SBV AHEAD

CANCER STEM CELLS – A BRIEF OVERVIEW

Dr. Sam Vijay Kumar J, *Scientist*
Central Inter-Disciplinary Research Facility,

Sri Balaji Vidyapeeth, Mahatma Gandhi Medical College and Research Institute Campus
Pillaiyarkuppam, Puducherry - 607403, India
Email: samvijaykumar@cidrf.res.in

Abstract ▶ Cancer is a disease where there is aberrant cellular behaviour characterized by uncontrolled growth and cellular signalling. Cancer though is viewed as a homogeneous pathology, does not show uniformity at the cellular level - there is difference in the characteristics within cells of a tumour. A major caveat in understanding the biology of cancer is the paucity of information on the origin and perpetuation of cancers. Towards salvaging these two models of cancer genesis and progression have been proposed: 'Stochastic' and 'Cancer stem cell' theories. The stochastic model holds that all cells in a tumour are identical while the cancer stem cell theory supports the existence of a subset of cells called cancer stem cells in a tumour that are responsible for the origin and perpetuation of the disease. Cancer stem cells are implicated in various aspects of cancer including metastasis, recurrence and therapeutic resistance. Though cancer stem cells have been reported from many cancers methods to identify and characterize them still rely on animal transplantation models along with surface protein studies. However better techniques of characterization of these cells would play a positive role in elucidating these cells better. The characterization of cancer stem cells would play an important role in the research and clinical management of the disease.

Key Words: Cancer, Stem cells, Perpetuation, Stem cell theory, Markers.

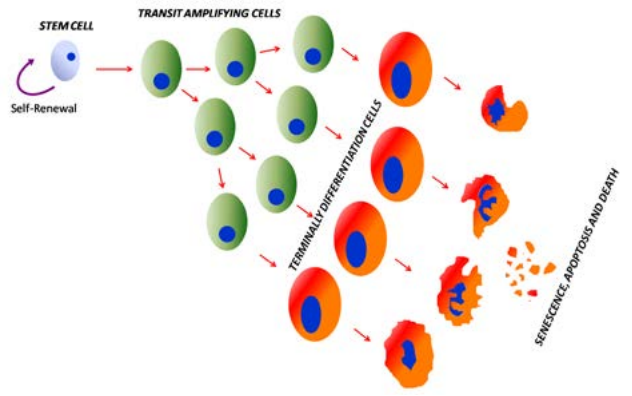
Stem cells

Human body is made up of 10^{12} to 10^{16} cells. Recently with a bibliographical and mathematical approach the total number of cells in the human body was averaged to be 3.72 x 10^{13} [1]. A preprint data from Weizmann Institute, Israel, hints at a revised estimate of the number of human cell in a 'reference man' of 70kg to be 3 x 10^{13} [2]. An estimated 10^{8} cells die in an adult per day[3] and have to be replaced daily. With such huge number of cells turned over it is of paramount importance to maintain the number and quality of cells. Homeostasis is important as there is constant loss of differentiated cells because of their limited lifespan. This is verified under *in vitro* conditions where cultured normal somatic cells undergo only a certain number of cell divisions beyond which they undergo senescence and apoptosis; a phenomenon called 'Hayflick's limit'[4]. Loss of cells and the subsequent need for replenishment in the body might also be due to normal wear and tear, injury or degeneration. The production and replacement of cells undergoing senescence and apoptosis with healthy and viable cells is a process that is well regulated, where a small population of cells called 'stem cells' serve as the reservoir, which can give rise to proliferating progenitors and terminally differentiated cells.

Stem cells are a small subset of cells within any kind of tissue in the body that have the capacity for long term self-renewal, asymmetric cell division, and differentiation

into one or more lineages[5, 6]. These cells subscribe to a hierarchical model[7,8] wherein a small fraction of stem cells having the above mentioned capacities maintain their cell numbers by self-renewing and when required as in tissue injury or homeostasis, can give rise to daughter cells that can proliferate extensively to accommodate the need for high cell numbers (Figure 1). Examples of well-studied systems in the body for the hierarchical stem cell model are the haematopoietic system[9] and gut[10].

Figure 1 – Stem Cell Hierarchy



There exists a stem cell which has the capacity for long-term self-renewal and thus maintaining the stem cell pool. This stem cell can divide and give rise to transit amplifying cells which are committed progenitor cells which differentiate into the terminally differentiated cells of the tissue of origin. After their limited life span these cells undergo senescence, apoptosis and death. Thus stem cells are characterized by three properties: (1) long term self-renewal, (2) extensive proliferation and (3) differentiation into multiple lineages.

