Cancer stem cell markers as potential targets for epithelial cancers

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In recent years, the role of tumor-initiating cells (popularly known as cancer stem cells) in tumor development and availability of novel cancer stem cell/tumor initiating cell markers promises a new arena in understanding their role in developing novel targeted molecules. It is important to identify and understand the relevance of cancer stem cells (CSC)/tumor initiating cells (TIC) in tumor development and to design appropriate strategies for CSCs and TICs elimination, which is crucial to future cancer prevention and treatment. In this review, we attempt to define various potential markers of cancer stem cells and potential exploration as therapeutic targets for epithelial cancer prevention and treatment.

Keywords: Cancer stem cells, CD133, DCAMKL-1, LGR5, Mushahi-1, Tumor initiating cells

Cancer is one of the leading causes of death across the globe. It is estimated that about 13 million people were diagnosed with cancer and about eight million men and women died of cancer in the year 2010 (Ref 1). A total of 1,596,670 new cancer cases and 571,950 deaths are projected to occur in the United States in 2011 (Ref 2). There have been significant improvements in diagnosis and treatment of several cancers, particularly an increased survival rate for cancer patients who are diagnosed at early stages. Regardless, in most cancers where diagnostic, surgical and therapeutic procedures have not yet evolved, cancer elimination and prevention are still a major challenge. For many decades, cancer drug developmental strategies led to several promising drugs, some of which have proven to be successful in cancer prevention and treatment. Despite the advances in the drug development, clinical intervention options are still limited for many types of human cancers. Particularly, tumor recurrence due to incomplete surgical elimination and resistance to anticancer agents are major problems. Typically, cancers are treated with drugs that inhibit tumor cell proliferation and are mainly targeted against DNA synthesis and key signaling molecules that contribute to tumor cell growth. In recent years, tumor-initiating cells (TICs) or cancer stem cells (CSCs) have gained significant interest due to their role in tumor development and recurrence. Therefore, it is important to identify and understand the differences between normal stem cells (SC) vs CSCs/TICs and design strategies for CSCs elimination, but not normal stem cells.

Stem cells

Stem cells (SCs) are important for normal cell turnover and tissue repair and are found in almost every organ. SCs are undifferentiated cells that exist in low numbers in each organ. They are characterized by their unique ability to divide indefinitely, generating copies of their own, as well as progenitors cells or transient amplifying cells (TACs) which further divide to produce progeny cells that differentiate into various cell lineages. Stem cells may undergo symmetric cell division by which they produce daughter cells, both of which may become stem cells or divide asymmetrically to generate one stem cell and one progenitor cell that further divides to generate differentiated cells. They are reported in various tissues such as bone marrow, brain, spinal cord, dental pulp, skeletal muscle, skin and digestive epithelium, lung, breast, bladder, cornea, retina, liver, and pancreas. In many tissues, they are dispersed such as hematopoietic stem cells that are present in bone marrow where they generate different types of blood cells, while in some tissues, like the intestinal epithelia (crypt base) or hair follicle (hair bulb), the stem cell niche is very much localized.

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Stem cells are mostly identified based on certain protein marker expression patterns that are generally cell surface antigens, while some may be cytoplasmic proteins. Specific antibodies generated against these marker proteins are used to identify or locate stem cells in tissue sections by immunohistochemistry or immunofluorescence. These antibodies identify stem cells by binding to cells expressing SC marker protein, however they rarely bind to non-stem cells, as some protein marker expression is not very specific. Routinely, stem cells are isolated by FACS method and grown in vitro to demonstrate generation of multiple cell types that make up tissue. In vivo, stem cells are labeled using transgenic technology and tracked to study the cell lineage patterns.

**Tumor initiating cells/cancer stem cells**

CSCs/TICs hypothesis was first proposed several decades ago and many aspects of this hypothesis remain speculative and evolving. A minimal operational definition of CSCs/TICs is: tumor cells that have the ability to re-grow the tumor from which they were isolated or identified. This implies that TICs can only be defined experimentally in vivo. Generally, CSCs/TICs are viewed as cells at the apex of tumor hierarchy, which exhibit frequent multipotency (pluripotency) of lineage differentiation. In myeloid (ex: leukemic) cell lineage the above assertion seems to be well supported, and recent results from a growing number of solid tumor transplantation experiments can be interpreted in favor of CSCs/TICs theory.

