Mini Review

Stem cell-like cancer cells in cancer cell lines

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There is increasing evidence that malignant tumors, such as leukemia, breast cancers, and brain cancers, contain cells that maintain the characteristics of tissue-specific stem cells and are malignant. Malignant glioma, for example, contain both proliferating cells expressing stem cell markers and differentiating cells expressing either neuronal markers or glial markers, raising the possibility that the tumors may contain neural stem cell (NSC)-like cells. This idea is supported by recent findings that malignant glioma can be generated from both NSCs and glial lineage cells, such as oligodendrocyte precursor cells or astrocytes, which behave as NSC-like cells in appropriate conditions. Additional evidence also exists indicating that malignant tumors might contain stem cell-like cancer cells, called "cancer stem cells" (CSCs). Although a number of anti-cancer drugs and irradiation have been successful in eliminating cancers, some cancer cells survive and the cancer recurs, indicating that the surviving cells are not only resistant to such anti-cancer drugs and irradiation but are also malignant. Previous studies have shown that various ATP binding cassette transporters, such as the protein encoded by the multi-drug resistant protein and the breast cancer resistant protein 1 (BCRP1), contribute to drug resistance in cancers. Interestingly, some of these transporters are also expressed in many kinds of normal stem cells. BCRP1, for example, excludes the fluorescent dye Hoechst 33342, identifying a side population (SP), which is enriched for stem cells. Together, these findings suggest that cancers might contain an SP that is enriched for cells that have the characteristics of CSCs. Taking advantage of the common characteristics between stem cells and cancer cells, several groups have demonstrated that such stem cell-like cancer cells, although not other cells, in tumors or cancer cell lines can self-renew, express well-known stem cell markers such as CD133, and form tumors when transplanted *in vivo*, suggesting that tumors contain CSCs and that stem cells might be the primary target of tumorigenesis. In order to develop effective therapy against tumors, characterizing and finding ways to kill CSCs is essential.

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The origin of cancers

Cancers have traditionally been thought to arise from either differentiated cells or their proliferating precursor cells, which have acquired oncogenic mutations. Since stem cells have been discovered in adult tissues, however, it has been suggested that tissue-specific stem cells (TSCs) might be a principal target of

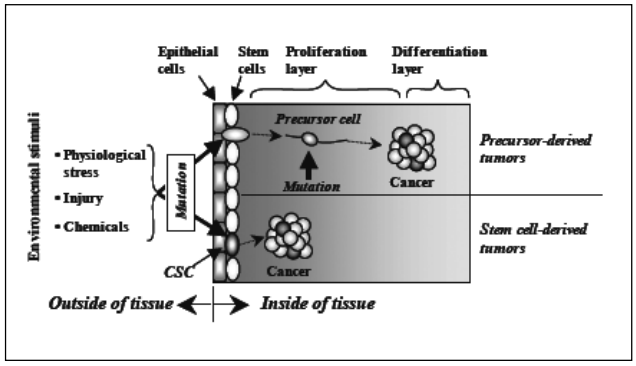

Fig.1 Origin of tumor-initiating cells

As stem cells usually exist just below epithelial cells, they are affected by environmental stimuli, physiological stress, chemicals, injuries and other factors, and may accumulate mutations. The mutated stem cells become cancer stem cells (CSCs) and form tumor. Proliferating precursor cells, however, may also accumulate mutations, become CSCs, and form tumor.

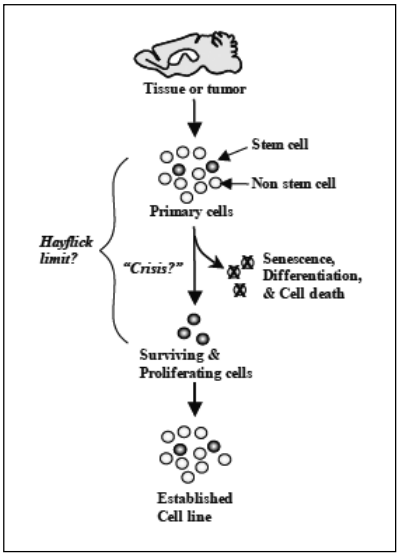

Fig.2 Establishment of cell lines

To establish cell lines, cells from a tissue or a tumor are dissociated and expanded in culture. It was generally believed that a small subpopulation of cells would acquire unlimited proliferation capacity via "crisis". As TSCs have now been found in tissues, however, it may be possible that a small subpopulation of cells is derived from TSCs, which originally proliferate indefinitely.

