REVIEW ARTICLE

Stem Cells in Solid Tumors: Accumulated Evidence and Future Directions

Deepthi Venkatachalapathy[1], Parveen Doddamani[2], Aradya V Hirriyannaiah[3], Prashant Vishwanath[4], Suma Nataraj[5], Akila Prashant[6]

Received on: 31 January 2024; *Accepted on:* 20 February 2024; *Published on:* 20 April 2024

Abstract

Cancer stands as a leading global cause of human mortality, predominantly driven by solid tumors that can ravage vital organs. The scientific community has made significant strides in comprehending the molecular and cellular underpinnings of cancer. However, translating these discoveries into effective, targeted therapeutics has proven challenging. This gap highlights the existence of critical bottlenecks hindering the journey from fundamental research findings to fully realized anticancer drugs. Recent insights into cancer biology have illuminated one potential bottleneck: the presence of cancer stem cells (CSCs). These represent a minority subset of cells within a tumor, believed to orchestrate the relentless growth of the entire tumor. The concept of CSCs has gained substantial traction, thanks to advancements in stem cell research. Developing more potent and precise cancer therapies hinges on our ability to identify and understand these cancer-initiating cells within solid tumors. It is essential to discern how CSCs differ from other cancer cells coexisting in the same tissue. This review endeavors to compile the accumulated scientific evidence supporting the existence of CSCs, elucidate the cell surface markers employed for their isolation, dissect the pathways governing their self-renewal and differentiation, and outline future directions for harnessing them as therapeutic targets to eradicate tumor growth comprehensively.

Keywords: Cancer stem cells, MicroRNA, Tumor initiating cell, Tumor microenvironments.

Indian Journal of Medical Biochemistry (2024): 10.5005/jp-journals-10054-0225

Background

Epithelial cancers such as cancer of the breast, lungs, colon, and prostate are the most common cancers being reported in adults. The mature cells in each of these tissues are constantly replenished primarily by a small population of cells called stem cells.[1] Stem cells in adult tissues produce large numbers of progenitor cells called transiently amplifying progenitor cells.[1] These transiently amplifying progenitor cells encounter a limited series of mitotic cycles before entering a fully differentiated post-mitotic state. Hence, the large numbers of differentiated progeny can be amplified with a relatively small number of stem cells. Mutations in these stem cells or the early progenitor cells might transform them into cancer stem cells (CSC) (Fig. 1). Immortal tumor-initiating cells that can self-renew and have pluripotent properties are attributed to the CSCs. They are found in various multiple malignancies like leukemia and many other solid cancers. Their, remarkable properties, CSCs are thought to play a major role in tumor initiation, development, metastasis, and recurrence. Al-Hajj et al. was the first one to report and demonstrate the presence of CSCs in solid tumors and breast cancers respectively.[2]

Frequent failure of the treatment is one of the major limitations of the present cancer therapeutic strategies due to the resistance developed towards chemotherapy and radiotherapy. Cancer stem cells with the capacity for unlimited self-renewal and the ability to initiate and drive tumor progression would seem the most probable candidates responsible for chemoresistance.[3] Moreover, therapeutic strategies fail in targeting CSCs in particular, affecting the healthy tissues. Recurrence of cancer in these current strategies is common since they remain unsuccessful in eliminating CSCs.[4,5] Thus, it is important that these cells are identified and isolated from the total cancer cell population and studied in detail. Technical innovations have recently allowed the identification, isolation, and growth of these cells in the laboratory, and it has become clear that they have properties that are distinct from both the bulk of tumor cells and the cancer cell lines usually used to test anti-cancer drug candidates. Eradication of cancer, therefore, requires targeting and elimination of CSCs. Also, it becomes necessary to identify the early events

[1,2,4]Center of Excellence in Molecular Biology and Regenerative Medicine, Department of Biochemistry, JSS Medical College, JSS Academy of Higher Education & Research, Mysuru, Karnataka, India

[3]Department of Gastroenterology, JSS Medical College, JSS Academy of Higher Education & Research, Mysuru, Karnataka, India

[5,6]Center of Excellence in Molecular Biology and Regenerative Medicine, Department of Biochemistry, JSS Medical College; Special Interest Group on Human Genomics and Rare Disorders (SIG-HGRD), JSS Academy of Higher Education & Research, Mysuru, Karnataka, India

Corresponding Author: Akila Prashant, Center of Excellence in Molecular Biology and Regenerative Medicine, Department of Biochemistry, JSS Medical College; Special Interest Group on Human Genomics and Rare Disorders (SIG-HGRD), JSS Academy of Higher Education & Research, Mysuru, Karnataka, India, Phone: +91 9008097970, e-mail: akilaprashant@jssuni.edu.in

How to cite this article: Venkatachalapathy D, Doddamani P, Hirriyannaiah AV, *et al*. Stem Cells in Solid Tumors: Accumulated Evidence and Future Directions. Indian J Med Biochem 2024;28(1): 13–24.

Source of support: Nil

Conflict of interest: Dr Akila Prashant is associated as Editor-in-Chief of this journal and this manuscript was subjected to this journal's standard review procedures, with this peer review handled independently of the Editor-in-Chief and her research group.

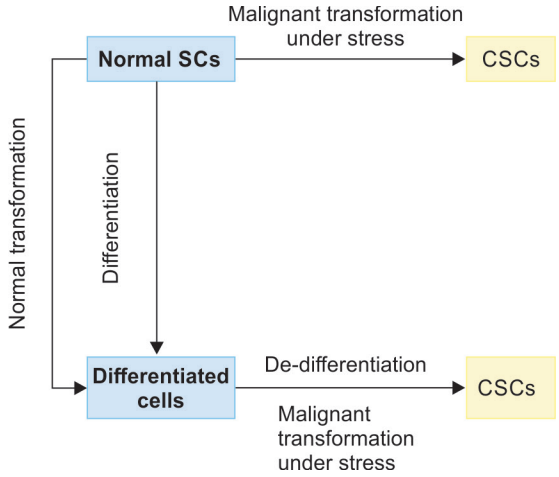

Fig. 1: Various mechanisms for the generation of CSC: (1) Normal stem cells transform to form differentiated cells. (2) Under the influence of stress, normal stem cells accumulate mutations or promote oncogenic activation to form CSCs. (3) The differentiated cells are de-differentiated into CSCs under genetic, epigenetic, or environmental influence. The process of differentiation and de-differentiation generating the CSCs is referred to as cellular plasticity

involved in the process of malignant transformation of these cancer-initiating cells. Signaling involved in carcinogenesis is complex and each cancer has a unique set of signals and group of cells involved in its development. Hence, strategies have to be developed, which protect the normal stem cells while eliminating CSCs.

Evidence for the Presence of CSCs

In the year 1875, the hypothesis of CSCs called "embryonal-rest theory" was proposed by Cohnheim.[6] This theory implied that traces of embryonic-like cancerous cells were present in adult tissues which later developed into cancer. Similarly, in the year 1994, Dick and his colleagues illustrated the presence of leukemia-promoting stem cells in the blood of leukemia patients and when it was transplanted into non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice these cells were capable of inducing leukemia.[7] This was the first experiment that supported the existence of CSCs. The CSC's presence is also studied in breast and brain cancer supporting the above theory.[8] Research suggests that treatment for any type of cancer yields poor results due to the presence of CSCs and their interaction with the tumor microenvironment making cells survive and develop resistance by evolving into more aggressive phenotypes.[8] In the case of normal stem cells, which are maintained by a specialized niche become activated and divide only upon reception of an external stimulating signal under normal physiological conditions. When the stem cells encounter genetic mutation initiating independent growth signals, they develop resistance against growth signals resulting in uncontrolled proliferation and tumorigenesis.[8]

Tumors are composed of heterogeneous cell populations with different biological properties.[9] They often seem to correspond to different stages of development. Previously it was thought that the observed tumor cell heterogeneity was due to the influence of the microenvironment and genomic instability that generate genetic and epigenetic changes and prevent the faithful and accurate replication and transmission of stable genotypes and phenotypes. Two prevailing models illustrate the organization of CSC; the stochastic and hierarchy models. The stochastic model, proposed by Nowell and colleagues, posits that every cancer cell harbors the ability for self-renewal and differentiation. Conversely, the hierarchy model suggests the presence of a heterogeneous cancer cell population, with only specific subpopulations capable of regeneration.[10] Recent reports indicate an interrelation between these models, demonstrating that non-stem cells (NSCC) can undergo reversible conversion into CSCs. Research by Iliopoulos D et al. has shown that CSCs can maintain equilibrium within the transformed cell population over numerous generations.[11] Identifying CSCs within tumors remains a formidable challenge. The lack of distinctive cell-surface markers and the plasticity of CSC-related phenotypes necessitate alternate methods for their identification. Functional assays, such as *in vivo* tumor transplantation assays, and lineage-tracing assays, play pivotal roles in evaluating CSC's growth through self-renewal and differentiation.[12]

CSCs in Breast Cancer

Breast cancer, a heterogeneous disease, has seen significant advancements in our understanding of CSCs, which play a pivotal role in tumor initiation and progression. Several groundbreaking studies have shed light on the identification and characterization of CSCs within breast cancer. Pioneering the field, Al-Hajj et al.[2] conducted seminal research that isolated tumorigenic cells within breast cancer. These cells were characterized by the $CD44^+/CD24^{-/low}Lineage^-$ phenotype, capable of initiating tumor growth in mice even when present in minimal numbers. This discovery challenged conventional thinking about the heterogeneity of cancer cell populations and understood the significance of a specific subset in tumor propagation. Building on Al-Hajj's work, Ponti D et al.,[13] further elucidated the properties of breast cancer-initiating cells. They established breast carcinoma cell lines that exhibited the $CD44^+/CD24^-$ characteristics. These cells not only demonstrate self-renewal capabilities but also prove to be highly tumorigenic. This research provided valuable insights into the intrinsic plasticity of breast cancer cells and their capacity to drive tumor formation. Another noteworthy contribution came from Shackleton M et al. who identified a distinct population enriched in Mammary stem cells (MaSCs). These cells were characterized as ($Lin^-CD29^{hi}CD24^+$), and they exhibited key attributes associated with stem cells. This finding added depth to our understanding of the stem cell hierarchy within the mammary gland and its relevance in breast cancer.[14] Most recently, innovative protocols have been developed to generate CSCs from induced pluripotent stem cells (iPSCs). These CSCs, derived from iPSCs, have shown remarkable tumorigenic potential. This approach not only provides a unique model system for studying breast cancer but also raises intriguing questions about the plasticity and reprogramming potential of CSCs.[15] Pioneering studies have uncovered specific markers and characteristics of breast CSCs, challenging traditional views of cancer cell populations. The identification and understanding of these cells hold great promise for developing targeted therapies and improving breast cancer treatment outcomes.