Generally stem cells are categorized as 'Embryonic stem cells' and 'Adult stem cells'[6]. Embryonic stem cells were first described almost three decades ago[11,12] and are seen in the inner cell mass of the blastocyst. These cells are pluripotent and can form any organ of the body but not the whole organism. While embryonic cells are predominantly responsible for the complete development of the foetus, 'Adult stem cells' or 'Somatic stem cells' take active role in meeting the body's cell requirements after birth. These adult stem cells have been demonstrated in almost all of the tissues of the human body including rapidly dividing intestinal tract[13-15], bone marrow[16] as well as slowly recycling tissues like muscle[17]. One of the ways of identifying normal adult stem cells from various human tissues is by their surface markers for e.g. normal colonic stem cells are reported to be positive for Lgrr5 expression on the surface which is a Leucine rich protein receptor[13,18], Haematopoietic stem cells are characterized by CD34+CD38-[19,20]. Underlying these differences are multiple molecular signalling pathways involved in a well regulated physiology from stem cell to differentiated cell distribution in any tissue.

Cancer

The World Health Organization (WHO) defines cancer as a generic term for a large group of diseases that can affect any part of the body, the defining features of which are the rapid creation of abnormal cells that grow beyond their usual boundaries, and invade adjacent parts of the body and spread to other organs[21]. Cancer is the second largest non-communicable disease; a leading cause of death around the world with 70% of related mortality seen in low and middle income countries. While 30% of cancers could be prevented, deaths due to cancer worldwide are projected to increase to an estimated 12 million deaths in 2030 (The global burden of disease: 2004 update, WHO). There were 7.6 million deaths related to cancer in 2008 alone. One in four deaths in the United States is due to cancer[22]. In India cancer incidence is reported to be increasing[23].

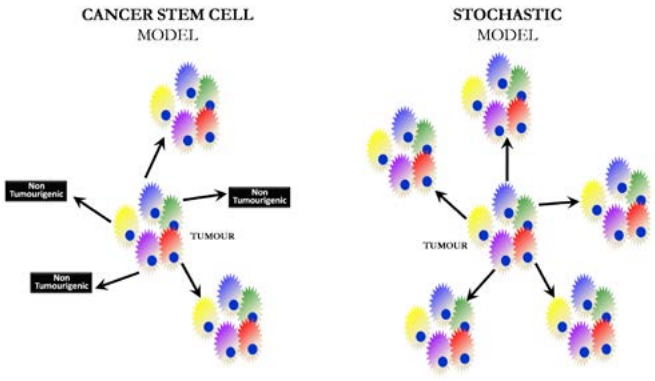
Six hallmark features of cancer were described by Hanahan and Weinberg more than a decade ago: Self-sufficiency in growth signals, insensitivity to anti-growth signals, resistance to apoptosis, limitless reproductive potential, sustained angiogenesis, and tissue invasion and metastasis[24]. Over the years the understanding of the hallmarks have been furthered with the addition of two more features: re-programming of energy metabolism and evasion of immune destruction[25]. There is also the emerging concept of metabolic hallmarks of cancer that serves to elucidate the disease from the perspective of energy and nutrition. A recent perspective describes six metabolic hallmarks of cancer[26] that serves to elucidate more on the energy dynamics in cancer. These hallmarks are also believed to be influenced by the extracellular matrix associated with tumour cells[27]. There is also the contrarian view that that proposes that carcinogenesis is due to interaction of cells with extracellular matrix[28]. Taken together, all these point to the growing complexity of the disease, its origin which ultimately has therapeutic ramifications.

Cancer perpetuation

Though there are many reports describing the aberrant cellular and molecular signalling in cancers and in the process of carcinogenesis, the putative cell that succumbs to the initial transformation event is yet to be elucidated clearly. There is paucity of information on the exact origin

of the disease. In spite of the various hallmark features of cancer and the factors associated with carcinogenesis being described in many reports, the initial transformation event which results in tumorigenesis is still not clear. Towards reckoning this lack of clarity about the biology and genesis of cancer two broad models have been posited. These two models that attempt to explain carcinogenesis or the initiation of cancer are the '*stochastic*' and the '*cancer stem cell*' models[29,30] (Figure 2).

Figure 2 – Theories of cancer genesis



The stochastic model states that every cell in a tumour has the capability to re-initiate and maintain a tumour whereas the cancer stem cell model attributes this capacity only to a limited subset of cells within the tumour bulk.

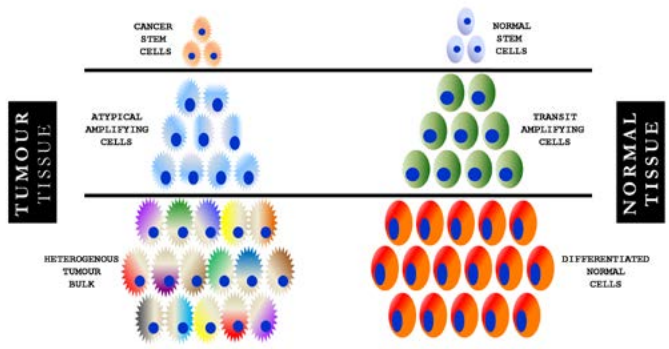
Stochastic theory

The 'stochastic model' does not subscribe to the speciality or uniqueness of any particular cell subtype and states that every cell in a tumour bulk is equally endowed with the potential to propagate the tumour and form new tumours. This model associates randomly accumulating genetic changes in the DNA along with micro environmental selections with carcinogenesis and cancer progression. Thus this model aims to address tumours as a heterogeneous mixture of cells but without any the biological differences which could have any implication on tumour progression or recurrence. Thus this model attributes no inherent differences between the constituent cells in a tumour to maintain the disease.

