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# Cancer stem cells: understanding tumor hierarchy and heterogeneity

Jeremy N. Rich, MD, MHSc*

**Abstract**

Heterogeneity within and between tumors is a well-known phenomenon that greatly complicates the diagnosis and treatment of cancer. A large body of research indicates that heterogeneity develops through time as tumor-initiating stem cells, also known as cancer stem cells (CSCs), evolve genetic or epigenetic alterations that allow them to differentiate into multiple tumor cell types. Similar to normal stem cells, CSCs can self-renew and possess long-term repopulation potential. However, unlike normal stem cells, CSCs are not subject to the usual controls that limit growth. Different models have been postulated to explain the heterogeneity of tumors, but it is widely agreed that interactions between tumor cells and their microenvironment create niches that promote CSC properties and enable their survival. Within the microenvironment, CSC self-renewal, replication, and differentiation are postulated to produce a hierarchy of cells constituting the tumor mass. Increased understanding of the factors that create and contribute to tumor heterogeneity may support the design of therapies that affect CSC function and their microenvironments.

**Abbreviations:** ALL = acute lymphoblastic leukemia, AML = acute myeloid leukemia, CE = clonal evolution, CRC = colorectal cancer, CSC = cancer stem cell, EMT = epithelial-mesenchymal transition, GBM = glioblastoma, HGF = hepatocyte growth factor, IL = interleukin, NSC = normal stem cell, VEGF = vascular endothelial growth factor.

**Keywords:** cancer stem cell, differentiation, heterogeneity, plasticity, self-renewal, stochastic model, tumorigenicity

## 1. Introduction

It has long been known that tumors are not homogenous masses of cells. Given the assortment of cell and tissue types known to exist within tumors, recent research has focused on the generation of tumor heterogeneity. Among the early investigators of the 1800s, Virchow and Cohnheim postulated the existence of cancer stem cells (CSCs) that arise from what they believed to be "activation of dormant embryonic tissue remnants."[1]

Ultimately, proof of the existence of CSCs came in 1997 when Bonnet and Dick isolated a set of stem cells from acute myeloid leukemia (AML) and the samples were capable of transferring AML to immunosuppressed mice.[2] As further advances have enabled deeper and more detailed investigations, tumor heterogeneity has been found to exist at the molecular and genetic levels, even among cancer cells that appear microscopically or otherwise identical.[3] Although the existence of CSCs is now well known and accepted, their properties, role in various tumors, and frequency with which they occur in various tumors still remain the subject of active debate and investigation.

This review will discuss: Models of tumor heterogeneity, Historic perspective on CSCs, Functional properties of CSCs, Similarities to and differences from normal stem cells (NSCs), and the Role of the microenvironment.

## 2. Tumor heterogeneity overview

Tumor heterogeneity can be described as intertumoral heterogeneity or intratumoral heterogeneity. Intertumoral heterogeneity can be defined as variations observed between tumors of different tissue and cell types; variations observed between tumors of the same tissue type from diverse patients; and variations observed between different tumors within the same individual. Intratumoral heterogeneity refers to variations observed within a single tumor.[4]

Numerous experiments have pointed to the existence of tumor heterogeneity. Tumor cell populations differed in such properties as[3]:

- Cell surface markers
- Genetic or epigenetic changes
- Genetic stability
- Resistance or susceptibility to therapy
- Growth rates

### 2.1. Intertumoral heterogeneity

Intertumoral heterogeneity provides the basis for classifying cancer types and subtypes by differences in histology, genetic profiles, protein signatures, or expression of specific markers. Many of these variables provide clinically prognostic and/or predictive information.[5] On the other hand, intratumoral heterogeneity complicates cancer prognosis and treatment.[6]

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*Department of Stem Cell Biology and Regenerative Medicine, Lerner Research Institute, Cleveland Clinic, Cleveland, OH.*

* *Correspondence: Jeremy N. Rich, Cleveland Clinic, Cleveland, OH 44195 (e-mail: richj@ccf.org).*