CSCs in Sarcoma

Osteosarcoma, in particular, presents an intriguing case in which CSCs are believed to play a pivotal role in disease initiation and progression. The initial oncogenic events may likely occur within a subset of cells known as the side population or SP that exhibit characteristics reminiscent of CSCs and are recognized for their role in drug resistance. This resistance, in part, is attributed to their quiescent, persistent, and dormant nature, making them particularly challenging to target with conventional therapies. Osteosarcoma CSCs have shown remarkable survival mechanisms and are

sustained by various factors, including the transcription factor Sox2, which is a key player in stem cell maintenance. Additionally, Sox2 has been found to inhibit the Hippo pathway, a crucial regulatory pathway involved in cell growth and differentiation.[16] These survival mechanisms underline the resilience of CSCs with the tumor microenvironment. These CSCs also exhibit increased tumorigenicity both *in vitro* and *in vivo*. In laboratory settings, they have the capacity to form spheres indicating their self-renewal abilities. This self-renewal potential is closely associated with their tumorigenic nature *in vivo*, where they drive the growth and progression of osteosarcomas. It also expresses high levels of aldehyde dehydrogenase (ALDH) that have been attributed to resistance against chemotherapy. This resistance further underscores the importance of targeting CSCs to enhance treatment efficacy. Recent studies have unveiled the intricate regulatory mechanisms governing CSCs in osteosarcoma. MicroRNAs, play a crucial role in controlling the phenotype of CSCs and regulate specific molecular pathways, including PTEN, Jagged1, and Wnt which have implications for CSC behavior and tumor progression.[16] Thus, osteosarcoma presents an intriguing landscape in which CSCs, particularly those within the side population, exert significant influence. Their unique survival mechanisms, tumorigenic potential, and resistance to chemotherapy pose challenges for effective treatment. Understanding the molecular intricacies and regulatory networks governing these CSCs holds promise for developing target therapies and improving outcomes for individuals battling osteosarcoma.

CSCs in Brain Tumors

Brain tumors, a complex and challenging category of cancers, have also been the subject of intense study concerning the existence and characterization of CSCs. Uchida et al. conducted pioneering research by isolating human neural stem and progenitor cells using the cell surface marker CD133. These cells were obtained from fresh human fetal brain tissue and were isolated through the use of antibodies to cell surface markers and fluorescence-activated cell sorting. The resulting population of clonogenic human central nervous system stem cells (CNS-SC) was characterized by the expression of CD133 while lacking CD34 and CD45. These sorted $CD133^+/CD34^-/CD45^-$ cells exhibited remarkable capabilities, including the initiation of neurosphere cultures and the capacity to differentiate into both neurons and glial cells. Upon transplantation into the brains of immunodeficient neonatal mice, these CNS-SC demonstrated robust engraftment, proliferation, migration, and neural differentiation.[17] Ignatova et al. isolated the clonogenic cells from cortical gliomas with an abnormal p53 status. The heterogeneity of CSC sub-populations is evident, with only a fraction possessing colony-forming ability, identified as clonogenic cells.[18] These clonogenic cells exhibited a unique phenotype, expressing markers associated with both neuronal and glial cytoskeletal markers. Remarkably, these cells bore similarities to neural stem cells (NSCs) typically found in neurogenic regions of the normal human brain thus referring to them as "stem-like," Singh SK et al. reported a breakthrough in the identification and purification of a distinct cell population within primary human brain tumors.[19] These cells displayed marked capacities for proliferation, self-renewal, and differentiation. CD133, a cell surface marker, was utilized for their identification and they represented a minority of the overall tumor cell population. Intriguingly, these $CD133^+$ cells did not express neural differentiation markers but were essential for tumor proliferation and self-renewal in culture. Additionally, these cells demonstrated the ability to differentiate *in vitro* into cell phenotypes closely resembling those found in the original tumor microenvironments.[20] To ascertain the tumorigenic potential of $CD133^+$ human brain tumor cells, Singh SK et al. conducted *in vivo* experiments. Comparing $CD133^+$ with $CD133^-$ tumor cells in NOD/SCID mouse brains, they found that injection of as few as 100 $CD133^+$ cells resulted in the formation of tumors that could be serially transplanted. These tumors faithfully recapitulated the characteristics of the patient's original tumor, while injections 10^5 CD133- cells did not yield tumor formation. Notably, recent research highlights that CD133 may have context-dependent information and the potential for false-negative results when identifying CSCs.[21] It is important to acknowledge the evolving understanding of CSCs in brain tumors, with studies indicating that the presence of specific markers like CD133 may not be universally definitive for identifying CSCs. Beier D et al. underscores this complexity, as they demonstrated that even $CD133^-$ cells could fulfill the criteria of stem cells *in vitro* and exhibit tumorigenicity *in vivo*.[22] Thus, the study of CSCs within brain tumors has unveiled a complex landscape of cells with unique properties. These discoveries hold promise for advancing our understanding of brain cancer biology and exploring targeted therapeutic approaches to address these challenging malignancies.

CSCs in Colorectal Cancer

Colorectal cancer, the second leading cause of cancer-related mortality, is unique in its comprehensive understanding from a genetic standpoint. Researchers, inspired by the emerging evidence of undifferentiated cell populations driving tumor formation and maintenance in breast and brain tumors, turned their attention to colorectal cancer. This endeavor, led by Ricci Vitiani et al. and O'Brien et al., aimed to identify and enrich colon CSCs using Fluorescent-activated cell sorting (FACS) and the putative stem cell marker CD133.[23,24] Transplantation of both $CD133^+$ and $CD133^-$ cells into the renal capsule of immunodeficient NOD/SCID mice was instrumental in identifying human colon cancer-initiating cells (CC-IC). Significantly, $CD133^+$ cells exhibited the ability to initiate tumor growth whereas $CD133^-$ cells were unable to do so. Within the $CD133^+$ population, CC-ICs displayed the remarkable capacity to self-maintain, differentiate, and re-establish tumor heterogeneity through serial transplantation. *In vitro*, $CD133^+$ colon cancer cells demonstrated exponential growth as undifferentiated tumorspheres in a serum-free medium, retaining the capability to engraft and reproduce the original tumor's morphological and antigenic characteristics. Shmelkov et al. made a significant contribution by uncovering that CD133 expression within the colon was not exclusive to stem cells but was ubiquitously expressed on the differentiated colonic epithelium in both adult mice and humans.[25] Furthermore, both $CD133^+$ and $CD133^-$ metastatic tumor subpopulations displayed the formation of colony spheres in *in vitro* cultures and demonstrated long-term tumorigenic potential in a NOD/SCID serial xenotransplantation model. Collectively, this data challenged the notion that CD133 was a definitive marker of colon CSCs, leading to the exploration of alternative, more robust markers for CSC isolation. Dalerba et al.[26] proposed alternative markers, EpCAM and CD44, as more reliable indicators of colon CSCs than the previously reported marker CD133. CD44 appeared to be informative in tumors that did not express CD133, offering the potential for further enrichment of colon CSC within the $CD133^+$ subset. Though enrichment strategies provided a solid foundation for the identification of more specific biomarkers, isolation of CSCs

solely relying on these has shown to be insufficient in some cases. Thus, the quest for improved strategies to isolate colon CSCs continues, underscoring the dynamic nature of this field of research.

Isolation of CSCs and its Methods

Various *in vitro* approaches have recently been published to reach the CSC population. Based on this literature, the CSCs can be recruited mainly from cancer cell lines or primary tumors by specific surface marker expression, reprogramming, selecting anoikis-resistant cells, or specific culture condition applications. Initially, the functional assay is performed *in vitro* for the enrichment of CSCs followed by descriptive assays performed *in vivo* for verification. The initial *in vitro* steps comprise non-adherent and serum-free conditions for the enrichment of CSCs in the population. Whereas *in vivo*, there is serial transplantation of CSCs into immunocompromised mice to examine the potential tumorigenic properties. For the detection of drug resistance properties in the cell population, the ALDH which is a dye exclusion assay is used for the enrichment of CSCs. However, the utmost care has to be taken to maintain experimental conditions during the isolation steps to obtain a pure CSC population. Since there are similarities between normal and CSCs, it is possible to identify CSCs based on the surface markers, formation of spheres in a non-adherent medium, and the nature of excluding some dyes. As a result, various assays have been introduced accordingly to identify CSCs such as sphere formation assay, Hoechst dye exclusion assay, Aldefluor assay, migration assay, signaling pathway identification, and detection of surface markers. Since these assays are based on the similar properties shared between normal and cancer cells, there is a requirement for *in vivo* assays to be performed which claims to be the gold standard for the identification of CSCs.

