

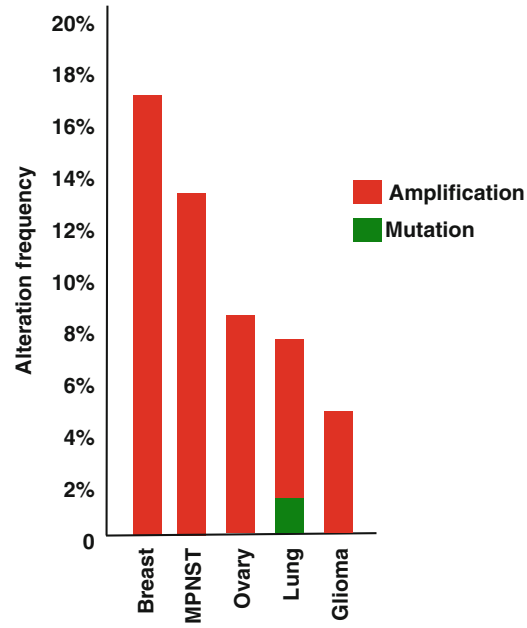

Fig. 1 NANOG alterations in various cancers

In support of these observations, embryonic stem cell-like gene expression signatures were found to be enriched in most aggressive tumors [46]. Myeloid leukemia stem cells have been shown to employ a transcriptional program similar to embryonic rather than adult stem cells [47]. Moreover, increased levels of NANOG protein has been demonstrated in CSCs of various tumor types, which were isolated by differential expression of surface markers [28, 48, 49]. Consistent with these association studies, NANOG knockdown is sufficient to decrease tumorigenicity and clonogenic growth of several breast, prostate, and colon cancer cell lines [50]. Whereas overexpression of NANOG is sufficient to induce self-renewal, tumor initiation, and drug resistance in prostate and breast cancer cell lines [40]. This change in cell behavior is also accompanied by a more stem-like phenotype with increased expression of CD133, CD44, and ABCG2 [40].

These findings indicate that NANOG is expressed in CSCs and confers pluripotency similar to observed effects in embryonic stem cells. Moreover, NANOG expression promotes malignant transformation and tumor progression.

3.1.2 NANOG-GFP Reporter Enriches CSCs

Originating from the abundant expression and importance of NANOG in cancer, a novel reporter system was developed using the NANOG promoter to control green fluorescent protein expression to track CSCs in established cancer lines [40, 41, 51]. Under this paradigm, cells expressing bright green signal by green fluorescence imaging or flow cytometry are presumed as putative CSCs,

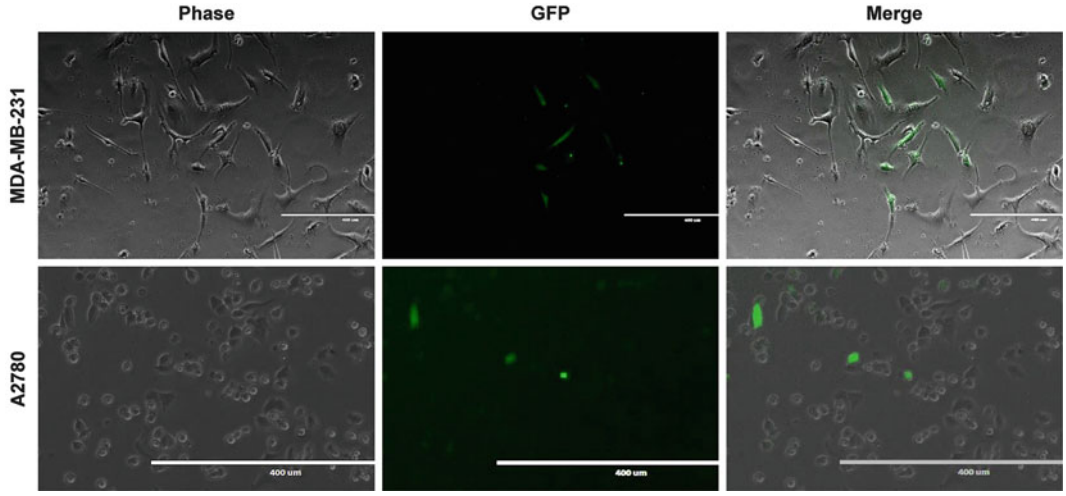

Fig. 2 Transduction of breast and ovarian cancer cells with NANOG promoter-driven GFP reporter

whereas cells expressing dim green fluorescence are considered nonCSCs.

To generate stable cell lines harboring NANOG-GFP reporter, MDA-MB-231 and HCC70 breast cancer cells [41], and A2780/CP70 ovarian cancer cells (data not published) were transduced with recombinant lentiviruses and monitored for green fluorescence (Fig. 2). Lentivirus production was generated using established methods.

