Cancer Stem Cells: *The Real Enemies within Cancer?*

Annapoorni Rangarajan

Abstract | That cancers are heterogeneous has been known to cancer biologists since the invention of microscope. Yet, the existence of functional heterogeneity within cancers has come to be appreciated only recently, with the discovery of cancer stem cells. The cancer stem cell hypothesis posits that a small subpopulation of cancer cells, with stem-like properties of self-renewal and differentiation, is central to the growth of cancers. While the rest, or the bulk of cancer cells, may be easily targeted by chemotherapy, this subpopulation of cancer stem cells remains refractory, thus leading to cancer relapse. Therefore, future anti-cancer therapies should be aimed at eradicating the cancer stem cells. In this article, I will review the origin and current status of the cancer stem cell hypothesis, and its therapeutic implications.

1 Introduction

Peering through a microscope, a pathologist can yield a wealth of information from a thin section of cancer biopsy. This has served as the basis for grouping cancers under various categories and subtypes. Yet, what escapes the eyes is which of these cells is likely to fuel a new cancer growth, and therefore, needs to be eradicated. It is this functional heterogeneity that sets apart the real enemies within cancers from the ones that can be tamed currently.

1.1 *Inequality within cancer*

Is there a reason to think that all cells of a cancer are not equal when it comes to their carcinogenic properties? The answer stares from the observations gathered by cancer biologists for decades using two carcinogenicity assays—an in vitro soft agar colony formation assay and an in vivo tumorigenicity (xenotransplantation) assay in mice (Fig. 1). The former tests the prowess of solid tumor cells to grow in an anchorage-independent fashion, outcompeting the normal epithelial cells that typically require adhesion for survival and growth. The latter additionally tests the ability of cancer cells to invoke neo-angiogenesis, a property that caters to the nutrition and oxygen demands of a budding tumor. The poor efficiency of colony formation (in the range of 0.01–0.1%) by freshly derived cancer cells from tumor biopsies, and the requirement of the order of a million cells to initiate tumor formation when injected in mice, together indicated that not all cells of a cancer have in them the ability to initiate colonies in vitro, or generate tumors in vivo. These data, although ignored for a long time, reveal that perhaps some cancer cells are more empowered, and thus possibly more dangerous, than the others.