such mutations[1]. This speculation is supported by a number of different findings: Firstly, it is likely that cancers arise from epithelia, which are in contact with the external environment and contain a wide variety of TSCs. Secondly, many cancers have been immunolabeled for TSC markers and for differentiation markers. Thirdly, while TSCs survive and continue to proliferate throughout life, differentiated cells do not, suggesting that TSCs are more susceptible to accumulating oncogenic mutations. Finally, stem cells and precursor cells, which are transformed with oncogenic genes, have been shown to as developing cancer *in vivo*. Taken together, these findings suggest that either TSCs or amplifying precursor cells can be seen as the origin of malignant tumors (Fig.1).

Establishment of cancer cell lines

We established cell lines from a number of normal tissues and cancers (Fig.2). Tissues and cancers were initially dissociated using trypsin and the dispersed cells (primary cells) were then cultured on plates at high cell densities. Most of the cells died following several divisions but a small number of cells were able to proliferate and formed colonies. These colony-forming cells (secondary cells) divided a limited number of times (50-100 times for human cells and 20-50 times for mouse cells) before dying (the Hayflick limit)[2]. A few cells, however, were able to pass through the "crisis" to become established (immortal) cell lines, suggesting that the cell lines were derived from a small population of TSCs or had subsequently acquired unlimited proliferation ability. No normal human cell lines have yet been established, with most of the human cell lines having been derived from either benign or malignant tumors. Although the mouse 3T3 cell line was successfully established from the normal mouse embryo, they are not normal cells as a result of their chromosome number having increased to roughly 65 chromosomes (normal mouse cells contain 46 chromosomes). Many cell lines contain the cells expressing TSC markers, such as CD133 in glioma cell lines and CD44 in breast cancer cell lines, and the cells expressing specific products of differentiated cells, such as adrenocorticotrophic hormone (ACTH) in pituitary tumor cell lines and albumin in liver tumor cell lines. Taken together, these findings also suggest that cancer cell lines maintain the characteristics of cancer stem cells (CSCs).

Definition of cancer stem cells (CSCs)

CSCs were initially defined by their extensive self-renewal capacity, tumorigenicity and multipotentiality. As a number of oncogenes, including inhibitor of differentiation (Id), hairy and enhancer of splits (Hes) and Notch, are expressed in CSCs as well as TSCs and block cell differentiation, then it remains un-

certain as to whether CSCs actually give rise to multi-lineage cells. Further evidence also exists suggesting that cancer cells co-express a number of lineage-specific markers, each of which is exclusively expressed in normal differentiated cells, such as neurofilaments in neurons, glial fibrillary acidic protein in astrocytes and galactocerebrocide in oligodendrocytes, raising the question of whether such lineage-marker positive cells are in fact differentiated cells. Seen against this light then the obvious definition that can be applied to CSCs might be their unlimited self-renewal, expression of TSC markers, and tumorigenicity.

Niche for cancer stem cells

The number of TSCs is precisely regulated by both intrinsic mechanism and extracellular signals derived from specialized microenvironment "niche". For example, it was demonstrated that niche provides a limited number of physical anchoring sites, including beta1-integrin and N-cadherin, for TSCs and secretes both growth factors and anti-growth factors, including Wnt, FGF, hedgehog, bone morphogenic proteins and Notch[3,4]. Moreover, it was shown that the ablation of niche results in loss of TSCs. It seems likely that CSCs also need niche for tumorigenesis. Kaplan and his colleagues have elegantly demonstrated that bone marrow derived progenitors form the pre-metastatic niche in the tumor-specific pre-metastatic sites before cancer cells arrive and that the ablation of the niche prevents tumor metastasis[5]. However, since transplanted cancer cells form tumors in any area *in vivo*, CSCs might be independent of the niche regulation or have a capability to make a new niche by recruiting bone marrow stem cells and other component cells.

