Progress in isolation and enrichment of cancer stem cells

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Cancer stem cells (CSC) are a group of cells with capacity of self renewal and can proliferate into a heterogeneous bulk with cancer progeny population. This is the exactly primary reason for metastasis and recurrence of tumors. Strategies for studying cancer’s targeted therapy and biological property mainly include its isolation and enrichment. In the coming era it will also be the focus of significant value. We will summarize the current strategies used for isolation and enrichment of CSC, including serum-free suspension culture, feeder cell layers, immunoselection based on cell surface markers, side population cells, resistance to radiation treatment and chemotherapy, combined utilization of various strategies and a three dimension (3D) in vitro model in this review.

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Introduction

It is well known that cancer is the world's highest rates of disease. Mainly because current chemotherapeutic agents and radiation therapy largely target proliferating and differentiated cells that form the bulk of the tumor but not the relatively quiescent cancer stem cells [1]. Accumulating evidences suggest that cancers may have a small population of cells that have the distinctive capability to initiate and maintain tumor’s growth and heterogeneity in successive transplantation experiments [2]. The most likely source of normal stem cells is cancer stem cells. Both share the similar features of potential of self-renewal and multilineage differentiation and have the same signaling pathways, such as Wnt, SHH, Notch pathway [3]. The concept that CSCs may be the source of recurrence of tumor is not a fresh one [4]. As a matter of fact, researchers proposed that a rare population of cells with stem-like properties may be the initiation of tumor. At the moment it has been respectively identified from selected types of human cancer, such as, colon [8-9], breast [10], brain [11-12], pancreatic cancer [13] and head and neck [14].

Thus, the way to isolate CSC efficiently and identify them from a heterogeneous tumor mass plays a vital role in CSCs’ research. In this review, we will shortly discuss the progress made in isolation and enrichment of CSC during the past few decades.
Serum-free suspension culture

SFM is the natural medium. Compared with the traditional media, SFM contains no creatural serum or other animals' extraction, still can meet the need of the reproduction and cells in vitro growing in a long time. It can solve the microbial contamination, immunogenicity and limited sources of problems exist in creatural origin serum culture medium.

In 1992, Reynolds et al. established a model of serum-free culture method to acquire neurospheres from brain tissues [15]. This is the foundation of the cancer stem cells' isolation. Without serum, normal cells will die for lack of nutrition, only tiny cancer stem cells will survive, then suspend in the medium and proliferate consistantly to differentiate into tumor spheres. The characteristics of this model is adding EGF, bFGF growth factor in the medium. This uncomplicated and convenient method for NSCs’ enrichment has been extensively applied to study neurogenesis and also been applied for CSCs’ isolation from brain tumors and other types of cancers with different combinations of complementary agents [16]. Many scholars applied the method of serum-free culture to isolate breast cancer stem cells from breast cancer [17-18]. ZHAO et al. cultured NSCLC A549 to obtain spheres with more invasiveness and highly expression of Oct4, Sox2 and CD133 [19]. SUN et al. found that tumor spheres exhibit more strengthful proliferation, faster cell cycle progress, and stronger resistance, high expression of multiple lung cancer drug-resistant related [20]. Kimet et al adopted this method to isolate cancer stem cells from primary head and neck tumors and for the generation of orospheres [21]. The orosphere assay is intended to evaluate the stemness, self-renewal, and tumorigenicity of CSCs, and to discuss courses involved in their chemoresistance to drugs [22]. Recently many cell lines have formed cancer stem cell spheres through SFM condition including HCT116, HT29, LOVO, SW480 and DLD-1 [23]. However, the use of the sphere assay for CSC isolation arises several problems. The most prominent issue is the low content. So it is still a big challenge to improve the purity of cancer stem cells.

