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Cancer Stem Cells: The Role of the Environment and Methods to Identify Them

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1. Introduction

The study and investigation of cancer stem cells (CSCs) or tumour initiating cells (TICs) have received enormous attention over the last 15 years. CSCs are rare, quiescent and capable of self-renewing and maintaining tumour growth and heterogeneity. Better understanding of CSCs will lead to a new era of both basic and clinical cancer research, re-classification of human tumours and development of novel therapeutic strategies. Therefore, the biological properties of CSCs, the relevance of CSCs to cancer therapy, methodologies to identify them are essential in order to address real and efficacious therapeutic strategies to eradicate the cancer.

Primary tumours are responsible for 10% of cancer deaths. In most cases, the main cause of mortality and morbidity is the formation of metastases in sites distant from tissue in which the primary cancer is formed. The cancer cell detach from primary tumour and, through blood and/or lymphatic vessels, colonises new sites in which forms the secondary tumour. Accumulating evidences suggest that a subpopulation of tumour cells with distinct stem-like properties is responsible for tumour initiation, invasive growth and possibly dissemination to distant organ sites (Reya et al., 2001; Brabletz et al., 2005). These few cells can divide asymmetrically, producing an identical daughter cell and a more differentiated cell, which, during their subsequent divisions, generate the vast majority of tumour bulk (Caussinus & Gonzalez, 2005; Clevers, 2005). Many names have been used to identify this subpopulation, but the term "Cancer Stem Cells" (CSCs) has received wide acceptance.

CSCs hypothesis states that the cancer stem cell is a cell within a tumour possessing the capacity of self-renewal and to cause the heterogeneous lineages of cancer cells that comprise the whole tumour (Ailles & Weissman, 2007; Lobo et al., 2007). Experimentally, this population is identified by its ability to form new tumours through serial transplantations in immunodeficient non-obese diabetic (NOD)/severe combined immunodeficient (SCID) mice, re-establishing tumour heterogeneity (Sarry et al., 2011).

There are two basic topics that underline the hypothesis that CSCs may originate from normal tissue stem cells. First of all, CSCs share many features with normal stem cells, including self-renewal, differentiation, drug resistance and migration capacity. Secondly, the longevity of stem cells make them susceptible to accumulate genetic and epigenetic damages in such a way to make them good candidates for the emergence of the neoplastic transformation.

Existing therapies have enhanced the length of survival after diagnosis of cancer, but completely failed in terms of recovery. Cancer therapy failures may be due to inefficient effects of current therapy upon potentially quiescent CSCs, which remain vital and retain the capacity to regenerate the tumour (Dean et al., 2005). In most cases, current therapeutic strategies are developed to target the bulk of cancer and likely do not eradicate CSCs completely. CSCs are more resistant to therapies, due to survival advantage with increased anti-apoptotic activities and drug resistance due to increased levels of drug efflux pumps such as BCRP (breast cancer resistance protein) and MDR (multi-drug resistance) complexes (Steinbach & Legrand, 2007; Chuthapisith et al., 2010; Dean et al., 2005).

Therefore, the rarity of CSCs will require therapeutic strategies different from conventional ones. Specific recognition of CSCs from the tumour mass will be the first challenge (Bao et al., 2006; Woodward et al., 2007). The identification of CSCs-specific antigens may help develop specific targeting. Since the origins of CSCs vary from cancer to cancer, the development of therapeutic strategies targeting different CSCs populations will also be necessary. Future therapeutic strategies will need to integrate inhibition of these resistant mechanisms with CSCs killing components. Finally, multiple pathways/mechanisms will likely need to be targeted together for their effective elimination. Therefore, with further improvements in understanding of CSCs biology, we will be able to develop better diagnostic and therapeutic methodologies, with which to classify, treat and cure cancer.

Research on CSCs has its roots in the second half of the nineteenth century when, in 1858, Virchow (Virchow, 1858.) argued that all cells come from other cells, and all organisms are made up of cells providing scientific basis for cancer origin. With Virchow, the concept of cellular hierarchy, a column in the investigation of the cancer stem cells, was born. In 1875, after the birth of experimental embryology, Cohnheim (Cohnheim, 1875) hypothesized that stem cells 'misplaced' during embryonic development were the source of tumours that formed later in life. The first experimental evidence of the existence of normal hematopoietic stem cells was supplied by Till & McCulloch in 1961 (Till & McCulloch, 1961). They eradicated the blood system of recipient mice with whole-body ionizing radiation and then injected donor bone marrow cells into the tail vein of irradiated mice to assay how many cells were required to restore blood production. They detected colonies of blood cells in the spleens of the recipient mice containing all the different mature blood cells. The same results were obtained with transplantations of spleen colonies into secondary recipients. On the basis of these studies on the normal blood system hierarchy, the framework for investigation of cancer stem cell was set. Bruce and Van Der Gaag (Bruce & Van Der Gaag, 1963) demonstrated that only approximately 1% of transplanted malignant blood cells could form colonies in the spleen of mouse as well as Hamburger and Salmon (Hamburger & Salmon, 1977) showed that only a small subset of tumour epithelial cells could produce in vitro colonies. Since then, researchers speculated that these cancer cells were cancer stem cells. Later studies involving teratocarcinomas further showed that a single tumour cell can give rise to a new tumour and generate heterogeneous progeny, providing strong evidence for the clonal origin and self renewal of tumours (Makino, 1956; Bruce, 1963; Lewis, 1964). During the twentieth century, much attention was placed both on physical and chemical carcinogenesis and genetic mechanisms that underlie cancer development. In the 1980s, Fialkow (Fialkow, 1981, 1990.) demonstrated that a single progenitor-cell gives rise to clones that sequentially acquire additional mutations and generate a tumour both in Chronic Myelogenous Leukemia (CML) and Acute Lymphoblastic Leukemia (ALL). Although all these studies, the work that laid the foundations for all subsequent cancer stem cells

research was provided by John Dick in 1997 (Bonnet & Dick, 1997). Dick and co-workers showed that, in human acute myeloid leukaemia (AML), a rare malignant cell with ability to repopulate the entire original disease over serial transplantations, implying self-renewal and capacity to differentiate, was only found within the immature $CD34^+CD38^-$, and not $CD34^+CD38^+$, subpopulation (Bonnet & Dick, 1997; Lapidot et al., 1994). This work represented the foundation from which started the research on CSCs in both hematologic malignancies and solid tumours.

The first identification of CSCs in solid tumours was made by Al-Hajj in 2003 (Al-Hajj et al., 2003) who identified and isolated CSCs from breast cancer by using CD44 and CD24 markers. Since, CSCs have been identified in a variety of solid tumours such as glioblastomas (Singh et al., 2003), melanoma (Fang et al., 2005), sarcoma (Tirino et al., 2008; Tirino et al., 2011), prostate (Collins et al., 2005), ovarian (Bapat et al., 2005), gastric (Takaishi et al., 2009) and lung cancers (Eramo et al., 2008; Tirino et al., 2009) as reassumed in Table 1.

Tumour type	CSCs phenotype	References
Breast	CD44+CD24-/low	Al-Hajj, 2003
Brain	CD133+	Singh, 2003
Melanoma	CD133+ CD20+	Fang, 2005
Sarcoma	CD133+	Tirino, 2008, 2011
Prostate	CD44+ $\alpha2\beta1$+ CD133+	Collins, 2005
Ovarian	Spheres	Bapat, 2005
Gastric	CD44+	Takaishi, 2009
Lung	CD133+	Eramo, 2007; Tirino, 2009

Table 1. CSCs identification in human solid cancer

2. Normal and cancer stem cells

Human development follows a predetermined program by which the zygote develops into a multicellular organism. The zygote gives rise to a totipotent ball of cells that further differentiates into the three germ layers: endoderm, ectoderm, and mesoderm (McClay, 1991) developing in turns into all tissues in the adult body. Tissues in which malignancies originate, such as the blood, brain, breast, skin, and gut, are organized as a cellular hierarchy with a small population of tissue-specific stem cells responsible for both development and maintenance/regeneration of tissues for the human lifetime (Cairns, 1981). A normal adult stem cell is defined as a somatic cell that can undergo extensive cell division and has the potential to give rise to both stem cells and cells that differentiate into specialized cells. Adult stem cells possess two unique characteristics: multipotency, which allows mature cells to compose specific organs or tissue, and self-renewal, which supplies an organ with an adequate number of cells to maintain the organ's function.

The first characteristic is the self-renewal, a special cell mitotic division, that enables a stem cell to produce another stem cell with essentially the same development and replication potential. The ability to self-renew enables expansion of the stem cell compartment in response to systemic or local signals, which trigger massive proliferation and maintenance of a tissue-specific undifferentiated pool of cells in the organ or tissue.

