*Cancer Heterogeneity and Plasticity*
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Perspective

# Developing a novel therapeutic strategy against cancer stem cell heterogeneity and plasticity

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**Abstract**

Cancer consists of heterogeneous cells, including cancer stem cells (CSCs), cancer cells, and tumor-associated cells, such as immune cells and vascular cells. Considering that these diverse cell types influence one another directly and indirectly through membrane proteins and secretion factors, such as exosomes and growth factors, the overall heterogeneity affects tumorigenicity and resistance to therapy. This review explores cancer heterogeneity, focusing on CSC heterogeneity, and discussed how the heterogeneity emerges by the intrinsic mechanism and the external factors and affects response to therapy. Additionally, as a potential therapeutic strategy to address this heterogeneity, I propose new Adeno-associated virus carrying a miRNA-dependent CSC eradication system that targets all types of CSCs with minimizing side effects.

**Keywords:** Glioblasoma (GBM); GBM-initiating cells (GICs); heterogeneity; stem cell factors; microRNA (miR); Adeno-Associated virus (AAV)

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## 1. Introduction

Efforts to develop effective cancer treatments have revealed numerous aspects of the disease, including oncogenes, tumor suppressor genes, signaling pathways, CSCs, secretory factors (such as exosomes), and immune checkpoints [1]. Despite these advancements, successful cancer treatment remains challenging. One reason is that the complete cellular communication network involved in tumorigenesis has not been fully understood. By utilizing advanced technologies like single-cell RNA sequencing (scRNAseq analysis) and spatial transcriptomic analysis of tumors, it has become apparent that cancer consists of heterogeneous

cell populations, including CSCs, cancer cells, and neighboring cells such as immune cells, blood vessels, and tissue-specific resident cells [2,3].

Furthermore, a tumor is likely to contain multiple types of CSCs that are generated during the process of tumorigenesis and by the reversion of cancer cells to CSCs induced by the microenvironment factors, such as Transforming growth factor β1 (TGFβ1) [4–6]. This indicates that each CSC in a certain cancer may have a different molecular profile. While CSCs generally possess strong tumorigenic abilities and give rise to various types of cancer cells, each individual CSC may exhibit different resistance to radiation and anticancer drugs, contributing to multidrug resistance in the tumor and likelihood of recurrence [7–11]. Therefore, gaining a comprehensive understanding of the mechanisms underlying CSC heterogeneity and plasticity throughout the entire cancer is crucial for the development of effective therapies.

Many researchers have extensively studied and characterized cancer stem cells (CSCs) and have identified potential therapeutic targets (Table 1). However, it is important to note that these targets also play a crucial role in maintaining the normal function of tissues and stem cells. For instance, CD133, a well-known marker for CSCs, is expressed in various normal adult stem cell types and is essential for the survival of photoreceptor cells [12–17]. Another membrane protein, CD44, found in CSCs, serves as a homing factor for immune cells and a cell-adhesion protein in keratinocytes [18,19]. Additionally, many other membrane proteins, such as CD24 [20,21], CD49f [21–23], CD15 [24,25] and LGR5 [26,27], are expressed in various types of CSC, while ABC transporters [28,29] and Aldehyde Dehydrogenase (ALDH) [30,31] act as detoxification factors in CSC as well as normal adult stem cells. Dihydroorotate Dehydrogenase (DHODH), a key factor in pyrimidine synthesis, is considered a potential therapeutic target for CSCs [32–38]. However, it is important to note that DHODH is also crucial for the proliferation of blast cells, which protect the body against infectious diseases and cancer [39]. Therefore, there is a concern about potential side effects if these CSC factors are targeted directly without a specific delivery method for CSCs.

Emerging evidence suggests that cancer cells acquire CSC status in the specific environment, such as the hypoxia [40]. Additionally, studies have shown that oligodendrocyte precursor cells (OPCs) and astrocytes (ASTs) can acquire multi-potency similar to neural stem cells (NSCs) under specific culture conditions [41,42]. Understanding which cancer cells revert to CSCs and uncovering the mechanisms of plasticity are crucial steps in developing novel therapeutic strategies.

This review discusses how cancer heterogeneity is generated and how it contributes to therapy resistance. Furthermore, we will explore potential methods for eradicating CSCs beyond the scope of CSC heterogeneity.