Cancer stem cell theory

On the other hand, the Cancer Stem Cell (CSC) model is a hierarchical model[31,32] where a particular cell called the 'cancer stem cell' has the capacity to self-renew and proliferate thus giving rise to a host of transit amplifying cells which form the bulk of the heterogeneous tumour mass; this feature parallels the normal stem cell biology in a tissue, where they maintain their cell number in low frequency but also proliferate and give rise to terminally differentiated cells as in case of an injury or normal homeostasis. The CSC model posits the existence of a small subpopulation of CSC within a tumour that exclusively harbours this capacity to initiate and propagate the disease. This model attributes the qualities of tumour initiation, maintenance and propagation to only a biologically distinct subpopulation of cells occurring in low frequency within a tumour called CSC[33]. A corollary of this has been that these CSC are also responsible for metastasis and for resistance to conventional chemotherapy and radiotherapy Like normal stem cells which self-renew and proliferate to produce terminally differentiated cells, CSC also have the capability to maintain their numbers by self-renewal and by proliferation give rise to a host of transit amplifying cells which form the heterogeneous tumour bulk[34] (Figure 3). To surmise this model subscribes to the theory that a tumour arising from a cancer stem cell is composed of cancer stem cells in low frequency and the non-stem cells as the tumour bulk.

Figure 3 – Stem cells in tumours and normal tissue



The stem cell hierarchy in the tumour tissue parallels that of normal tissue. Like normal tissue the cancer stem cells model also states the existence of a small subset of stem cells which gives rise to the heterogeneous tumour populations and the atypical cells of the tumour.

Cancer stem cells markers

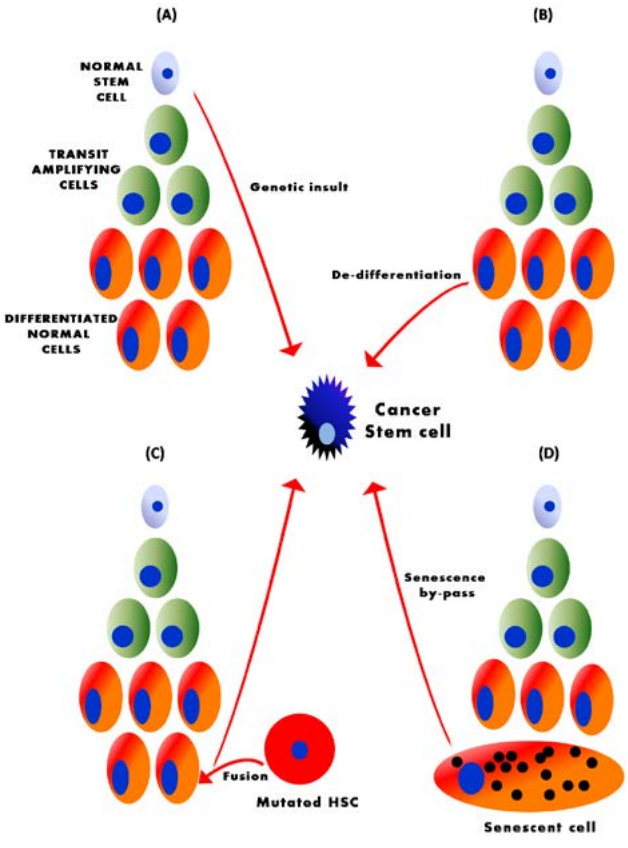
One of the earliest hints for the cancer stem cell theory came from the publication of the 'Trophoblast theory of cancer' in the year 1902[35]. This theory states that cancer is a germ cell disorder wherein remnant foetal trophoblasts in

the adult can get activated to form cancer upon sufficient activation by environmental and chemical cues thus hinting at the stemness nature in cancer. Though this theory was not promptly accepted at that time, in retrospect its proponent John Beard is considered one of the pioneers of the present day theory of cancer stem cells[36].