Methods used in the separation of CSCs

Several groups have recently succeeded in separating CSCs from cancers and cancer cell lines using the common characteristics of TSCs, such as cell surface markers, side population (SP), and/or a floating sphere formation method.

1) Cell surface markers

Dick and colleagues have been able to show that the acute myeloid leukemia (AML) initiating cells are found in primitive $CD34^+$ and $CD38^-$ populations, in which hematopoietic stem cells are enriched[6,7]. Al-Hajj et al. have successfully separated tumorigenic breast CSCs from mammary tumors and breast cancer cell lines as $CD44^+$ $CD24^{-/low}$ Lineage- cells. As few as one hundred $CD44^+$ $CD24^{-/low}$ Lineage- cells formed tumors in NOD/SCID mice, while tens of thousands of other cancer cell populations did not[8,9]. Another study by Singh et al. reported their success in separating brain CSCs from human medulloblastoma and glioblastoma multiforme (GBM) using an anti-CD133 antibody that recognizes a variety of different stem cells. Here, as few as one hundred $CD133^+$ GBM cells, although not $CD133^-$ cells, formed tumors in NOD-SCID brain[10]. A very recent study has also revealed that colon CSCs are enriched in a $CD133^+$ population[11,12]. This is in addition to prostate CSCs being found to be enriched in $CD44^+$alpha2beta1^{hi}$CD133^{+}$[13]. It therefore seems likely that cell surface markers, in particularly CD133, are useful in separating CSCs from many types of tumors (Fig. 3A).

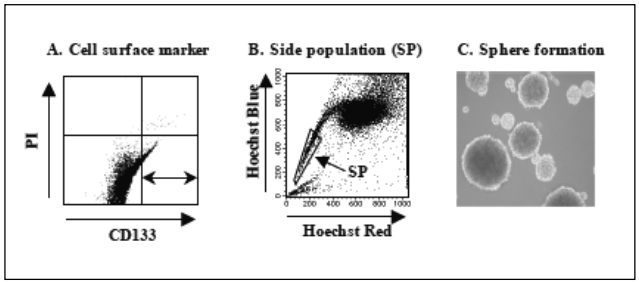

Fig.3 Methods involved in CSC separation
(A, B) Several types of CSCs are enriched in CD133+ fraction (A) and in SP (B). (C) CSCs as well as normal TSCs tend to form floating spheres in a serum-free medium with mitogens, such as bFGF and EGF, and are enriched in the spheres.

2) Side population

A number of anti-cancer drugs have been successful in eliminating cancers, however, some cancer cells survive and the cancer recurs, indicating that the surviving cells are not only resistant to such anti-cancer drugs but are also malignant. It has been shown that glutathione and its related enzyme apparatus, topoisomerase II, O6-methylguanine-DNA- methyltransferase, dihydrofolate reductase, metallothioneins, and various ATP binding cassette (ABC) transporters, such as the protein encoded by the multi-drug resistant gene (MDR), the multi-drug resistant protein (MRP), and the breast cancer resistant protein (BCRP1), contribute to such drug resistance in cancers[14]. Interestingly, some of these transporters are expressed in many kinds of normal stem cells. BCRP1, for example, excludes the fluorescent dye Hoechst 33342, identifying a side population (SP)[15], which is enriched for the various types of TSCs - although some research has shown that TSCs exist in both SP and non-SP and that SP cells do not express stem cell markers[16,17]. A number of research groups have found that some established cancer cell lines, which have been maintained in culture for decades, and tumors, such as AML, neuroblastoma and ovarian cancer, con-

tain a small SP (Fig.3B). These studies have demonstrated that SP cells — but not non-SP cells — self-renew in culture, are resistant to anti-cancer drugs including Mitoxantrone, and form tumors when transplanted *in vivo*[9,18-22]. Seen in this light, therefore, SP in cancers and cancer cell lines contain cells with characteristics of both stem cells and cancer cells.