Two models have been put forth to explain the conundrum of why all cancer cells are not equally cancerous (Fig. 2). The 'stochastic model' predicts that all cells of a cancer are homogeneous in that they do possess the ability to generate colonies or initiate tumors; however, chance or stochasticity determines which cells will actually do so at any given point of time.[1] The 'heterogeneity model' on the other hand, posits that, to begin with, cancer is heterogeneous; some cells have the ability to generate colonies or tumors, while others do not.[1] If the heterogeneity model were true, then one should be able to separate cells from a cancer such that some of them, when injected into mice, will enable tumorigenic growth while the rest will not. In order to do so, first one needs to be able to identify which
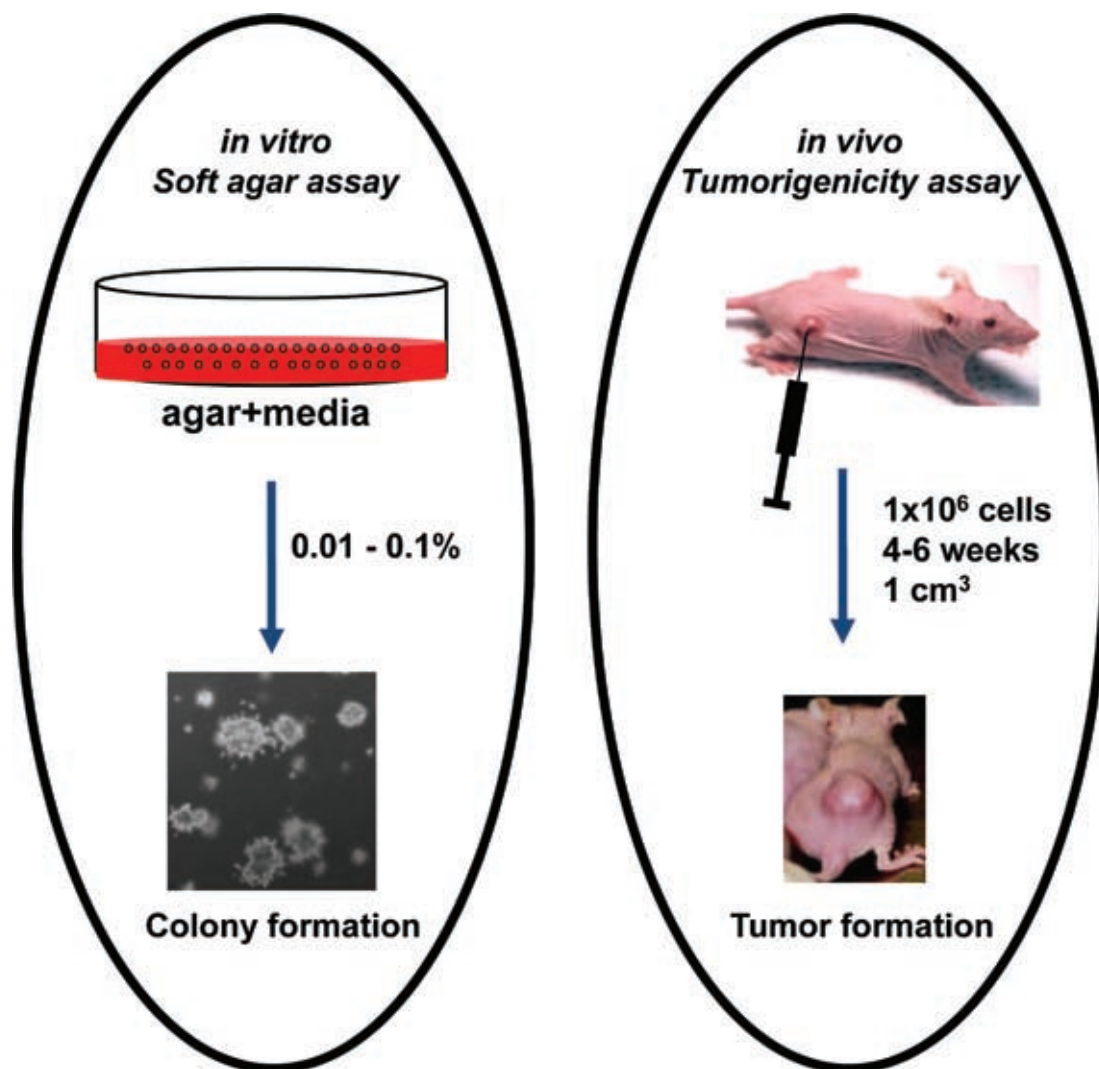
Figure 1: Assays of tumorigenicity: In the in vitro soft agar colony formation assay, single cells of the cancer (either obtained from fresh cancer biopsies or cancer cell lines) are admixed with molten agar in a suitable media and poured onto plates. As the agar solidifies, the cells get trapped in an anchorage-deprived and cell-cell contact deprived fashion. Few cancer cells, typically less than 1%, grow out to generate 3-dimensional colonies. In the in vivo tumor formation assay, typically a million cells are injected either subcutaneously or orthotopically into immune-compromised mice, leading to the generation of tumor growth in ~4–6 weeks of time.

Cell surface marker: Proteins present on the cell surface that uniquely identify a specific set(s) of cells. Often antibodies that specifically recognize this protein are used in conjunction with flow cytometry to isolate these cells.

of the cells of a cancer can initiate a new tumor growth and which cannot. The problem that gripped cancer researchers for a long time was one of how to identify markers that would help the segregation of *tumor-initiating (TICs)* and *non tumor-initiating cells (non-TICs)* from within a cancer.