Differentiation is the second function of a stem cell and involves the production of daughter cells that become tissue-specific specialized cells. For example, the hierarchy in the blood system leads stem cells that first differentiate into transiently amplifying progenitors cells, then these cells rapidly proliferate for a short time and produce terminally differentiated cells, such as macrophages or basophiles.

Cancer stem cells have been defined as 'a cell within a tumour that possess the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumour' (Clarke & Fuller, 2006). These two definitive biological properties are what make the CSC the prime candidate for initiation of relapse, thereby becoming a crucial target for the development of novel therapies.

The CSC is commonly assumed to be developed from a normal tissue stem cell and, as such, thought to be the cell from which a malignancy originated. There is an ongoing debate over whether CSCs represent a mature tissue stem cell which has undergone malignant change or whether more differentiated cells re-initiate a 'stemness' programme as part of, or following, malignant transformation. Despite the multitude of regulatory systems that prevent abnormal proliferation during biologic normal processes, mutations that result in aberrant mitoses can occur. Most of the mutations leading to cancer regards the cell division, DNA damage, and aberrant signal transduction pathways. Stem cells may be preferential targets of initial oncogenic mutations because in most tissues in which cancer originates, they are the only long-lived cell populations and are therefore exposed to more genotoxic stresses than their shorter-lived, differentiated progeny (Pardal et al. 2003, Reya et al. 2001).

The cancer stem cell theory proposes that tumours have a cellular hierarchy that is a caricature of their normal tissue counterpart because they reflect the multipotency of the originally transformed cell.

The basis for this functional heterogeneity has been explained by two models. The stochastic model predicts that a malignancy is composed of a homogeneous population of cells, which generate their heterogeneity in response to particular combinations of endogenous and exogenous factors. Endogenously these would include gene dosage effects, transcriptional and translational control mechanisms, whereas exogenously cytokine concentrations, cell–cell interactions and particularly niche environment would all be important. Therefore, all tumour cells may give rise a tumour.

The CSCs model predicts that a malignancy is organised in a manner analogous to the normal tissue hierarchy with cancer/tissue stem cells able to produce identical daughter stem cells with self-renewal capacity, and committed progenitor daughter cells with limited, although potentially still significant, potential to divide. Therefore, the CSC model has a rare CSC at the apex, by which tumour heterogeneity originate as result of a random process of genetic changes and selective advantage. Although this, in the stochastic model, stemness exists as a functional phenotype, which could be shown by any member of the malignant population given the appropriate endogenous and exogenous factors. Most plausibly, having occupied a suitable niche, a cell now able to express its self-renewal programme and producing daughter cells which differentiate to populate the bulk malignancy, can become a CSC. The stochastic model does not yet predict whether stemness is found truly within each population, or whether cells first undergo a process of de-differentiation to a more tissue specific stem cell-like phenotype, reacquiring stemness. This plasticity within a cell lineage, between the CSC and non-CSC compartments, is known as bi-directional interconvertibility (Gupta et al., 2009). Another model that we consider is one in which cancer cells, stem cells included, are subject to a process known as clonal evolution. In clonal evolution, new clones

continuously develop, emerging with new genetic, and potentially epigenetic, changes. Environmental pressures result in constantly adapting cancer cell populations. These adaptations may change proliferation, metastatic potential or drug resistance, for example. It is also possible that evolution could generate novel clones with self-renewal potential, providing a rather more 'hard-wired', albeit evolving, route to the development of CSCs than does the process of interconvertibility described above as shown in primary and relapsed leukaemias by Mel Greaves (Greaves et al., 2010), albeit at the level of a limited number of known targets.

3. Cancer stem cells and niche

Stem cells are found in specific areas of an organ where a special microenvironment called the niche maintains stem cell functions. Stem cells and niche cells interact with each other via adhesion molecules and paracrine factors. They exchange molecular signals that maintain the unique characteristics of the stem cells. Therefore, stem cells, their progeny, and elements of their microenvironment make up an anatomical structure that coordinates normal homeostatic production of functional mature cells by cellular mechanisms that regulate the balance of self-renewal and differentiation. The microenvironment surrounding normal and cancer stem cells, which provides the stem cell niche, plays multiple roles including as a mechanical anchorage for the stem cells and in cross-talk communication mediated by direct contact and/or indirect extracellular factors.

For instance, Wnt ligands are produced and released from both stem cells and niche cells, BMP and Sonic hedgehog (Shh) are released from niche cells and epithelial cells respectively, and Notch signaling is transmembranously transmitted between neighboring. The microenvironment may also provide signaling via the cell receptor integrin as suggested by its expression.

The existence of a stem cell niche, or physiological microenvironment, has been studied for mammalian adult stem cells in the intestinal, neural, epidermal, and hematopoietic systems (Iwasaki & Suda, 2009; Shaker & Rubin, 2010).

Bone marrow (BM) hematopoietic stem cells (HSCs) are the best characterized stem cell population. Single HSCs are multipotent, highly self-renewing, and cycle with slow kinetics. Bone and marrow are intrinsically linked with HSCs, and their primitive progeny is located proximal to the endosteal surface of trabecular bone (Fig. 1).

Studies have shown that osteoblast (OB) cells are required for this localization. Genetically engineered increases in OB numbers lead to elevated HSC numbers without changes in committed progenitor populations. The Bmpr1a and activated parathyroid hormone–related protein receptor (PPR) studies provide mechanistic insights into OB-mediated HSC expansion. The Bmpr1a studies identified a specific subset of N-cadherin–expressing OBs that form an N-cadherin/β-catenin adherens complex with HSCs, perhaps mediating the attachment or adhesion of HSCs within their niche. N-cadherin is negatively regulated by c-Myc in differentiating HSCs, perhaps promoting displacement from the endosteum (Wilson, 2004). In the PPR studies, Notch signaling was implicated, because the Notch ligand Jagged 1 was highly expressed in OBs and Notch activated in HSCs. Also, Wnt protein was shown to promote HSC proliferation (Reya et al., 2003; Willert et al., 2003), and now, an additional study has shown that Notch and Wnt inputs are integrated by HSCs. Although this, the exact roles of Wnt and Notch signaling will require further analysis. The maintenance of HSC levels by these Wnt, Notch and Hedgehog signaling pathways could lie in controlling

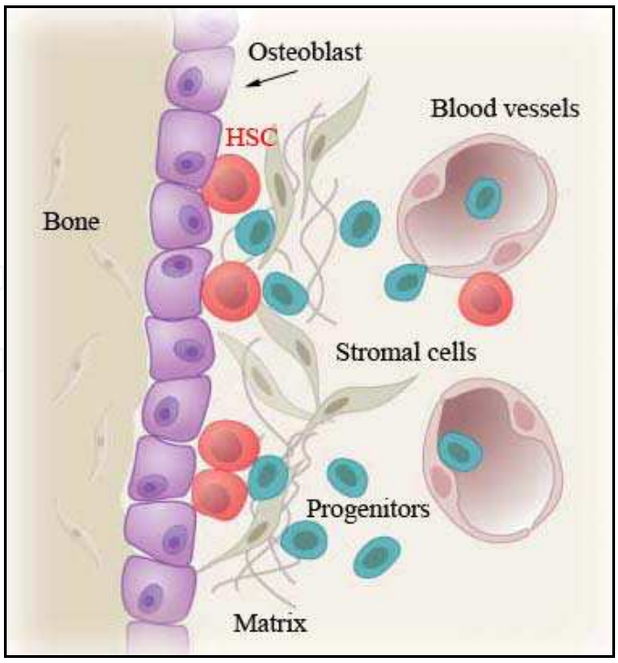


Fig. 1. Overview of Bone Marrow niche in which HSC are indicated. (Moore, Lemischka, 2006)

asymmetric cell division. Other mediators of HSC self-renewal have been identified; such as p21 (Cheng et al., 2000), p18 (Yuan et al., 2004) and bmi-1 (Park et al., 2003); but how these are controlled by extrinsic signals from the niche has not been determined. In addition, HSCs can be found in tissues that have no OBs (Taniguchi, et al., 1996). Thus, although BM-HSC niches are at least in part composed of OBs, other cell types may also provide this function. The contribution of other cellular elements, such as stromal cells or perivascular cells, is yet to be defined. It is has been shown that HSCs can be recruited to a ''vascular niche'' in the BM (Heissig et al., 2002). Such vascular structures could serve as components of extra-medullary niches. One intriguing study has demonstrated that HSCs express a calcium sensing receptor. Stem cells lacking this receptor fail to localize to the endosteal niche and do not function normally after transplantation (Adams et al., 2006). This study highlights the importance of the ionic mineral content of the bone itself and of the bone-derived matrix in the lodgment and retention of HSCs within the endosteal niche.

