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REVIEW ARTICLE

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Cancer Stem Cells Converted from Pluripotent Stem Cells and the Cancerous Niche

Kasai T[1], Chen L[2], Mizutani A[1], Kudoh T[1], Murakami H[1], Fu L[3], Seno M[1]

Nowadays, the cancer stem cells are considered to be significantly responsible for growth, metastasis, invasion and recurrence of all cancer. Cancer stem cells are typically characterized by continuous proliferation and self-renewal as well as by differentiation potential, while stem cells are considered to differentiate into tissue- specific phenotype of mature cells under the influence of micro-environment. Cancer stem cells should be traced to the stem cells under the influence of a micro-environment, which induces malignant tumors. In this review, we propose this micro-environment as a 'cancerous niche' and discuss its importance on the formation and maintenance of cancer stem cells with the recent experimental results to establish cancer stem cell models from induced pluripotent stem cells. These models of cancer stem cell will provide the great advantages in cancer research and its therapeutic applications in the future.

Introduction

The regulation of cell proliferation and growth is disordered in cancer. And cells show uncontrolled growth, which is caused commonly by genetic damages, including mutations of oncogenes and tumor suppressor genes. Thereby, cancer cells are historically considered driven from a single cell leading into the idea that they are clonal. However, the individual cells as the component of cancer tissue exhibit significant heterogeneity in such as their morphology, cell surface antigens, genetic alterations, pattern of gene expression profiles, epigenetic modifications, and etcetera. One possible explanation of their heterogeneity is that cancer is a cellular hierarchy with cancer stem cells (CSCs) at the apex, just like normal tissue development with their tissue stem cells (Wang and Dick, 2005). The CSC concept derives from the fact that the unlimited growth of cancer tissues depends on a small number of distinct cells of which proliferation is unlimited. A CSC is currently defined as a cell within a tumor that possesses the capacity to self-renew and to exhibit the heterogeneous lineages of cancer cells that comprise the tumor. Cancer stem cells can, thus, only be defined experimentally by their ability to recapitulate the generation of a continuously growing tumor (Clarke *et al*., 2006). However, characterization and analysis of these cells are limited due to the small number of CSCs in a tumor, and technical difficulty of isolation as a homogenous population of cancer stem cells from clinical samples. Appropriate model cells recapitulating cancer stem cell properties would accelerate not only the investigation of cancer stem cells, but also the development of new clinical cancer therapy by establishing a screening system for anti-cancer stem cell agents. We introduce here, in this review, the recent research works by us and by others on the generation of cells with cancer stem cell properties *in vitro*. We also discuss the concept of cancer stem cells with the results obtained from originally established cancer stem-like cells as well as the future applications of the model to basic and clinical fields.

Micro-environment to Develop Malignant Tumors (Cancerous Niche)

The pluripotent stem cells such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are promising sources of differentiated cells for transplantation in the field of regeneration therapy. When exposed to appropriate environments, the stem cells should be directed to differentiate into the progenitor cells, which are independently destined to differentiate into each mature cell such as macrophage, monocyte, neural cell, cardiac cell, and pancreatic β-cell. The behavior of stem cells is tightly regulated by the signals from surrounding micro-environment, so called 'niche' which supports the self-renewal of stem cells keeping some stem cell number. Simultaneously, a niche regulates the differentiation, in turn, maintains tissue homeostasis (Moore and Lemischka, 2006). Thus the cell fate is determined by the events and factors present in the range of a niche. Taking the pluripotency of stem cells into consideration, it should be hypothesized that malignant neoplasm is one of the tissue types differentiated from stem cells. In this context, a CSC could be described as a progenitor cell that is destined to differentiate into a cancer cell. This might be called 'canceration' rather than tumor initiation. Hereby, we propose the niche that directs stem cells into CSCs as 'cancerous niche' (Fig. 1).

Author Names in full: Tomonari Kasai[1], Ling Chen[2], Akifumi Mizutani[1], Takayuki Kudoh[1], Hiroshi Murakami[1], Li Fu[3], Masaharu Seno[1]

[1]Department of Biotechnology, Graduate School of Natural Science and Technology, Okayama University, Okayama, Japan; [2]Department of Pathology, Tianjin Central Hospital of Gynecology Obstetrics, Tianjin, China; [3]Department of Breast Cancer Pathology and Research laboratory of Cancer Hospital, Tianjin Medical University, Tianjin, China.