1. Genetically engineered plasmid containing reporter gene (i.e., GFP) under the control of human NANOG promoter, and puromycin resistance gene under the control of EF-1α promoter were purified from *E. coli*.
2. Low-passage HEK293T/17 cells were transfected with this plasmid and the lentivirus packaging plasmids (psPAX2 and pMD2.G). HEK293T/17 cell line, which is a derivative of 293T cell line, is known for its high transfectability.
3. After transfection, cells should be incubated at 3 % CO_2, 37 °C overnight (16–20 h). Incubation at 3 % CO_2 is absolutely required as $CaPO_4$ precipitation (and therefore transfection efficacy) is highly dependent on media pH.
4. Then, media were removed and cells were incubated with 6 mL PBS (37 °C) per dish for 10 min. After that, 10 mL medium was added and cells are incubated at 5 % CO_2 incubator.
5. On the following day, cells were reaching confluency. More than half of the cells were expressing GFP.
6. Cell medium from HEK293T/17 was collected and centrifuged at 3000 × *g* for 15 min to remove debris. Then, supernatant was transferred to a sterile vessel and recombinant

lentivirus was precipitated from culture medium by using PEG-it virus precipitation solution (**Note 5**).

7. Supernatant/PEG-it mixture was centrifuged at 1500 × *g* for 30 min at 4 °C. After this centrifugation, lentiviral particles appeared as a beige pellet at the bottom of the vessel. Supernatant was aspirated off and lentiviral pellet was resuspended in 400 μL cold and sterile 1× PBS.
8. Breast and ovarian cancer cells were infected with the virus and 5 μg/mL Polybrene was used to enhance viral entry into cells.
9. After 2 days, transduction efficacy was >80 % and cultures were selected with puromycin in 4 μg/mL concentration for 3 days.
10. Transduced cell cultures were flow sorted and GFP+ cells were enriched. Upon 1–2 weeks of culture, GFP+ cells repopulated the culture heterogeneity and stably transduced cancer cell lines were subsequently expanded up to 20 passages.

Once the GFP reporter construct is incorporated, cancer cells can readily be sorted to obtain GFP+ CSCs and GFP− non-CSCs. Ideally, the histograms should demonstrate an even distribution or two peaks of GFP− and GFP+ ends. In the latter, the difference is usually clear and isolation of GFP+ and GFP− is simple. However, in case of a Gaussian distribution, GFP− population can be discriminated by using non-transduced parental cell line as negative control. For example, assuming that 30 % of all events have GFP signal overlapping with parental cells and hence sorted as GFP− cells, one can take the upper 30 % with bright GFP signal and designate as GFP+ population. It is good practice to eliminate cells with intermediate GFP signal. However, the optimal way to determine a cutoff percentage point is to divide the histogram into ten columns from the dimmest to brightest end with each having 10 % of cells, and sort these cells for in vitro self-renewal assay. This critical percentage point should demonstrate a significant difference in self-renewal between GFP high vs. low populations. Further validation requires demonstration of a difference in expression of pluripotency markers, including NANOG, SOX2, and OCT4.

In our experience, GFP+ cells isolated from both triple negative breast cancer (TNBC) [41] and ovarian cancer cell lines (unpublished data) had higher expression of NANOG, SOX2, and OCT4 as well as GFP as compared to their GFP− counterparts. Moreover, we compared the power of NANOG-GFP reporter system with other markers used to enrich CSCs. The reporter was more efficient than CD44, CD49f, and ALDH in isolation of self-renewing population in TNBC [41].

3.1.3 NANOG-GFP Reporter Is a Dynamic System

The novel NANOG-GFP reporter system provides a facile method to isolate CSCs and eliminates the need for antibodies, thus is free of antibody specific-related biases. At the same time, it is also a

functional imaging approach for CSC identification and enables visual tracking of putative CSCs within a population of cultured cancer cells. This system has a range of applications, including but not limited to migration and invasion assays, time lapse imaging to study CSC biology, and monitoring changes in CSC state upon treatment with cytokines, drugs, or inhibitors. We have also taken advantage of NANOG-GFP reporter system to identify CSC-specific molecular pathways by using high-throughput flow cytometry screen in TNBC [41]. This enabled us to discover a novel CSC marker, junctional adhesion molecule (JAM)-A, in TNBC [41]. Similarly, we utilized the reporter system to study the differential effects of cisplatin and certain targeted therapies on CSCs compared to non-CSCs with visual tracking of changes via time-lapse imaging (unpublished data). Therefore, the NANOG-GFP reporter system is a dynamic tool that enables interrogation of CSC state in real time.