1.2 *Identification of tumor-initiating cells*
The very first evidence supporting the heterogeneity model came from studies in leukemia, cancers of the blood cells, in the middle to late 1990s. It was uncovered that only 1 in 20,000 of acute myeloid leukemia (AML) cells carried in them the ability to re-launch a new disease when injected into a surrogate host, the mice,[2] revealing that the tumor-initiating potential rested with only a rare subset of cells. Later it was uncovered that these leukemia-initiating cells carried the same cell surface marker phenotype as that of the normal hematopoietic stem cells (HSCs), i.e., $CD34^+CD38^-$.[3]

Then arose the question—is the existence of such rare TICs restricted to cancers of the blood lineage, or do they represent a wider prototype for other types of cancers like solid tumors too? Almost a decade later, the answer came in the form of identification of a subpopulation of lin$^-$CD44^{high}-CD24^{low} cells as the breast cancer-initiating cells.[4] While as yet unclear what made the researchers
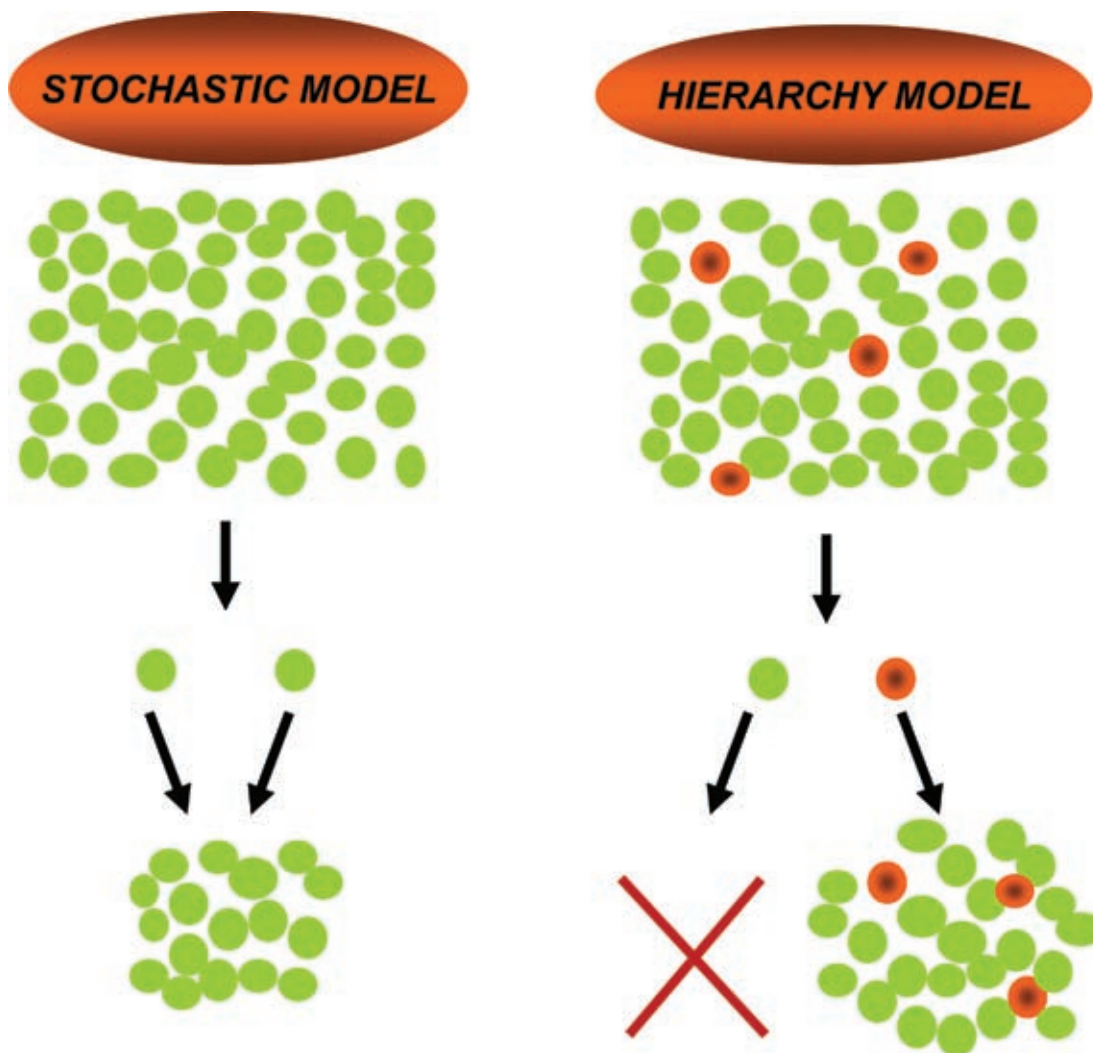
Figure 2: Models to explain functional heterogeneity within cancers: The stochastic model predicts that tumors are homogeneous. Every cell within the tumor (green circles) is potentially tumor-initiating and has equal propensity of forming a tumor. The heterogeneity models predicts that the tumor is heterogeneous to begin with (harboring green and orange circles), and only a limited number of cells (orange circles) are capable of tumor initiation.