Although it has been initially thought that every cell in the tumor possess uniform tumorogenic potential, the first experimental evidence against this has been reported when it is found that less than 0.1% of leukemic cells can develop into colonies in soft agar. This laid foundation for the thought that cell proliferative capacity is not uniform within the tumor. Since then similar observation have been made in other tissues as well. Other evidence, in favor of tumor initiating cells (TICs) existence come from the observations in cancer patients undergoing anticancer therapy, where tumors are initially found to shrink/die upon chemo and radiation treatment, but are found to reappear and be more aggressive. Therefore, it is proposed that each tumor harbors a small number of cells, highly resistant to drugs as well as radiation, that have the ability to survive therapies and may lead to tumor relapse. These cells referred to as tumor initiating cells or cancer stem cells (CSCs). This hypothesis is supported by the observations that some solid tumors and leukemia contain small numbers of cells that have increased potential to proliferate in vitro, to form colonies/microspheres in soft agar assays and to induce tumors when transplanted in NOD/SCID mice, when compared to other cells of the tumor. Targeted deletion of the tumor suppressor gene APC (Adenomatous Polyposis Coli) in Lgr5+ CBCs representing intestinal stem cells results in rapidly growing adenomas, whereas targeted deletion of APC in the higher-positioned transit amplifying cells (TACs) fails to induce significant adenoma growth, illustrating that a mutation in a stem cell population is most effective for tumor initiation.

Cancer is, therefore, now considered by most people as a stem cell disease. This is mainly due to the fact that they are the only cells in most tissues that are long lived and constantly dividing, therefore more susceptible to accumulate genetic changes that result in cells with tumorogenic potential. Cancers are believed to arise from a series of sequential mutations that occur because of genetic instability and/or environmental factors. Therefore, CSCs/TICs are the cells within the normal tissues that have undergone and retained these mutations, which makes them tumorogenic. It has now become clear that tumors are made of a heterogeneous population of cells that vary greatly in terms of their genetic and tumorogenic nature. However, it is still unclear whether cancer stem cells are the direct progeny of mutated stem cells or more mature cells that re-acquire stem cell properties during tumor formation.

**Markers for cancer stem cell**

In many studies, known stem cell markers have been used to identify CSCs within tumor tissues (Table 1). Standard procedures for isolating CSCs from tumor tissues involve cell sorting (FACS) of a sub-population based on SC markers and confirmation of tumor-initiating activity in xenograft transplantation assays. In addition, in vitro propagation of CSCs applying adult stem cell culture conditions have also been reported. Various studies have shown that CSC populations isolated and expanded from a variety of tumors including glioblastomas, melanoma, breast, lung, ovarian and colon cancers, frequently grow as non-adherent, three-dimensional (3D) tumor spheres under serum-free conditions. Despite this evidence, it remains contradictory whether in vitro-
Cultured CSCs retain their original phenotype. Similarly, controversies have also surrounded the use of certain stem cell surface markers to isolate cancer stem cells that do not show specificity thereby identify non-stem cell population. However, with certain markers there has been consistent and reliable evidence in support of their use. The following are some of the most popular and widely used cancer stem cell markers.