Some of the potential challenges or drawbacks faced during the identification and isolation of CSCs using surface markers are that these markers being non-specific require the use of a combination of markers.[27] The cell viability exclusively depends upon how fresh the sample is and the probability of acquiring the optimum result is maximum when fresh specimens are processed within 30 minutes. Sometimes there might be distinct CSC markers for a subset of a given tumor type. Identification of CSCs using surface markers is valid using FACS, and not by other methods like immunohistochemistry (IHC). Tumors with reduced malignancy fail to produce tumors in animal models despite expressing the CSC surface markers questioning the ability of these markers to correctly identify the CSCs. Human tumors and their signaling networks are complex to analyze using CSC markers.[27] Isolation of CSCs using FACS, although has high specificity and multiparameter separation, it requires a large number of cells. There is no universal marker for the identification of CSCs, their analysis, and FACS results interpretation is complicated that requires expertise. There should always be a check of the system for clogging, and sterile conditions should be maintained to avoid contamination of the cells. It is both time-consuming and expensive as well.[28,29]

Functional Isolation Using Hoechst 33342

Most tissues contain multiple populations of stem cells expressing different markers. Apart from the cell surface markers, various other methods have been employed by researchers to enrich the stem cells, like efflux fluorescent dyes. Hoechst 33342 is a dye that binds to the AT-rich regions of the minor groove in DNA. Its fluorescent intensity is dependent on the DNA content, chromatin structure, and position of the cell within the cell cycle. Although the uptake of Hoechst 33342 and other lipophilic dyes is universal, the ability to efflux the dye actively appears to be restricted to a subset of cells. The FACS profile of the cells that actively efflux Hoechst 33342 has a characteristic appearance and has been termed the 'side population' or SP. Based on the hypothesis that stem cells contain drug efflux transporters, flow cytometry can be used to isolate this side population subset containing CSCs.[30] Goodell et al. were the first to use the Hoechst 33342 dye exclusion method for the isolation of hematopoietic stem cells. The Hoechst-purified SP cells comprised 0.1% of the bone marrow and were highly homogeneous concerning cell surface markers.[31] They also showed that this purification strategy relies on a high level of multidrug resistance protein (MDR), a member of the ATP-binding cassette (ABC) transporter transmembrane protein or MDR-like activity present in stem cells. Even though the regulation of stem cells by ABC transporters emerged as an important new field of investigation, the specific molecules involved had to be defined. Zhou et al. showed that the expression of the breast cancer resistance protein 1 (Bcrp1) (also known as Abcg2 murine/ABCG2 human) gene is a conserved feature of stem cells from various sources and it had increased mRNA expression in primitive murine hematopoietic stem cells and was gradually decreased with differentiation. Thus they identified the expression of the Bcrp1/ABCG2 gene is attributed to the SP phenotype determinant and also serves as a marker for stem cells from different sources.[32] Haraguchi N et al. identified and isolated SP cells from gastrointestinal cancers by using flow cytometry and Hoechst 33342. These cells showed evidence of self-renewal and generated both SP and non-SP cells. The whole-genome DNA microarray analysis of these cells provided insight into the existence of key molecules such as carcinoembryonic antigen-related cell adhesion molecule (CEACAM6), AREG, ABCC2/GSTA1 genes which may be associated with chemoresistant properties of CSCs.[33] However, the toxicity associated with the Hoechst 33342 dye has limited its use for the selection of cell populations for functional stem cell assays *in vivo*.[34] Another small molecule that has been reported to be effluxed by stem cells is Rhodamine 123 (Rho). Rho that binds to the mitochondrial membrane is used as a substrate of the ABCB1/P-GP transporter and is actively pumped out of the cells by ABC transporters. Liu et al. have shown Rho to be non-toxic to cells even at high concentrations and it can be an alternative to the use of Hoechst.[35]

Isolation of Stem Cells by ALDH Activity

The focus then shifted to conserved stem cells and progenitor functions for finding shared stem cell markers. These functional markers are inherited by the malignant stem cells and pass through various histological subtypes of cancer arising from the same tissue. Aldehyde dehydrogenase1 was one such marker that fit the description and was used to identify the stem/progenitor population in both human hematopoietic tissue and normal mammary glands.[36] The cellular activity of ALDH1 was demonstrated using fluorescent substrate Aldefluor and flow cytometric analysis. An increase in ALDH activity is observed in mesenchymal, neural, endothelial, and hematopoietic stem cells. Recent studies observed various ALDH isoforms in the progenitor pancreatic and breast showing Aldefluor positivity.[37] The Aldefluor-positive population which was isolated from fresh mammoplasty samples, was potential enough to produce mammospheres with 5000 cells/mL density in the suspension culture. Also, these cells formed invasive ductal carcinomas when transplanted orthotopically in the humanized cleared fat pad of NOD/SCID mice,

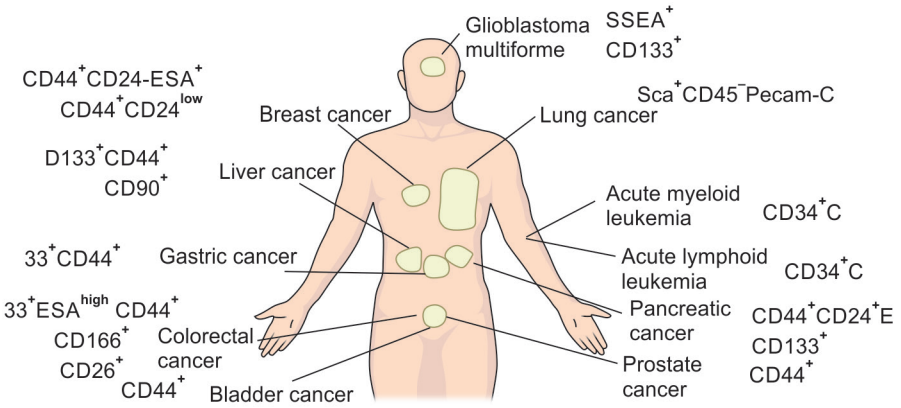

Fig. 2: Cell surface markers for types of cancers: The most widely used cell surface markers for the classification of cancer stem cell (CSCs) subpopulations and various cancer types

without cultivation *in vitro*. in these three tumors represented 3 to 10% of the total cell population was observed to be Aldefluor-positive population. When combined with FACS analysis for CD44/CD24/lin the Aldefluor$^+$/CD44$^+$/CD24$^{-/low+}$/Lineage$^-$ phenotypes had a high tumorigenic capacity and generated tumors from as few as 20 cells. By contrast, the Aldefluor-negative cells bearing the CD44$^+$/CD24$^-$/lin$^-$ phenotype, were not tumorigenic, even when implanted in numbers of 50,000 cells/fat pad, suggesting that this population may lack tumorigenic cells. Stem and progenitor cells are proved to be the primary targets during the transformation since the segregation and identification of both stem/progenitor cells are done based on the same cell-surface marker, supporting the cancer stem cell hypothesis.[36] One of the limitations of this assay is higher toxicity which however can be reduced by decreasing the concentration of the dye and standardizing the method as it avoids the usage of other markers to identify CSCs.[37]

CSC Enrichment by Expression of Surface Markers

For the enrichment of CSCs from tumors, cell surface antigens are most commonly used (Fig. 2). Marker CD133 expression in various types of cancer has been observed. Also, CD44 which is an adhesion molecule is found to be associated with phenotypes of CSCs for example, in breast, pancreatic, colorectal, and ovarian cancer. Isolating and identifying tumor-initiating cells in various cancers is possible by sorting the cells based on cell surface markers and transplanting them into animal models. The first evidence of such CSC was found in acute myeloid leukemia (AML) in which the cells having the surface makers CD34$^+$CD38$^-$ were identified and purified. These cells were capable of transferring AML from human patients to NOD/SCID mice proving their clonogenic capacity, whereas the introduction of cells that did not bear this phenotype was non-tumorigenic.[38,39] From the hematopoietic system, the CSC hypothesis has been extrapolated to solid cancers, supporting data for which is growing rapidly. In future years, there may be extensive use of markers for identifying and isolating CSCs, although there are 40 CSC markers currently in extensive use. Glycoprotein markers of the glycosylation process have also been explained to influence the behavior of the CSCs.[39]