Feeder cell layer

At the beginning of the original generation or training phase, the stem cells depends on the feeding layer and the secretion of growth factors, otherwise their survival rate and clone formation rate will be very low. For this point, feeding cell layer is a key factor in initial stage of stem cells. Feeder layer cells refers to some specific single layer cells after occurring mitosis blocking. In 1981, Evans et al. and Martin et al. first successfully used the cultivation of fibroblast feeder layer system to establish mouse embryonic stem cell lines [24-25]. After that fibroblast cells breeding layer is widely used in clonal culture research of embryonic stem cells. Unger et al. derivated Human Skin Fibroblast Lines as feeder cells for culturing Human Embryonic Stem Cells [26]. Xu Y et al. established a culturing method of lung cancer stem cells with autologous tumor fibroblasts as feeder cells [27]. After that fibroblast cells feeding layer is widely used in embryonic stem cells. Of course there are other cells can also be used as feeding layers. Such as STO cells, Sertoli cells, marrow stroma cells. Feeding layer must be carried on the pretreatment before using, in case of losing abilities of proliferation on the basis of guaranteed secretion, lest competed with stem cells for a long time. Now the most commonly used are silk crack enzyme factor C and gamma irradiation. It is difficult to obtain high purity and activity of cancer stem cells. But feeder cell layer provides a simple and feasible laboratory cultural way and amplification method for research on cancer stem cells and offers resources for the clinical application of cancer stem cells.

Selection based on cell surface marker

fluorescent cell sorting

Flow cytometry sorting is characterized by the specific combination of fluorescently labeled antibodies and cell surface antigens. By flow cytometry cells with specific fluorescence can be sorted out and at the same time can be tagged by a variety of fluorescence. This surface marker-based approach has been the most widespread strategy to isolate CSCs from heterogeneous tumor cells and has made a great contribution to the research of cancer stem cells. Now various kinds of surface markers have been applied in isolating CSCs. In1994, Dick firstly proved the existence of CSCs derived from acute myeloid leukemia by fluorescence activated cell sorting (FACS) based on CD34 and CD38 (CD34+ CD38-) surface marker expression [3, 28]. Ginestier et al. sorted out the cells based on acetaldehyde dehydrogenase 1(ALDH1+) to prove the property of cancer stem cells [29]. Beier et al. sorted out cells based on CD133(CD133+) and inoculate 1000 CD133+ cells in nude mice in vivo, finally they found CD133+ cells tumorigenic ability was more stronger than CD133-cells [30]. Flow cytometry can also sort out cells with compound positive expression. Bonnet et al. proved that using flow cytometry sorting out the AML cells with expression of CD34+/CD38-, confirmed the characteristics of cancer stem...
cells. Li et al. sorted out the CD44$^+$ CD24$^-$ ESA$^-$ cells from pancreatic cancer cells, only 500 cells can form tumor in the body of nude mice. Collins et al. sorted out the CD44$^{+}\alpha2\beta1^{high}$CD133$^+$ cells from prostate cancer cells and found that these were prostate cancer stem cells. Many CSC markers are shared with a wide variety of tumor types. They may also be expressed in organs of normal SCs, the origin of these tumors. Even through a lot of surface markers has been applied in this technology, lots of criterions and accurate markers can not be found and the numbers of cells sorted out are very rare. Therefore, the way to choose the correct marker is very crucial and definitely will save time and resources.

Magnetic activated cell sorting

This is a quick and efficient way for isolating cells with highly purity from a complex mixture. Now it has been put into all kinds of detection methods. With this method, Richardson et al. selected CD133$^+$ cells from the prostate cancer and confirmed the characteristics of tumor stem cells. Beier et al. selected CD133$^+$ cells from glioma by this way, and shew that these were the brain glioma stem cell. Hermann et al. selected CD133$^+$ cells from pancreatic cancer and verified the greater abilities of metastasis and invasiveness and properties of tumor stem cells. In fields of cancer stem cells’ separation, this application of the technology is very extensive. It has the characteristic of high specificity and sensitivity, but the most obvious weakness is expensive device and unable to reuse and always gets single positive cells.