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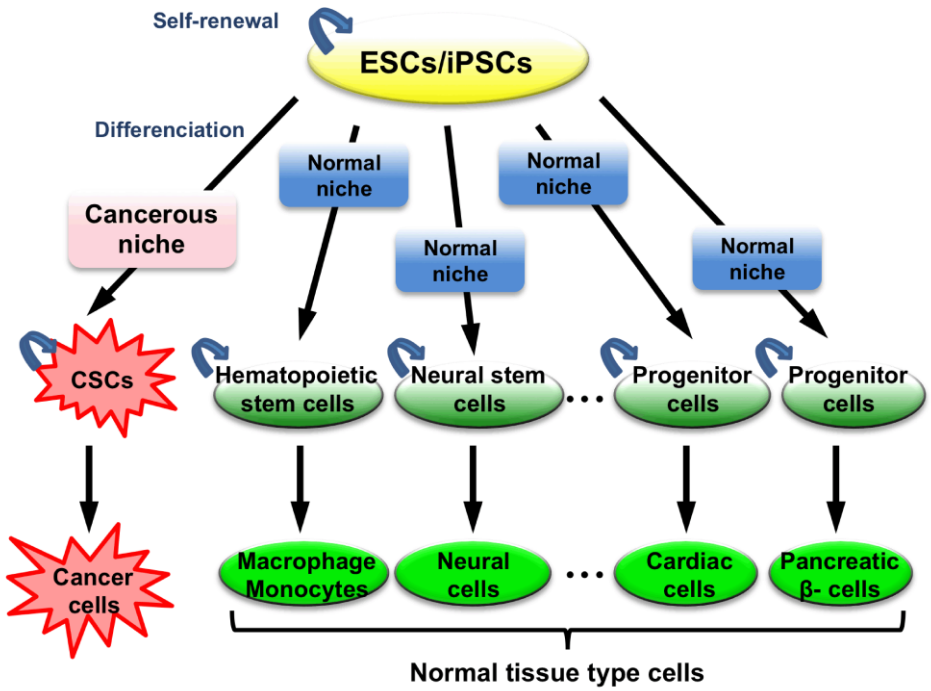

Figure 1. *Hierarchy of differentiation of embryonic stem cells (ESCs) and induced stem cells (iPSCs). Stem cells are considered to undergo differentiation depending on unique microenvironment, so called niche, as well as self-renewal. Normal tissue type cells are produced under the effect of "Normal niche". Cancer stem cells (CSCs) are hypothetically considered induced from ESCs/iPSCs to produce cancer cells.*

But the cancerous niche could hardly be defined because normal tissue or body should have normal niche in majority. The question to be asked is how and where we can find the cancerous niche. We designed unique experiments to convert pluripotent stem cells into the cells, which have the characteristic properties of CSCs, using established cancer cell lines (Fig. 2).