Molecular Profiling of Cancer Stem Cells

Characteristic surface markers like CD133, CD44, CD38, CD34, CD24, LGR5, nestin, α6–integrin, and β_1 integrin act as molecular markers which help to identify the CSC, sub-classify cancer and also act as prognostic markers.[3,40–43] These specific markers help to precisely isolate the cells and target treatment regimens. The rationale behind this type of profiling lies in its usage as therapeutic targets and helps to provide early-stage interventions. The surface markers are divided into two groups: Tissue-specific CSC surface antigens which only indicate the presence of CSC and cytoplasmic/protein markers which aid in identifying the behavior of CSC, such as active cell cycling process, loss of differentiation, and invasiveness of the respective tumor cells.[44] Although there are various markers used for different types of cancer as shown in (Fig. 2), the future perspective of the utilization of these surface markers lies in the development and evolution of sensitive methods for the identification of these markers, especially in cases of follow-up and remission for better prediction of clinical outcomes.[4] Studies have associated elucidation of gene signatures like BMI-1 to 10 different types of human malignancies which have been associated with poor prognosis.[45,46] Certain stem cell populations exhibit the expression of proteins or are indicated by the very low level of their expression or even absence. Categorization of the stem cell population based on the presence or absence of this combination of markers has been standardized. For the identification of neural stem cells, markers that are stage-specific like CD24, and CD15 are used. In the case of epidermal stem cells, there are no efficient markers, however, markers like β1 integrin are involved in conferring adhesiveness to the epidermal cells. This facilitates the maintenance of the stemness of keratinocytes via the mitogen-activated protein kinase (MAPK) pathway.[47] The expression of the integrins is also found in breast, prostate, and colon CSCs (Table 1).[48] They are attributed as adhesion molecules and transmembrane receptors are composed of α and β units that bind to the extracellular matrix (ECM). This binding to the ECM induces a wide range of intracellular signaling and cellular functions like proliferation, migration, and cellular behavior favoring the microenvironment. Evidence shows that integrin dysfunction results in the development of cancer. Mice studies exhibiting the absence of subunits of integrins are shown to promote tumorigenesis. A laminin binding receptor α6 (CD49f) is one of the most studied integrin markers which is shown to express in embryonic and neural stem cells. In the case of glioblastoma, a combination of α6 and CD133 is found to enhance the detection of CSCs.

The hyaluronic acid receptor, CD44 is the most commonly studied CSC marker. It is involved in the initiation of migration in normal cells and shows increased expression in cancer cells.

Table 1: Functions of various subtypes of integrins in different cancer types

Subtypes of integrin	*Types of cancer*	*Integrin functions*
β4	Triple-negative breast cancer	Identifies CSC population.
	Pancreatic-ductal adenocarcinoma	An increase in β4 indicates an increase in stemness and EMT.
	Prostate	Sustaining self-renewal and initiating tumorigenesis.
α6	Glioblastoma	Identifies enriched-CSCs population and co-expression with glioblastoma CSC markers.
α7	Oral squamous cell carcinoma	Identifies enriched-CSCs population and co-expression with glioblastoma CSC markers.
β3	Breast cancer	It is a luminal epithelial progenitor marker. Identifies CSCs in the mouse model.
β8	Glioblastoma	Overexpression in the CSCs population initiates radio-resistance associated with poor prognosis.
αvβ3	Breast cancer	Adult mammary regulation during pregnancy, activating Slug genes in BC cells and increasing CSCs features -tumor formation.
α6 and β3	Breast cancer	Identifies CSCs-enriched population. Develops drug resistance in mouse models.
α2β1	Non-small cell lung cancer	Low levels of miR-34c-3p result in upregulation of α2β1, initiating invasion and migration.
	Colon cancer	Development of stemness metastatic property development through PI3K/AKT/Snail axis.
β1	Head and neck squamous cell carcinoma	Induces stemness, and resistance to chemotherapy.
	Oral squamous cell carcinoma	Overexpression in CSCs population and inducing cell proliferation and migration.

In cancer cells, CD44 is involved in alternative splicing and encodes proteins of cancer subtypes accordingly. Therefore, it is used as a surface marker in breast, prostate, pancreas, colorectal, and ovarian cancers. The CD24, a small cell surface marker protein, was initially found to be a heat-stable antigen in mice. It is a protein molecule with increased glycosylation due to which it is involved in cell-cell and cell-matrix interactions and acts as a ligand for various cancer types like breast, ovarian, prostate, and bladder. The increased expression of CD44 is more common when compared to CD24. The CD24 expression, its distribution among various cancers, and its specificity are yet to be discovered. Although there is extensive research on these CSC markers and their functions, there are several literature studies favoring and opposing their utility in various cancer types. Collectively, the research with CSC markers has ambiguity and is still a complicated concept to be discovered.[49]

Challenges and Limitations of Working with CSC

In FACS, the tumor under examination is exposed to enzymes that aid the degradation of intercellular junctions. The cell suspension is incubated with a specific antibody against the respective antigen tagged with fluorescent dye. It is made to flow in a narrow passage or tunnel leading to the separation and formation of single-cell during the flow by breaking into droplets. The fluorescing cells or opsonized single-cells are captured by the detector and laser beam when passed through it with a suitable wavelength. Further, these cells are subjected to an electrostatic field and those cells exhibiting electrostatic deflections are isolated or collected.[50]

Challenges in the Maintenance of Properties of the Stem Cells In Vitro

The stem cells maintained *in vitro* should be fed daily by completely changing the medium to renew the lost nutrients. The process of feeding every day maintains these stem cells healthy and undifferentiated. It is compulsory to clean the culture on a daily basis by removing or picking the few differentiated regions manually. The cultured stem cells when visualized under a microscope should be examined for colony size, density, quality, the color of the media, and contamination.[51] Differentiation of stem cells in the culture media may be triggered if the cells are exposed to any inappropriate stimulus.

The stem cells have all the potential to self-renew and differentiate into various types of specialized cells according to their origin. The traditional practice of stem cell culture as a monolayer 2-dimensional using culture plates needs xenogenic materials, substrates for attachment, growth factors, cytokines, and serum. The drawback of 2-dimensional cultures is that they are susceptible to pathogens, limiting the reproducibility potential due to the presence of xenogenic materials used. It often requires passaging for the maintenance and equal nutrition distribution for the self-renewing process which would be a limitation for large-scale purposes. Although 2-dimensional culture has been in use for the primary cell lines, for the standardization and analytical assays, it possibly distorts the geometry of the cell leading to the change in nuclear shape, and internal cytoskeleton which in turn results in a change in protein expression. Altogether, 2-dimensional stem cell culture practice lacks functional derivatives, has inaccurate animal physiology, and is not adequate for the validation of drug discoveries.

Differentiation of Stem Cells In Vitro

The 2-dimensional culture plates are provided with ECM materials called feeder layers facilitating their attachment. Supplementation like fibroblast growth factor-2 and transforming growth factor β1 are used for human PSCs. It is possible to generate specific single-cell lineages by the use of growth factors, ECM, and attachment substrates. There are various protocols in use for the stepwise culture and differentiation of stem cells into layers but most of them show differentiated cells with mixed populations. In the case of 3-dimensional culture, the primary step is the formation of spheroids which consists of proliferative and non-proliferative cells, and apoptotic cells. PSCs are initiated to undergo differentiation by the spheroids called the embryoid body (EBs). These EBs have

the potential to differentiate into three germ layers mimicking the development of the embryo and inducing heterogeneous differentiation. Variable size and population of the formed EBs facilitate the differentiation of germ layers accordingly. Small EBs are destined to differentiate into ectoderm and larger ones into endoderm and mesoderm layers. There is also the use of various types of natural biomaterials for the *in vitro* stem cell culture which exclusively maintains the stem cell niche, and also the self-renewal and differentiation of cells. The use of the materials will aid in incorporating the inductive signals, the integrity of scaffolds, and cell adherence. Therefore, natural biomaterials like fibronectin, agarose, collagen type1, chitosan, and synthetic polymers like polyethylene glycol, polyglycolic acid, and polycaprolactone are in use for *in vitro* stem cell culture for maintenance, expansion, and differentiation of stem cells.[52]

CSCs Metastasis

Now that CSCs characteristics and their potential to induce tumor and cellular plasticity are known these CSCs also play a vital role in establishing metastasis in various cancers. Despite no evidence of CSCs initiating metastasis, many studies favor this mechanism of metastasis being induced by CSCs. There are several supporting data exhibiting metastatic properties when there is the existence of a subpopulation of CSC markers in colorectal, pancreatic, and breast cancers. According to Fidler et al., their experiments, imply that metastasis is remarkably ineffective with <0.1% of cancer cells administered to induce metastasis. This indicates that only a few specialized cells having self-renewing properties would direct the tumorigenesis process. Hence, the CSC model favors various such experiments and studies which explain that only a specific cellular population would initiate metastasis when injected orthotopically or directly into the circulatory system. The signaling pathways regulating the self-renewing property of normal stem cells have been revealed to participate in CSCs during stemness and metastasis as well. These pathways are Sonic Hedgehog (Shh), Notch signaling, Wnt, etc. Also, the genes of cell determinants during embryogenesis are responsible to establish metastasis. The genes *KLF4, NANOG, SOX2, OCT4, SOX9*, and *SNAIL* are the promoters of stemness and also metastasis.[40] It is also thought that the primary tumors with CSCs metastasize through the EMT process.[53,54]

Epithelial to Mesenchymal Transition and Stemness

Epithelial-mesenchymal transition (EMT) is a process wherein the epithelial cells during embryonic development escape the structural constraints imposed on them and adopt a phenotype that provides more cell mobility. The EMT-like process is seen to occur during the progression of carcinoma to invasive and metastatic disease.[55] The epithelial cells generate cells having stem-like properties during the EMT process. The first link between the EMT and CSCs was established in breast cancer.[56] Later it was demonstrated that cancer cells acquire stem cell properties through a fusion mechanism with bone marrow-derived mesenchymal stem cells.[57] Chronic inflammation facilitates this fusion. These cells have properties of both EMT and self-renewing stem cells which enable the tumor cells to disseminate from the primary site and form colonies at the secondary site. Further exploring the common characteristics and the mechanisms behind the two processes will help us establish a strong basis for the development of improved anticancer therapies.