Side population cells

In 1996, Goodell et al. first found a small population of cells called SP cells which were based on these cells’ different ability to pump out Hoechst 33342. Hoechst 33342 is a kind of dye of binding DNA specifically and it can be pumped out of membrane by ABC (ATP--binding cassette). Side population cells were defined as a small group of cells rich in characteristics of stem cells, and with clonogenic capacity, tumorigenicity, multipotency, long-term proliferating property, and chemoresistance. Handnagy et al. firstly used SP analysis verifying the CSCs, proved that this method can be used in the detection of tumor stem cells, especially when some kinds of cancer stem cell markers are not clear. Kondo et al. isolated the SP cells and the non SP cells from the glioma cell line C6, shew that the similar characteristics between SP cells and stem cells. Wu et al. isolated SP cells from the gastric cancer cell line BGC-823 and demonstrated that these cells had stem-like property. These all are the proof of SP cells are likely to stem cells. In recent years, many researches have indicated that the SP cells may be useful for the FACS-based identification and isolation of CSCs from human cancers. Olmsted-Davis et al. isolated SP cells from rat bone marrow cells, then observed the SP cells that made almost depleted bone progenitor cell pool acquire the ability of rebirth. Moreover, the potential cytotoxicity of Hoechst 33342 is disputable, and some researchers have put forward that the different clonogenicity and tumorigenicity between SP and non-SP cancer cells might be the dye itself. Also several studies have questioned the characteristics of CSC of SP cells and the instability and cell toxicity of this methods and offer the experimental basis, it is undeniable that the emergence of SP cells brought a new dawn for our clinical therapy.

Chemotherapy drugs and radiation stimulation

Several experiments demonstrated that CSCs can resist apoptosis induced by chemotherapy drugs and radiation stimulation through multiple complicated mechanisms. Traditional chemotherapy drugs aimed at destructing tumor cells in the proliferation. Cancer stem cells are mostly in G0 period, a relatively steady state, so that they can avoid the effect of chemotherapy drugs. By utilizing the characteristic, the cancer stem cells can be enriched. After radiation effect or chemotherapy drugs, most common tumor cells will die, cancer stem cells in stationary stage will survive; this part of the cells could avoid the effect of radiation and chemotherapy and exists in tumor tissues. When the radiotherapy or chemotherapy takes a stop, those cells will occur recurrence and metastasis. Lagadec et al. found that the number of ALDH1 cells increases with the increasing irradiation dose in a certain radiation dose, which is the first time that radiotherapy can promote breast cancer cells turn into breast cancer stem cells. It is discovered that anti angiogenesis drug sunitinib can increase mice breast cancer stem cells in proportion. Hu et al. found that when low-dose carboplatin treat with liver cancer cell lines, the number of cancer stem cell increased obviously. Therefore, it is feasible to enrich cancer stem cells by radiotherapy or chemotherapy, or even the combination of the two methods. However, different types of cancer cells’ enrichment by chemotherapy or radiotherapy or the requirements of different concentration or doses of drugs limit the implements.
Combined utilization of various strategies for isolation of CSC

In the current study, the selection of cancer stem cells is not adopting one single way, but with two or more than two ways in order to obtain CSCs of more quantity and higher quality [65-66]. If only using FACS, the quantity is not much, the way of expanding CSCs is very complexing, and use SFM alone, although after several generations of training to get more tumor cells, contains no more tumor stem cells. Only with serum-free culture method, tumor stem cells were cultured for up to a certain number. If you combine SFM with FACS, the quantity and purity can satisfy the requirements of further experiments. Hypoxia is a common phenomenon in microenvironment of tumor. In a study, the SFM method was improved and optimized by mean of hypoxia as a way of intervention, which was combined with the serum-free suspension culture. The anoxic condition can improve the speed and efficiency, and increase the diameter of the spheres and the stability of the structure of the spheres. In addition, it still can improve the proportion of positive cells with some special surface markers.