3.2 Other Reporter Systems

3.2.1 SOX2

Sex determining region Y box 2 (SOX2) is a transcription factor and expressed in various cancers including gastric cancer, small cell lung cancer, melanoma, esophageal squamous cell carcinoma, and breast cancer [53, 54]. SOX2 is known to be an essential factor for inducing and maintaining stemness, namely pluripotency and self-renewal [54]. SOX2 promoter-driven reporter system has first been used in breast cancer [54]. Liang et al. demonstrated that cells with high SOX2 promoter activity had higher expression of stemness-related genes including CD44, ABCB1, NANOG and TWIST1 [54]. Subsequently, the efficacy of this reporter in enriching CSCs has also been shown in cervical cancer [55], glioma [56], and squamous cell carcinoma (SCC) of skin [53]. In a mouse model of glioblastoma, Stoltz, et al. showed that Sox2-EGFP reporter successfully isolated CSCs, which were phenotypically similar to patient-derived tumor initiating cells with respect to modes of cell division, self-renewal, niche location, and differentiation [56].

Of interest, SOX2 has been shown to be upregulated in pre-neoplastic skin tumors and it is much highly expressed in human SCC. Using a SOX2-GFP knock-in mice, Boumahdi, et al. demonstrated that SOX2-high SCC cells were greatly enriched in tumor-propagating cells, and they elucidated the role of SOX2 in initiation and progression of primary skin tumors [53].

Taken together, SOX2 promoter-driven reporter constructs have been shown to enrich CSCs in different solid tumors and utilized as a powerful tool to track CSCs real time with both in vitro and in vivo models.

3.2.2 OCT4

Similar to NANOG and SOX2, OCT4 is a master regulator of pluripotency and is upregulated in human solid tumors [57]. OCT4-promoter activity-dependent CSC enrichment strategy has been validated in melanoma [57], hepatocellular carcinoma [58],

and soft tissue sarcomas [59]. This reporter system was used in two studies to investigate the preclinical activity of patient-derived cytokine-induced killer (CIK) cells against CSCs and non-CSCs of melanoma and soft tissue sarcoma cells [57, 59]. In hepatocellular carcinoma, OCT4+ cells demonstrated classical CSC features including higher expression of stemness associated transcription factors, in vitro sphere formation, tumor initiation in immunocompromised mice, and resistance to sorafenib [58]. Therefore, OCT4 promoter-driven reporter system is a promising novel way to isolate CSCs in solid tumors.

3.2.3 SORE6

SORE6 is a novel reporter system generated by Tang, et al. which is a flexible CSC reporter system in which six tandem repeats of a composite OCT4/SOX2 response element were used to drive expression of a fluorescent protein [39]. This reporter is special as it responds to two core stem cell transcription factors. Breast cancer stem cells enriched by SORE6 reporter exhibited higher self-renewal, tumor initiating capability, and resistance to chemotherapeutics [39]. Tumor cell subpopulation selected by SORE6 reporter could generate a heterogeneous offspring, which is a cardinal characteristic of CSCs. Utility of this reporter in other solid tumors is under investigation.

3.2.4 NOTCH

NOTCH signaling pathway is involved in the development of many multicellular organisms. In humans and other mammals, four distinct receptors (NOTCH 1–4) are involved in a downstream proteolysis pathway that eventually releases the Notch Intracellular Domain (NICD) protein, which enhances gene transcription [60]. In addition to its critical role in embryogenesis and fate decision of various cellular systems, NOTCH pathway has also been implicated in a wide variety of malignancies [61]. For example, overexpression of NOTCH has been shown in non-small-cell lung cancer (NSCLC) and blockade of this signaling pathway had direct effects on tumor growth, apoptosis, and cellular proliferation [62, 63].

NOTCH-GFP reporter construct has been used to enrich for self-renewing cell population in non-small-cell lung cancer [64]. NOTCH 2 and 4 receptor expressions were higher in the GFP-bright population, while the GFP-dim population showed higher expression of NOTCH-1 and NOTCH-3 receptors. GFP-bright cells had higher tumor initiation capacity as assessed by serial in vivo xenotransplantation assays in NOD/SCID mice. Cells with high NOTCH activity were more resistant to chemotherapy and NOTCH activity correlated with poor clinical outcome. NOTCH reporter system has also been used in breast cancer cell lines and successfully enriched CSCs with higher tumor initiating capacity [42].

3.2.5 TERT

Telomerase is known to be essential in maintaining cancer stem/progenitor cells. Human telomerase reverse transcriptase (hTERT) is the catalytic subunit of the telomerase enzyme and hTERT reporter has been used to enrich CSCs in osteosarcoma [38]. It was found that a subpopulation of osteosarcoma cells expressed higher levels of telomerase, which was read out by bright GFP expression linked to the hTERT promoter. Telomerase-positive cells had enhanced ability to form sarcospheres and demonstrated higher levels of epithelial–mesenchymal transition marker expressions.