Many plausible mechanisms for the origin of CSC have been put forth[29,37-39]. One of the ways suggested for the origin of CSC is said to be the activation of a normal resident stem cell. In a report by Dean[37] activation of a stem cell is proposed to be a good target for subsequent genetic hits leading to a complete transformation resulting in an autonomous growth and acquisition of cancer cell phenotype. This cell is transformed while retaining the stemness property. Activation of a stem cell according to this theory can occur in ways: (a) the stem cell might be naturally dividing as in an embryo or haematopoietic system thus rendering it vulnerable to accrue genetic errors during replication, (b) hormonal activation of stem cells can be another way as in the case of oestrogen and ovarian cells and lastly (c) tissue damage caused by injury, inflammation, infection or chemical exposure like asbestos etc. In all these conditions a resident stem cell is activated and stimulated to divide thus increasing the probability of acquiring genetic mutations due to errors of DNA repair. Even the dedifferentiation of a mature cell with concomitant acquisition of self-renewal ability can result in the formation of a cancer stem cell. Another way of a cell acquiring CSC phenotype reported in this paper as well as in Costea *et al*[38] is the dedifferentiation of mature differentiated cell which acquires the ability of self-renewal. Apart from direct stem cell transformation and dedifferentiation Costea *et al*[38] have also report two additional ways in which the origin of CSC can be envisaged to happen in the context of oral squamous cell carcinoma: (a) fusion of a mutated haematopoietic stem cell with a keratinocyte (differentiated cell) can result in a heterokaryon and might give rise to a CSC and (b) Exposure of senescent cells to chemicals can also result CSC like cells (Figure 4).

> Plausible mechanisms of the origin of cancer stem cells include (a) Genetic mutation in a resident tissue stem cell, (b) De-differentiation of a mature differentiated cell in a tissue (c) Fusion of a mutated haematopoietic stem cell with a mature differentiated cell and (4) Senescence by-pass wherein a senescent giant cell undergoes multiple genetic changes and results in a transformed cancer stem cell.

Figure 4 – Plausible mechanisms of cancer stem cell genesis

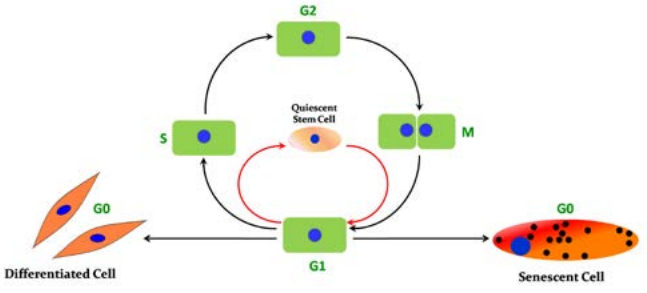


In 1937 one of the early proofs for the existence of cancer initiating property within subpopulation of cells came of the work of Furth and co-workers[40], where inoculation of single cells from inbred leukaemic mice into mice of the same type resulted only a small fraction (5%) of animals developing leukaemia. In the early nineteen fifties some studies with solid tumours also yielded similar results indicating the involvement of a subset of cells in the process of carcinogenesis[41,42]. The actual term 'cancer stem cell' was popularized after Carney *et al*[43] demonstrated the tumorigenicity of patient derived lung cancer cells grown as tumour cell colonies on soft agarose, when injected into athymic nude mice. It was just around the same time that the involvement of stem cell compartment in leukaemogenesis was reported[44,45]. The first definitive description of such a cell was in acute myeloid leukaemia, where it was shown that cells which were characterized to be CD34+/CD38-, when transplanted into SCID mice, could stably re-initiate and sustain the disease[46]. There have subsequently been many reports trying to prove and identify these cells in tumours of diverse origin including breast (CD44+/CD24-/Lineage-)[47], pancreas (CD44+/CD24+/ESA+)[48], colon (CD133+, CD44 and Lgr5+)[49-53], and prostate (CD44+/β2β1 Integrin-hi/CD133+)[54].

CSC reported in many cancers corresponds to the stem cell theory in being of low percentages in the tissue. Ricci-Vittiani *et al*[55] and Dalerba *et al*[56] showed that the percentages of CSC in colon identified by CD133 and CD44 respectively were around 0.7 – 2.5%. In the samples analyzed by O'Brien *et al*[49], CSC identified by Lgr5 were also found to be as high percentage as high as 24.5% in one of the samples. Similarly CSC in breast, identified by CD44+C,CD24-,ESA+ was found to be 2-4%[47]. CSC in AML identified by CD34+CD38- was reported to be 0.2%[57]. CSC percentages have been reported in many other cancers including liver[58], pancreas[48,59], prostate[60], kidney[61]. It is generally accepted that the percentages of CSC in tumours are low because these cells are quiescent, slow cycling and express high levels of anti-apoptotic proteins[29,39], all of which are also implicated in resistance to conventional chemotherapy.