**Table 1** CSC markers.

| Typical CSC marker | Cancer type | Enrichment method | Function | References |
|---|---|---|---|---|
| CD133 (Prominin 1, Prom1) | Multiple cancer, including Glioblastoma, Colorectal cancer | Flow cytometry | Survival and proliferation | [12-17] |
| CD44 | Multiple cancer, including Breast cancer, Head and neck cancer, Glioblastoma | Flow cytometry | Cell-cell interaction, Activation of PI3K-4EBP1-SOX2 pathway by binding with hyaluronic acid | [18,19] |
| CD24 | Multiple cancer, including Breast cancer, Colorectal cancer | Flow cytometry | B-cell activation and proliferation, ERK1/2activation | [20,21] |
| CD49f (Integrin subunit alpha 6, ITGA6) | Multiple cancer, including Breast cancer, Glioblastoma | Flow cytometry | Multiple functions, including laminin receptor, sperm-egg fusion and Insulin growth factor signalling | [21−23] |
| CD15 (Lewis x, Fucosyltransferase4 (FUT4), Stage-Specific Embryonic Antigen 1 (SSEA1) | Glioblastoma, Medulloblastoma | Flow cytometry | Cell-cell interaction, adhesion | [24,25] |
| LGR5 (Leucine Rich Repeat Containing G Protein-Coupled Receptor 5, G-Protein Coupled Receptor 49 (GPR49) | Multiple cancer, including Colorectal cancer, Gastric cancer, Liver cancer | Flow cytometry | Wnt signal | [26,27] |
| ABC transporters (e.g. ABCG2) | Multiple cancer, including Acute myeloid leukemia, Breast cancer, Glioblastoma, Colorectal cancer, Pancreatic cancer, *etc.* | Flow cytometry | Exclusion of chemicals and dyes, such asHoechst33342 and cytotoxic drugs | [28,29] |
| Aldehyde Dehydrogenase (ALDH) | Multiple cancer, including Acute myeloid leukemia, Breast cancer, Glioblastoma, Colorectal cancer, Pancreatic cancer, *etc.* | Flow cytometry | Detoxification | [30,31] |

## 2. Cancer heterogeneity

Analysis of specimens using scRNAseq revealed that cancer consists of heterogeneous cells [43–45]. In the case of glioblastoma (GBM), for example, GBM stem-like cells (GSCs) commonly express central stemness factors, such as Oct4, Sox2, and Myc. The combined expression of tumor-related factors, including Nuclear factor-κB (NF-κB), RAC-Alpha Serine/Threonine-Protein Kinase (AKT), Hypoxia inducible factor (HIF), TGFβ, Signal Transducer and Activator of Transcription 3 (STAT3), Hedgehog, mechanistic Target of Rapamycin (mTOR), Wingless-Type MMTV Integration Site Family (Wnt), p53, Rat Sarcoma Viral Oncogene Homolog (Ras), and Notch, as well as cell lineage-specific genes (such as CD24 for neuronal precursor cells (NPCs), S100β for AST, Platelet-derived growth factor receptor α (PDGFRα) for oligodendrocyte precursor cells (OPCs), and CD44 for mesenchymal cells, MES), reveals the heterogeneity of GSC [46]. These heterogeneous GSCs communicate with one another and the tumor microenvironment through membrane proteins and secretion factors to promote maintenance, proliferation, and the production of cancer cell (Figure 1). Furthermore, heterogeneous GSCs in GBM were found to have amplifications in the copy number of Cyclin-dependent kinase 4 (CDK4), Epidermal growth factor receptor (EGFR), and PDGFRα loci as well as mutations in the Neurofibromin 1 (NF1) locus [43]. This demonstrates that tumors are more complex and heterogeneous than initially believed.

Abdelfattah *et al.* demonstrated that GBM consists of approximately 40% cancer cells, 45% myeloid cells, and 10% T cells, based on scRNAseq analysis of over 200,000 cells from 44 specimens [47]. The molecular characteristics of GBM further confirmed that each tumor contained a mixture of GSCs with NPC-like, OPC-like, AST-like, and MES-like features at different ratios. T cells were found to consist of eight clusters, including three CD8+ clusters, two CD4+ clusters, one naive T cell cluster, and two NK cell clusters. One of the CD4 clusters expressed regulatory T cell markers like FOXP3 and CTLA4, while expression of PD1 was low in NK and T cells across all samples, indicating an immunosuppressive environment in GBM [48]. Additionally, myeloid cells, the largest stromal compartment in GBM, comprised nine clusters, which did not correlate with the *in vitro*-defined macrophage states (M0, M1, or M2), but rather included both anti-tumorigenic and immunosuppressive macrophages. The study also found that microglia clusters had an anti-tumorigenic effect, whereas macrophage and MES-derived stem cell clusters were pro-tumorigenic.