A model of cancer stem cells

We have demonstrated that mouse iPSCs (miPSCs) could acquire characters of CSCs when miPSCs were cultured in the presence of conditioned medium prepared from various cancer cell lines (Chen *et al*., 2012). The CSCs established from miPSCs (miPS-CSCs) through epigenetic regulations affected by the conditioned medium formed spheroids when they were cultured under non-adherent condition, implying they have capacity of self-renewal. The genes associated with stem cell properties and an undifferentiated state such as *Nanog*, *Rex1*, *Eras*, *Esg1* and *Cripto1*, were expressing in miPS-CSCs. Most importantly, miPS-CSCs exhibited high tumorigenicity with rapid growth in nude mice, while parental miPSCs provided only benign tumors, teratomas. Among established miPS-CSCs, miPS-LLCcm cells, which were derived from miPSCs cultured in the media containing conditioned medium of Lewis lung carcinoma (LLC) cells, showed highly angiogenic and typically malignant phenotype after transplantation into nude mice. The procedure of establishment and assignment of this cell to a CSC is briefed below. Based on our hypothesis that the 'cancerous niche' could generate CSCs by transforming or differentiating normal stem cells (Fig. 1), we cultured miPSCs with conditioned medium of LLC cells in the absence of mouse embryonic fibroblasts (MEF) as feeder cells and leukocyte inhibitory factor (LIF). After 4 weeks of culture, survived cells were expanded in the normal medium without MEF and LIF. Interestingly, in this condition, approximately 30–50 % of cells retained GFP expression, which was under the control of *Nanog* promoter (Okita *et al*., 2007), indicating those cells should be undifferentiated. This implies that the mechanism(s) for maintenance of undifferentiated state of miPS-LLCcm cells should not depend on MEF or exogenous LIF. To evaluate the self-renewal capacity of the cells, we examined the growth of the cells in suspension culture. The formation of spheroids pressing GFP was observed. When the spheroids were dissociated, individual cells formed new spheroids during serial passage in the suspension culture. miPS-LLCcm cells in either adherent or suspension culture formed adenocarcinomas in nude mice exhibiting cells with high nuclear to cytoplasmic ratio, nuclear pleomorphism, aberrantly high mitotic rates, and multiple pathological mitotic figures. Furthermore, CD31 positive staining in the tumor showed multiple vascular vessels, indicative of angiogenesis (Fig. 2). Totally, the histology revealed that the tumor formed by miPS-LLCcm cells were malignant. It is noteworthy that 30–50 % of the cells were GFP-positive in the tumors derived from miPS-LLCcm cells. The characters of self-renewal capacity and tumorigenicity observed in miPS-LLCcm cells are consistent with the definition of CSCs (Clarke *et al*., 2006). Thus, we are proposing miPS-LLCcm cell as a model of cancer stem cell. Furthermore, the section from the tumor derived from miPS-LLCcm showed that more than half of the mesenchymal cells in the stroma of tumor turned out to be GFP negative. Also, the gland-like structure was extensively stained with anticytokeratin antibodies (Chen *et al*., 2012). From these observations, we concluded miPS-LLCcm cells have the potential of differentiation, showing the heterogeneous linage in the tumor. As for the differentiation potential of miPS-LLCcm cells, we will discuss again in the following section together with the previous reports on the differentiation of CSCs in glioblastomas (Ricci-Vitiani *et al*., 2010; Wang *et al*., 2010; Soda *et al*., 2011).