Quiescence is one of the properties that are seen in stem cells and essential for maintaining their numbers. Quiescence enables the stem cells to go into a reversible state of minimal metabolic behaviour without cell division, the deregulation or loss of which could affect the number of resident stem cell numbers in a tissue and can lead to depletion of the same. G0 phase in the cell cycle is an irreversible state where cells like those undergoing senescence or differentiation, while stem cells go into quiescence which is a reversible G0 phase[62] (Figure 5). As indicated previously in Costea *et al*[38] it is at this point that malignant transformation can occur where a senescent cell or differentiated cell can through multiple genetic hits acquire the property to enter the cell cycle again thus activating their ability for quiescence . There are many factors that regulate quiescence. This confers the cells with the ability to escape any cytotoxic agent acting via DNA replication mechanism or cell division protein inhibition. Quiescence has been well described in hematopoietic stem cells[63,64] and the various factors having an influence on quiescence.

Figure 5 – Quiescence in stem cells



Normally a cell undergoing cell cycle goes into G0 phase upon reaching a point of senescence (a dysfunctional state reached because of limited life span or accumulated errors) or differentiation into mature cells. This is an irreversible stem except for the cancer stem cell genesis. Normal stem cells have the property of entering and leaving the G0 phase as dictated by the homeostatic cues. Both normal and cancer stem cells by entering into quiescence are shielded from agents targeting cell cycle components.

Consequently characterization of these cells are of paramount importance in the context of therapeutic oncology; and these cells unlike other tumour cells need unique techniques of isolation and characterization which include *in situ*, *in vitro* and *in vivo* approaches.

Cancer stem cells characterization

In Situ Identification using Surface Markers

Identification of CSC using surface markers is one of the widely employed techniques because of the availability of a repertoire of antibodies. One of the techniques exploiting the surface antigen chemistry of cells is flow cytometry which depends on treating live or fixed cells with monoclonal antibodies tagged with fluorescent tags[65,66] such as fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) and peridinin-chlorophyll protein Cy5.5 (PerCP Cy5.5). Identification using surface markers gives the advantage of specificity and sensitivity. The use of flow cytometry assisted cell surface profiling gives the advantage of qualitative as well as quantitative analysis of cells based on surface markers. This approach would not only help elucidate the presence or absence of markers; this can also successfully identify fluctuations in the marker expression. Flow cytometry based surface profiling has been used in studying CSC in cancers affecting various tissues including oral mucosa[67], colon[68] and breast[47].

Fluorescence microscopy[69] is another important tool that is successfully used for the study of CSC. This technique gives us the flexibility of analyzing a fixed and stored tissue and gives us the advantage of analyzing single cells visually. Immunofluorescence based study of CSC has been employed for studying the topographical distribution of cells of our interest in a tissue. Thus Immunofluorescence microscopy helps us to correlate the location, frequency and distribution of stained cells with the tissue histology. This makes it a vital tool for studying CSC.

Surface markers and their utility in studying CSC have been used in another technique: magnetic activated cell sorting (MACS). It uses surface protein differences to differentially enrich or deplete cells of concern. The best functional study for CSC is animal transplantation and MACS serves purify cells of our interest based on surface markers. Thus surface marker based identification is one fruitful avenue for the study of different types of CSC.

In Vitro Assays for Cancer Stem Cells

Many techniques and ways have been reported to isolate and study cancer stem cells. Chiou *et al* suggest three ways in which CSC can be isolated and studied: Immunophenotyping by flow cytometry, Hoechst 33343 exclusion based side population (SP) assay and Sphere formation[70].

Immunophenotyping by flow cytometry is certainly a strong tool for studying CSC because of the ease of performing and also because of the availability of a wide range of antibodies against a wide range of surface proteins. This technique easily gives signature surface profiles across multiple samples and hence is a valuable tool in the study of CSC as well.

Hoechst 33342 is DNA binding dye which can be used to stain live cells. Hoechst 33342 dye based SP assay exploits the fact that ABC transporters especially ABCG2 efflux this dye from cells. Thus there would be differential staining between stem cells expressing high levels of ABCG2 and non-stem cells showing low levels of this transporter when treated with this chemical[71]. This protocol was first established for bone marrow derived haematopoietic stem cells which is successfully adapted to other types of stem cells and also to cancer stem cells.

Sphere formation assay is another important way to study CSC from any tissue. This technique relies on the fact that non stem cells fail to survive and grow under an anchorage independent or serum starved condition with growth factors, while normal stem cells and CSC remain not only remain viable in these conditions but also form spheres of cells indicating their ability to proliferate and clonally expand. The ability of these cells to self-renew can also be inferred by generating secondary spheres from dissociated primary spheres[72].

CSC have been successfully identified by label retaining assays like the DNA intercalating bromodeoxyuridine (BrdU) as these cells have a low turnover thus labelling the long term non-dividing cells[64]. Tritiated thymidine, which exhibits a similar DNA binding ability, has also been used to label slow cycling cells or quiescent cells[73].