3) Sphere formation assay

An increasing amount of evidence points to the fact that CSCs as well as TSCs, such as NSCs and mammary gland stem cells, can form floating aggregates (spheres) and be enriched in the spheres when cultured in serum-free medium with proper mitogens, such as bFGF and EGF (Fig.3C)[9,18,19,21]. Although many CSC researchers use sphere formation methods to concentrate their CSCs in culture, it is of interest to investigate exactly why CSCs—as well as normal stem cells—are enriched in the spheres.

It is therefore crucial to characterize *bona fide* CSCs in any attempt to reach a curable therapy. As it seems likely that the cells separated by one of the three methods remain a mixture of CSCs and other cells, then the combination of these methods might help in purifying CSCs; failing this we will have to establish new methods to obtain pure CSCs.

Cancer cell lines as alternative sources of CSCs

Although it is obvious that tumors are the primary source of CSCs for their characterization, cancer cell lines may have the potential to act as alternative sources of CSCs. Many cancer cell lines can be maintained indefinitely in culture and form tumors like the original one when transplanted *in vivo*. As such cell lines were derived from single cancer cells, it seems likely that they do not contain any contaminating normal stem cells, such as hematopoietic stem cells, bone marrow (BM)-derived mesenchymal stem cells, or NSCs - all of which are recruited to tumors *in vivo*. BM-derived mesenchymal stem cells, for example, promote angiogenesis and support tumorigenesis; when they are eliminated *in vivo*, the growth of a transplanted tumor is significantly inhibited, suggesting that both endogenous (CSCs) and exogenous stem cells (normal stem cells) contribute to tumorigenesis *in vivo*. Moreover, brain CSCs from human GBM are able to form neurospheres in the presence of bFGF and EGF, even after they have been cultured in medium with 10% serum or in serum-free medium without both cytokines for 14 days, suggesting that CSCs in GBM can be maintained in normal culture conditions, in which all normal NSCs quickly lose their multipotentiality and differentiate into neurons and glia[23]. Together, these findings make cell lines attractive models for investigating the characteristics of CSCs.

Several groups have already shown that many cancer cell lines contain CSCs. Using Hoechst 33342 staining and flow cytometry, a number of studies have shown that a number of established cancer cell lines, including the rat C6 glioma cell line, human glioma cell lines, breast cancer cell lines, prostate cancer cell lines and neuroblastoma cell lines—all of which have been maintained in culture for decades — contain a small SP[9,18-22]. These studies also demonstrated that the SP cells, but not the non-SP cells, self-renew in culture, are resistant to the anti-cancer drug Mitoxantrone, and form tumors when transplanted *in vivo*. In this way, the SP in cancer cell lines contains cells with characteristics of both stem cells and cancer cells. However, since Hoechst dye is toxic for the cells, we cannot exclude the possibility that CSCs also exist in non-SP. Therefore it is needed to confirm that ABC-transporter-expressing cancer cells are enriched for CSCs. Another study revealed that CD133 is expressed in a small subset of cancer cell lines and such $CD133^+$ cells retain the characteristics of CSCs: $CD133^+$ cells predominantly formed tumors when transplanted *in vivo* [24] and were resistant to irradiation[25]. It was also shown that multiple myeloma cell lines also retain $CD138^-$ CSCs (<5%), which had greater clonogenic potential than corresponding $CD138^+$ cells[26]. Taken together, it can be seen that cancer cell lines are potentially useful tools for investigating the characteristics of CSCs under controlled experimental conditions.

Perspective

Although the concept of CSCs was first proposed several decades ago, it has yet to gain broad acceptance. The continuing significant progress of stem cell research has given rise to the identification of a number of stem cell markers and new techniques have been developed to identify stem cells. The use of stem cell markers, including CD133, and techniques such as Hoechst staining, has revealed that cancers contain a small population of CSCs, which are involved in tumorigenesis and recurrence. The next challenge involved in this process is to purify *bona fide* CSCs, characterize them, and subsequently look for their targets, which can be then applied for use in therapy.

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Conflict of Interest Statement

None declared

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