Epithelial-mesenchymal transition is almost exclusively represented as a dedifferentiation process in various cancer studies. EMT-acquired cells in breast cancer exhibit mesenchymal stem cell properties leading to the differentiation process by forming multiple lineages. As compared to normal cells acquiring EMT, this cancer-associated EMT gives rise to the cells having increased migratory properties and increased stemness. Therefore, cells enhanced by EMT exhibit 10-fold more sphere-forming capability. Studies related to breast, pancreatic, and colorectal cancer reveal that there is inhibition of CD24 by Twist. Twist and SNAIL are the EMT-inducing transcription factors that are overexpressed during the transition process implying dedifferentiation after EMT (Fig. 3).[58]

Correlation between CSCs and EMT in Cancer

One of the features of CSCs is that they can undergo both EMT and MET. Nestin, one of the EMT regulating neural stem/progenitor cells, also CSC markers in pancreatic cancer, increases the expression of E-cadherin when nestin is inhibited and vice versa when nestin is activated. Thus, to avoid metastasis, the current focus is on the EMT undergoing CSCs. Also, recent studies claim that expression or forced expression of EMT transcriptional factors like Snail, and Twist initiates CSCs features in the cancer cells present in breast cancer. In the case of pancreatic cancer, EMT induction leads to the potential gaining of self-renewal ability in the side population of the cancer cells which induces CSCs. It is becoming more apparent that there is an overlapping of EMT and CSC phenotypes facilitating tumor invasion, apoptotic resistance, and survival properties.[59]

CSC's Signaling Network

Recent studies performed an advanced in-depth analysis of dysregulated signaling pathways that govern the tumor cell progression, activation, and renewal of CSCs. The signaling pathways that have been elucidated so far are Notch, Hedgehog, Wnt/β catenin, Epsom, TGF–β family, BMI1, mullerian inhibiting substance (MIS), PTEN Lin28, and let-7 (Fig. 4).[3,41,46,60] The CD47/SIRPα has been identified as an anti-phagocytic signal contributing to tumorigenesis.[42] Any form of dysregulation or breakage in these pathways due to genetic or epigenetic alterations leads to the formation of CSCs, and are also held responsible for chemoresistance and radioresistance. Thus a detailed meticulous analysis of these pathways would help to identify the potential targets for treating malignancy. For example, Hedgehog pathway inhibitors like cyclopamine have shown dramatic results in the form of tumor regression and low rates of recurrence in mouse models with medulloblastoma, the other promising inhibitor of this pathway is HBAntag which is more efficacious than cyclopamine. These inhibitors have been non-toxic when given for a brief period.[41] Notch pathways are regulated by the enzyme γ-secretase, the inhibitors of this enzyme have been successful in counteracting the CSCs in breast cancers where this pathway is overexpressed.[43,46] The other notch inhibitors that are used as targets include amino-terminal enhancer of split (AES) and adenomatous polyposis coli (APC) genes which regulate this pathway and have shown promising results in colon cancer. Novel drug regimens include the inclusion of monoclonal antibodies like cetuximab and panitumumab or a cocktail of antibodies, to target certain mutants responsible for liver metastasis.[61,62] An effort has to be made in this direction wherein the identification of such antibodies, i.e. formulation of immunotherapy and their inclusion in combination with other signal inhibitors would be more efficacious in targeting CSCs.[62] The future of CSCs lies in formulating a combination of drug regimens with tolerable drug dosages which can effectively target different signaling pathways.

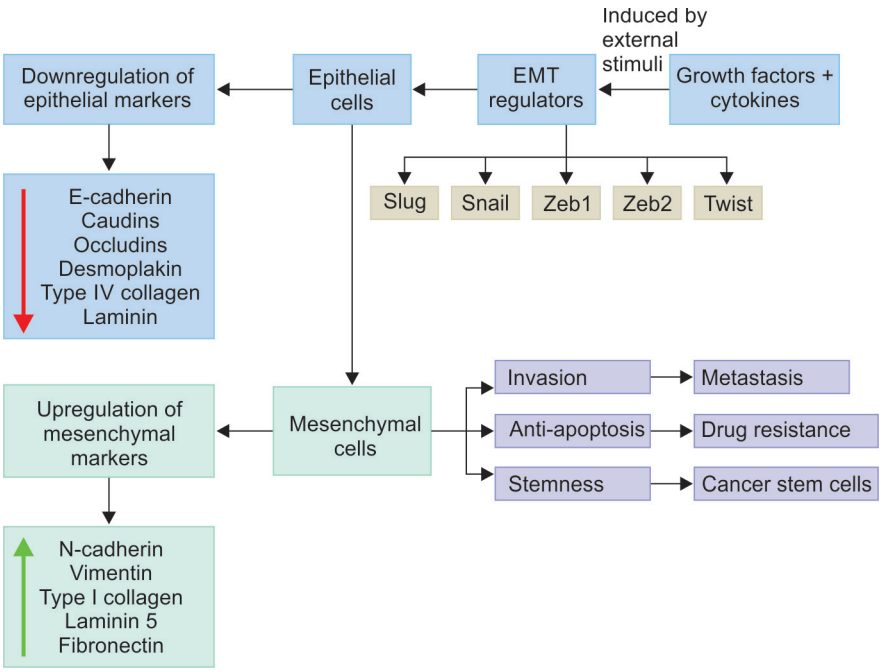

Fig. 3: Process of EMT: Diagrammatic representation of EMT initiation by external stimuli emerging from the microenvironment consisting of growth factors, cytokines, extracellular matrix, and cancer-associated fibroblast. This induced EMT transforms the epithelial cells into mesenchymal cells resulting in the downregulation of EMT markers. Therefore, promoting invasion, and anti-apoptosis stemness give rise to metastasis, resistance to drugs, and cancer stem cells respectively

STAT3 Pathway

The STAT3 pathway is of a STAT protein family, facilitates signaling by growth factors, IL-6, and IL-10 cytokines, and controls a set of genes participating in invasion and metastasis, maintaining the population of CSCs and initiating EMT in solid tumors.

Wnt/β Catenin Pathway

The Wnt/β catenin pathway is said to have a role in the maintenance of the stemness of CSCs and embryonic stem cells. This pathway involves an increased level of β-catenin and its translocation into the nucleus upon binding of Wnt to its cell Frizzled surface receptor Frizzles. β-catenin is a part of the Cadherin complex which helps in cell adhesion and migration activity. The Wnt pathway is observed to be closely associated with the EMT mechanism in CSCs.

Notch Signaling Pathway

It is involved in proliferation, cell differentiation, and apoptosis, and in the case of CSCs, it is responsible for maintaining the population of the CSCs as seen in breast and pancreatic cancers. When the notch is bound to ligands called Delta-like or Jagged, translocation of the intracellular part of the Notch into the nucleus occurs. As a result, signaling molecules like cell-cycle regulatory proteins, p^{21}, and c-Myc are produced. Studies say that an increased level of Notch aids in maintaining the stemness of CSCs and decreased levels promote progenitor amplification.[63]

Other signaling pathways include the Shh pathway and the NF-kB pathway which also participate in the facilitation of cell differentiation, proliferation, and development of chemoresistance of CSCs.

Tumor Microenvironment

The tumor microenvironment is constituted of non-malignant cells like fibroblast cells, endothelial cells, non-cellular matrix, and immune cells.[64] The presence of CSCs or progenitor cells alters the original environment to specific microenvironmental conditions that are most efficient for them. As a result, there will be an increase in tumor growth, whereas the same CSC or progenitor cells located in the non-invasive environment will not be seen initiating the tumor growth. Also, there may exist multiple niches coexisting in a single type of tumor and inducing various CSCs. The tumor microenvironment is said to regulate the destiny of treatment. Hence, there is a requirement to reconstitute the tumor condition *in vitro* to study and look for better therapeutic strategies.[65]

This *tumor niche* surrounding the cells aids in inducing the self-renewal of CSCs, also recruiting stromal cells, and immune cells to secrete various other factors initiating the invasiveness of the tumor-promoting angiogenesis. The tumor niche facilitates cell-cell communication by adherent junctions, ECM communication by integrin, and hormonal signaling. There is more literature evidence regarding this tumor microenvironment where the normal fibroblast cocultivation along with tumor cells initiated the growth of tumor cells. Another feature of this tumor microenvironment is the inflammation that promotes tumor cell proliferation and thereby metastasis. This is achieved by inhibiting the cyclin-dependent kinases (CDKs) and suppressing immunosurveillance activity by natural killer (NK) cells by recruiting other secreted factors into the microenvironment. Cancer stem cells take advantage of these factors in the normal niche for their survival support and promote the EMT pathways.

The microenvironment is governed by three factors hypoxia, angiogenesis/vasculogenesis, and reactive oxygen species (ROS) which implicate the progression and sustenance of CSCs. Thus microenvironment support provides the much-needed survival niche to CSCs against various modalities of treatment and results in recurrence and relapse of cancer.