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### 2.2. Intratumoral heterogeneity

Early evidence of intratumor heterogeneity came from experiments in which tumor cells from murine cancers were extracted and introduced into syngeneic hosts.[7,8] It was determined that not all transplanted cells could reconstitute tumors.[7,8] Similarly, varying numbers of metastatic colonies resulting from injection of syngeneic mice with murine melanoma tumor-derived cells suggested that the parent tumor harbored cells with diverse metastatic potential.[9]

In small sets of studies in patients with terminal cancer, tumors were autotransplanted subcutaneously and even then did not initiate new tumors in every case.[10,11] In glioblastoma (GBM), analysis of tumor fragments from the same tumor revealed various gene losses and amplifications.[12] Additionally, results from mitotic heterogeneity experiments and gene expression signature analysis demonstrated complex cell clonal population hierarchies and the presence of multiple GBM subtypes within a single tumor, respectively. These results clearly indicated the presence of multiple clonal cell populations within the tumors.[12]

Intratumor heterogeneity was strongly demonstrated at the genetic level in renal cell carcinoma. Study results showed that samples from different regions of the same tumor often displayed varying mutations and chromosomal imbalances. In addition, gene expression signatures of good and poor prognosis were found in different regions of the same tumor, suggesting that intratumor heterogeneity also exists at the RNA-expression level.[13]

Intratumor heterogeneity is postulated to develop across time as CSCs divide and differentiate asymmetrically.[14,15] The loss of normal cellular controls allows the development and propagation of genetic or epigenetic alterations that give the cells novel properties associated with metastasis, self-renewal, treatment resistance, and recurrence.[14,16]

As stated, intratumoral heterogeneity complicates cancer prognosis and treatment. Two reasons are: biopsy samples used for diagnosis are taken from small regions of tumors that may not be representative of the entire lesion[6]; and cells may adopt new functional properties and biomarker expression patterns as the disease progresses.[5,17] Thus, treatment choices based upon a single biopsy taken at a single time point may only be effective for some cells within a tumor population. This may result in the expansion of treatment-resistant cell populations through time.[6,18]

As the presence of multiple clonal subpopulations within the same tumor imparts different phenotypes, such as growth advantages or treatment resistance, a substantial therapeutic challenge exists, as only some cells within a tumor would be affected by any one treatment.[18] Logically, combination therapeutic regimens targeting both the bulk of tumor cells as well as CSCs could be an effective approach to improve long-term treatment outcomes.[19]

Recent research has shown an increase in the number and activity of CSCs in response to radiation.[20,21] The effect of radiation on CSCs was counteracted with agents shown to inhibit mammosphere formation or expression of stemness-related genes.[20,21] In both studies, use of these agents led to reductions in the number of radiation-induced CSCs.[20,21]

## 3. Models of tumor heterogeneity

Two models currently predominate to explain or envisage the origin, maintenance, progression, and heterogeneity of tumors (Fig. 1).[5,6,22] They are known as the stochastic or clonal evolution (CE) model, and the hierarchy or CSC model.[5,6,22] Although these models are dissimilar and place differing weight on the importance of stem cells and the microenvironment, they are not mutually exclusive.[5,6,22,23]

### 3.1. CE model

According to the CE model, malignant cells are biologically equivalent initially.[24] Because the cells are genetically unstable, they can, however, accumulate genetic and epigenetic alterations through time; these changes may increase tumor aggressiveness, invasiveness, treatment resistance, or other characteristics.[5,6] Selection for these new cellular traits then drives tumor progression and increases tumor heterogeneity.[5,6] A corollary of the CE model is that tumor-initiating activity cannot be isolated or enriched by cell-sorting methods based upon intrinsic characteristics of a subpopulation of tumor cells.[24]