There has been lot of technological advancement and histone protein based labelling systems have been developed like H2B-green fluorescent protein (H2B-GFP)[74] where the histone protein are genetically modified by the addition of a green fluorescent protein for easy visualization. Some other approaches that identify CSC include RNA content, lack of proliferation markers, elevated anti-apoptotic proteins etc. These techniques though pick up slow cycling cells and help elucidate the quiescent behaviour it has become apparent that other techniques to identify CSC are used in tandem to get significant results.

In Vivo Assays for Cancer Stem Cells

Normal stem cells are endowed with properties of self-renewal and lineage capability. CSC paralleling on normal stem cells have properties of self-renewal and tumour propagation. Thus a technique that can successfully demonstrate these features would certainly be of a huge impact[75]. Thus amidst all the other techniques serial orthotopic xenotransplantation is still hailed as the gold standard in experimental CSC biology[75]. Though no xenotransplantation model exactly replicates the host tissue environment, they give the advantage of studying the putative CSC under question in an environment that gives a milieu and microenvironment at least distantly similar to native tissue from which the CSC is derived. One of the concerns to bear with while using this assay is the inherent difference in the transplantation site especially the lack of stromal cell signals, which are also described to impact the development and propagation of CSC[73]. This caveat can be addressed and improved by co-engrafting the putative CSC with stromal cells. Another factor to be aware of is the cell preparation and the process of transplantation; these can to some degree introduce some mechanical stress but in any case this would true of other assays as well. Xenotransplantation is nevertheless considered the best functional test for CSC.

Another important development is the newer and better models of in vivo experimentation are being designed from time to time for tumour transplantation studies[76]. Development of mice models that more immunocompromised than SCID and NOD/SCID show greater transplantability with cancer cells. This suggests altogether a different perspective on the methodologies to estimate the frequencies of CSC. Quintana *et al*[77] have shown this in melanoma by injecting single melanoma cells into two types of immunocompromised animals (NOD/SCID and NOD/SCID with Interleukin 2 deficiency) and showing that the former model can underestimate the frequency of CSC. Thus a combined and informed way of isolating and studying CSC is imperative.

Conclusion

Development and perpetuation of cancers is highly debated with both stochastic and stem cell model of cancer being identified as plausible models to explain the genesis. Cancer stem cell model has been repeatedly supported by multiple studies attempting the isolation of cancer stem cells from tumours. However a unique, all-encompassing marker for isolation, characterization of CSC has not yet been available which engenders the necessity for the use of multiple techniques – both *in vitro* and *in vivo* – in the analysis of CSC. Thus elucidation of CSC would require a host of techniques and further validation of presently available techniques towards harnessing the knowledge of stem cells in cancer research and therapy.