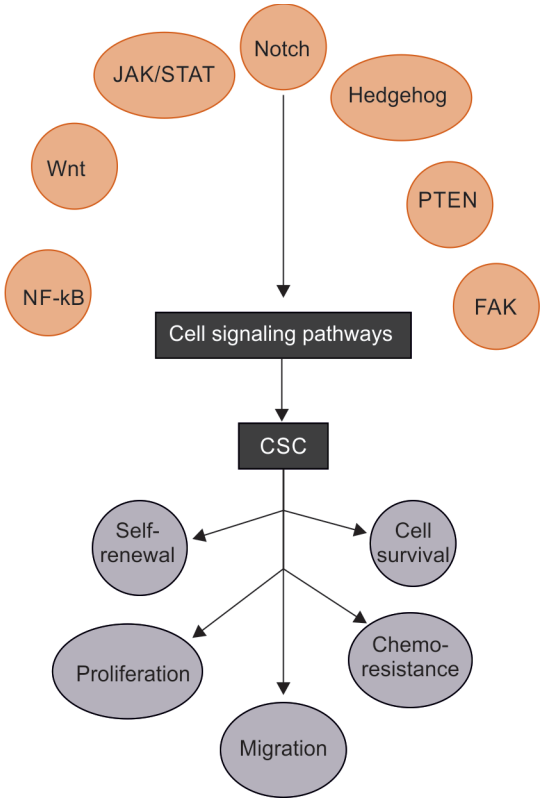

Fig. 4: Signaling pathways for the maintenance of CSCs: The signaling pathways- NF-kB, Wnt, Jak/Stat, Notch, Hedgehog, PTEN, and FAK play a major role in chemoresistance, cell survival, self-renewal, proliferation, and migration. NF-kB pathway facilitates signaling by TNF-α, toll-like receptors. Wnt, PTEN, and FAK are majorly involved in the regulation and self-renewal of stem cells and CSCs respectively. JAK/Stat and Notch pathway is initiated by growth factors, interleukins, and Delta-like, jagged receptors respectively, for the maintenance of the CSCs population. The hedgehog pathway facilitates the stemness of CSCs by responding to the excessive expression of the Hh ligand

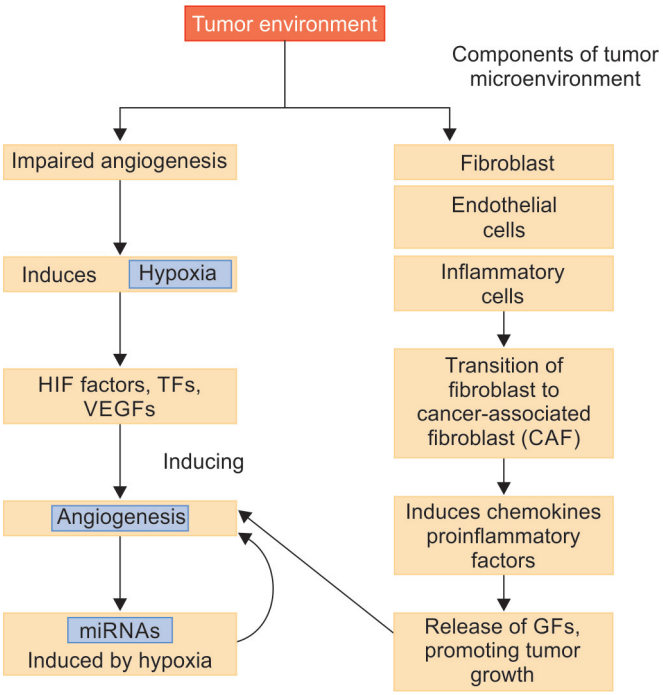

Fig. 5: Explaining the cross-talk between the tumor microenvironment and its associated factors: The relationship between the tumor microenvironment regulating factors and its components resulting in self-renewal, proliferation, and metastasis of the tumor

Hypoxia

The increased availability of ROS and activation of anti-phagocytic signals in CSC's niche preferentially result in activation of pro-survival signals, like increased production of ATP which in turn, up-regulates hypoxia-inducible factor-1α (HIF-1α) signaling. HIF-1α results in the attenuation of pro-apoptotic signals, and stimulation of certain transcriptional responses, resulting in three important results: relapse of the tumor, angiogenesis, and clonal selection of apoptosis resistance tumor cells.[4,40] This paves the way for systematic therapeutic intervention by selective treatment of hypoxic areas to eradicate CSCs by delivering selective doses of radiotherapy to avoid excess production of ROS.[4] But as the response of cells to radiotherapy is dependent on the availability of O_2, certain animal studies have been carried out using synthetic heme-based O_2 carriers which have been proven to be successful to a great extent.[40]

Angiogenesis

Tumor growth and metastasis are the two mechanisms that are dependent on angiogenesis. In the case of cancer, the formation or sprouting of new blood vessels is established by pre-existing vasculature of the tumor endothelial cells. This vascularization occurs from the subpopulation of cancer cells termed as CSCs as documented by several studies. Angiogenesis forms an important aspect of the microenvironment support system for CSCs. Studies have shown the preferential association of CSCs, especially in solid tumors near proliferating blood vessels. The proliferation of CSCs around blood vessels is partly dependent on endothelial cell signals.[4] Vascular endothelial growth factor (VEGF) is considered to be one of the most studied pathways during angiogenesis. Hypoxia is noted to be the major stimulus for the activation of angiogenesis by the induction of hypoxia-inducible factors (HIFs). These HIFs are then attributed to promoting transcription factors for the activation of the VEGF gene.[66] Studies show that there is an increase in the expression of VEGF in hypoxic conditions in the CSCs population (Fig. 5).[67]

Anti-angiogenic therapy has been a comparative novice therapeutic target and very little data is available to support the concept of anti-angiogenic therapy for hepatic and breast CSCs but has been successful to some extent in treating colorectal cancers.[4,68] The actual association between the CSCs and angiogenesis is however less understood.[69]

Reactive Oxygen Species (ROS)

Reactive oxygen species like superoxide and hydrogen peroxide responsible for both chemoresistance and radioresistance are produced in excess due to various metabolic pathways and act as a cancer-promoting factor by causing tumor initiation, increased activity of transport channels, and activation of nonphagocytic macrophages resulting in cell proliferation and survival.[5] The role of ROS's imbalance in epithelial to mesenchymal transition, dysregulated miRNA expression, and hypoxia is pointing toward the increasing need of including antioxidant agents in treating malignancies.[5,68] More evidence is drawn wherein the ROS induces oncoproteins that stimulate anti-apoptotic pathways. Thus it is

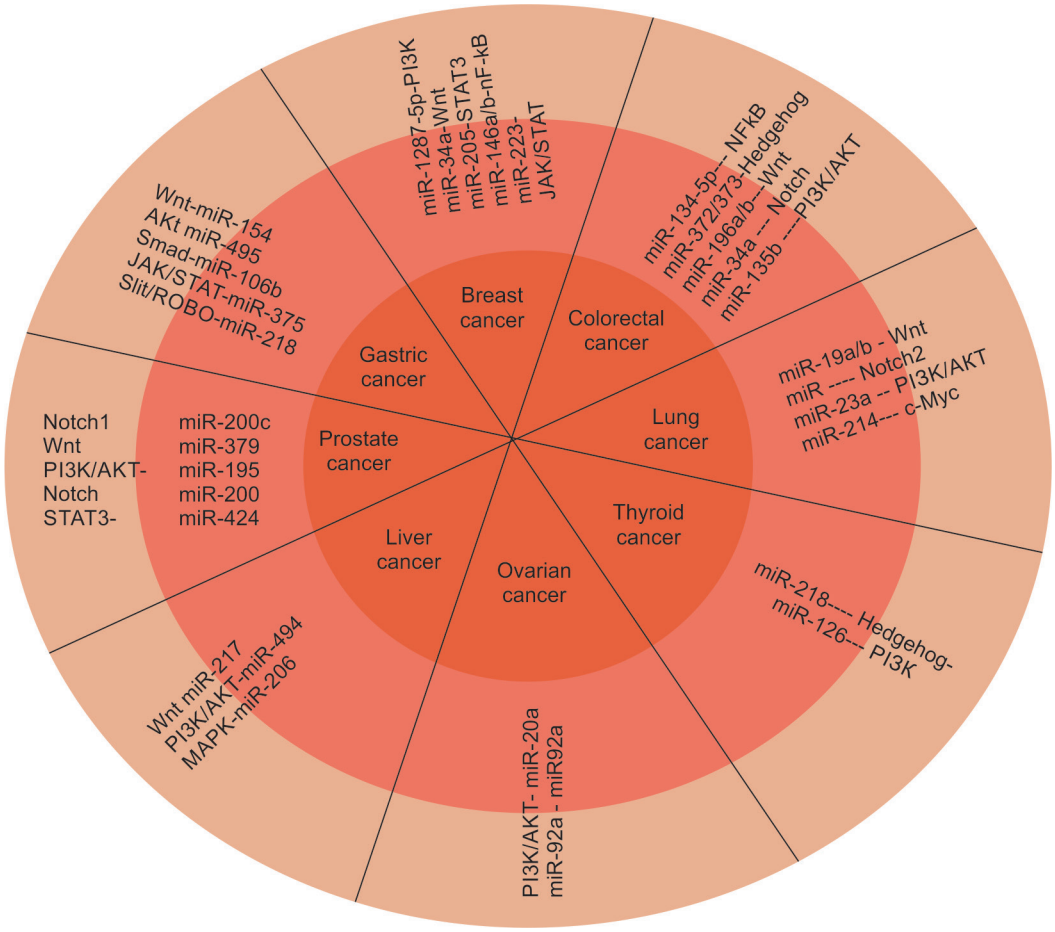

Fig. 6: Representation of miRNAs involved in regulating CSCs in different types of cancer through their respective signaling pathways: Depicting the majority of miRNAs that are deregulated in various types of cancers and their responsible signaling pathways

this imbalance in redox potentials that is critical for the survival of CSCs. $CD133^{+}$ CSCs in brain cancer have been identified to utilize the above dysregulated ROS pathway for its recurrence.[3]

One of the promising approaches to alter the microenvironment and make it less favorable for CSCs has been the effective utilization of oncolytic viruses as vectors to carry transgenes to alter the microenvironment and thus effectively eradicate the CSCs in addition to slaying general tumor cells. The mechanism of action of virotherapy primarily depends on three factors: induction of tumor-specific cytotoxic T lymphocytes, tumor vascular shutdown, and chemosensitization. Adenovirus-based oncolytic vectors or replicative adenovirus (CRAd) have been able to fulfill all these three factors with favorable phase I to phase III clinical trial results but with certain accompanying disadvantages.[60] The components of the tumor microenvironment consist of an array of fibroblasts, inflammatory cells, and endothelial cells. The fibroblasts are driven to a cancer-associated state by inducing chemokines and inflammatory factors. Apart from these tumor microenvironment factors, miRNA dysregulation is reported in the transition of fibroblasts into cancer-associated fibroblasts. Their characteristic pattern of expression may serve as a signature in identifying various cancer types.