### 3.2. CSC model

In contrast with the CE model, the CSC model holds that a minority subpopulation of stem cells within a tumor are able to self-renew and differentiate into a variety of cell types, each with its own abilities and phenotypes.[5,6,22,25] As a result of the processes of differentiation of stem cells, tumors are composed of a hierarchy of cell types that include highly tumorigenic CSCs that give rise to intermediate progenitors and terminally differentiated progeny.[5] These CSCs are, therefore, the source of tumor initiation and heterogeneity.[6] Further in contrast with the CE model, the CSC model predicts that intrinsic properties of CSCs can be used to identify and purify CSCs from the total tumor cell population.[24]

### 3.3. Plasticity model

As stated earlier, the CE and CSC models are not mutually exclusive. An alternative model of reversible, cellular plasticity provides a unifying framework to tie the CE and CSC models together by postulating that cancer cells can interconvert between stem cell and differentiated states (Fig. 1).[22]

According to the plasticity model, intrinsic tumor cell processes and/or stimuli within the tumor microenvironment could influence differentiated tumor cells to reacquire stem cell characteristics.[6,23,26] Conversely, these processes could also drive CSCs toward differentiation into nonstem cancer cells.[23]

Cancer cells in general display higher intrinsic or spontaneous plasticity than normal cells and observation has linked plasticity and stemness with regulation of the epithelial-mesenchymal transition (EMT) process.[17,26]

Experimental evidence from several studies has demonstrated conditions that can induce the transition of nonstem cancer cells to a CSC phenotype. Such results show that tumor stemness and the EMT process can be transient and reversible in CSCs and nonstem cancer cell populations.

- Human lung cancer cell lines expressing both epithelial and mesenchymal markers switched to a stem cell-like state following treatment with transforming growth factor beta 1, an EMT stimulator.[26]
- Immortalized and transformed human mammary epithelial cells switched between nonstem and CSC-like states according to modulation of ZEB1, an EMT transcription factor.[27]
- Tumor-associated myofibroblasts secreted hepatocyte growth factor (HGF), a potent EMT inducer, to induce stemness phenotypes in nonstem colon cancer cells.[28]