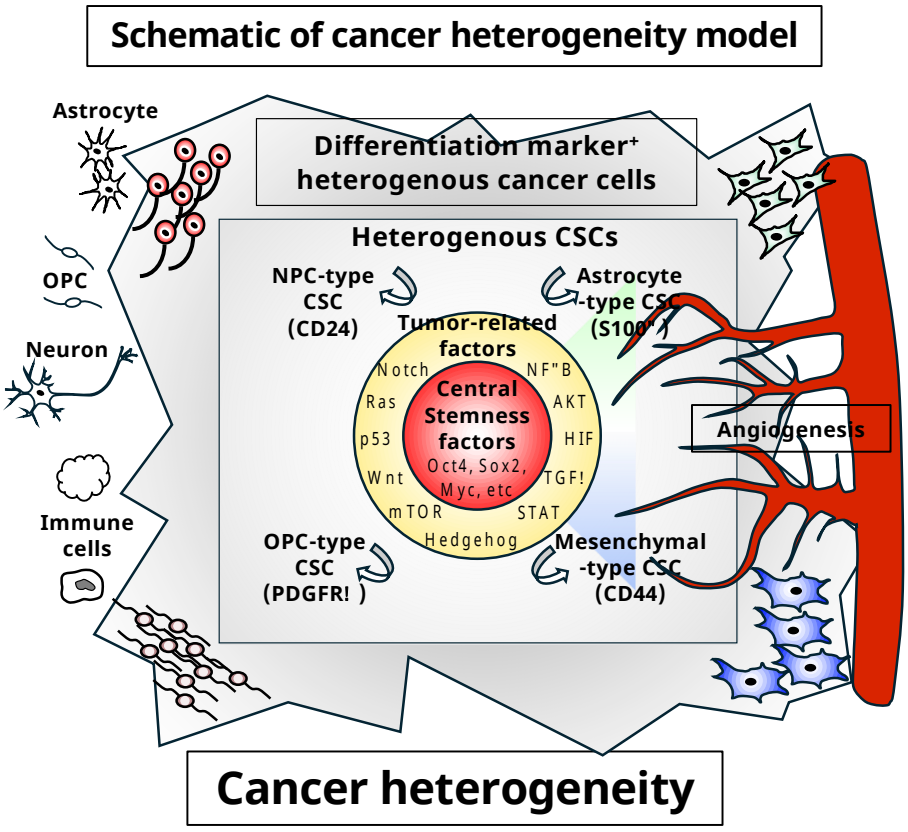


**Figure 1** Schematic of cancer heterogeneity model. Glioblastoma is composed of various types of cells, including Cancer stem cells (CSCs), cancer cells, and their surrounding cells. These surrounding cells consist of immune cells (macrophages, Natural Killer cells, T and B cells), cells that make up the Blood Brain Barrier (pericytes and endothelial cells), and differentiated neural cells (neurons, astrocytes (ASTs), and oligodendrocytes), as well as their precursor cells. Communication among these heterogeneous cells is facilitated through membrane proteins, such as Notch and Integrin, as well as secretion factors, such as exosomes and growth factors. Additionally, cancer is likely to contain heterogeneous CSCs that express central stemness genes, including Oct4, Sox2, and Myc, tumor-related genes, including Nuclear factor-κB (NF-κB), Transforming growth factor β (TGFβ), Signal transducer and activator of transcription 3 (STAT3), p53, Ras, and Notch, as well as lineage markers, such as CD24 for Neuronal precursor cell (NPC)-type, S100β for ASTs, Platelet-derived growth factor receptor α (PDGFRα) for Oligodendrocyte precursor cells (OPCs), and CD44 for mesenchymal (MES) cells. The combination of these factors further contributes to the heterogeneity of the cancer.

Cytometry using Time-of-Flight (CyTOF) mass cytometry is another technology used for analyzing tumor heterogeneity. Wagner *et al*. prepared a single-cell atlas for tumors and immune cells in breast cancer using mass cytometry [49]. They discovered phenotypic abnormalities and phenotype dominance in breast cancer cells.

Through immunohistochemical analysis, they also observed a relationship between PD-L1+ tumor-associated macrophages and exhausted T cells in high-grade tumors, revealing characteristics of ecosystems related to immunosuppression and poor prognosis. One limitation of mass cytometry analysis is the use of established antibodies (up to 40) in one experiment. Additionally, this technique cannot determine which cells are bona fide CSCs, as the membrane proteins specific to pan-CSCs have not yet been identified.