A three dimension (3D) in vitro model

The occurrence of cancer is a multi-step process and is regulated by the microenvironment [67]. Tracking in the body really requires a lot of time and many unforeseen difficulties exist in the process. Therefore, the establishment of a model to study the molecular mechanisms of development and evolution of tumor has become a priority. Conventional 2D models lead to a lack of interaction with surrounding cells and extracellular matrix which are associated with the occurrence, evolution and metastasis of the tumor [68]. Animal models also cannot repeat the characteristics of human body very well because of human species differences. In order to solve the problems, 3D model arises at this historic moment. In recent years, many experiments were conducted to study the maintenance and differentiation of stem cells by 3D such as Embryonic Stem Cells are much easier differentiated into epithelial cells in collagen 3D culture [69]. In 3D culture, prostate progenitor cells (NHPtE1) with characteristics of stem cells appeared to be as dendritic growth and prostate epithelial stromal cells (BHIPtE1) were not, suggesting that the former contains a higher proportion of stem cells / progenitor cells [70]. In the 3D culture model, it was found that breast cancer cells (MCF-7) can attract human stem cells (hMSCs) and enhance their abilities of migrating. And hMSCs can also enter the breast cancer spheres formed by the breast cancer cells to affect the growth and development of the latter and their immune response [71]. Additionally, the researchers used cell adhesion force different of human embryonic stem cells (hESCs) or induced pluripotent stem cells (iPSCs) different with others to separate from the matrix with technology of micro fluid [72]. The 3D culture is a new technology and exists some imperfections but it has its unique advantages. It provides a unique and effective model for the development of mechanism of cancer, CSCs and new drug screening. The 3D culture technology is expectedly to become a new standard and a tool for tumor in vitro research.

Conclusions and perspectives

The development of methods of isolation and identification of Cancer stem cells is the first step in the course of applying CSC theory. On this basis, further research about the tumor stem cells, including the biological characteristics of cancer stem cell, specific surface markers, look for the key protein of cancer stem cell, tumor stem cell formation in the process are in the need as well. Not only to realize the early diagnosis of tumors, but also to design specific surface markers, tumor stem cells, the enzymes required in the process of proliferation, the main signaling pathways in the process of cell self-renewal, cell cycle regulatory proteins and miRNAs as target to restrain and kill cancer stem cells so as to ultimately achieve the goal of radically cure cancer. Even though the theory of CSCs still has a lot of controversy issues and difficulties of research, not only it can enrich the theory of traditional tumor, but also shed new light on the mechanism of tumorigenesis and suggest better models of treatment. It should pay attention that curing cancer can not only kill cancer cells, but also should at the same time carry out the corresponding treatment of killing tumor stem cells, so as to achieve the aim of eradicating the tumor.

There are all kind of ways for the isolation and/or enrichment of different types of CSCs. However, the range, advantages, and restrict of these different strategies remain to be explained. But as a result of biological genetic diversity, cells of the group of different subtypes of CSCs are different in the expression of genes, invadeness, metastasis and drug sensitivity [16]. These suggest that different phenotypes may exist in the overlapping tumorigenic population; thus, the method of combining a variety of ways rather than single strategy is needed to enrich or isolate CSCs.

Although we have had a deeper understanding through in-depth study of cancer stem cells, still we faces lots of difficulties in the study: (1) The study of cancer stem cells is
still directly from a lot of researches of stem cells, most are markers of normal stem cells as well as cancer stem cells, and this may result in toxic and side effects on normal stem cells as targeted therapy kills cancer stem cells. (2) Because of the different subtypes, the separation of the methods has a lot of restrictions in the final analysis. (3) Whether the cancer stem cells we isolated can maintain its characteristics or whether it has evolved into tumor cells is still unknown. (4) The origin of tumor stem cells is still unknown, whether it is caused by stem cell differentiation or mutation, or by mutations in common somatic cells. (5) About the purity and efficiency of separation of CSCs is still waiting to be solved.

In summary, research into CSCs is still in its infancy. Isolation and enrichment of cancer stem cells are viewed as the foundation and starting point for better understanding the characteristics of this specific subpopulation. Its comprehensive application value in the field of cancer research can not be indelible. Additionally it should be underlined that the hypothesis of CSCs is based on the functional identification in vivo with the serial xenotransplantation assay, which is thought as the gold standard of identification of CSCs[16].

Conflicting interests

The authors have declared that no conflict of interests exist.

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Author contributions

C.Z. and Q.S. wrote the manuscript.

Abbreviations

CSC: Cancer stem cells; 3D: three dimension; SFM: serum free medium; SC: stem cells; NSC: neural tumor stem cell; FACS: fluorescence activated cell sorting; SP: side population cells; 2D: two-dimensional; hMSCs: human stem cells; IPS: induced pluripotent stem cells; ABC: ATP-binding cassette.

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