**Aldehyde dehydrogenase (ALDH)**

Aldehyde dehydrogenases (ALDH) are a group of NAD(P)+ dependent detoxifying enzymes that oxidize a wide variety of intracellular aldehydes to their weak carboxylic acids thereby, conferring resistance to alkylating agents\(^{26-29}\). ALDH also converts retinol to retinoic acid, a modulator of cell proliferation. There are several ALDH genes in humans that are widely distributed in the body with highest expression in kidney and liver. Stem cells, in order to maintain their longevity, need to have strong detoxifying activity therefore, ALDH has been tested and identified as a marker for progenitor cells in various tissues. Cells expressing ALDH are identified by immuno-histochemistry using specific antibodies and the functional activity of ALDHs may be assessed in living cells using ALDEFLUOR assay. Now ALDH is one of the well recognized and most widely used stem cell marker in different tissues. ALDH has been first reported to identify hematopoietic stem cell in humans. It has been shown that as few as 10 hematopoietic cells are capable of reconstituting bone marrow\(^{30}\), similarly ALDH+ murine brain cells have been found to be capable of differentiating into multi-lineage cells\(^{31}\). ALDH1 is also used to identify cancer stem cells in colon\(^{32}\), breast\(^{33}\), blood\(^{34}\), liver\(^{35}\), bladder\(^{36}\) and prostate\(^{37}\). In the colon, during the progression of normal epithelial cell to adenoma as a result of APC mutation, ALDH1+ cells have been found to increase in number. These cells develop into tumors upon xenografting to a NOD-SCID mice while the ALDH1-negative cells fail to induce tumor formation. ALDH1 has also been found to co-segregate with other known colon SC markers CD133 and CD44 during cell sorting indicating they all recognize the same set of cells\(^{32}\). ALDH expression and activity has been analyzed in a huge collection of normal and epithelial tumor tissue samples and a distinct pattern of ALDH expression has been observed in tumors compared to normal tissue. ALDH+ cells are also found to be resistant to chemotherapy\(^{38}\). These studies indicate that ALDH1 not only identifies CSCs, but also plays a role in their survival. Hence, it may be used as a preferable marker for identification of adult SCs and for targeting CSCs in epithelial tissues like blood, breast, lung, ovarian and colon.

**Prominin1 (Prom1 or CD133)**

Prominin1, also known as CD133 is a five-transmembrane-domain containing glycoprotein that has been first described as a surface antigen specific for human hematopoietic stem and progenitor
cancer drug development. A desirable candidates of CSC identification and initiating potential of the CD133+ cell, they are data in favor of the stem cell nature and tumor fractionate stem cells. However, in view of enormous cell, thus complicating the use of these reagents to the differentiation and transformation status of the glycosylation-dependent epitopes that vary with thought to be due to CD133 antibodies recognizing of its wide use, there have been few reports where to their respective newly diagnosed tumors GBM tissue obtained from patients as compared to their non-CSCs counterparts and clonogenic potential and sustain expression of been found to have higher capacity for self-renewal and medulloblastoma. Msi1 expression has been reported in stem and progenitor cells from tissues, suggesting that Msi1 may be higher in stage III than stage I and II colon cancer expression has been found in the majority of colorectal adenoma and carcinoma. Msi1 protein expression has been reported to be significantly higher in stage III than stage I and II colon cancer tissue samples, suggesting that Msi1 may be involved in tumor invasion and metastasis. Msi1 over-expresses in 55% of the human breast cancer cells with a strong correlation with ErbB and CD133 expression has been found to be significantly higher in recurrent GBM tissue obtained from patients as compared to their respective newly diagnosed tumors. In spite of its wide use, there have been few reports where CD133 identified non-stem cells, which is mainly thought to be due to CD133 antibodies recognizing glycosylation-dependent epitopes that vary with the differentiation and transformation status of the cell, thus complicating the use of these reagents to fractionate stem cells. However, in view of enormous data in favor of the stem cell nature and tumor initiating potential of the CD133+ cell, they are a desirable candidates of CSC identification and cancer drug development.

Musashi-1 (Msi1)

Musashi protein has been identified as an RNA-binding protein required for two successive asymmetric divisions of sensory organ precursor cells in Drosophila. The first homolog of this highly conserved gene, Msi1, has been identified in mammalian CNS stem cells. Later its expression has been reported in stem and progenitor cells from different tissues that decreases as cells commit to lineage differentiation. The undifferentiated neural precursor cells identified by Msi1 generate a variety of cell types including neurons, astrocytes and oligodendrocytes. Msi1 has been shown to be a positive regulator of Notch signaling through its interaction and translational repression of mammalian Numb [mNumb mRNA (an inhibitor of Notch signaling)]