Spatial transcriptomics analysis is a latest technology. Yu *et al.* combined scRNAseq with multi-sector biopsies of gliomas to generate a geographical gene-expression dataset of the tumor, revealing the cellular status at the single cell level in the tumor and the surrounding microenvironment [50]. They found that each GBM contains four GSC subtypes: NPC-type, OPC-type, AST-type and MES-type, at different ratio. They also discovered that high interaction between OPC-type and MES-type, particularly in the hypoxia subtype, correlated with worse prognosis for the patients. Furthermore, Ravi *et al*. combined spatial transcriptomics with metabolomics and proteomics and showed that GBM is organized by spatial segregation of lineage states based on the locoregional tumor-host interdependence and the selection pressure of environmental stress [51].

Considering that these heterogeneous cells communicate with each other directly through cell membrane proteins and indirectly through secretion factors to maintain cancer cell homeostasis, this complex environment poses challenges for the development of effective therapeutics.

## 3. CSC as an origin of intratumor heterogeneity

How is cancer heterogeneity generated? At the beginning of tumorigenesis, an initial genetic or epigenetic mutation occurs in the original cancer cell (Figure 2). If tissue specific stem cell (ts-SC) is the cell-of-origin of cancer, then the ts-SC with a genetic or epigenetic mutation (pre-CSC) acts as a seed of intratumor heterogeneity and can produce multiple pre-cancer precursor cell (pre-CPC) types. During proliferation, pre-CSCs may accumulate further random mutations over time, eventually transforming into various CSCs with different characteristics. This is one of the processes that result in the expansion of intratumor heterogeneity. Conversely, pre-CPCs are thought to either transform into a lineage-committed CPC, which forms a low-grade tumor, or become CSCs by acquiring both stemness and tumorigenic properties after accumulating additional hits [52,53]. These processes also increase intratumor heterogeneity.

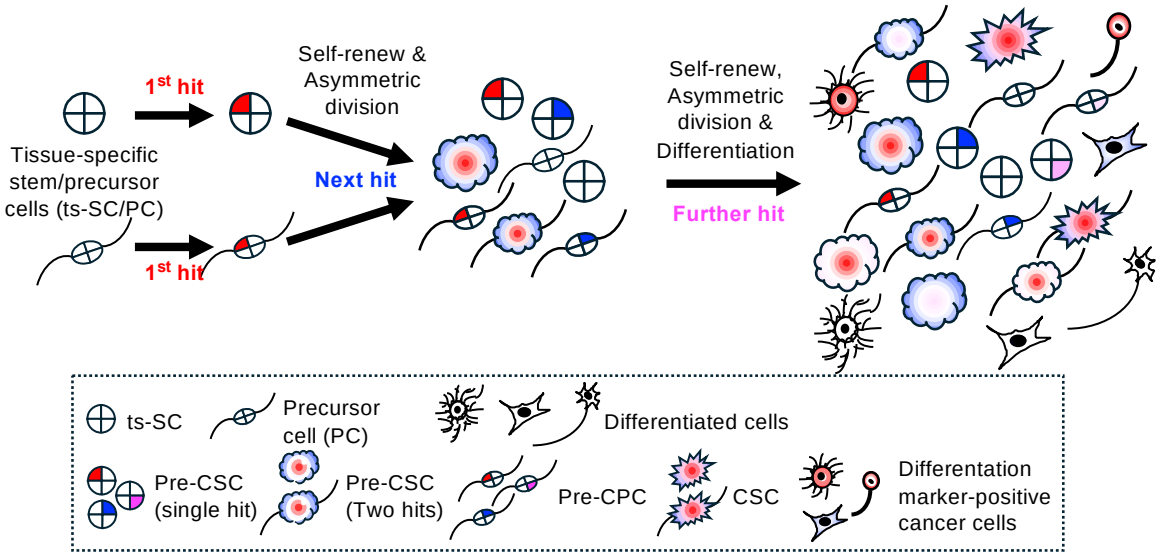


**Figure 2** CSC Heterogeneity is likely generated by the cell-of-origin, the combination of gene mutations, and the reversion to stem cell. The first "hit" (represented by red) occurs in the cell of origin of CSC, such as tissue-specific stem/ precursor cells (ts-SC/PC). During self-renew and asymmetric division, mutant cells not only differentiate into functional cells but also acquire next hit (represented by blue), resulting in the generation of various types of cells, including ts-SC, pre-CSC with either single or two "hits", precursor cell (PC) and pre-cancer PC (pre-CPC). Eventually, this process leads to the formation of CSCs. It is important to note that pre-CPCs acquire stemness when transform into CSCs.