Another example of niche is the intestinal niche. The epithelial villus/crypt structure and its surrounding pericryptal fibroblasts and mesenchyme in the small intestine make up an anatomical unit that generates four cell lineages: absorptive enterocytes and the goblet, enteroendocrine, and Paneth cells of the secretory lineage. The crypt is a contiguous pocket of epithelial cells at the base of the villus. Intestinal stem cells (ISCs) and transit amplifying (TA) cells within the crypt regenerate the entire villus every 3 to 5 days (Potten & Loeffler, 1990) (Fig. 2).

Genetic marking shows that crypts are derived from individual or few ISCs and that each villus is the product of cells from several adjacent crypts (Gordon et al., 1992). There are four to six ISCs per crypt that are located in a ring about four cell diameters from the crypt bottom. Progeny of activated ISCs migrate upwards to become TA cells. When they reach the top of the crypt, TA cells stop proliferating, differentiate, and assume their appropriate positions within the villus structure. As such, proper cell-fate decisions are organized within

the microanatomy of the crypt structure. Asymmetric cell division mediated by oriented mitotic planes, together with defined migratory activities within the overall crypt structure, could produce the correct localization of distinct differentiated cell types. Although asymmetric cell division along the vertical crypt axis is an attractive mechanism, this process has yet to be rigorously demonstrated in the ISC system. Mesenchymal cells surround the crypt. It is likely that the mesenchymal signals that mediate different cell fates along the vertical crypt axis are spatially organized into distinct domains. The canonical Wnt pathway regulates ISCs. A Wnt gradient is predicted by the distribution of nuclear versus cytoplasmic β-catenin along the crypt axis (van de Wetering et al., 2002). A comprehensive study has now shown that Wnt signaling components are expressed by both crypt epithelial cells and surrounding mesenchymal cells, predicting an even broader role for this pathway in normal homeostasis as indicated by genetic studies (Gregorieff et al., 2005). There is also evidence that Wnt inhibitors such as Dkk3 may be expressed in a graded manner in this tissue, suggesting an intricate quantitative balance between positive and negative regulators of this pathway (Byun et al., 2005).

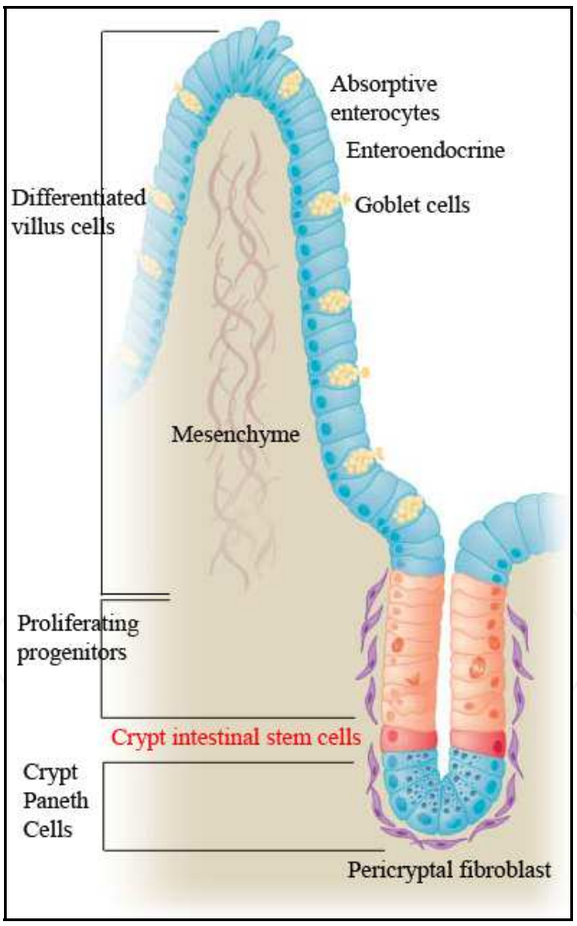


Fig. 2. Overview of intestinal niche in which the epithelial villus/crypt structure and its surrounding pericryptal fibroblasts and mesenchyme in the small intestine make up an anatomical unit that generates four cell lineages: absorptive enterocytes and the goblet, enteroendocrine, and Paneth cells of the secretory lineage. (Moore, Lemischka, 2006)

In light of these findings, it has been proposed that a "cancer stem cell niche" also exists and that interactions with this tumour niche may specify a self renewing population of tumour cells. The surrounding microenvironment (stromal fibroblasts, adipocytes, and endothelial cells, as well as the extracellular matrix) and the immune system are known to play important roles in cancer progression.

As already described above, the two cardinal characteristics of stem cells are the capacity to self-renew, in order to produce stem cells, and to differentiate in order to obtain the full repertoire of specialized cells that comprise the tissue in question.

Achieving a delicate balance between these two opposing processes is critical in the adult organism for maintaining proper tissue homeostasis and for repair and regeneration of tissues after injury. Excessive differentiation at the expense of self-renewal, for instance, can deplete the stem cell pool, whereas excessive self-renewal could lead to aberrant expansion and even tumorigenesis.

Niche cells provide a sheltering environment that sequesters stem cells from differentiation stimuli, apoptotic stimuli, and other stimuli that would challenge stem cell reserves. The niche also safeguards against excessive stem cell production that could lead to cancer. Stem cells must periodically activate to produce progenitor or transit amplifying (TA) cells that are committed to produce mature cell lineages. Thus, maintaining a balance of stem cell quiescence and activity is a hallmark of a functional niche. In fact, it is widely known that in general stem cells are in a quiescent state (G_0 phase in the cell cycle) and that this quiescence prevents the stem cells from entering into the cell cycle and undergoing differentiation. Different mechanisms ensure a proper balance between the production of stem cells and/or progenitor cells and differentiated cells. These could include Wnt, Notch, and Hedgehog signaling. Disregulation of these pathways can lead to tumour formation. Cancer and normal stem cells have much in common with regard to the maintenance system within their niches.

A representative example of cancer stem cells niche is observed in acute myeloid leukemia (AML) and its niche in BM. Dick and colleagues showed that anti-CD44 antibody treated non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice transplanted with AML cells exhibited a significantly lower rate of disease onset (Jin et al., 2006). Also, Van Etten and colleagues showed that there was impaired induction of chronic myeloid leukemia (CML)-like myeloproliferative disease among recipient mice that received transplanted *BCR–ABL1*-transduced CML progenitors from CD44-mutant donors (Krause et al., 2006). These results indicate that for both AML and CML, CD44 is essential for the homing and engraftment of the cancer stem cells to the niche. In other words, CD44-expressing leukemic stem cells adhere to the niche and bind to hyaluronic acid expressed by cells on the surface of sinusoidal endothelium or endosteum in BM; this binding is crucial for the niche's maintenance of the stem cells. Interestingly, this molecular mechanism resembles that of the interaction between normal HSC and the vascular niche described earlier.

Gilbertson and colleagues showed that brain tumour cells coexpressing Nestin and CD133, the fraction believed to contain the cancer stem cell, were found near the capillaries in the brain tumour (Calabrese et al., 2007). When these cells were cocultured, the cancer stem cells selectively adhered to the endothelial cells. This suggested that the endothelial cells secreted factors necessary to maintain the cancer stem cells and showed that cancer stem cells of brain tumours rely on endothelial cells, which form a vascular niche that maintains the capacity of the cancer stem cells for self-renewal, differentiation, and proliferation. In addition to its role in brain tumours, CD133 has been extensively studied in other kinds of

cancer, such as colon, prostate, and pancreatic cancers, and is now considered to be a tumour marker for those cancers (Table 1). Despite the growing interest in CD133, the functional role of CD133 itself remains unclear. CD133 is a cholesterol-binding pentaspan membrane glycoprotein and is associated with a membrane microdomain. The microdomain in the stem cells or progenitors has been proposed to be a carrier of important molecular factors necessary for the maintenance of stem cell properties. Therefore, it is hypothesized that the localized distribution of CD133 during cell division might reflect the localized distribution of the microdomains that determine the daughter cell's fate, that is, whether it remains as a stem cell or undergoes differentiation (Bauer et al., 2008).