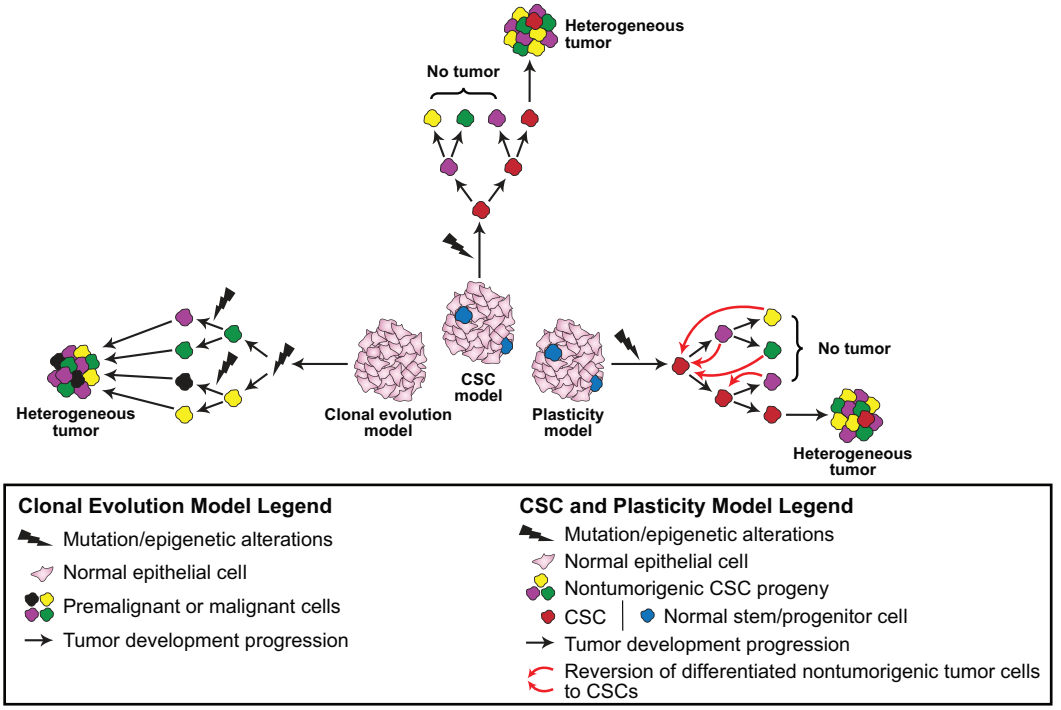


**Figure 1.** CE (stochastic) vs. CSC (hierarchy) vs. plasticity models—in the CE model, mutations accumulate through time and any cell may have tumorigenic potential.[6,22] However, this potential cannot be isolated or enriched.[24] In the CSC model, only stem cells possess tumorigenic potential while differentiated cells have little or none.[6,22,25] According to the plasticity model, differentiation can be bidirectional so that differentiated nontumorigenic cancer cells may revert back to CSCs. CE = clonal evolution, CSC = cancer stem cell.[22,23]

## 4. Historic perspective on CSCs

The theory that cancers arose from a small population of stem cells was first proposed >150 years ago by Virchow and Cohnheim, who believed that these CSCs were reactivated from "dormant embryonic tissue remnants." Many decades later, researchers in the 1970s advanced the theory that tissue-specific stem cells might be the cells of origin for specific cancers. Further technical advances were needed before it would be possible to finally produce decisive, experimental proof for the CSC hypothesis.[1]

Proof came in 1997 with the work of Bonnet and Dick,[2] who demonstrated the existence of "leukemic-initiating cells" in AML. These cells possessed the abilities to differentiate, proliferate, and self-renew. A subsequent study suggested that similar to NSCs, leukemia CSCs vary in self-renewal potential and exist in a developmentally regulated hierarchy. These findings supported the idea that CSCs derive from malignant transformation of NSCs.[29] Further research demonstrated the existence of CSCs in many other tumor types, including brain, prostate, lung, breast, and multiple myeloma.[29]

The existence of CSCs has become widely accepted and researched to understand their properties and how they may function in disease and be targeted for therapy. Alternative terms for CSC that may be used in the literature include "tumor-initiating cell" and "tumorigenic cell." In 2006, a workshop of the American Association for Cancer Research established the definition of a CSC as "a cell within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor."[30]

## 5. Functional properties of CSCs

CSCs are defined primarily by their functions, although much research has been conducted to discover phenotypic markers that can be used to identify and separate CSCs from other tumor cells.[31]

The major functional capacities of CSCs are self-renewal, tumor initiation, and long-term tumor repopulation potential.[22,32] For example, injection of approximately 3 AML CSCs into immunosuppressed mice could initiate tumor formation and yield a 30- to 100-fold expansion of the cells in engrafted AML for up to 8 weeks, indicating significant self-renewal.[2] In a *Wnt-1* proto-oncogene activation mouse breast cancer model, CSCs made up 1% to 4% of primary tumor cells and subsequent engraftment of as few as 50 CSCs into secondary and tertiary mice resulted in tumors with similar ratios of tumorigenic to nontumorigenic cell populations as those in primary tumors. The data indicate that breast CSCs can repeatedly initiate tumor formation to repopulate tumors with heterogeneity similar to that of primary tumors across long periods.[33]

Because of their capacity for self-renewal and uncontrolled amplification, CSCs can differentiate into large heterogeneous populations of tumor cells with altered phenotypes that impart treatment resistance and propagate and maintain tumors.[14] In glioblastoma, unfractionated patient tumor cells isolated from radiation-treated mouse xenografts were reported to be significantly enriched in CSCs, and formed tumors with reduced latency in secondary mice compared with untreated controls.[34] CSCs isolated from irradiated xenograft tumors formed more colonies, had lower rates of apoptosis, and displayed enhanced DNA damage response compared with untreated controls.[34] These