MicroRNA Expression

MicroRNA plays a vital role in gene expression by interacting with mRNA, and hence deregulation of the miRNA expression is a ubiquitous feature associated with cancer and forms a part of the CSC's microenvironment supporting system (Fig. 6). Some of the factors governed and regulated by miRNA expression are epigenetic modifications, EMT, affecting the cell sensitivity to different chemotherapeutic agents thus implied in drug resistance, radioresistance, acting as oncogenes, inducing CSC dysregulation resulting in more aggressive tumor progression and tumor relapse.[4,40,70–72] These factors thus have opened the gates for the utilization of miRNA as new therapeutic targets for treating cancer. Drugs that affect the feedback loop of miRNA expression might be a promising treatment modality for cancer and hence clinical trials are being designed with miRNA as a target, especially in cases of drug resistance. Epigenetic therapy has been an emerging treatment modality with drugs such as demethylating agents designed to tackle the epigenetic changes seen in glioblastoma stem cells (GSCs) which potentially activate the silenced tumor suppressor genes.[4,12]

Experimental evidence accumulated in the past few years supports the existence of CSC and the role played by them in tumor growth, metastases, and the development of drug resistance. Isolating CSCs and developing therapies to eliminate them may have a remarkable clinical outcome. As a whole CSC's support system is a complex network system that has to be embarked upon with utmost provision to:

- Identification of specific gene signatures so that it is easier to differentiate the molecular variances between CSC and tissue-specific stem cells to ensure less damage to normal

somatic stem cells but at the same time warrant selective targeting of CSC,
- Discovery of drugs which target key signaling pathways, kill differentiated cells, specifically and selectively target CSC's, disrupt the self-protection potential of CSC, avoid toxic effects,
- Devise better modes of drug delivery like nanotubes and liposomes,
- Look into newer modalities of treatment such as disruption of the microenvironment, epigenetic therapy, immunotherapy, endocrine therapy and virotherapy to combat cancer more successfully.

Conclusion

Cancer stem cells drive most cancers, necessitating their identification and targeting for remission. Despite diverse treatments like chemotherapy, surgery, and radiotherapy, CSCs resist, posing a challenge. Research increasingly focuses on CSCs due to treatment failure leading to tumor progression, recurrence, and metastasis. Relapse cases are aggressive, highlighting the need to target CSCs for tumor eradication. However, complexities including surface markers, stem cell signaling, microenvironment cues, and miRNA expression pose challenges. Deciphering and targeting this signaling network is critical for the future of cancer treatment.

References

1. Terskikh AV, Easterday MC, Li L, et al. From hematopoiesis to neuropoiesis: Evidence of overlapping genetic programs. Proc Natl Acad Sci USA 2001;98(14):7934–7939. DOI: 10.1073/pnas.131200898.
2. Al-Hajj M, Wicha MS, Benito-Hernandez A, et al. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 2003;100(7):3983–3988. DOI: 10.1073/pnas.0530291100.
3. Tang C, Ang BT, Pervaiz S. Cancer stem cell: Target for anti-cancer therapy. FASEB J 2007;21(14):3777–3785. DOI: 10.1096/fj.07-8560rev.
4. Zhao L, Zhao Y, Bao Q, et al. Clinical implication of targeting of cancer stem cells. Eur Surg Res 2012;49(1):8–15. DOI: 10.1159/000339610.
5. Bao B, Azmi A, Li Y, et al. Targeting CSCs in tumor microenvironment: The potential role of ROS-associated miRNAs in tumor aggressiveness. Curr Stem Cell Res Ther 2013;9(1):22–35. DOI: 10.2174/1574888x113089990053.
6. Sell S. Infection, stem cells and cancer signals. Curr Pharm Biotechnol 2011;12(2):182–188. DOI: 10.2174/138920111794295675.
7. Franco S, Szczesna K, Iliou MS, et al. In vitro models of cancer stem cells and clinical applications. BMC Cancer 2016;16(Suppl2):738. DOI: 10.1186/s12885-016-2774-3.
8. Ayob AZ, Ramasamy TS. Cancer stem cells as key drivers of tumour progression. J Biomed Sci 2018;25(1):20. DOI: 10.1186/s12929-018-0426-4.
9. Hope KJ, Jin L, Dick JE. Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. Nat Immunol 2004;5(7):738–743. DOI: 10.1038/ni1080.
10. Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: Accumulating evidence and unresolved questions. Nat Rev Cancer 2008;8(10):755–768. DOI: 10.1038/nrc2499.
11. Iliopoulos D, Hirsch HA, Wang G, et al. Inducible formation of breast cancer stem cells and their dynamic equilibrium with non-stem cancer cells via IL6 secretion. Proc Natl Acad Sci USA 2011;108(4):1397–1402. DOI: 10.1073/pnas.1018898108.
12. Ishiguro T, Ohata H, Sato A, et al. Tumor-derived spheroids: Relevance to cancer stem cells and clinical applications. Cancer Sci 2017;108(3):283–289. DOI: 10.1111/cas.13155.
13. Ponti D, Costa A, Zaffaroni N, et al. Isolation and *In vitro* propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. Cancer Res 2005;65(13):5506–5511. DOI: 10.1158/0008-5472.CAN-05-0626.
14. Shackleton M, Vaillant F, Simpson KJ, et al. Generation of a functional mammary gland from a single stem cell. Nature 2006;439(7072):84–88. DOI: 10.1038/nature04372.
15. Hassan G, Afify SM, Nair N, et al. Hematopoietic cells derived from cancer stem cells generated from mouse induced pluripotent stem cells. Cancers (Basel) 2019;12(1):82. DOI: 10.3390/cancers12010082.
16. Schiavone K, Garnier D, Heymann MF, et al. The heterogeneity of osteosarcoma: The role played by cancer stem cells. Adv Exp Med Biol 2019;1139:187–200. DOI: 10.1007/978-3-030-14366-4_11.
17. Uchida N, Buck DW, He D, et al. Direct isolation of human central nervous system stem cells. Proc Natl Acad Sci USA 2000;97(26):14720–14725. DOI: 10.1073/pnas.97.26.14720.
18. Ignatova TN, Kukekov VG, Laywell ED, et al. Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers in vitro. Glia 2002;39(3):193–206. DOI: 10.1002/glia.10094.
19. Singh SK, Clarke ID, Terasaki M, et al. Identification of a cancer stem cell in human brain tumors. Cancer Res 2003;63(18):5821–5828. PMID: 14522905.
20. Tsai RYL. A molecular view of stem cell and cancer cell self-renewal. Int J Biochem Cell Biol 2004;36(4):684–694. DOI: 10.1016/j.biocel.2003.10.016.
21. Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumour initiating cells. Nature 2004;432(7015):396–401. DOI: 10.1038/nature03128.
22. Beier D, Hau P, Proescholdt M, et al. $CD133^+$ and $CD133^-$ glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. Cancer Res 2007;67(9):4010–4005. DOI: 10.1158/0008-5472.CAN-06-4180.
23. O'Brien CA, Pollett A, Gallinger S, et al. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 2007;445(7123):106–110. DOI: 10.1038/nature05372.
24. Ricci-Vitiani L, Lombardi DG, Pilozzi E, et al. Identification and expansion of human colon-cancer-initiating cells. Nature 2007;445(7123):111–115. DOI: 10.1038/nature05384.
25. Shmelkov SV, Butler JM, Hooper AT, et al. CD133 expression is not restricted to stem cells, and both $CD133^+$ and $CD133^-$ metastatic colon cancer cells initiate tumors. J Clin Invest 2008;118(6):2111–2120. DOI: https://doi.org/10.1172/JCI34401.
26. Dalerba P, Dylla SJ, Park IK, et al. Phenotypic characterization of human colorectal cancer stem cells. Proc Natl Acad Sci USA 2007;104(24):10158–10163. DOI: 10.1073/pnas.0703478104.
27. Medema JP. Cancer stem cells: The challenges ahead. Nat Cell Biol 2013;15(4):338–344. DOI: 10.1038/ncb2717.
28. Akbarzadeh M, Maroufi NF, Tazehkand AP, et al. Current approaches in identification and isolation of cancer stem cells. J Cell Physiol 2019;234(9):14759–14772. DOI: 10.1002/jcp.28271.
29. Dobbin ZC, Landen CN. Isolation and characterization of potential cancer stem cells from solid human tumors—potential applications. Curr Protoc Pharmacol 2013;63:14.28.1–14.28.19. DOI: 10.1002/0471141755.ph1428s63.
30. Kondo T, Setoguchi T, Taga T. Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. Proc Natl Acad Sci USA 2004;101(3):781–786. DOI: 10.1073/pnas.0307618100.
31. Goodell MA, Brose K, Paradis G, et al. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 1996;183(4):1797–1806. DOI: 10.1084/jem.183.4.1797.
32. Zhou S, Schuetz JD, Bunting KD, et al. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med 2001;7(9):1028–1034. DOI: 10.1038/nm0901-1028.
33. Haraguchi N, Utsunomiya T, Inoue H, et al. Characterization of a side population of cancer cells from human gastrointestinal system. Stem Cells 2006;24(3):506–513. DOI: 10.1634/stemcells.2005-0282.
34. Balicki D. Moving forward in human mammary stem cell biology and breast cancer prognostication using ALDH1. Cell Stem Cell 2007;1(5):485–487. DOI: 10.1016/j.stem.2007.10.015.