Li and Neaves studied the dependence of stem cells on their niches and they hypothesized that the behaviors of cancer stem cells and normal stem cells are regulated by the niche to different degrees (Li & Neaves, 2006) The cancer stem cell is engendered by an intrinsic mutation that leads to its high proliferation. This highly proliferative state itself can alter the signaling balance between the niche and stem cells. Namely, the characteristics of the niche that function to maintain quiescence become relatively ineffective, and the characteristics of the niche that function to support proliferation and differentiation become more dominant. This model is supported by some clinical symptoms, one of which is the blast crisis of CML. It is important to note that many signaling pathways involved in the interaction between normal stem cells and their niches are also involved in the interactions between cancer stem cells and their niches, and can play a role as promoters of tumourigenesis and cancer proliferation. An identical set of proteins under slightly different conditions can deliver totally different results. Thus, the purpose of the niche is not only the cradling of existing cancer stem cells, but also the cradling of future incoming cancer stem cells. The niche is constantly sending passive signals of invitation to remote cancer stem cells. Matrix metalloproteinases (MMP) are well-known factors, not only for their contribution to the repair of inflammation and wounds, but also for their involvement in cancer invasion and metastasis. For example in lung cancer, vascular endothelial growth factor secreted by primary cancer cells induces specific MMP9 expression in lung endothelial cells and macrophages via vascular endothelial growth factor receptor (VEGFR) tyrosine kinase, resulting in the formation of the cancer stem cell niche. This means that the cancer cells can produce their own favorable microenvironment, the future cancer stem cell niche, from a distance by secreting factors that influence the protein composition at that site. Bone metastasis of prostate cancer has been shown to be supported by urokinase-type plasminogen activator (uPAR) or prostate specific antigen (PSA) secreted by prostate cancer cells through alteration of the growth factors in the bone microenvironment, thus enhancing the proliferation of the osteoblasts that serve as the cancer stem cell niche (Logothetis et al., 2005). Lung metastasis of breast cancer via secreted protein acidic and rich in cysteine osteonectin or MMP2 has also been found to be based on this mechanism (Minn et al., 2005). From both scientific and clinical viewpoints, the biology of the normal and cancer stem cell and their niche is expected to be one of the most promising fields of research to address an efficacy therapeutic strategy in order to cure and heal the cancer.

4. Signaling in normal and cancer stem cells niche

4.1 Wnt/β-catenin pathway

Wnt/β-catenin pathway is a conserved molecular system that plays a major role in development and homeostatic tissue self-renewal (Rattis et al., 2004). This pathway takes its

name from the wingless gene in Drosophila, homologue of human gene int-1 and was characterized for the first time by Rijsezijk et al., in 1987. The Wnt ligands activate a signaling pathway that induces changes in gene expression, physiology, cell adhesion, and polarity of stem cells. In mammals, the Wnt protein family comprises 19 highly conserved molecules. At least three pathways have been described, including the main one that interacts with two distinct families of cellular receptors: the Frizzled family of receptors (Fz) and the family of proteins related to the LDL receptor (LRP). In a physiological state, β-catenin level remains low due to constant turnover mostly by the destruction complex APC/Axin/GSK3b. Phosphorylation by this complex causes β-catenin to be degraded by the proteasome. When the Wnt binds to the cell surface receptor Frizzled and activates disheveled (Dsh), glycogen synthase kinase 3b (GSK3b) is dissociated from the destruction complex. As a result, free β-catenin accumulates and translocates into the nucleus. There, β-catenin binds to T cell factor (TCF) releasing it from a repressed state and initiates transcription of its target genes (Fig. 3).

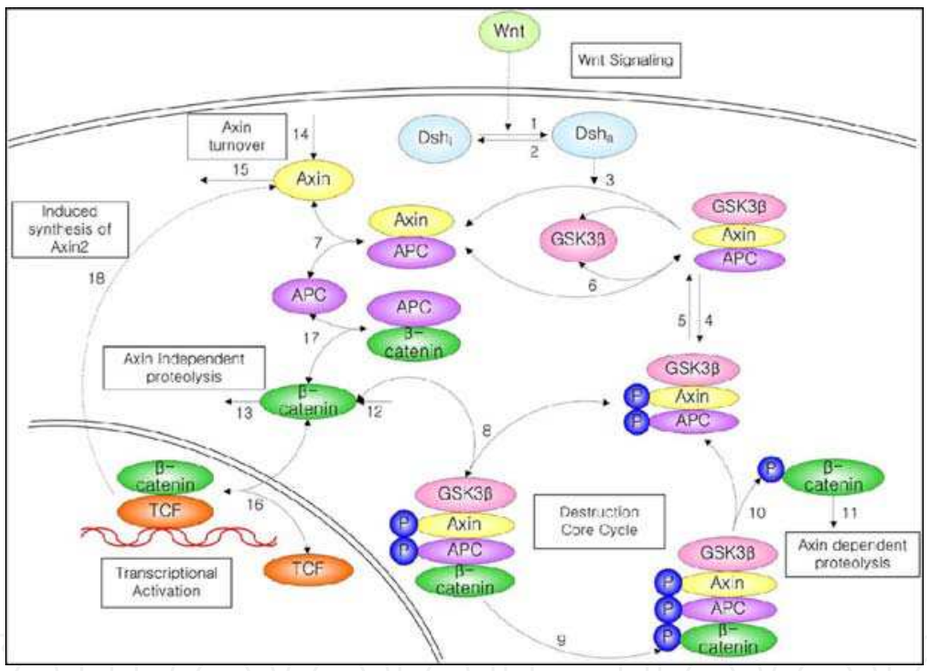


Fig. 3. Brief overview of Wnt/β-catenin pathway (Cho et al., 2006)

C-myc, cyclin D1, MMP7, and CD44 are some of the known target genes that may be relevant for a tumour. Among the factors that regulate the release of Wnt family proteins, there are also the BMPs. For example, intestinal tissue is produced by mesenchymal cells outside the crypts. A defect in this setting may cause an expansion of stem and progenitor cells that leads to a state-onset juvenile polyposis. Wnt proteins may also play a role through activation of II kinase calmodulin and protein kinase C, causing an increase of intracellular Ca2 + or Jun N-terminal kinase (JNK), under the control of cytoskeleton rearrangements of cell polarity.

Recently in the development of research on "cancer stem cells" a great interest in the Wnt pathway has taken mainly regarding the regulation mechanisms of self-renewal of stem cells, repair and tissue regeneration that are involved in cancer development when Wnt

signaling is activated in aberrant manner (Polakis, 2000; Reya & Clevers, 2005). In this context, recent experimental evidence together with encouraging clinical trials have focused attention on the role of molecular signaling pathway in tumours.
In summary, the organs in which Wnt proteins influence the process of stem cell self-renewal are the same organs that are studied as Wnt-dependent tumours. In fact, numerous studies have shown aberrant activation in many human tumours, such as colorectal, melanoma, head and neck and leukemia. This aberrant activation may be caused by mutations and/or deregulation of many different components of the Wnt signaling.

4.2 Notch pathway

Notch is known to promote the survival and proliferation of non neoplastic neural stem cells and to inhibit their differentiation.
Notch signaling mechanism consistis of four membrane receptors (called Notch1, 2, 3 and 4) and five ligands (eg Delta-like 1, Delta-like 2, Delta-like 4, Jagged1 and Jagged2). Notch receptors are eterodimeric proteins formed by NEC (extracellular subunit) and NTM (transmembrane subunit). Interaction of Notch receptors (Notch 1 to 4) with their ligands (Delta like 1, -3, -4, Jagged-1 and -2) leads to cleavage of the transmembrane Notch receptor, giving rise to the Notch intracellular domain (NICD) that migrates into the nucleus (Weinmaster, 1998). In the nucleus, NICD associates with a transcription factor, RBP-Jk (also known as CSL for *C*BF1/*S*u(H)/*L*ag-1) (Weinmaster, 1998; Artavanis-Tsakonas et al., 1999; Egan et al., 1998; Greenwald, 1998; Mumm & Kopan, 200) and activates transcription from the RBP-Jk DNA binding site (Fig.4). The NICD–RBP-Jk complex upregulates expression of primary target genes of Notch signaling, such as hairy and enhancer of split (HES)-1, -5, -7 and more recently isolated HES-related repressor protein (HERP)-1 to -3 in mammals (Davis & Turner, 2001; Iso et al., 2003). The HES and HERP families are basic helix–loop–helix-type transcriptional repressors and appear to act as Notch effectors by negatively regulating expression of downstream target genes (Chen et al., 1997; Ishibashi et al., 1995; Ohsako et al., 1994; Van Doren et al., 1994). Thus, many ligands, receptors, and effectors are involved in this pathway.