select these specific markers for the identification of the tumor-initiating population within the breast tissue, groups working on brain tumors used the argument that if normal hematopoietic stem cells and leukemia-initiating cells shared the same cell surface marker expression, then perhaps brain cancers will share the same marker as normal brain stem cells. In line with these thoughts, when a marker that identifies normal brain stem cells, CD133, was applied to glioma, a form of highly aggressive brain cancer, it indeed divided the cancer into two populations: the CD133+ cells that were capable of initiating new tumors in mice, and the CD133− cells which lacked this potential.[5,6] Thus, similar to some blood cancers, breast and brain tumors too seemed to carry a subset of cells

that alone had the ability to initiate a new tumor when injected into surrogate host animals.

2 Cancer Stem Cells (CSCs): The Modern Terminology

Further, in the context of both breast cancer and glioma, injection of a few hundreds of the isolated tumor-initiating cells sufficed for tumor initiation in xenotransplantation assays in mice, as against requirement of millions of cells earlier when it was the norm to inject the unsegregated population, indicating that indeed this subset of cells is enriched for its tumor-initiating potential. Moreover, the tumors generated by the injection of these sorted cells (lin−CD44^{high}CD24^{low} and CD133+ cells) mirrored the parent tumor heterogeneity in

that they once again carried both the TICs and the non-TICs, revealing the ability of the sorted cells to generate 'other' types of cancer cells. Moreover, such flag bearers could be serially transplanted from one animal to another, suggesting that indeed these cells possessed the ability of long-term self-renewal. Thus, the identification of a distinct subset of tumor-initiating cells within cancers revealed the existence of a hierarchical organization within cancers, much akin to the normal tissue (Fig. 3). Based on this, and other phenotypic and functional similarities that the TICs share with normal stem cells, these tumor-initiating cells have been termed as the cancer stem cells. Thus, much like the normal stem cells that are responsible for normal tissue growth and maintenance, CSCs appear to be central for fuelling cancer growth.

Using similar approaches involving cell surface markers and xenotransplantation assays, the identification of CSCs in breast and brain cancers was soon followed in several other human cancers including colon cancer, pancreatic cancer, and ovarian cancer.[7] Together, these data suggested that several types of cancers follow a hierarchical organization with CSCs at their apex (Fig. 3). The close resemblance of CSCs with normal stem cells has led several researchers to suggest that perhaps cancers originate in normal stem cells. Since progenitor cells