Musashi was recognized as a possible marker representing a stem cell within the intestinal epithelium of mice. Recently, the functional role of Msi1 in cancer has attracted increasing interest. It has been found to be overexpressed in tumor tissues and cell lines such as medulloblastoma, glioma, retinoblastoma, endometrial carcinoma, hepatoma cell lines and cervical epithelial cells. A high level of Msi1 expression has been found in the majority of colorectal adenoma and carcinoma. Msi1 protein expression has been reported to be significantly higher in stage III than stage I and II colon cancer tissue samples, suggesting that Msi1 may be involved in tumor invasion and metastasis. Msi1 over-expresses in 55% of the human breast cancer cells with a strong correlation with ErbB and CD133 expression. Msi1 expression in primary tumors shows a direct correlation with metastasis and inversely correlated with the 5yr survival. Overexpression of Msi1 in oligodendrocyte and mammary precursors induces cell proliferation, arrests differentiation, and prevents apoptosis. Furthermore, Msi1 knockdown results in tumor growth arrest in xenografts, reduces cancer cell proliferation, and increases apoptosis, alone as well as in combination with radiation injury. Effect of Msi1 knock-down has also been studied in cell lines of breast cancer, colon cancer and medulloblastoma. The results indicate that knock-down leads to reduced cell proliferation, in vitro spheroid formation and reduced tumor size when xenografted into nude mice. Msi1 seems to be an attractive target for cancer treatment due to its significant over-expression and role in various cancers progression.
Leucine-rich-repeat containing G-protein coupled receptor 5 (LGR5)

G-Protein-coupled receptor GPR49, also known as LGR5/HG38/FEX, belongs to the leucine-rich repeat containing G-protein-coupled receptors (LGRs) structurally similar to glycoprotein hormone receptors including thyroid-stimulating hormone receptor, follicle-stimulating hormone receptor, and luteinizing hormone receptor. LGRs are grossly divided into subgroups; glycoprotein hormone receptors (GPR48, GPR49 and LGR6) and relaxin family ligand receptors (LGR7 and LGR8). GPR49 knockout mice exhibit neonatal lethality characterized by ankyloglossia and gastrointestinal distension indicating that they play vital role in embryo development. Role of LGR5 during ileal development has been analyzed using LGR5 null/LacZ–NeoR knock-in mice and found that after villus morphogenesis, Lgr5 expression becomes restricted to dividing cells clustered in the intervillus region and is more pronounced in the distal small intestine. Further, LGR5 deficiency leads to premature paneth cell differentiation in the small intestine without detectable effects on differentiation of other cell lineages, nor on epithelial cell proliferation or migration. Interestingly, LGR5 has evolved as a reliable stem cell marker in the small intestine following the development of Lgr5-EGFP-IREScreERT2 mice and lineage tracing study where these cells have been shown to give rise to other cell forms that constitute the intestinal crypt. Although the function of GPR49 in cancer is poorly understood, overexpression of GPR49 has been reported in some studies. In particular, it is overexpressed in some hepatocellular carcinoma with b-catenin mutation. Several of the colorectal cancer cell lines (mostly metastatic tumor derived) and all of the sporadic colonic adenomas show significant overexpression of Lgr5. In silico analysis of GPR gene expression in primary human tumors of non-small cell lung cancer, breast cancer, prostate cancer, melanoma, gastric cancer and diffused large B cell lymphoma identified GPRs that are up-regulated in primary or metastatic cancer cells. GPRs, such as neuropeptide receptors, adenosine A2B receptor, P2Y purinoceptor, calcium-sensing receptor and metabotropic glutamate receptors have been found to be expressed at a significantly higher level in cancer tissue. Induction of APC mutation in a Lgr5+ crypt stem cells has been found to be potential enough to induce long-living adenoma, while a similar mutation in Lgr5- cell failed to do so. This observation strongly supports the notion that stem cells can be the origin of cancer in many tissues and therefore are an important target for cancer treatment.