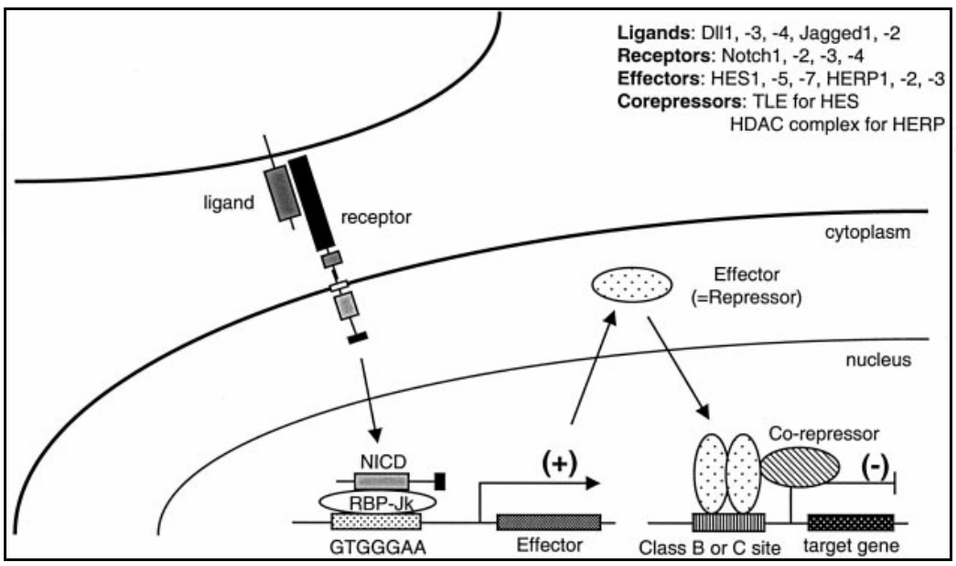


Fig. 4. Brief overview of Notch pathway (Iso et al., 2003)

Through this mechanism, the Notch signals provide an indication on how much of ancestors will remain undifferentiated or differentiate into different cell types. This type of signal is present in many different cell lines, including the nervous system and T lymphocytes (Hajdu et al., 2010).

4.3 Hedgehog pathway

Hedgehog (hh) signaling plays a role in many processes during embryonic development and remains active in the adult where it is involved in the maintenance of stem cell populations. Here, aberrant Hedgehog signaling in some cases can lead to certain forms of cancer. The hedgehog gene was originally identified in flies, where it is first required for patterning of the early embryo (Nusslein-Volhard & Wieschaus, 1980). In mammals, the Hh family consists of three different members, Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh) (Riddle et al., 1993; Roelink et al., 1994). Shh is the most broadly expressed member and is involved in the patterning and growth of a large variety of organs, including the brain, skin, lung, prostate, gastrointestinal tract, and skeletal system (Pasca di Magliano & Hebrok, 2003). Hedgehogs are secreted glycoproteins, which undergo posttranslational modifications, including autocatalytic cleavage and lipid modification, before binding to a transmembrane receptor in responding cells. Hh ligands act through the transmembrane proteins Patched1 (Ptch1) and Smoothened (Smo) to trigger an intracellular signal transduction pathway that results in the activation of the Gli zinc finger transcription factors (Fig. 1). The current model of ligand receptor signaling proposes that in the absence of Hh ligands, Ptch1 blocks the function of Smo. The binding of Hhs to Ptch1 releases this basal repression of Smo. As a consequence, Smo initiates an intracellular signaling cascade that is regulated by a multimolecular complex, leading to the action of the Gli proteins (Fig. 5).

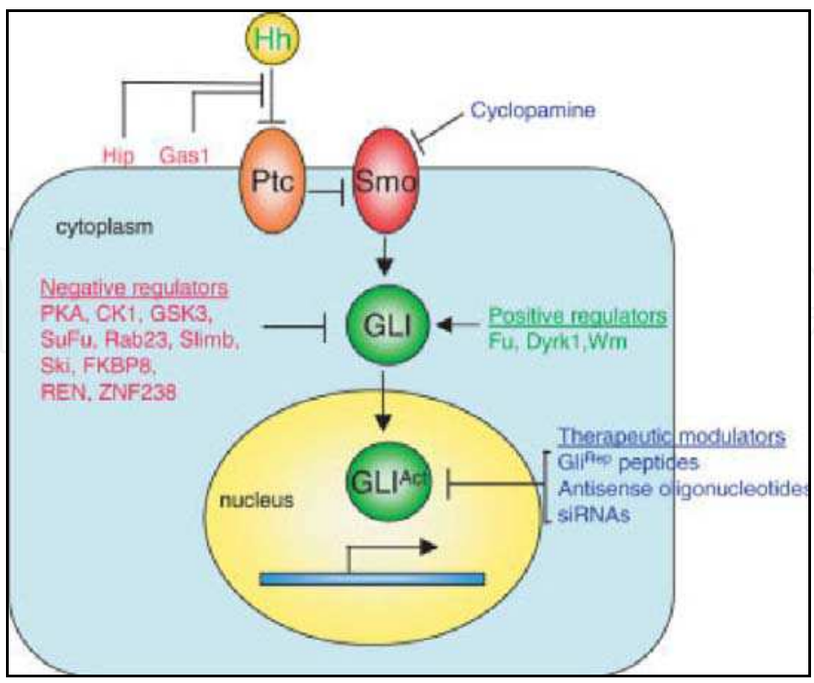


Fig. 5. Brief overview of Hedgehog pathway (Stecca et al., 2005)

5. Detection, isolation and characterization methodologies of cancer stem cells

5.1 Current methodologies

The CSCs can be identified and isolated by four main methodologies:

1. isolation of CSCs by flow cytometry according to CSC-specific cell surface markers (Al-Hajj et al., 2003; Singh et al., 2003; Fang et al., 2005; Tirino et al., 2008; Collins et al., 2005; Bapat et al., 2005; Takaishi et al., 2009; Eramo et al., 2008; Tirino et al., 2009; Dean et al., 2005);
2. detection of side population phenotype by Hoechst 33342 exclusion (Goodell et al., 1996; Song et al., 2010; Fukuda et al., 2009; Moserle et al., 2008);
3. ability to grow as floating spheres (Rybak et al., 2011; Zhong et al., 2010);
4. aldehyde dehydrogenase (ALDH) activity (Ma & Allan, 2010; Awad et al., 2010).

None of the methods above mentioned are exclusively used to isolate the CSCs, highlighting the imperative to delineate more specific markers or to use combinatorial markers and methodologies. Therefore, the "cancer stem cell" will be the cell that shows the following characteristics:

- expression of stemness markers;
- side population phenotype;
- capable of forming spheres;
- capable to form new tumours in mice.

5.2 Isolation and *in vitro* expansion of cells from tumour specimens.

5.2.1 Glioblastoma primary cell culture

Biopsy specimens are put in ice-cold Leibowitz-15 medium (L-15), washed in L-15 and mechanically dissociated using 2 scalpels. The dissociation into single cells is achieved by incubation in trypsin-EDTA solution and mechanical dissociation. Thereafter, trypsin-EDTA solution is blocked using 2 mg/mL BSA and washed in L-15 twice. Cells are cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, at 37 °C, 5% CO_2, in humidified atmosphere.

5.2.2 Breast primary cancer cell culture

Breast lesions, within 30 minutes of surgery, are immediately mechanically disaggregated and then enzymatically digested in a 1:1 solution of III Type collagenase/hyaluronidase. The digestive solution is incubated at 37°C for 4-18 hours on shaking bath. After filtration through a 30 µm pore filter, the cells are plated in DMEM or RPMI at 10% FBS, at 37 °C, 5% CO_2, in humidified atmosphere.

5.2.3 Lung primary cancer cell culture

Surgical specimens are washed several times and left overnight in DMEM–F12 medium supplemented with high doses of penicillin/streptomycin and amphotericin B to avoid contamination. Tissue dissociation is carried out by enzymatic digestion by 20 mg/ml collagenase II, for 2 h at 37°C (Eramo et al., 2008). Recovered cells (Fig. 6) are cultured in Bronchial Epithelial Cell Growth Medium or RPMI at 10% FBS, at 37 °C, 5% CO_2, in humidified atmosphere.

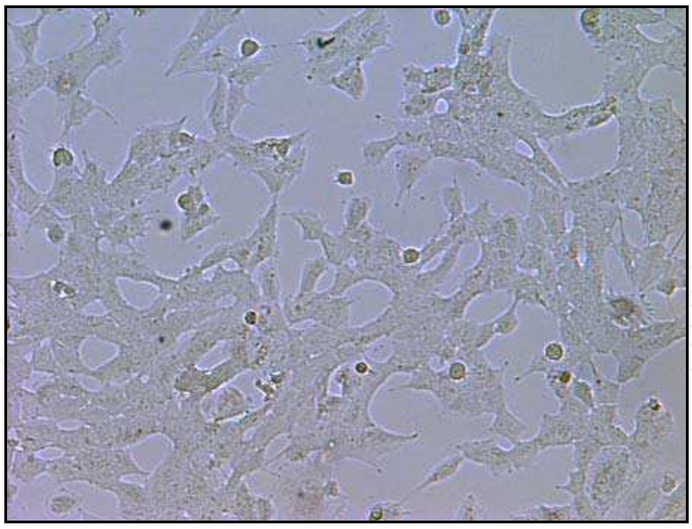


Fig. 6. Example of tumour lung cells obtained from lung biopsy (original magnification 100x)

5.2.4 Sarcoma primary cell culture

Tumours biopsies are dissected, minced and washed in PBS. After all visible clumps are removed, the cells are digested with 10mg/ml Collagenase IV or I (soft tissue or bone sarcoma, respectively), and 3mg/ml Dispase at 37 °C over night. Then cells are washed with PBS twice and filtered through a 70-µm filter to generate cell lines. Cells are cultured in DMEM at 10% FBS at 37 °C, 5% CO_2, in humidified atmosphere.