that are derived by the asymmetric division of stem cells (Fig. 3) also possess limited self-renewal potential, it followed that mutations occurring in stem or progenitor cells may actually lead to the genesis of cancer and cancer stem cells. Limited experiments in hematological malignancies do support a role for both normal hematopoietic stem and progenitor cells as the cell of origins of these cancers.[8] Yet, this topic is hotly debated for most solid tumors. Experiments in mice have revealed a stem cell origin for the gut tumors since only the *Lgr5+* stem cell compartment could initiate tumors, while cells of the more differentiated compartments could not.[9] Studies from my laboratory have highlighted that compared to the in vitro transformation of adherent cells (that are enriched in differentiated cells), transformation of mammospheres that are enriched in breast stem/progenitor cells led to the generation of invasive ductal adenocarcinoma, the most commonly encountered breast cancer in the oncology clinics, suggesting that perhaps this predominant form of breast cancer finds its origin in stem/progenitor cells.[10] Thus, even though circumstantial evidence strongly points towards the tissue-resident normal/progenitor stem cells as the cell-of-origin for human cancers, more direct and concrete evidence is lacking at this moment. Also, the ability of more differentiated cells to acquire mutations that restore
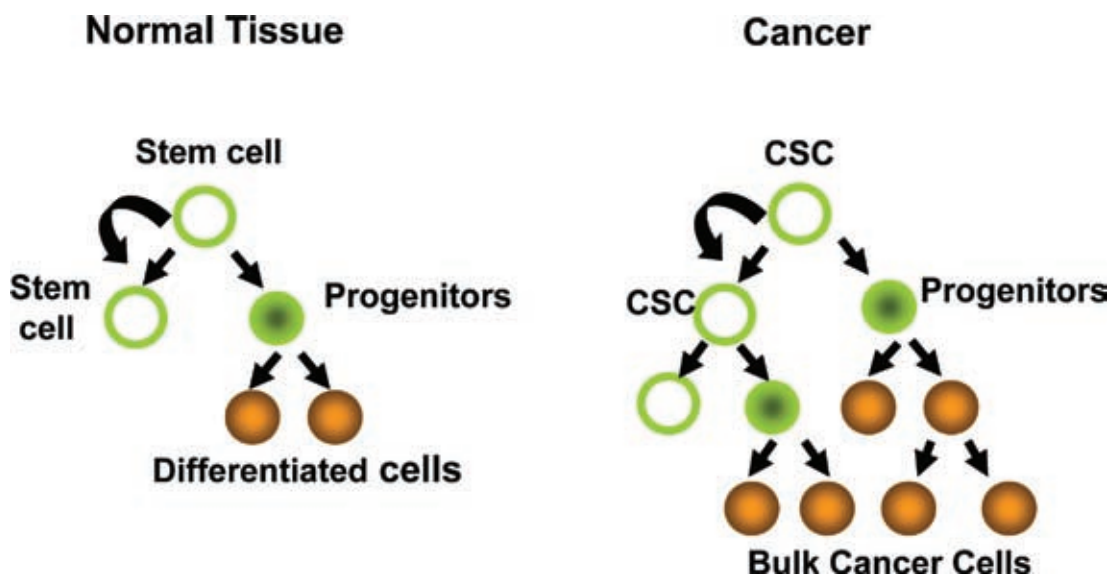
Figure 3: Hierarchical organization of normal tissue and cancers: Normal tissue has few tissue-resident healthy stem cells (with long term self-renewal and proliferation potential) that undergo asymmetric cell division to generate one cell of its own kind, and a progenitor cell (with limited self-renewal and proliferation potential) which then undergoes terminal differentiation to generate the diverse cell types of the specific tissue. According to the cancer stem cell model, cancer is organized in a similar hierarchical fashion with the cancer stem cells (bearing long term self-renewal and proliferation potential) at the apex, leading to the generation of progenitors (or transit amplifying cells) which then divide rapidly and 'differentiate' to generate the bulk cells of the cancer cells.

their self-renewal properties, and undergo tumorigenic conversion, cannot be ruled out altogether.