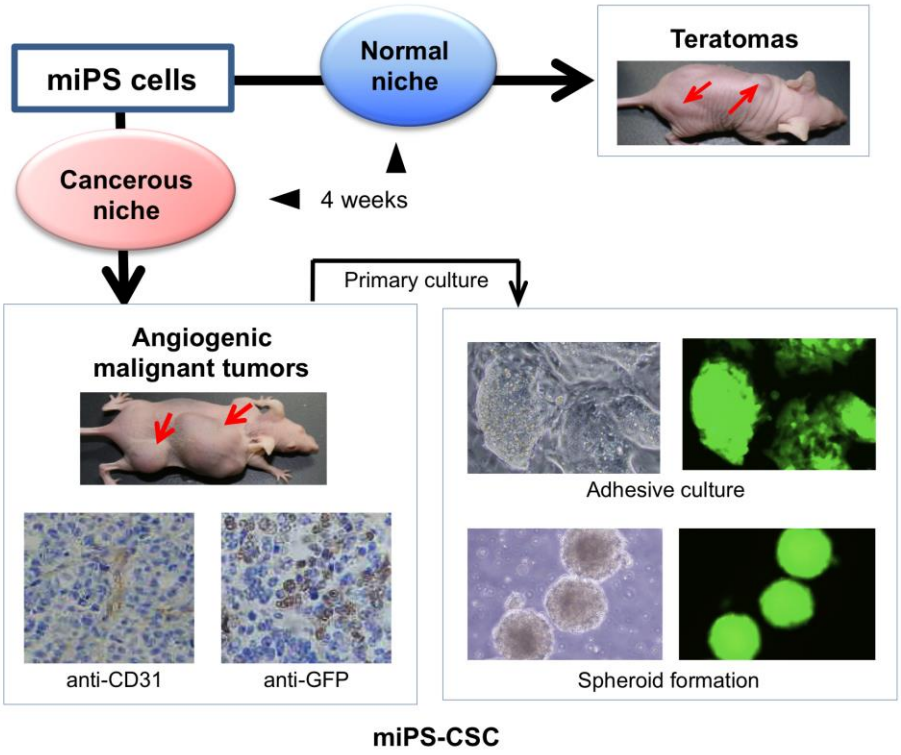

Figure 2. *A model of cancer stem cells. iPSCs were converted to CSCs (miPS-CSCs) under the treatment with conditioned medium from cancer derived cells regarding as "Cancerous niche" while normal culture condition as "Normal niche" (Chen et al., 2012). miPS-LLCcm cells, which was developed with the conditioned medium of Lewis lung carcinoma cells, showed angiogenic malignant tumor in nude mice in vivo. Primary culture from the tumor exhibited both potential of self-renewal and differentiation. Since GFP expression was directed under the control of Nanog promoter, the cells expressing GFP are judged undifferentiated.*

Cancer Stem-Like Cells in Other Literatures

Several cell lines that exhibit cancer stem-like features have been reported. A human cell line *in vitro* with cancer stem cell properties was established (Scaffidi and Misteli, 2011). In contrast to the niche-induced conversion demonstrated in our miPSCs, they transformed human primary skin fibroblasts by retroviral introduction of the genes for telomerase, oncogenic H-RasV12, simian virus 40 large T, and small T antigens. After transfection, they isolated the stage-specific embryonic antigen (SSEA-1) positive cells as in vitro generated cancer stem cells. These cells were assessed for the multipotency of both self-renewal and tumor initiation. SSEA-1 is considered as not only a marker for human ESCs at the early stage of differentiation, but also a cancer stem cell marker in brain tumors (Son *et al*., 2009). Variants of hESCs have been isolated and characterized to establish the safety standard in regenerative therapies (Werbowetski-Ogilvie *et al.*, 2009). During long-term cultivation, hESCs acquired genetic and/or epigenetic abnormalities, which lead the cells to transformation. The variants of hESCs showed some features of neoplastic progression such as high proliferation, growth factor independence, increased frequency of tumorigenicity, and aberrant linage specification. However, the authors concluded that these variants were benign because no obvious metastasis was found *in vivo*. In the variants of hESCs, the expression of stem cell markers was enhanced. Forced expression of Yamanaka factors or microRNA was applied in a series of cancer-specific iPSCs, so called induced pluripotent cancercells (iPCCs) (Miyoshi *et al*., 2010; Carette *et al*., 2010; Lin *et al*., 2008; and reviewed by Sun *et al.*, 2011). iPCCs exhibit not only the similarity with iPSCs/ESCs in pluripotency/ multipotency and teratoma formation, but also some oncogenic memories or surface markers, which their parental cancer cells possess. While some iPCCs formed teratomas in immunodeficient mice, others acquired malignancy after long-term cultivation for more than three months *in vitro* probably due to their oncogenic memories or re-activation of oncogenes (Nagai *et al*., 2010). Thus, iPCCs behave like CSCs mimicking the capacity of differentiation into cancer cells. The cancer stem-like cells established *in vitro* including our miPS-CSCs have been derived from stem cells or reprogrammed cells. It is worthwhile noticing that some of these cancer stem-like cells described above were not necessarily introduced with the genes such as for cell surface markers that are commonly used to characterize and isolate cancer stem cells. In contrast, CD44 gene was introduced into the $CD44^-$ subpopulation isolated from several $CD44^+$-colon cancer cell lines resulting in the cells exhibiting cancer stem cell phenotype (Su *et al.*, 2011). CD44 is well known as a marker for several cancer stem cells, including breast, prostate, pancreatic, and colorectal cancers (reviewed by O'Brien *et al.*, 2010). Although the cell surface markers are widely used for isolation of cancer stem cells, the functional role of the markers is still unclear. Especially, the relationship between their expression and transformation/maintenance of cancer stem cell is under investigation. It might be noteworthy to mention that CD44 was translocated into nuclei functioning as a transcription factor, and led reprogrammed cancer cells into cancer stem cells in the case of colon cancer cell lines. CD44 was demonstrated to bind the promoter region of *twist1*, one of the markers for epithelial-mesenchymal transition, which is associated with the acquisition of the cancer stem cell properties (Mani *et al.*, 2008).