5.3 Markers expression and cell sorting by flow cytometry

Within 7 days of primary cultures, cells are detached with 0.5% BSA and 2 mM EDTA in PBS, counted and washed in 0.1% BSA in PBS. At least 200,000 cells are incubated with 1 µg/ml of fluorescent-labelled monoclonal antibodies or respective isotype controls at 4 °C for 30 min in the dark. After washing, the labelled cells are analysed by flow-cytometry using a cytometry cell sorter. The same procedure is also performed on spheres. The antibodies used are: mouse anti-human CD133/2 PE, mouse anti-human CD326 (EpCAM) FITC and PE, mouse anti-human cytokeratin (CK3-3E4) FITC, mouse anti-human CD24 PE conjugated, mouse anti-human CD29 PE-CyTM5, mouse anti-human CD44 FITC, mouse anti-human CD90 FITC conjugated and mouse anti-human CD45 CY (Fig. 7). CD133 positive and negative fractions are sorted. The purity of sorted populations is routinely 90%. Aliquots of $CD133^+$ and $CD133^-$ sorted cells are evaluated for purity by flow cytometry. $CD133^+$ and $CD133^-$ sorted cell populations are cultured in standard medium, used for *in vivo* and *in vitro* experiments and spheres formation assay.

5.4 Side population assay

Cells are resuspended at 2.0×106 cells/ml in pre-warmed standard culture medium and divided into two portions. A portion is treated with 50 µM verapamil and the other is left untreated. Both portions are incubated in standard culture medium with 5 µg/ml Hoechst 33342 for 90 minutes at 37°C on shaking bath. Mix the cells well, and place in the 37°C water bath for 90 minutes exactly. Make sure the staining tubes are well submerged in the bath water to ensure that the temperature of the cells is maintained at 37°C. Tubes should be

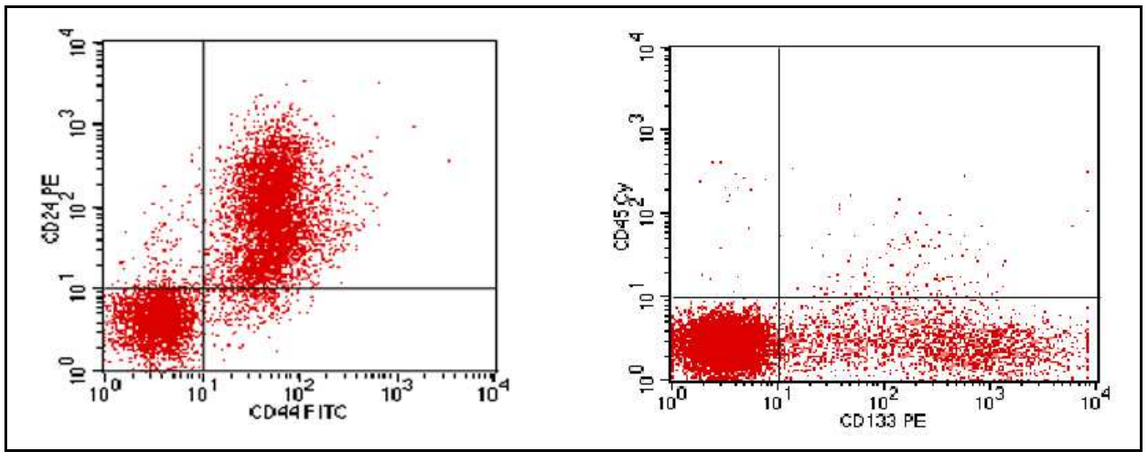


Fig. 7. Figure showing an example of cancer stem cells in breast cancer by expression of CD44 and CD24 and in lung cancer by expression of CD133

mixed several times during incubation. After 90 minutes, spin the cells down in the cold and re-suspend in cold PBS. All further proceedings should be carried out at 4°C to prohibit leakage of the Hoechst33342 dye. Add 2 µg/ml of 7-AAD or PI to the suspended cells and mix about 5 minutes before FACS analysis. This will allow for the discrimination of dead versus live cells as 7-AAD or PI permeates only cells that do not have an intact membrane. The Hoechst 33342 dye is excited at 350 nm ultraviolet and resultant fluorescence is measured at two wavelengths using a 424/44 BP and 675 LP filters for detection of Hoechst blue and red, respectively (Fig. 8). Side population (SP) positive and negative fractions are sorted. Aliquots of SP^+ and SP^- sorted cells are evaluated for purity by flow cytometry. SP^+ and SP^- sorted cell populations are cultured in standard medium, used for *in vivo* and *in vitro* experiments, analyzed for stemness markers and spheres formation assay.

5.5 ALDH activity

The ALDEFLUOR kit (StemCell Technologies, Durham, NC, USA) is used to isolate the population with a high ALDH enzymatic activity. Cells are suspended in ALDEFLUOR assay buffer containing ALDH substrate (BAAA, 1 µmol/l per 1×10^6 cells) and incubated during 40 minutes at 37°C. As negative control, for each sample of cells an aliquot is treated with 50mmol/L diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor. The sorting gates are established using as negative controls the cells stained with PI only. ALDH positive and negative fractions are sorted. Aliquots of $ALDH^+$ and $ALDH^-$ sorted cells are evaluated for purity by flow cytometry. $ALDH^+$ and $ALDH^-$ sorted cell populations are cultured in standard medium, used for *in vivo* and *in vitro* experiments, analyzed for stemness markers and spheres formation assay.

5.6 Spheres

5.6.1 Mammospheres

Single cells are plated at 1,000 cells/mL in ultra-low attachment plates (Corning) in serum-free DMEM-F12 supplemented with 10 ng/mL basic fibroblast growth factor (bFGF), 20 ng/mL epidermal growth factor (EGF), 5 µg/mL insulin, and 0.4% BSA. Cells (Fig.8) grown in these conditions as non-adherent spherical clusters of cells (usually named "mammospheres") were enzymatically dissociated by incubation in a trypsin-EDTA