REFERENCES

1. Bianconi E, Piovesan A, Facchin F, Beraudi A, Casadei R, et al. An estimation of the number of cells in the human body. Ann Hum Biol. 2013;40:463-71.
2. Sender R FS, Milo R. Revised estimates for the number of human and bacteria cells in the body. bioRxiv. 2016;1(036103).
3. Renehan AG, Booth C, Potten CS. What is apoptosis, and why is it important? BMJ. 2001;322:1536-8.
4. Shay JW, Wright WE. Hayflick, his limit, and cellular ageing. Nat Rev Mol Cell Biol. 2000;1:72-6.
5. Rippon HJ, Bishop AE. Embryonic stem cells. Cell Prolif. 2004;37:23-34.
6. Blau HM, Brazelton TR, Weimann JM. The evolving concept of a stem cell: entity or function? Cell. 2001;105:829-41.
7. Perez-Losada J, Balmain A. Stem-cell hierarchy in skin cancer. Nat Rev Cancer. 2003;3:434-43.
8. Preston SL, Alison MR, Forbes SJ, Direkze NC, Poulsom R, et al. The new stem cell biology: something for everyone. Mol Pathol. 2003;56:86-96.
9. Ema H, Morita Y, Suda T. Heterogeneity and hierarchy of hematopoietic stem cells. Exp Hematol. 2014;42:74-82.
10. Radtke F, Clevers H. Self-renewal and cancer of the gut: two sides of a coin. Science. 2005;307:1904-9.
11. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981;292:154-6.
12. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A. 1981;78:7634-8.
13. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature. 2007;449:1003-7.
14. Van der Flier LG, Clevers H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. Annu Rev Physiol. 2009;71:241-60.
15. Li H, Jasper H. Gastrointestinal stem cells in health and disease: from flies to humans. Dis Model Mech. 2016;9:487-99.
16. Gunsilius E, Gastl G, Petzer AL. Hematopoietic stem cells. Biomed Pharmacother. 2001;55:186-94.
17. Goldring K, Partridge T, Watt D. Muscle stem cells. J Pathol. 2002;197:457-67.
18. Schuijers J, Clevers H. Adult mammalian stem cells: the role of Wnt, Lgr5 and R-spondins. EMBO J. 2012;31:2685-96.
19. Plesa A, Chelghoum Y, Mattei E, Labussiere H, Elhamri M, et al. Mobilization of CD34(+)CD38(-) hematopoietic stem cells after priming in acute myeloid leukemia. World J Stem Cells. 2013;5:196-204.
20. Schuurhuis GJ, Meel MH, Wouters F, Min LA, Terwijn M, et al. Normal Hematopoietic Stem Cells within the AML Bone Marrow Have a Distinct and Higher ALDH Activity Level than Co-Existing Leukemic Stem Cells. PLoS One. 2013;8:e78897.
21. Available from http://www.who.int/cancer/en/. Accessed on 05-May-2016.
22. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. CA Cancer J Clin. 2009;59:225-49.
23. Marimuthu P. Projection of cancer incidence in five cities and cancer mortality in India. Ind J Cancer. 2008;45:4-7.
24. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100:57-70.
25. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646-74.
26. Pavlova NN, Thompson CB. The emerging hallmarks of cancer mtabolism. Cell Metab. 2016;23:27-47.
27. Pickup MW, Mouw JK, Weaver VM. The extracellular matrix modulates the hallmarks of cancer. EMBO reports. 2014;15:1243-53.
28. Sonnenschein C, Soto AM. The aging of the 2000 and 2011 Hallmarks of Cancer reviews: a critique. J Biosci. 2013;38:651-63.
29. Jordan CT, Guzman ML, Noble M. Cancer stem cells. N Engl J Med. 2006;355:1253-61.
30. Marjanovic ND, Weinberg RA, Chaffer CL. Cell plasticity and heterogeneity in cancer. Clin Chem. 2013;59:168-79.
31. Matsuda S, Yan T, Mizutani A, Sota T, Hiramoto Y, et al. Cancer stem cells maintain a hierarchy of differentiation by creating their niche. Int J Cancer. 2014;135:27-36
32. Werner B, Scott JG, Sottoriva A, Anderson AR, Traulsen A, et al. The cancer stem cell fraction in hierarchically Organized tumors can be estimated using mathematical modeling and patient-specific treatment trajectories. Cancer Res. 2016;76:1705-13.
33. Nguyen LV, Vanner R, Dirks P, Eaves CJ. Cancer stem cells: an evolving concept. Nat Rev Cancer. 2012;12:133-43.
34. Tysnes BB. Tumor-initiating and -propagating cells: cells that we would like to identify and control. Neoplasia. 2010;12:506-15.
35. Gurchot C. The trophoblast theory of cancer (John Beard, 1857-1924) revisited. Oncology. 1975;31:310-33.
36. Moss RW. The life and times of John Beard, DSc (1858-1924). Integr Cancer Ther. 2008;7:229-51.
37. Dean M. Cancer stem cells: redefining the paradigm of cancer treatment strategies. Mol Interv. 2006;6:140-8.
38. Costea DE, Tsinkalovsky O, Vintermyr OK, Johannessen AC, Mackenzie IC. Cancer stem cells - new and potentially important targets for the therapy of oral squamous cell carcinoma. Oral Dis. 2006;12:443-54.
39. Lin EH, Jiang Y, Deng Y, Lapsiwala R, Lin T, et al. Cancer stem cells, endothelial progenitors, and mesenchymal stem cells: “seed and soil” theory revisited. Gastrointest Cancer Res. 2008;2:169-74.
40. Furth J, Kahn MC, Breedis C. The transmission of leukemia of mice with a single cell. American J Cancer. 1937;31:276-82.
41. Makino S. Further evidence favoring the concept of the stem cell in ascites tumors of rats. Ann N Y Acad Sci. 1956;63:818-30.
42. Hewitt HB. Studies of the quantitative transplantation of mouse sarcoma. Br J Cancer. 1953;7:367-83.
43. Carney DN, Gazdar AF, Bunn PA, Jr., Guccion JG. Demonstration of the stem cell nature of clonogenic tumor cells from lung cancer patients. Stem Cells. 1982;1:149-64.
44. Martin PJ, Najfeld V, Hansen JA, Penfold GK, Jacobson RJ, et al. Involvement of the B-lymphoid system in chronic myelogenous leukaemia. Nature. 1980;287:49-50.
45. Greaves MF, Verbi W, Reeves BR, Hoffbrand AV, Drysdale HC, et al. “Pre-B” phenotypes in blast crisis of Ph1 positive CML: evidence for a pluripotential stem cell “target”. Leuk Res. 1979;3:181-91.
46. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature. 1994;367:645-8.
47. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A. 2003;100:3983-8.

48. Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, et al. Identification of pancreatic cancer stem cells. Cancer Res. 2007;67:1030-7.
49. O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature. 2007;445:106-10.
50. Saigusa S, Inoue Y, Tanaka K, Toiyama Y, Matsushita K, et al. Clinical significance of LGR5 and CD44 expression in locally advanced rectal cancer after preoperative chemoradiotherapy. Int J Oncol. 2012;41:1643-52.
51. Vermeulen L, Todaro M, de Sousa Mello F, Sprick MR, Kemper K, et al. Single-cell cloning of colon cancer stem cells reveals a multi-lineage differentiation capacity. Proc Natl Acad Sci U S A. 2008;105:13427-32.
52. Kemper K, Prasetyanti PR, De Lau W, Rodermond H, Clevers H, et al. Monoclonal antibodies against Lgr5 identify human colorectal cancer stem cells. Stem Cells. 2012;30:2378-86.
53. Todaro M, Gaggianesi M, Catalano V, Benfante A, Iovino F, et al. CD44v6 is a marker of constitutive and reprogrammed cancer stem cells driving colon cancer metastasis. Cell Stem Cell. 2014;14:342-56.
54. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res. 2005;65:10946-51.
55. Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, et al. Identification and expansion of human colon-cancer-initiating cells. Nature. 2007;445:111-5.
56. Dalerba P, Dylla SJ, Park IK, Liu R, Wang X, et al. Phenotypic characterization of human colorectal cancer stem cells. Proc Natl Acad Sci U S A. 2007;104:10158-63.
57. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nature Med. 1997;3:730-7.
58. Suetsugu A, Nagaki M, Aoki H, Motohashi T, Kunisada T, et al. Characterization of CD133+ hepatocellular carcinoma cells as cancer stem/progenitor cells. Biochem Biophys Res Commun. 2006;351:820-4.
59. Li C, Lee CJ, Simeone DM. Identification of human pancreatic cancer stem cells. Methods Mol Biol. 2009;568:161-73.
60. Lawson DA, Xin L, Lukacs R, Xu Q, Cheng D, et al. Prostate stem cells and prostate cancer. Cold Spring Harb Symp Quant Biol. 2005;70:187-96.
61. Bruno S, Bussolati B, Grange C, Collino F, Graziano ME, et al. CD133+ renal progenitor cells contribute to tumor angiogenesis. Am J Pathol. 2006;169:2223-35.
62. Cheung TH, Rando TA. Molecular regulation of stem cell quiescence. Nat Rev Mol Cell Biol. 2013;14:329-40.
63. Passegue E, Wagers AJ. Regulating quiescence: new insights into hematopoietic stem cell biology. Dev Cell. 2006;10:415-7.
64. Glauche I, Moore K, Thielecke L, Horn K, Loeffler M, et al. Stem cell proliferation and quiescence--two sides of the same coin. PLoS Comput Biol. 2009;5:e1000447.
65. Mahnke YD, Roederer M. Optimizing a Multicolor Immunophenotyping Assay. Clin Lab Med. 2007;27:469-85.
66. Brown M, Wittwer C. Flow Cytometry: Principles and Clinical Applications in Hematology. Clin Chem. 2000;46:1221-9.
67. Dalley AJ, AbdulMajeed AA, Upton Z, Farah CS. Organotypic culture of normal, dysplastic and squamous cell carcinoma-derived oral cell lines reveals loss of spatial regulation of CD44 and p75 NTR in malignancy. J Oral Pathol Med.42:37-46.
68. Ernst A, Aigner M, Nakata S, Engel F, Schlotter M, et al. A gene signature distinguishing CD133hi from CD133- colorectal cancer cells: essential role for EGR1 and downstream factors. Pathology.43:220-7.
69. Lichtman JW, Conchello J-A. Fluorescence microscopy. Nat Meth. 2005;2:910-9.
70. Chiou SH, Yu CC, Huang CY, Lin SC, Liu CJ, et al. Positive correlations of Oct-4 and Nanog in oral cancer stem-like cells and high-grade oral squamous cell carcinoma. Clin Cancer Res. 2008;14:4085-95.
71. Goodell MA, McKinney-Freeman S, Camargo FD. Isolation and characterization of side population cells. Methods Mol Biol. 2005;290:343-52.
72. Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer. 2008;8:755-68.
73. Pedersen EA, Shiozawa Y, Mishra A, Taichman RS. Structure and function of the solid tumor niche. Front Biosci (Schol Ed). 2011;4:1-15.
74. Fuchs E, Horsley V. Ferreting out stem cells from their niches. Nat Cell Biol. 2011;13:513-8.
75. Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CHM, Jones DL, et al. Cancer Stem Cells - Perspectives on current status and future directions: AACR workshop on cancer stem cells. Cancer Res. 2006;66:9339-44.
76. Beckhove P, Schutz F, Diel IJ, Solomayer EF, Bastert G, et al. Efficient engraftment of human primary breast cancer transplants in nonconditioned NOD/Scid mice. Int J Cancer. 2003;105:444-53.
77. Quintana E, Shackleton M, Sabel MS, Fullen DR, Johnson TM, et al. Efficient tumour formation by single human melanoma cells. Nature. 2008;456:593-8.