Acquiring stemness associated with transformation can be reproduced in culture. For example, our study demonstrated that when p53 knockout (p53KO) OPCs overexpressed oncogenic H-RasL61 (where glycine at codon 61 is replaced with leucine), they acquired both tumorigenicity and stemness [53]. These H-RasL61 overexpressing p53KO OPCs (OPCL61) lost their original characteristics, such as the expression of Olig2 and Nkx2.2, but began to express neural stem cell (NSC) markers, including High mobility group AT-Hook 2 (Hmga2) and Prom1. Moreover, OPCL61 formed a GBM-like brain tumor with features such as necrosis, hemorrhaging, and angiogenesis, even when just ten cells were injected into the brains of nude mice. These results indicate that pre-CPC, such as p53KO OPC, serve as a seed for CSC. Interestingly, it has been observed that p53 knockdown fibroblasts readily acquire pluripotency when Yamanaka factors are overexpressed [54–56], suggesting a significant connection between stemness and transformation.

Hide *et al.* isolated tumorigenic single CSC clones from H-RasL61-overexpressing p53KO NSC (NSCL61). These clones, when transplanted into the brains of nude mice, formed GBM-like brain tumors. These single CSCs were capable of generating both CSCs and differentiation marker-positive cells expressing either the neuronal marker microtubule-associated protein or the AST marker glial fibrillary acidic protein, both in culture and *in vivo*. This finding suggests that each CSC

has the potential to generate heterogenous cancer cells within the tumor [57]. Additionally, the same group discovered that NSCL61 and OPCL61 exhibited different drug resistance capacities. While the combination of gefitinib and celecoxib inhibited tumorigenesis of OPCL61, it had no effect on NSCL61 [53]. This highlights that the difference of cell-of-origin of CSC is one of the factors contributing CSC heterogeneity and multiple drug resistance.

CSC heterogeneity has also been observed in mouse models, such as p53KO mice, which initially develop but eventually suffer from various types of cancer. Zhang *et al.* showed that breast cancer, which formed in p53KO mice, consisted of four different tumorigenic populations, characterized as $Lineage^{-}(Lin^{-})CD29^{high}(CD29^{H})CD24^{H}$, $Lin^{-}CD29^{H}CD24^{Low}(CD24^{L})$, $Lin^{-}CD29^{L}CD24^{H}$, and $Lin^{-}CD29^{L}CD24^{L}$. This suggests that pre-CSC and/or pre-CPC transform into different types of CSCs over time [20]. Due to the presence of multiple CSC types with distinct mutations, these cancers are likely resistant to various therapies.

Additionally, single-cell RNA sequencing (scRNAseq) analysis has revealed the heterogeneity of GSCs in GBM specimen (see Table 2). Patel *et al.* demonstrated that individual GBMs contain four heterogenous GSCs: proneural, neural, classical and MES types, identifying using CD133 as a GSC marker. They also observed hybrid states with two subtypes, either classical and proneural or MES and neural, revealing the existence of GSCs with abnormal developmental programs [58].

**Table 2** GSC heterogeneity.

| Number of heterogeneous GSC in cancer | GSC markers | GBM microenvironment | Major finding | Reference |
|---|---|---|---|---|
| Four (proneural, neural, classical mesenchymal) | CD133 (PROM1) | Hypoxia | Different types of GSCs co-existed in one GBM. | [58] |
| Two to seven | SOX2, FUT4, PROM1, CD44 L1CAM | | Outer radial glial-like cancer cell was identified as a new GSC. | [59] |
| Sixteen | CD133, CD44, A2B5, CD15 | Hypoxia | Heterogeneity arised from reversible state transitions instructed by the microenvironment. | [40] |

Bhaduri *et al.* also demonstrated that single GBM contains multiple subtypes of GSCs, using four GSC markers: PROM1, Fucosyltransferase 4 (FUT4), CD44, L1 cell adhesion molecule (L1CAM) and SOX2. Among the subtypes, they focused on the outer radical glia-like GSCs (oR-GSCs), as radial glia has not been identified in adult brain even using scRNAseq analysis [59]. They demonstrated that the oR-GSCs contribute to the cellular composition and invasive behavior of glioblastoma.

Furthermore, Dirkse *et al.* found that GBM contains sixteen types of GSCs, which express different sets of GSC markers, CD133, CD44, A2B5 and CD15, in patient specimen. They demonstrated that hypoxia endows the cancer cells with the CSC status and that the phenotype is reversible [40]. They concluded that understanding the mechanism of phenotype transition is crucial for therapy.

Considering the heterogeneity of CSC, including the diversity of cells-of-origin, reversion to CSC, cell-cell interaction, environment stress, and varying therapy resistance, characterizing the heterogeneity in whole cancers will lead to the development of new therapeutic strategies.