35. Liu W Hui, Qian N Song, Li R, et al. Replacing Hoechst33342 with Rhodamine123 in isolation of cancer stem-like cells from the MHCC97 cell line. Toxicol In Vitro 2010;24(2):538–545. DOI: 10.1016/j.tiv.2009.11.008.
36. Ginestier C, Hur MH, Charafe-Jauffret E, et al. ALDH1 Is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 2007;1(5):555–567. DOI: 10.1016/j.stem.2007.08.014.
37. Bandhavkar S. Cancer stem cells: A metastasizing menace! Cancer Med 2016;5(4):649–655. DOI: 10.1002/cam4.629.
38. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 1997;3(7):730–737. DOI: 10.1038/nm0797-730.
39. Snyder V, Reed-Newman TC, Arnold L, et al. Cancer stem cell metabolism and potential therapeutic targets. Front Oncol 2018;8:203. DOI: 10.3389/fonc.2018.00203.
40. Oishi N, Wang XW. Novel therapeutic strategies for targeting liver cancer stem cells. Int J Biol Sci 2011;7(5):517–535. DOI: 10.7150/ijbs.7.517.
41. Effendi K, Yamazaki K, Fukuma M, et al. Overexpression of leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) represents a typical wnt/β-catenin pathway-activated hepatocellular carcinoma. Liver Cancer 2014;3(3–4):451–457. DOI: 10.1159/000343873.
42. Chao MP, Weissman IL, Majeti R. The CD47–SIRPα pathway in cancer immune evasion and potential therapeutic implications. Curr Opin Immunol 2012;24(2):225–232. DOI: 10.1016/j.coi.2012.01.010.
43. Kaur S, Singh G, Kaur K. Cancer stem cells: An insight and future perspective. J Cancer Res Ther 2014;10(4):846–852. DOI: 10.4103/0973-1482.139264.
44. Tu L, Foltz G, Lin E, et al. Targeting stem cells-clinical implications for cancer therapy. Curr Stem Cell Res Ther 2009;4(2):147–153. DOI: 10.2174/157488809788167373.
45. Mikhail S. Stem cells in gastrointestinal cancers: The road less travelled. World J Stem Cells 2014;6(5):606. DOI: 10.4252/wjsc.v6.i5.606.
46. Wicha MS, Liu S, Dontu G. Cancer stem cells: An old idea—a paradigm shift. Cancer Res 2006;66(4):1883–1890. DOI: 10.1158/0008-5472.CAN-05-3153.
47. Kavyasudha C, Joseph JP, Jayaraj R, et al. Conventional and emerging markers in stem cell isolation and characterization. In 201913:1–14. DOI: https://doi.org/10.1007/5584_2019_475.
48. Xiong J, Yan L, Zou C, et al. Integrins regulate stemness in solid tumor: An emerging therapeutic target. J Hematol Oncol 2021;14(1):177. DOI: 10.1186/s13045-021-01192-1.
49. Jaggupilli A, Elkord E. Significance of CD44 and CD24 as cancer stem cell markers: An enduring ambiguity. Clin Dev Immunol 2012;2012:708036. DOI: 10.1155/2012/708036.
50. Fulawka L, Donizy P, Halon A. Cancer stem cells – the current status of an old concept: Literature review and clinical approaches. Biol Res 2014;47(1):66. DOI: 10.1186/0717-6287-47-66.
51. Kent L. Culture and maintenance of human embryonic stem cells. J Vis Exp 2009 Dec 22;(34):1427. DOI: 10.3791/1427.
52. McKee C, Chaudhry GR. Advances and challenges in stem cell culture. Colloids Surf B Biointerfaces 2017;159:62–77. DOI: 10.1016/j.colsurfb.2017.07.051.
53. Celià-Terrassa T, Jolly MK. Cancer stem cells and epithelial-to-mesenchymal transition in cancer metastasis. Cold Spring Harb Perspect Med 2020;10(7):a036905. DOI: 10.1101/cshperspect.a036905.
54. Brungs D, Aghmesheh M, Vine KL, et al. Gastric cancer stem cells: Evidence, potential markers, and clinical implications. J Gastroenterol 2016;51(4):313–326. DOI: 10.1007/s00535-015-1125-5.
55. Nieto MA. The ins and outs of the epithelial to mesenchymal transition in health and disease. Annu Rev Cell Dev Biol 2011;27(1):347–376. DOI: 10.1146/annurev-cellbio-092910-154036.
56. Mani SA, Guo W, Liao MJ, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 2008;133(4):704–715. DOI: 10.1016/j.cell.2008.03.027.
57. Xu MH, Gao X, Luo D, et al. EMT and acquisition of stem cell-like properties are involved in spontaneous formation of tumorigenic hybrids between lung cancer and bone marrow-derived mesenchymal stem cells. PLoS One 2014;9(2):e87893. DOI: 10.1371/journal.pone.0087893.
58. Wang H, Unternaehrer JJ. Epithelial-mesenchymal transition and cancer stem cells: At the crossroads of differentiation and dedifferentiation. Dev Dyn 2019;248(1):10–20. DOI: 10.1002/dvdy.24678.
59. Batlle E, Clevers H. Cancer stem cells revisited. Nat Med 2017;23(10):1124–1134. DOI: 10.1038/nm.4409.
60. Vincent Z, Urakami K, Maruyama K, et al. CD133-positive cancer stem cells from colo205 human colon adenocarcinoma cell line show resistance to chemotherapy and display a specific metabolomic profile. Genes Cancer 2014;5(7–8):250–260. DOI: 10.18632/genesandcancer.23.
61. Lindeman GJ, Visvader JE. Insights into the cell of origin in breast cancer and breast cancer stem cells. Asia Pac J Clin Oncol 2010;6(2):89–97. DOI: 10.1111/j.1743-7563.2010.01279.x.
62. Garza-Treviño EN, Said-Fernández SL, Martínez-Rodríguez HG. Understanding the colon cancer stem cells and perspectives on treatment. Cancer Cell Int 2015;15(1):2. DOI: 10.1186/s12935-015-0163-7.
63. Raggi C, Mousa HS, Correnti M, et al. Cancer stem cells and tumor-associated macrophages: A roadmap for multitargeting strategies. Oncogene 2016;35(6):671–682. DOI: 10.1038/onc.2015.132.
64. Roato I, Ferracini R. Cancer stem cells, bone and tumor microenvironment: Key players in bone metastases. Cancers (Basel) 2018;10(2):56. DOI: 10.3390/cancers10020056.
65. Klimkiewicz K, Weglarczyk K, Collet G, et al. A 3D model of tumour angiogenic microenvironment to monitor hypoxia effects on cell interactions and cancer stem cell selection. Cancer Lett 2017;396:10–20. DOI: 10.1016/j.canlet.2017.03.006.
66. Frentzas S, Lum C, Chen TY. Angiogenesis and its role in the tumour microenvironment: A target for cancer therapy. Current Cancer Treatment. 2020. DOI: 10.5772/intechopen.89667.
67. Markowska A, Sajdak S, Markowska J, et al. Angiogenesis and cancer stem cells: New perspectives on therapy of ovarian cancer. Eur J Med Chem 2017;142:87–94. DOI: 10.1016/j.ejmech.2017.06.030.
68. O'Brien CS, Farnie G, Howell SJ, et al. Breast cancer stem cells and their role in resistance to endocrine therapy. Horm Cancer 2011;2(2):91–103. DOI: 10.1007/s12672-011-0066-6.
69. Fujita K, Akita M. Tumor angiogenesis: A focus on the role of cancer stem cells. Physiologic and Pathologic Angiogenesis - Signaling Mechanisms and Targeted Therapy. 2017. DOI: 10.5772/66402.
70. Tsujii M. Search for novel target molecules for the effective treatment or prevention of colorectal cancer. Digestion 2012;85(2):99–102. DOI: 10.1159/000334678.
71. Safa AR, Saadatzadeh MR, Cohen-Gadol AA, et al. Glioblastoma stem cells (GSCs) epigenetic plasticity and interconversion between differentiated non-GSCs and GSCs. Genes Dis 2015;2(2):152–163. DOI: 10.1016/j.gendis.2015.02.001.
72. Shih JY, Yang PC. The EMT regulator slug and lung carcinogenesis. Carcinogenesis 2011;32(9):1299–1304. DOI: 10.1093/carcin/bgr110.