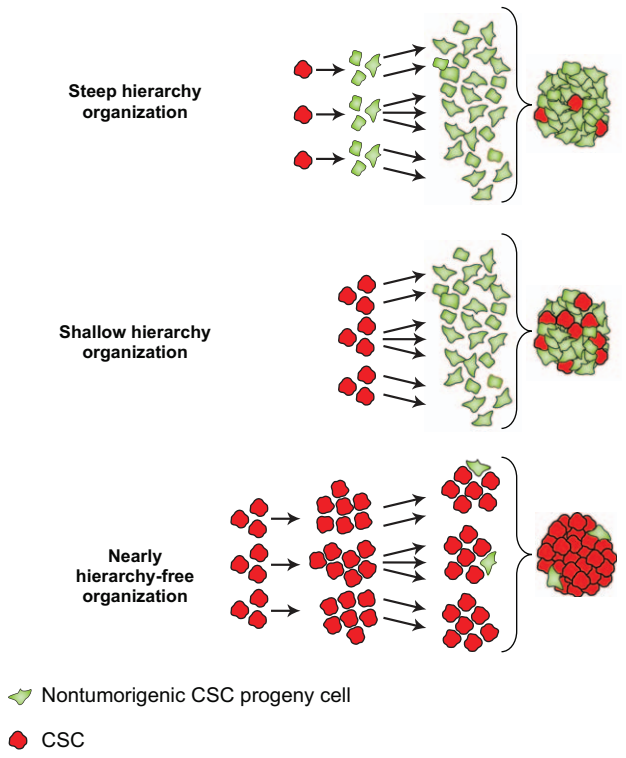


**Figure 2.** Tumor cell hierarchical organization. Tumor cellular hierarchies can vary in depth such that CSCs are relatively rare (top panel), common (middle panel), or even constitute the majority of tumor cells (bottom panel). CSC = cancer stem cell.[25]

data collectively indicate that radiotherapy-resistant glioma CSCs may be enriched after treatment and may support posttreatment disease recurrence.[34] CSCs have been demonstrated to be involved in drug resistance in other types of cancer. CSCs were found to be enriched after in vitro culturing of chemotherapy-treated, patient-derived primary breast tumor cells compared with cultures of chemotherapy-naïve controls.[35] Cell counts of paired pre- and postchemotherapy treatment clinical tumor biopsy samples indicated an approximate 10-fold increase in CSC frequency in posttreatment samples, suggesting that chemotherapy may promote CSC survival.[35] Similarly, CSCs were found to be enriched by approximately 2-fold after chemotherapy treatment of immunocompromised mice-bearing xenografted colorectal cancer (CRC) tumors derived from serially transplanted, in vivo-passaged CRC cell lines.[36] Furthermore, gene expression analysis of CSCs isolated from chemotherapy-treated tumors indicated high expression of the gene encoding aldehyde dehydrogenase, a known CSC marker.[36] Genetic ablation of the aldehyde dehydrogenase gene in unfractionated CRC cells sensitized tumors to chemotherapy without affecting tumorigenicity or pretreatment tumor growth kinetics.[36] These data indicate that CSCs may be enriched in CRCs following chemotherapy and that high aldehyde dehydrogenase expression in CSCs may mediate CRC chemotherapy resistance.[36]

The frequency of CSC varies broadly between different tumor types, ranging from small populations of <1% in AML and liver cancer up to 82% in acute lymphoblastic leukemia (ALL).[32,37] Moreover, the CSC fraction of tumors from the same tissue of origin may vary.[38] It should be noted that variation in the percentage of CSCs within a tumor across time[37] and methodological differences, such as choice of cell surface markers used to isolate CSCs,[38] may in part account for the wide range in CSC frequency reported in various studies.[37]