## 4. Potential therapeutic methods for overcoming CSC heterogeneity

Given the heterogeneity of cancer, the question is how we can develop novel therapeutic methods to conquer it. One promising approach is the use of cancer organoids directly prepared from patient specimens. These organoids likely consist of CSCs, cancer cells, and surrounding non-cancer cells [60,61]. Nonetheless, cancer organoids do not completely mirror original cancers because tumor-surrounding non-cancerous cells, such as immune cells, and systemic factors in the bloodstream are not continuously present in culture. There is no data regarding which culture conditions can maintain whole cancer cell heterogeneity *in vitro*. Based on the available methods, we cannot exclude the possibility that the defined culture medium selects specific cells, such as adaptable CSCs and their sister cells, for survival.

Although scRNAseq reveals cancer heterogeneity using directly prepared patient specimens, these data do not provide the essential information on which cells retain tumorigenicity and are potential therapeutic targets, because CSC marker-negative cells, such as CD133-negative cancer cells, have also been shown to be tumorigenic when transplanted into immunodeficient mice [62]. Thus, without establishing methods that can determine the phenotype of each single cell in a tumor, it is difficult to generate a useful CSC heterogeneity profile that may be used to develop tailor-made therapeutic strategies targeting each CSC type. Therefore, targeting common CSC factors is likely to be a realistic therapeutic method at this time if the off-target effects on normal cells, particular normal tissue-specific stem cells, can be minimized.

Gene therapy is widely used to treat various disorders. It can be used to manipulate target gene (knockdown or KO), overexpress exogenous genes in target cells using specific promoters and/or enhancers, and deliver therapeutic genes using viruses [63]. Compared to antibody-based therapeutic methods or the identification of chemicals to target disease specifically, constructing a vector for gene therapy is much

easier. However, if there is no specific promoter or enhancer to target cells specifically, it is impossible to express exogenous genes only in target cells. Additionally, the large size of the promoter and enhancer or having multiple promoters/enhancers poses another obstacle to successful gene therapy targeting specific cells.

Naldini and his colleagues established a new method that specifically regulates the expression of an exogenous gene only in the target cells in a microRNA (miR) expression-dependent manner (Figure 3). They elegantly demonstrated that endogenously expressed miRs, miR-142-3p specific to hematopoietic cells and miR-302a specific to human embryonic stem cells, suppress the expression of an exogenous gene when combined with four tandem complementary sequences of the miRs (miRCS) *in vitro* and *in vivo* [64,65].

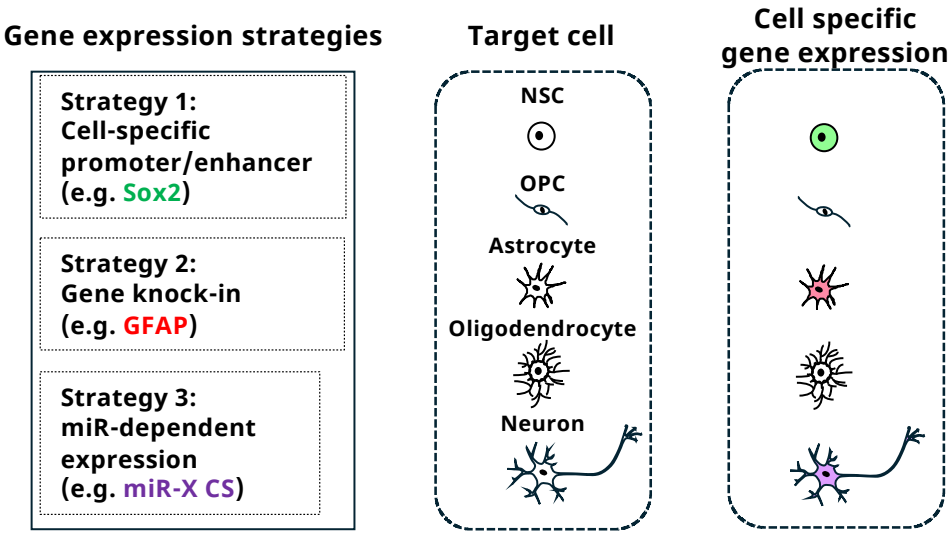


**Figure 3** Systems for cell-specific expression of exogenous genes. There are three potential systems for cell-specific gene expression systems. The first system involved using cell-specific promoter or enhancer (Strategy 1). While some of these promoters and enhancers have been well-studied, there are many genes for which the specific promoters and enhancers are unknown or not well-characterized. The second system utilizes homologous recombination (Strategy 2). One advantage of this strategy is that it allows the use of endogenous promoters. However, this system requires the use of two vectors: a Genome editing (GE) vector with a single-guide RNA expression cassette, and a targeting vector. The third system involves the use of consensus sequences (CS) of miR-X (Strategy 3). These sequences are expressed in non-target cells, but not in target cells. By using the short (~100 bp) size of the four tandem repeats of the miR-X consensus sequences (miR-XCS), it is possible to construct vectors that specifically express exogenous genes in target cells, even in mixed cell populations.