Potential of Differentiation and Angiogenesis

The capacity of differentiation in CSC should be considered with tumor angiogenesis. One of the definitive characters of CSC is the multipotency to create heterogeneous linages in tumors. Three independent groups have reported the origin of blood vessels in tumors (Ricci-Vitiani *et al*., 2010; Wang *et al.*, 2010; Soda *et al.*, 2011). In the glioblastoma, a subpopulation of endothelial cells was found to carry the same somatic mutation as found in tumor cells, indicating the endothelial cells arose from the neoplastic origin. A series of analyses indicated that glioblastoma stem-like cells could differentiate into vascular endothelial cells in tumors. Because of the extensive angiogenesis in the tumor derived from our miPS-LLCcm cells, the CSC model should be a critically important source to investigate precise mechanism of tumor angiogenesis (Chen *et al*., 2012). Our data showed miPS-LLCcm cells could differentiate into endothelial cells forming tubular structure *in vitro* (Matsuda *et al*., 2013). The results from our study with miPS-CSCs will reveal the molecular mechanisms of both differentiation of CSC and angiogenesis in tumors. Through the analyses, the physiological significance of the differentiation potency and self-renewal capacity in CSCs could be further clarified because endothelial cells are considered to create a stem cell niche promoting self-renewal of CSCs (Krishnamurthy *et al*., 2010; Zhu *et al.*, 2011).

Future Applications of CSC Model for Screening Anti-CSC Agents

It is widely known that CSCs show resistance against the conventional chemo- and radiation-therapy. The characters of the resistance are considered to be one of the reasons for recurrence in patients after clinical treatments. The mechanisms involved in this resistance are considered due to expression of ABC drug pumps, expression of anti-apoptotic proteins, resistance to DNA damage, and so on (Zhou *et al*., 2009). The CSC model generated *in vitro* could be useful as tools to ask why and how they acquired those resistances and what kinds of molecules are critically responsible for them. They also should be useful to screen new anti-cancer agents that would eliminate CSCs by restricting their survival and/or differentiation of CSCs to make them more sensitive to traditional drugs, because a large number of CSCs should be required to perform drug-screening in a high-throughput manner.

Future Applications of CSC Model for CSC Vaccine

Despite an attractive theory, cancer vaccination in clinical trials has not been satisfactory or successful. A reason of failure might be unexpected presence of CSCs in tumors, which could not be characterized in detail at the diagnosis. It has been shown that cancer vaccination induced the expression of *Nanog* in the tumor cells, and raised the relative quantity of immune-resistant stem-like cells in the tumor mass (Noh *et al.*, 2012). Although further investigation is necessary to elicit the molecular mechanisms for selection of *Nanog* expressing cells, this report implies the contribution of CSCs to the acquisition of immuno-tolerance/escape of tumor. In the meantime, enriched CSCs were described immunogenically more effective than the whole cells in the tumor to induce protective antitumor immunity (Ning *et al.*, 2012). Their results proposed the novel type of cancer immunotherapy against CSCs. Enough amounts of CSCs will be required as much as the drug screening process to generate vaccine in good quality and quantity. Thus, CSC models generated *in vitro* would have a great advantage as good sources of antigen.

Communication of Cells Between Stem Cells and Cancer Cells

Several studies have demonstrated that the ESC niche could have significant influence on the phenotype of aggressive cancer cells (Tzukerman *et al*., 2006; Postovit *et al.*, 2008; Costa *et al*., 2009). These results indicate that the malignant phenotype of cancer cells could be suppressed in embryonic niche, accompanied by alternative expression of miRNAs and by epigenetic regulation such as DNA methylation. The tumor micro-environment is supposed to play important roles in the initiation, progression and metastasis of cancer (Hu and Polyak, 2008; Laconi, 2007). It has been reported that tumor cells can inhibit p53 induction, one of the most famous tumor suppressor, in the fibroblasts adjacent to the tumor tissue. This suppression was considered to be dependent on the factor secreted from tumor cells (Bar *et al.*, 2009), which raised the possibility that the factors secreted from the cancer cells might confer cancerous properties to the adjacent stem cells. On the other hand, we have recently demonstrated the effects of secreted factors from Nanog-miPSCs on LLC cells that are found in the conditioned media (Chen *et al*., 2014). The miPS cells secrete factors that can convert the epithelia phenotype of LLC cells to a spindle-shaped mesenchymal phenotype, and can promote tumorigenisity, migration and invasion. Furthermore, LLC cells that have been exposed to miPS conditioned medium became resistant to apoptosis. These biological effects are so various that the micro-environment created by miPS contains factors that can promote an epithelial-mesenchymal transition (EMT) through an active Snail-MMP axis or by suppressing differentiation in LLC cells.