Metastasis, or the spread of tumor from the primary site to other parts of the body, is the major life-threatening component of cancer. It involves the detachment of cells from the primary site, entry into blood or lymphatics, exit from these into a secondary distant site, colonization of that area and initiation of a tumor growth in the new environment.[11] If only CSCs have in them a potential to re-initiate a tumor, then the cells capable of generating a successful metastasis must, by definition, be CSCs. Thus, it follows that perhaps a subset of CSCs have in them the additional potential to initiate metastasis, and such cells have been christened the circulating or migratory cancer stem cells.[12]

2.1 *Critical evaluation of CSCs*

While the proponents of the CSC theory were on a victory march, having identified CSCs in several cancers, critics argued that the very operational definition of cancer stem cells is flawed. The current gold standard for identification of CSCs involves in vitro selection based on cell surface marker expression using flow cytometry followed by testing their ability to form new tumors in xenotransplantation assays in mice. Critics quickly pointed out that these assays are conducted away from the native niche of the cancer cells, and are more likely to simply test the potential of cancer cells to acclimatize to the new host environment. In addition, the transplantation assays necessarily involve the creation of a wound environment, and the process of wound-healing might itself contribute to the outcome of the assay. Thus, while stem cells may be coerced to spawn diverse cell types in vitro or in vivo following explantation, what matters most is to understand their behaviour within their native environment.

The very concept of CSCs was put to test when a study using similar criterion as highlighted above demonstrated that melanomas, an aggressive form of skin tumors, may have one in four cells capable of initiating new tumors,[13] thus suggesting that CSCs may not be all that rare. However, the controversy further escalated when another study revealed that indeed melanomas also contained only few tumor-initiating cells.[14] Together, several such studies exposed the importance of the strategies employed for tissue processing, the site of injection, the genetic background and sex of the surrogate host mice, and perhaps other as yet undetermined parameters, as key determinants of the outcome of the xenotransplantation assays, and thus their interpretation. So, the frequency of CSCs identified within a cancer is likely to depend on several of these parameters.

2.2 *Resurrection of the CSC hypothesis*

Bombarded by the critics and with no new ammunition in their kitty, just when the fate of the CSC hypothesis was beginning to look bleak, three recent studies using lineage-tracking experiments in mice have brought the proponents of the CSC theory back into limelight. Three independent studies focusing on tumors of the skin,[15] the gut,[16] and the brain[17] used genetic approaches to elegantly track the cells that are responsible for tumor initiation in vivo. All three studies came to the same conclusion: that tumor growth is driven by a small subset of cells, the 'CSCs'. The skin study showed that most of the tumors came from a few cells, which resembled the normal stem cells that maintain the tissue. The gut study further showed that adenomas arise from cells that expressed *Lgr5*, a gene that is active in normal gut stem cells. The glioma study additionally showed how a subset of slow-dividing, stem-like cells remain dormant during standard chemotherapeutic treatment, only to strike back after withdrawal of the drug. In contrast, when these stem-like cells were suppressed, the tumor regressed to residual vestiges that bore no resemblance with the parent cancer. Together, these studies have unequivocally validated the concept of CSCs—that a small subset of cancer cells is responsible for fuelling tumor growth, and thus, their elimination is likely to lead to better cancer cure. Yet, how these lineage-tracking experiments performed in laboratory animals relate to the CSCs identified within human cancers by transplantation assays earlier on remains to be evaluated.

3 Therapeutic Implications of the CSC Hypothesis

Since its inception, chemotherapy has relied on the efficacy of a drug to shrink tumor size. Is it possible that while this practice selects for drugs that de-bulk the tumor (Fig. 4a), it leaves behind the subset of CSCs unscathed which can then launch a relapse? Is there evidence to support this? Indeed it turns out that the CD133+ brain CSCs are more chemoresistant than the non-CSCs.[18] Thus, the CSCs may be inherently drug resistant, in which case drug treatment will likely lead to their selective enrichment at the expense of the non-CSCs. Indeed this has been shown for several drugs in vitro including doxorubicin, cyclophosphamide, temozolomide and flurouracil. In human breast cancers patients too, an enrichment of the CSC population following chemotherapeutic treatment has been identified. Even though the mechanisms of drug resistance in CSCs remain ill understood, an increase in the expression of the ABC family of