3.2.6 s-SHIP

Stem-SH2-domain-containing 5′ inositol phosphatase (s-SHIP) expression has been previously shown to be linked to stem/progenitor cell activity [65]. In the context of cancer stem cells, the role of s-SHIP has been studied in human prostate cancer cell lines [66]. An s-SHIP promoter linked GFP reporter has been used to isolate a stem-cell like subpopulation of cells in the RWPE-1 cell line. Cells with high s-SHIP activity demonstrated the ability of anchorage-independent growth (i.e., forming spheres) and had higher expression of pluripotency transcription factors (OCT4, SOX2) and stem cell surface markers (TROP2, CD44, CD166).

3.2.7 AFP

Alpha-fetoprotein is a well-known marker for hepatic progenitor cells, but it has also been studied as a marker for CSCs in cholangiocarcinoma [67]. Four cholangiocarcinoma cell lines were transfected with AFP enhancer/promoter-driven EGFP gene and one out of four cell lines showed a population of cells expressing both AFP and EGFP. EGFP-positive cells had higher tumorigenic potential and Notch1 expression, which supported the role of Notch pathway in maintaining CSC characteristics.

3.3 Caveats of Using Reporters

The reporter systems are extremely useful tools to isolate CSCs and in the design of continuous functional experiments to monitor the CSC state in real time. However, there are some potential limitations. First, a limitation of the process is efficacy in transduction that may differ between different cell lines. Second, tumor heterogeneity might not be repopulated after initial enrichment of GFP high cells. Third, long promoter sequences might contain binding sites for various different transcription factors, which may adversely affect the specificity of reporter. Therefore, meticulous validation of sorted cells for differential self-renewal capability (both in vitro and in vivo) and expression of CSC markers (i.e., “core triad” of pluripotency markers) is imperative. Fourth, certain cells with aggressive phenotypes (e.g., chemotherapy resistant cell lines) might not be efficiently enriched for CSCs based on reporter systems. For example, although the NANOG-GFP reporter was very useful in isolating CSCs in cisplatin-naïve A2780 ovarian cancer cell line, it could not enrich CSCs in its isogenic cisplatin-resistant derivative,

CP70 (unpublished data). We later found out that CP70 cells had higher baseline expression of CSC markers and self-renewing capability than their cisplatin-naïve counterparts. In addition, GFP is a stable molecule and its half life may exceed 24 h. Therefore, there might be a time period between the onset of loss of CSC gene expression, and visualization of this by reporter read-outs. Finally, experiments incorporating fluorescent dyes should be designed carefully in order to prevent cross talk between GFP signal and other fluorophores. This is a common problem while comparing GFP+ and GFP− cells with commercially available assays (e.g., annexin V-FITC staining, BCECF-based assays).

3.4 Conclusions

The cancer stem cell model proposes that tumors contain a heterogeneous population of cells, which are hierarchically organized [1]. CSCs are at the highest level of this organization, and are important for tumor formation, maintenance, invasion, metastasis, and drug resistance [8]. Therefore, isolation of CSCs is critically important to discover drivers of tumorigenic properties and target them effectively. Reporter systems depend on CSC-specific pathways and transcriptomes, enable segregation of CSCs easily, and are dynamic tools that can track CSCs with functional imaging approaches. In our experience, they isolated CSCs with higher fidelity compared to conventional methods (i.e., surface markers, ALDH enzyme activity assays) and were utilized in screening approaches to identify CSC-specific markers as promising future therapeutic targets [41, 56].

4 Notes

1. Split HEK293T/17 cells to the 10 cm dishes, swirl the cells thoroughly as you seed them in order to obtain even distribution across the surface of the dish. Incubate at 37 °C overnight. Cells should preferably be of low passage number and their use is not recommended after passage 20 or if growth is slow. The cells should be 80–90 % confluent at the time of transfection.
2. For each 10 cm dish, prepare 2 eppi tubes with 0.5 mL 0.25 M $CaCl_2$ solution and 0.5 mL 2×BBS solution.
3. An alternative might be 3 vectors system (pPACKH1 Lentiviral packaging kit, System Biosciences) which is compatible with inducible pLVX vectors (Clontech).

pMDL/GAG	5.6 μg
VSU-G	3 μg
REV	2.13 μg

The amount of expression vector DNA to add in this setting is 9.27 μg.

4. Polybrene increases the efficacy of transfection by changing surface potential.
5. Lentivector-containing supernatants mixed with PEG-it virus precipitation solution are stable for up to 2 days at 4 °C

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