As CSCs replicate and differentiate, giving rise to progenitor cells, a hierarchy consisting of subpopulations of tumorigenic and nontumorigenic cells is created. Such hierarchies serve as one source of tumor heterogeneity. Just as the frequency of CSCs varies from one tumor to another, the depth or degree of cellular hierarchies also varies (Fig. 2).[25] Some hierarchies may be steep, with only rare tumorigenic cells; or shallow, with common tumorigenic cells; or almost nonhierarchical, with only rare nontumorigenic cells.[25]

## 6. CSC similarities with and differences from NSCs

Long-standing observations of the similarities between cellular mechanisms of normal, embryonic development and abnormal, neoplastic growth have led some to suggest that at least to some extent, "oncology may reflect ontogeny."[39,40] Research in gliomas found that tumors originated from neural stem cell-like cells and used signaling pathways of forebrain neurogenesis to control tumor aggressiveness.[41] The work also demonstrated that prognostic subtypes of glioma paralleled key stages in neurogenesis.[41]

When comparing CSCs with NSCs, it is obvious that from a functional viewpoint, they are very similar. Among the many characteristics they share, are[14,29]:

- Capacity for self-renewal
- Ability to differentiate into multiple progenitor cell types
- Angiogenic induction
- Active telomerase expression
- Increased membrane transporter activity
- Migratory and metastatic capacity
- Apoptotic resistance
- Long life spans

The critical differences between CSCs and NSCs are in the loss of "social control" manifested as alterations in the tumor microenvironment, and in failures of the genetic or epigenetic mechanisms that regulate stemness pathways.[14,42] As a consequence, CSCs replicate and differentiate in an uncontrolled fashion, producing progenitor populations with altered molecular and cellular phenotypes.[14]

Another major difference between CSCs and NSCs is seen in their progeny. NSCs generally differentiate into rapidly proliferating progenitor cells that subsequently mature into the various types of terminally differentiated functional cells.[17] Such fully differentiated functional cells no longer have the ability to proliferate, and typically only dedifferentiate or transdifferentiate in response to injury or experimental reprogramming.[17] Conversely, CSCs give rise to cancerous progeny that may possess essentially limitless proliferative and survival potential with more plasticity than NSC progeny.[17,42,43]

Similarities between CSCs and NSCs have led to the common assumption that CSCs originate from NSCs that have accumulated transforming mutations. Supporting findings for this belief have been found by using stem cell markers to trace stem cells in intestinal adenoma through the development of all other adenoma cell types. In AML, hematopoietic stem cells are considered to be the cells of origin for AML CSCs since both have identical markers.[44]

## 7. Role of the microenvironment

Ordinarily, NSCs are maintained within microenvironments that serve as reservoirs to replenish cells lost because of damage or aging.[45] During tumorigenesis, NSC microenvironments may be altered to become havens for CSCs,[45] and thus become tumor cell reserves that can cause, for example, tumor recurrence following treatment.[46]

The tumor microenvironment is a complex milieu that includes stromal cells, immune cells, endothelial cells, epithelial cells, smooth muscle cells, nerves, the extracellular matrix, vasculature, and local extracellular proteins.[5,42,47] These components comprise a regulatory niche that promotes CSC growth, maintenance, and differentiation.[42] Nurtured within such a favorable environment, CSCs can thrive, evolve, and generate diverse progenitor cells that constitute the rest of the tumor mass.[42]

Complex intercellular signaling networks between CSCs and constituents of their microenvironments modulate CSC intracellular signaling pathways to promote stemness, plasticity, EMT, and metastasis. Intercellular signaling mediators include myriad factors, such as receptors, oxygenation conditions, direct cell-to-cell contact, and secreted factors.[22] In some tumors, the relationship between CSCs and the microenvironment is bidirectional with elements of the microenvironment affecting the cellular behavior of CSCs, whereas CSCs reciprocally modify their microenvironment.[31]

At least 3 distinct processes are understood to operate as part of the microenvironment's role in tumor growth[5]:

### 7.1. Immune-mediated interactions

Within the tumor, chemokines and cytokines are secreted to recruit myeloid-derived suppressor cells, tumor-associated macrophages, and tumor-associated neutrophils that suppress immune-mediated cytotoxicity and surveillance functions.[22] Other functions of macrophages within the tumor include supporting angiogenesis and promoting tumor cell invasion.[48] Immune escape is also mediated by decreasing tumor-specific antigens and increasing anti-inflammatory cytokines and growth factors.[49]

### 7.2. Angiogenesis

CSCs and the tumor microenvironment can interact to increase production of proangiogenic factors.[49] For example, CSCs in GBM can secrete vascular endothelial growth factor (VEGF) to support local vasculature development.[31] Additionally, breast cancer-initiating cells, which were identified as being CD44+/CD24-/low, had higher levels of VEGF in the culture medium compared with the control.[50] Similarly, murine breast cancer cells, forced into an EMT-mediated CSC-like state, were shown to secrete high levels of VEGF protein and produce highly vascularized tumors.[51] Interestingly, in cutaneous squamous cell carcinomas, evidence has been found for the existence of an autocrine loop in which VEGF also promotes intrinsic self-renewal pathways in CSCs.[31] Paracrine signaling may also support CSC maintenance as skin papilloma CSC proliferation and renewal were demonstrated to be dependent on VEGF expression by tumor epithelial cells.[52]

CSCs may directly support angiogenesis by transdifferentiating into endothelial cells or endothelial progenitor cells.[53] A subpopulation of ovarian cancer cells with CSC-like characteristics formed vessel-like structures and increased expression of the endothelial marker VE-cadherin after in vitro differentiation, suggesting that CSCs have the ability to differentiate into endothelial cells.[54] CSCs in glioblastomas have also demonstrated the ability to transdifferentiate into vascular cells.[31] Glioblastoma neurospheres or patient-derived glioblastoma CSCs gave rise to in vitro tube-forming microvascular cultures expressing the endothelial markers CD31 and Tie2, whereas nonstem glioblastoma cells or adherent glioblastoma cell lines failed to do so. In vivo experiments of glioblastoma CSC-derived tumors in mice indicated the presence of human endothelial cells that had formed functional microvascular structures containing circulating erythrocytes in the inner portions of tumors. Furthermore, knockdown of the *Tie2* gene in in vivo experiments of glioblastoma neurosphere-derived tumors indicated the presence of apoptotic foci exclusively in the endothelial compartment, resulting in significant reduction in tumor size and vasculature. These data strongly suggest that glioblastoma CSCs transdifferentiate into functional endothelial-like cells to directly support tumor growth via angiogenesis.[55]

### 7.3. Secretion of regulatory factors

Cells of the microenvironment support CSCs with secretion of growth factors and cytokines such as stromal-derived factor-1, interleukin (IL)-6, and IL-8, all of which function to regulate CSC activity and promote treatment resistance.[45,56] It has been shown in CRC that myofibroblasts within the tumor-associated stroma secrete HGF that maintains CSC function by activating the Wnt pathway.[31] HGF is also a potent inducer of the EMT process[31] and studies suggest that the microenvironment can govern tumor cell stemness via HGF-mediated activation of the Wnt pathway.[45] This can induce CSC-like capacities in nonstem cancer cells with limited tumorigenic capacity.[31] Additionally, microenvironmental cues from cytokines and growth factors help to determine the fate of CSCs in nonsolid tumors.[31]

## 8. Summary

Cancer heterogeneity, especially intratumor heterogeneity, presents substantial challenges to cancer treatment. Developing a deeper understanding of heterogeneity and better models of how CSCs and nonstem cancer cells interrelate may improve efforts to treat cancer and prevent its recurrence.

With growing knowledge of CSC biology and tumor heterogeneity, it may become possible to design multimodal therapies to eradicate not only bulk tumor cells, but to target CSC intracellular and tumor microenvironment signaling.

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