a) Conventional Chemotherapy
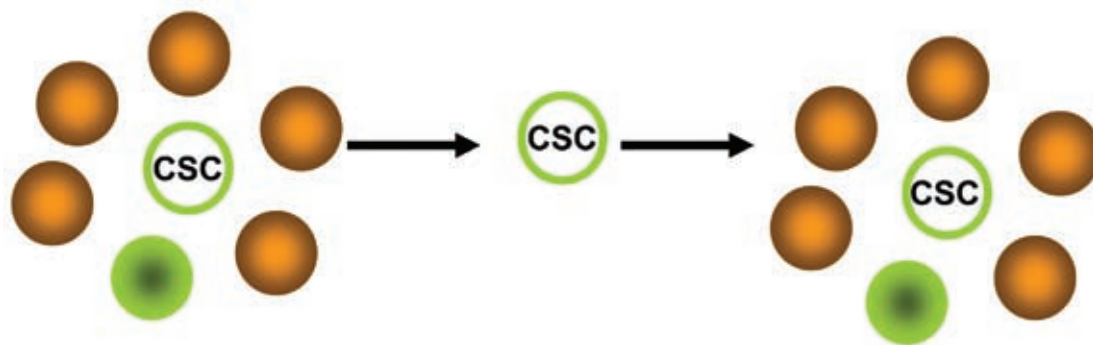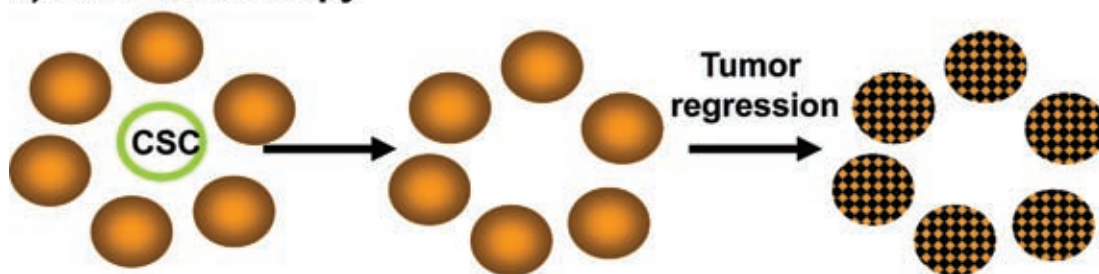

b) Anti-CSC therapy

Figure 4: Conventional versus CSC-specific chemotherapy: a) The conventional chemotherapy leads to tumor shrinkage by killing the bulk (orange) cells, but perhaps leaves behind the dangerous CSCs which then result in tumor relapse. b) In contrast, targeting CSCs would eliminate the cells responsible for tumor growth and initiation, thus leading to tumor regression. A combination of conventional and CSC-specific therapy is likely to offer better cancer treatment.

Niche: Refers to the local microenvironment/neighborhood in which a normal or a cancerous cell normally resides. The niche often secretes factors that are critical for the regulation of growth and differentiation of stem cells.

drug transporters that actively efflux drugs have been largely implicated in this process. It is this property that also enables the identification of both normal and CSCs based on their Hoechst dye efflux, thus appearing as Hoechst low side-population (SP) cells.[19] Treatment with chemotherapeutic agents enriches for the SP phenotype, suggesting that the conventional chemotherapy regimen probably has no ammunition against the CSCs.