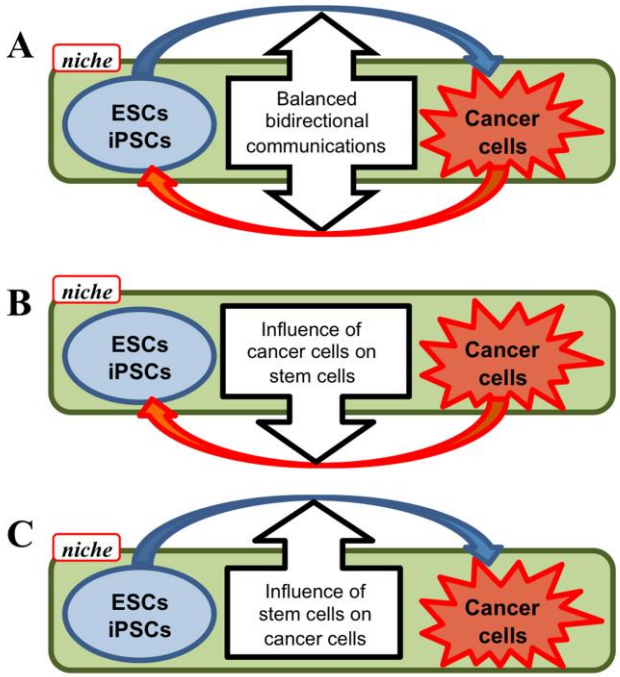

Figure 3. *Proposed cellular communication between ESCs/iPSCs and Cancer cells. cancer therapy and/or EMT. A, bidirectional communication balanced between stem and cancer cells. B, one-way influence of cancer cells on stem cells. C, one-way influence of stem cells on cancer cells. See text in detail.*

Our miPS-CSCs were obtained from the culture in the conditioned medium of cancer derived cells, but were hardly obtained in the co-culture with cancer cells (Chen *et al*., 2012). We also reported that miPS-LLCcm cells were autonomously balanced with stem-like cells and differentiated cells including vascular endothelial cells *in vitro* (Matsuda *et al*., 2013). The CSC properties appeared to be stable in the presence of the factor(s) secreted by the differentiated cells. The factor(s) activated Notch signaling and promoted self-renewal of CSCs, and appeared to regulate the differentiation lineage of CSCs. Therefore, miPS-LLCcm cells are considered to create their own niche to maintain themselves in the hierarchy of differentiating CSCs *in vitro*. Collectively, there appears the presence of bi-directional communications between cancer cells and ESCs (Fig. 3). Both communications should be regulating the activities of cancer cells and ESCs each other, so that the loss of either communication should make unbalanced regulations that would result in converting stem cells into cancer stem cells, and *vice versa.*

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Abbreviations:

CSCs: Cancer Stem Cells
EMT: Epithelial-Mesenchymal Transition
ESCs: Embryonic Stem Cells
GFP: Green Fluorecent Protein
hESCs: human ESCs
iPSCs: Induced Pluripotent Stem Cells
LLC: Lewis Lung Carcinoma
LIF: Leukocyte Inhibitory Factor
MEF: Mouse Embryonic Fibroblasts
miPSCs: Mouse iPSCs
SSEA: Stage-Specific Eembryonic Antigen

Corresponding Authors:

Prof. Tomonari Kasai & Prof. Masaharu Seno
Room 460, Bldg Eng-6, Okayama Univ. 3.1.1 Tsushima-Naka, Kita-ku, Okayama 700-8530 Japan. Phone/Fax +81-86-251-8216
e-mail: t-kasai@cc.okayama-u.ac.jp, mseno@cc.okayama-u.ac.jp