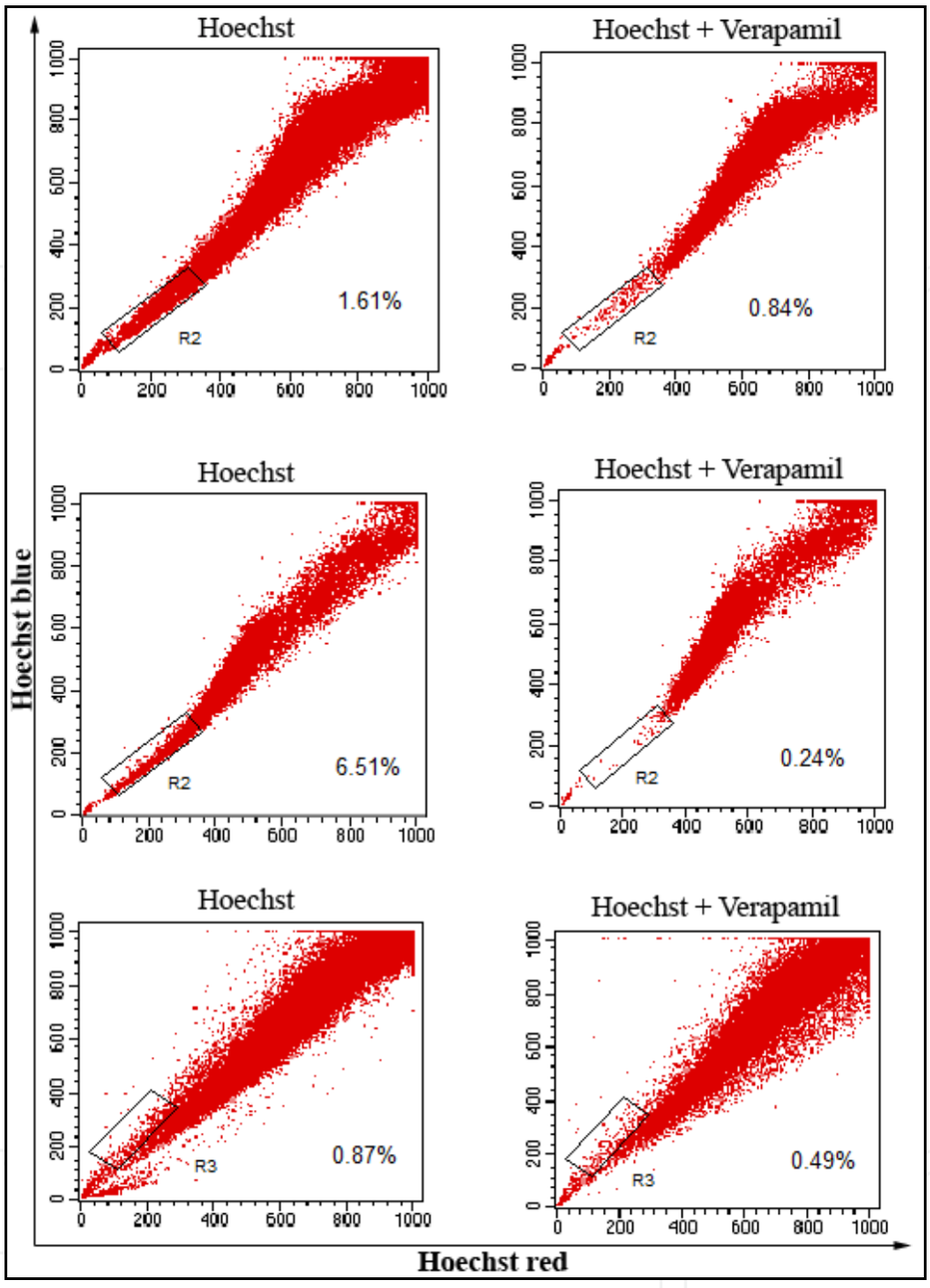


Fig. 8. Figure showing different samples in which side population is detected

solution or mechanically disaggregated every 3 days for 2 minutes at 37°C (Al-Hajj et al., 2003). Conversely, differentiation is induced by culturing mammosphere-derived cells for 8 days on collagen-coated dishes in DMEM-F12 supplemented with 10% FBS without growth factors.

5.6.2 Neurospheres

Tumour cells are resuspended in TSM consisting of defined serum-free neural stem cell medium, human recombinant EGF (20 ng/ml), bFGF (20 ng/ml), leukemia inhibitory factor

(10 ng/ml), Neuronal Survival Factor (NSF) (1x), and N-acetylcysteine (60 μg/ml). The cells are plated at a density of $3x10^6$ live cells/60-mm plate. Cells grown in these conditions as non-adherent spherical clusters of cells (usually named "spheres" or "neurospheres") are enzymatically dissociated by incubation in a trypsin-EDTA solution or mechanically disaggregated every 4 days for 2 minutes at 37°C (Singh et al., 2003). Conversely, differentiation is induced by culturing neurospheres-derived cells for 7 days on collagen-coated dishes in DMEM-F12 supplemented with 10% FBS without growth factors.

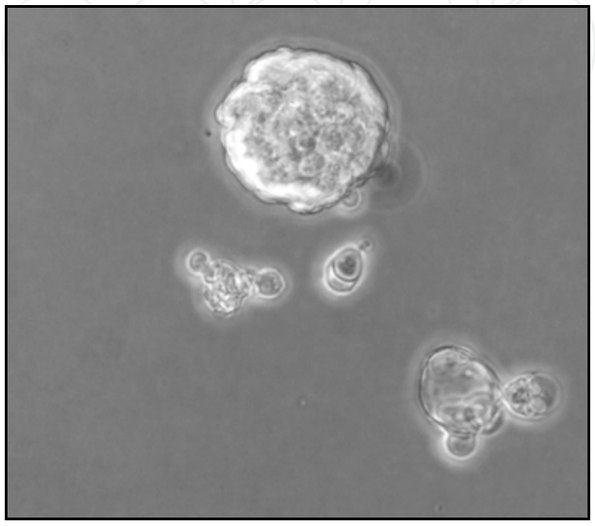


Fig. 8. Mammospheres (original magnification 40x)

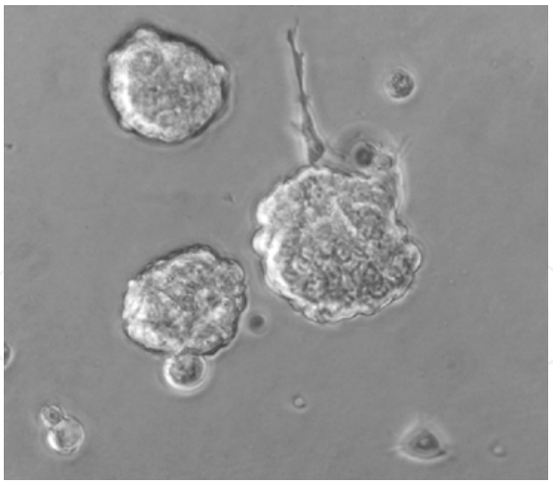


Fig. 9. Pneumospheres (original magnification 40x)

5.6.3 Pneumospheres

Tumour cells are cultured at clonal density in DMEM–F12 serum-free medium containing insulin (50μg/ml), apo-transferrin (100μg/ml), putrescine (10μg/ml), sodium selenite (0.03Mm), progesterone (2μM), glucose (0.6%), sodium bicarbonate (0.1%), BSA (0.4%), glutamine and antibiotics, and supplemented with 20μg/ml EGF and 10μg/ml bFGF. The

medium is replaced or supplemented with fresh growth factors twice a week until cells started to grow forming floating aggregates. Cultures are expanded by mechanical dissociation of spheres, followed by re-plating of both single cells and residual small aggregates in complete fresh medium. Cells (Fig. 9) grown in these conditions as non-adherent spherical clusters of cells (usually named "pneumospheres") were enzymatically dissociated by incubation in a trypsin-EDTA solution or mechanically disaggregated every 3 days for 2 minutes at 37°C (Eramo et al., 2007). Conversely, differentiation is induced by culturing pneumosphere-derived cells for 5 days on collagen-coated dishes in DMEM or RPMI supplemented with 10% FSB without growth factors. Another medium that are used to form pneumospheres is BEBM in ultralow attachment plates (Tirino et al., 2008).

5.6.4 Sarcospheres

Tumour Cells are plated at a density of 60,000 cells/well in 6-well ultra low attachment plates (Corning Inc., Corning, NY, USA) in DMEM/F12 cell medium, supplemented with 1% methylcellulose, progesterone (10 nM), putrescine (50 μM), sodium selenite (15 nM), transferrin (13 μg/ml), human recombinant insulin (10 μg/ml), human EGF (10 ng/ml) and human bFGF (10 ng/ml). Fresh aliquots of EGF and bFGF are added every day. After culture for 48–72 hours, spheres are visible at inverted phase-contrast microscope (Nikon TS 100, Nikon) (Fig. 4). Cells grown in these conditions as non-adherent spherical clusters of cells (usually named "spheres" or "sarcospheres") are enzymatically dissociated by incubation in a trypsin-EDTA solution every 3 days for 2 minutes at 37°C (Tirino et al., 2008). Conversely, differentiation is induced by culturing sarcospheres-derived cells for 2 days on collagen-coated dishes in DMEM supplemented with 10% FBS without growth factors.

5.7 Clonogenic assay

For clonogenic assays, spheres are mechanically disaggregated or detached with tripsyn-EDTA solution and the single cells obtained are plated in 96-well ultra low attachment plates for 20 days in sphere medium. The medium was changed twice a week to renew the growth factors. Singly dissociated primary sphere cells give rise to secondary spheres that, in turn, are able to form tertiary spheres. Undifferentiated spheres are passaged twenty times during the culture period.

5.8 Mesenchymal differentiating culture conditions for sarcospheres

To determine their differentiation potential, sarcospheres are cultured in osteogenic and adipogenic media supplemented with 10% FBS without EGF or bFGF. After one day of culture, spheres attached to the bottom of the flask and gradually migrated from the sarcospheres into adherent cells and after 15 days, they differentiated into mesenchymal lineages. Adipogenic medium: DMEM supplemented with 10% FBS, 1μM dexamethasone, 10μM recombinant human insulin, 200μM indomethacin, and 3-isobutyl-1-methyl-xantine (IBMX) for 15 days, changing the adipogenic medium twice a week. To detect adipocytes, immunocytochemistry for adiponectin (diluted 1:100 in PBS) was performed with a DAKO Cytomation En Vision+System-HRP kit (AEC), according to the manufacturer's instructions. Osteogenic medium: DMEM supplemented with 10% FBS, 0.1μM dexamethasone, 50μM ascorbate-2-phosphate, and 10mM β-glycerophosphate for 15 days. To detect osteoblasts, immunocytochemistry for osteocalcin (diluted 1:100 in PBS) was performed with the DAKO Cytomation En Vision+System-HRP kit.