If indeed CSCs are the only cells capable of initiating a new tumor, and represent the cells left behind following conventional chemotherapy, then newer anti-cancer strategies aimed at targeting these CSCs need to be designed in order to eradicate them (Fig. 4). This is a paradigm shift so far as cancer chemotherapy is concerned. In line with this, one could consider targeting the CSC itself. Some attractive strategies would include targeting the signaling pathways that regulate CSC self-renewal, such as the Notch, the Wnt, and the hedgehog pathways.[20] Indeed work from our laboratory,[21] and that of others[22,23] has established that targeting

Notch proteins can deplete the stem-like populations in breast and other cancers. Further, few small molecules have been identified that seem to specifically reduce the burden of CSCs. For e.g., a natural plant product, parthenolide, has been shown to specifically target leukemia-initiating cells. Salinomycin, the ionophore antibiotic was found to specifically deplete breast CSCs from patient samples.[24] Following treatment with current chemotherapeutic drugs (that would likely de-bulk or shrink the tumor), the left over CSCs could be eliminated perhaps by combinatorial approaches involving the inhibition of ABC transporters together with targeting self-renewal pathways.

Alternative approaches to directly targeting CSCs have also been proposed. This would include differentiating the CSCs into non-CSCs (which can then be targeted by conventional chemotherapy). For example, exposure to bone morphogenetic protein (BMP) led to the differentiation of the CD133+ brain CSCs, thus preventing their ability to initiate new tumors.[25] Yet another attractive strategy includes targeting the stem cell niche that

plays an important role in dictating the behaviour of stem cells.

4   The Challenges Ahead and Future Directions

The actual clinical relevance of CSCs has been questioned time and again. Can CSCs be targeted effectively without disturbing the normal stem cells? Since the CSCs are very similar in their appearance and function to normal, healthy stem cells, one of the biggest challenges in front of the cancer field right now is to find strategies to attack CSCs without harming their normal counterparts. The identification of the dependence of HSCs but not the LICs on the *Pten* pathway,[26] and that of cutaneous CSCs but not normal skin stem cells on the β-catenin pathway,[27] provides a ray of hope that indeed CSCs can be specifically targeted without harming the normal stem cells. This has further fuelled research interests into identifying mechanisms unique to cancer stem cells with an aim to distinguish them from the normal stem cells.

Functional heterogeneity may exist within CSCs, with some more capable than others in terms of generating new tumors. This is likely to compound the problem of identifying which cells within the CSCs need to be eliminated. Further, the CSCs may stay dormant or quiescent and thus escape any forms of treatment, in which case the initial step may include re-kindling them into an active state which can then be targeted. Additionally, the CSCs may represent a moving target, with the threat of inter-conversion of one population into another. Speculations that 'stemness' is not an entity, but rather a state,[28] further supports this line of thought.Although the ability of non-CSCs to give rise to CSCs has been shown only under culture conditions,[29] the possibility of continual regeneration of CSC from non-CSC populations would suggest the requirement of long term treatments with anti-CSC agents.

Even though recent lineage tracking studies have provided considerable evidence for the existence of CSCs, it still remains to be seen if what has been shown for the gut, brain and skin cancers will hold true for various other types of cancers too. Most importantly, these experiments were undertaken in mice, in the context of murine tumors. The paths that lead to carcinogenesis in murine cells could be very different from that in human cells.[30] Therefore, it still remains to be seen if these experiments will hold true for human cancers as well. Nevertheless, may what the skeptics say, the identification of CSCs, and the promises and challenges that are at stake, is likely to keep both the believer and the disbeliever looking for more.

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Annapoorni Rangarajan obtained her PhD. in the year 2000 from the National Centre for Biological Sciences TIFR Centre, Bangalore. She was also a *Visiting Research Scholar* at the Laboratory of Dr. Paolo Dotto, CBRC, Massachusetts General Hospital, Harvard Medical School, Charlestown, USA in July 1998–Nov. 1998 and Aug. 1999–May 2000 during her PhD. She completed her *Postdoctoral Fellowship* at the Laboratory of Dr. Robert Weinberg, Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology, Cambridge, USA. She was honoured with Wellcome-DBT India Alliance '**Senior Research Fellowship**' in the year 2010 and US-Army Dept. of Defense "**Breast Cancer Research Postdoctoral Fellowship**" in the year 2002.

Currently she is an *Associate Professor* at the Department of Molecular Reproduction, Genetics and Development, Indian Institute of Science, Bangalore.