Sayeg *et al.* also showed that miRCS of miR-128 and miR-221, both expressed in excitatory neurons, but not in inhibitory neurons, selectively induced the expression of an exogenous gene in inhibitory neurons in the brain when the expression vector was delivered with miRCS using a lentivirus [66].

Using miR-regulated expression system, Suzuki *et al.* have demonstrated that the herpes simplex virus encoding the thymidine kinase with target sequences of miR-122a, which expresses in hepatocytes, killed the tumor in liver with little hepatotoxicity, when injected intratumorally [67].

These findings suggest that posttranscriptional gene silencing by endogenous miR can specifically induce the expression of any exogenous gene with miRCS in miR-negative target cells, but not miR-positive others.

The miRNA Tissue Atlas (https://ccb-web.cs.uni-saarland.de/tissueatlas2/) revealed that the expression of miR-142-3p is highest in the lung, followed by the brain, whereas the expression of both miR-128 and miR-221 is low in non-brain tissues. This suggests that miRCS alone may not be enough regulate the expression of an exogenous gene restricted to the target cells *in vivo*. Nonetheless, miR-dependent gene expression systems are very attractive for generating new therapeutics by combining them with other innovative ideas and technologies.

A remaining question is how to selectively deliver the expression system selectively into CSCs. Various methods have been developed for delivering therapeutics into tumors [68]. In the case of brain tumors, different delivery methods such as viral vectors, liposomes, cationic polymers, gene guns, and cell-penetrating peptides have been evaluated [69]. Among these methods, dual-targeting nanoparticles that can pass through the blood–brain barrier (BBB) and target brain tumors show promise. However, there is a concern that these particles may also deliver chemicals to other tissues, particularly the liver [70].

Another potential option is the use of recombinant viruses, including Adeno-Associated Virus (AAV), Zika virus, and Herpes simplex virus, which can infect brain cancer cells and activate a cytotoxic signal to kill them *in vivo* [71–74]. AAV seems to be superior due to its low immune response, high yield, long-term expression in nonproliferating cells, and histotrophic ability [75,76]. Recent studies have identified brain tropic AAV capsids, such as PHP.eB, CAP-B10, and CAP-B22, which can target the brain and enable the analysis of gene function in both endogenous and exogenous gene in mice and nonhuman primates. However, it was found that these capsids also deliver a small number of virus particles to the liver [77,78]. Although AAV is not effective for long-term expression in rapidly dividing cells, such as cancer cells, due to its low genome integration, this problem could potentially be addressed by using a Cas9-dependent genome-editing (GE) system, as its transient expression is sufficient to knockout the target gene [79].