Bmi1

Bmi1 has been first identified in a mouse proviral insertion screen for lymphomagenesis. It is part of the Polycomb group gene family and specifically a member of polycomb-repressing complex 1 (PRC1). PRC1 has an essential role in maintaining chromatin silencing. Bmi1 knockout mice die before or near weaning from a defect in self-renewal of hematopoietic stem cells. Using a transgenic Bmi1Cre-ER/+;Rosa26LacZ/+ it has been observed that Bmi1 is predominantly expressed in four cells above the base of the crypt (+4 position) located near the bottom of crypts in the small intestine. Lineage tracing reveals that these cells proliferate, expand, self-renew and give rise to all the differentiated cell lineages of the small intestine epithelium as well as in pancreas, identifying it as an important stem cell marker. Bmi1 has been found to be crucial for the short-term survival of cancer cells and its loss is effective in suppressing cancer cell growth and significantly reduces tumorigenicity of the surviving cells. In breast cancer patients a significant correlation between Bmi1 expression in plasma and overall survival in advanced stages has been observed. Bmi1 altered expression has frequently been described in human tumors, mainly in hematological malignancies and correlates with poor prognosis parameters, in solid tumors such as lung cancers, medulloblastomas, neuroblastomas, liver, breast, colon, nasopharyngeal, prostate carcinomas and bladder.

Double Cortin CAM like Kinase 1 (DCAMKL1)

DCAMKL1 is a microtubule associated kinase expressed in post-mitotic neurons, intestine and pancreas. DCAMKL1 protein homology with the DCX protein strongly suggests a likely role for this gene in neuronal migration of a developing brain and perhaps the adult brain, microtubule polymerization and mediation of the phosphorylation-dependent signal transduction pathway. It has been also found that DCAMKL1 is expressed in the Apcmin adenomas and its selective blockade using Let7a miRNA leads to tumor growth arrest in a xenograft. There has been some controversy with the specificity of this marker.
since DCAMKL1 expressing cells do not express proliferation markers\textsuperscript{112} and also do not incorporate BrdU in pulse-chase experiments\textsuperscript{112,113}. It has been thus reported to identify tuft cells rather than intestinal stem cell\textsuperscript{117}. Therefore, DCAMKL1 still awaits more authentic confirmation from lineage tracing studies before its acceptance as a stem cell marker.

**Targeting CSCs for effective cancer prevention and treatment**

Traditional anticancer therapies such as radiation and chemotherapeutics have been in practice for many years. Tumors constitute CSCs and transient amplifying cells (TACs) that are differentially sensitive to chemotherapeutic drugs and/or radiation, although the extent to which this occurs widely varies\textsuperscript{118-121}. CSCs have also been postulated to be more resistant to hypoxic and acidic tumor micro-environment. Since large portions of the tumor consist of TACs, chemotherapeutic drugs that target these cells cause tumor regression initially, however they are known to become resistant at the later stage or relapse with more aggravation once the therapy is withdrawn. This can mainly be due to the fact that these therapies lead to enrichment of CSCs that are highly resistant and possess more tumorigenicity as demonstrated by many studies. Indeed, in its purest form, the CSC hypothesis implies that only CSCs need be eradicated as TACs will eventually be lost through attrition. The evidence from various studies targeting the expression of these cancer stem cell markers using knock-down strategies also suggests that CSC-directed anti-cancer therapy indeed are more effective in cancer treatment by eliminating the CSCs that are the root cause of cancer therefore depriving the tumor of a source that is believed to constantly supply cells that make up the tumor\textsuperscript{80,122-124}. Therefore, use of combinatorial therapies that target both rapidly proliferating cancer cells as well as relatively quiescent cancer stem cells will be a more effective way to cure cancer. There are several other marker proteins that have also been well established as stem cell and CSC markers in various cancers, such as CD24, CD34, CD44, C-kit, Jagged 1 etc which are also attractive candidates for anti-CSC therapies. Cancer stem cell markers, in spite of being an excellent and most desirable tools to study and target the tumor initiating cells, need to be selected with extra care as the targetted antibodies are known to identify non-CSC in some cancers. Further understanding of the distinction between normal stem cells and cancer stem cells can lead to the development of effective regimen that can either be used to eliminate the stemness of the cancer cells making them susceptible to standard therapies or eliminate the CSCs themselves.

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