5.9 Soft agar assay

One of the methods of analysing the transformed phenotype of the cells is the soft agar assay that measures anchorage-independent growth, which is an indicator for assessing cell transformation. In order to assess the anchorage-independent growth properties of spheres versus adherent cells or $CD133^+$ cells versus $CD133^-$ cells, soft agar assay is performed as following. Cells are detached with Tripsyn-EDTA solution (adherent cells) or mechanically disaggregated (spheres) for 5 minutes, counted and 500, 1000 and 5000 cells per well in 24-well were plated, in triplicate. The test is performed using 0.8% and 0.3% agar in IMDM as the base and top layers, respectively. Spheres and adherent cells are plated and incubated for 21 days at 37 °C in a humidified atmosphere at 5% CO_2 and 50 µl of standard medium are added twice a week. At the end of the incubation period, colonies (Fig. 10) is stained with nitrobluetetraziolium (NBT) at a concentration of 50 mg/100 ml in PBS and counted using an inverted microscope. The colony efficiency is calculated as proportion of colonies per total number of seeded cells. The data are analyzed by Image Pro Plus software.

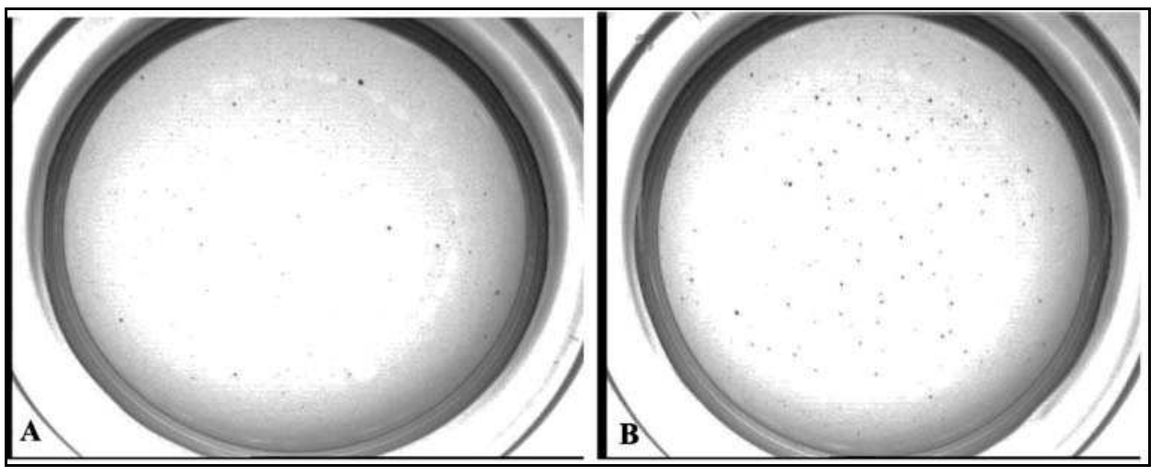


Fig. 10. Figure showing colonies derived from adherent cells (A) and spheres (B). Spheres formed colonies with major efficiency than adherent cells

5.10 *In vivo* tumourigenicity evaluation

In order to evaluate the tumourigenicity of spheres versus adherent cells or $CD133^+$ cells versus $CD133^-$ cells, *in vivo* experiments are performed using Nonobese diabetic (NOD)/severe combined immunodeficiency (SCID) mice. Cells are detached with Tripsyn-EDTA (adherent cells) or mechanically disaggregate (for spheres) for 5 minutes and counted. Cells are diluted in PBS, mixed with matrigel and injected subcutaneously in six-week-old female NOD/SCID mice at following serial dilutions: 1 and 5 x 10^2; 1 and 5 x 10^3; 1 and 5x10^4; 1 and 5 x 10^5; 1x10^6 cells. Mice are monitored every 3 days for the appearance of subcutaneous tumours. After 60-80 days, mice are sacrificed and the tumour tissue collected, in part fixed in buffered formalin and in part minced to re-obtain the cell line. Tumour volume is calculated by the formula (length x width^2)/2. Haematoxylin and eosin staining are performed to analyse tumour histology (Fig. 11). The injection experiments are in triplicate. Regarding to the possibility to regenerate tumours in mice, several publication have challenged the frequent assertion that CSCs are necessarily a rare phenomenon, by showing that assay conditions can have a significant effect on the engraftment of transplanted

malignancies. Limitations on the ability of recipient microenvironmental/niche factors to successfully provide the survival and growth signals required to support engraftment are compounded by damage to cells during isolation and preparation, the effect of residual recipient immunity and, in haematological malignancies, a lack of homing factors to allow leukaemic stem cells to engraft a suitable bone marrow niche environment. The development of mouse strains more heavily immune-suppressed than the SCID and NOD/SCID mice used in early AML studies has been a major step forward. NOD/SCID mice with additional knock out of the IL2-R γ chain (NSG and NOG mice) lack all B, T and NK cells and have deficiencies in macrophage and complement function and are the current gold standard species to regenerate tumour in *in vivo* experiments.

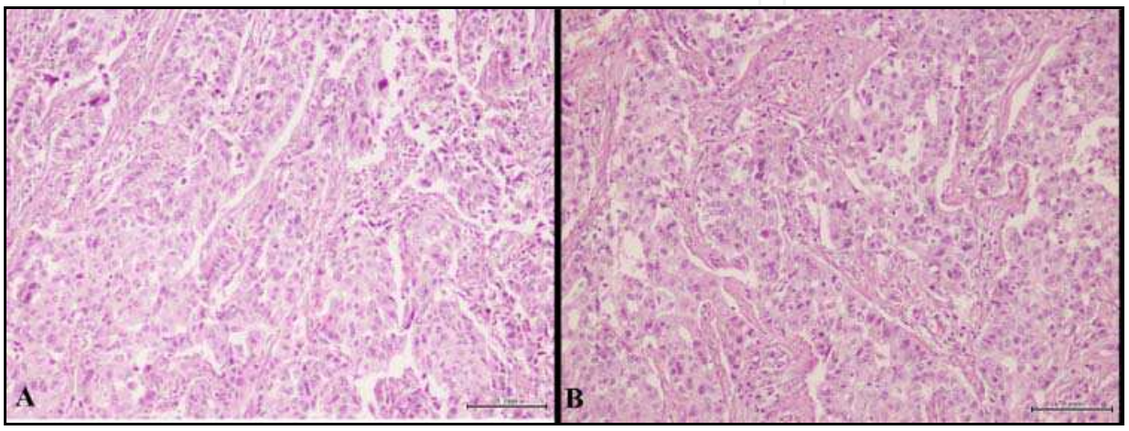


Fig. 11. Figure showing hematoxylin and eosin staining in human tumour (A) and tumour derived from xenograft (B)

5.11 Cryopreservation of tumour cells

For freezing, tumour cells or spheres are harvested by trypsin-EDTA solution for 2 minutes at 37°C and centrifuged at 800g. The pellet is washed once with fresh growth medium and cells are re-suspended in the standard medium containing 10% DMSO (freezing medium) to yield a final concentration of 1.5 x10^6cell/ml. Cells are transferred into 2-ml cryogenic vials and cells are gradually cooled at a rate of 1°C/min and stored in liquid nitrogen. For thawing, vials are quickly thawed by immersion in a 37°C water bath and cells are gently re-suspended in 12 ml of growth medium and rinsed twice with the same medium prior to re-plating under standard growth conditions.

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Over the last thirty years, the foremost inspiration for research on metastasis, cancer recurrence, and increased resistance to chemo- and radiotherapy has been the notion of cancer stem cells.The twenty-eight chapters assembled in Cancer Stem Cells - The Cutting Edge summarize the work of cancer researchers and oncologists at leading universities and hospitals around the world on every aspect of cancer stem cells, from theory and models to specific applications (glioma), from laboratory research on signal pathways to clinical trials of bio-therapies using a host of devices, from solutions to laboratory problems to speculation on cancersâ€™ stem cellsâ€™ evolution. Cancer stem cells may or may not be a subset of slowly dividing cancer cells that both disseminate cancers and defy oncotoxic drugs and radiation directed at rapidly dividing bulk cancer cells, but research on cancer stem cells has paid dividends for cancer prevention, detection, targeted treatment, and improved prognosis.

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