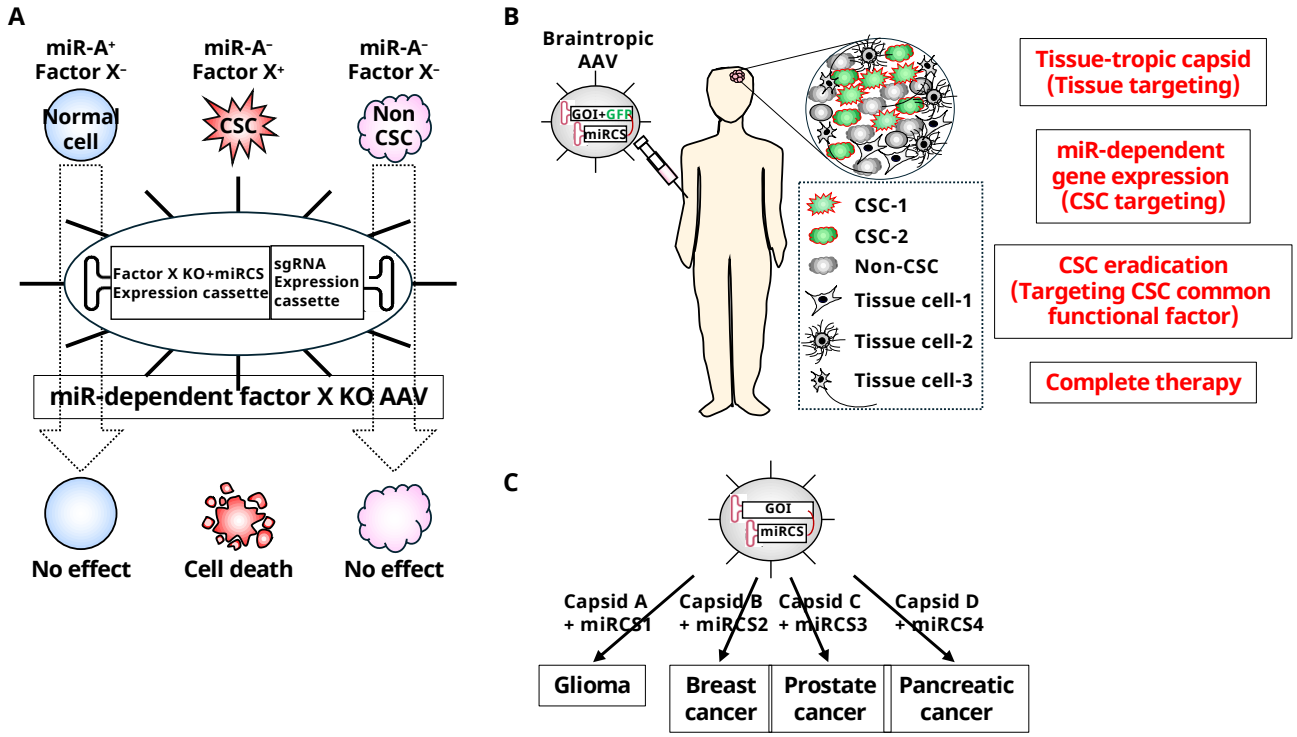


**Figure 4** Cancer Therapy using tissue-tropic AAV encoding miR-dependent gene expression system. (**A**) miR-dependent factor X Genome-editing (GE) Adeno-Associated Virus (AAV) encodes both the GE system for a Cancer Stem Cells (CSCs)-specific functional factor X and the consensus sequences of miRs (miRCS). These miRCS are not expressed in CSCs, but are expressed in normal cells and non-CSCs. The AAV can knock out factor X only in the CSCs, while leaving normal cells and non-CSCs unaffected. This results in the specific elimination of CSCs. (**B**) The miR-dependent factor X GE AAV coated with a brain tropic capsid can be delivered to the brain via systemic administration. It selectively expresses the GE system in CSCs and specifically kills these cells by knocking out factor X. This leads to the eradication of the cancer with minimal side effects. (**C**) The miR-dependent factor X GE AAV coated with the tissue-tropic capsid demonstrates high versatility. The AAV can be used to treat various types of cancer, such as glioma, breast cancer, prostate cancer, and pancreatic cancer, by replacing the capsid according to the specific cancer-bearing tissue. Additionally, the CS of the miR can be tailored to each type of CSC, allowing for targeted treatment.

By combining key factors of CSC function, selective miR-dependent gene expression systems specific to CSC, and AAV-based tissue targeting, it may be possible to eliminate different types of heterogeneous CSCs in tumors without causing harm to normal cells or non-CSCs (Figure 4). Recent research by Wan *et al.* has shown promising results in this area. They developed brain tropic AAVs that encode a miR-dependent GE system specifically targeting a common functional factor in GSC. Their experiments demonstrated that the virus selectively transduced the system into GSCs in the brain without affecting other tissues, including the liver, and effectively killed the GSCs *in vivo* when administered intravenously (unpublished observation). Although the tumor was not completely cured in the treated mice, further improvements to this system are needed before it can be translated into the clinical setting.

Nevertheless, this approach shows promise not only for eliminating different types of GSCs and overcoming CSC heterogeneity but also for eradicating various types of cancers by replacing the AAV capsid, miRCS, and CSC target genes.

## 5. Conclusions and perspectives

Technological advances, such as scRNAseq, CyTOF and special transcriptomics analysis, have revealed the heterogeneity of tumors, which consist of various types of cancer cells and non-cancer cells. However, this information alone is insufficient to develop therapeutic methods that target heterogeneous CSC, as there is currently no way to verify which cells retain tumorigenicity *in vivo*. As a result, a more realistic therapeutic approach is to target common CSC factors while minimizing the impact on normal cells, particularly ts-SC/PCs. One potential method is to use a tissue-tropic capsid-coated AAV that encodes a miR-dependent GE system for CSC factors. This AAV can be delivered to the target tissue and the GE system will only be expressed only in CSCs in a miR-dependent manner, effectively knocking out key CSC functional factor and eliminating heterogeneous CSCs without significant side effects.

## Ethics Statement

Not applicable.

## Consent for Publication

Not applicable.

## Availability of Data and Material

Not applicable.

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## Competing Interests

The author has declared that no competing interests exist.

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