Study on the Role of Niche for Cancer Stem Cells

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Abstract

Since Schofield R. proposed the existence of a niche or specialized location in 1978, for hematopoietic stem cells that would serve a key regulator of stem cell fate, a number of researchers started to focus on the function of the stem cell niche. Normally, stem cells depend on the integration of both intrinsic and extrinsic factors for proper, homeostatic tissue maintenance through achieving a delicate balance between self-renewal and differentiation processes. As the differentiated cells rarely divide, and rapid proliferating cells have poorly differentiated phenotype, cancer is thought to be the disease of stem cells. Recent studies showed, some forms of leukemia clearly come from true stem cells, but cancer can also arise from progenitor cells downstream of stem cells. Some studies have demonstrated that an aberrant microenvironment with locally derived growth-promoting signals rather than growth-inhibiting signals may contribute to the genesis of cancer stem cells (CSCs). CSCs are defined as cells within a tumor that possess the capacity to self-renewal and to give rise to the heterogeneous lineages of cancer cells that comprise the tumor. Cells with CSC properties have been isolated from various types of tumors and cancer cell lines, including glioblastoma, lung carcinoma, Ewing's sarcoma, and breast cancer. Despite many studies, the biological nature of CSC-niche remains largely unclear, especially in the context of the origin of CSCs.

Recently, we have reported the successful conversion of mouse induced pluripotent stem cells (miPSCs) into CSCs (miPS-LLCcm) following exposure to the conditioned medium (CM) of the mouse Lewis Lung Carcinoma (LLC) cell line serving as a cancerous niche. After subcutaneous injection into immunodeficient mice, the cells generated adenocarcinomas with extensive angiogenesis. Thus, we confirmed the endothelial differentiation ability of the CSCs in this cell line. Since the puromycin

resistant gene is under the control of Nanog promoter, I applied puromycin in the culture medium to remove the differentiated cells for 1 week, followed by removing the puromycin and allowed the cells to differentiate for another 1 week, as one cycle. I found the tube formation ability was decreased along with puromycin selection cycles, indicating the endothelial differentiation ability of CSCs decreased without differentiated cells. I concluded the differentiated cells could form a niche for the regulation of CSC behaviors.

The CM of LLC can convert miPSCs to active CSCs, suggesting the LLC CM could serve as cancerous niche to induce normal stem cells to malignant counterpart. Findings pertinent to secreted vesicle-based intercellular communication, has provide a potential mechanism involved in niche regulation of cell fate/behavior. Here, I focused on the contribution of tumor-derived extracellular vesicles (tEVs), including exosomes/microvesicles that are secreted from LLC cells to induce the transformation of miPSCs into CSCs. tEVs were isolated from the CM of LLC cells, and then the differentiating miPSCs were exposed to tEVs for 4 weeks. The resultant tEV treated cells (miPS-LLCev) were immortalized. When miPS-LLCev cells were subcutaneously transplanted into Balb/c nude mice, malignant liposarcomas with extensive angiogenesis developed. Additionally, some of the cells owned metastatic ability to transfer into mesentery of the mice. All the data above indicate the tEVs can contribute to the cancerous niche to induce malignant transformation.

In light of cancer stem cell hypothesis, a subset of cells, which possesses stem cell properties, has been found in several kinds of tumors. According to this hypothesis, in solid tumor, only the small part of stem-like cells could produce all kinds of cells, who are necessary to repopulate a tumor. In the current study, miPS-LLCev cells proliferated endlessly and, interestingly, started to express GFP, which is a signal of stemness in the iPS cells used here. Immunoblotting analysis showed the cells started to express Nanog, Oct3/4 and CD133 proteins during tEV treatment. In suspension

culture, both the bulk cells and puromycin selected cells formed spheroids in the serum free medium, indicating the self-renewal capacity of the cells. By serial transplantation, the cells developed similar liposarcoma, suggesting the cells possessed replication potential *in vivo* to maintain the unique properties. Quantitative RT-PCR analyses showed the embryonic stem cell markers, such as Oct3/4 and c-Myc, are expressed comparably to miPS cells; Sox2 and Klf4 are significantly higher than the parental miPS cells. Moreover, the cells were showed to differentiate to the cells expressing CD34, CD36, PPARγ2 and CD31, indicating the adipocyte and endothelium differentiation of the CSCs. Thus, miPS-LLCev cells were defined as a CSC cell line, and tEVs from LLC cell line can serve as a CSC genetic niche, which is responsible for the CSC origination.

The role of stem cell niche in regulation of stem cell behaviors has been shown in increasing studies. As collecting all the data above, I showed the cancer stem cells differentiated to endothelial cells, and the differentiated cells contributed to a niche, in turn regulate the differentiation ability of the CSCs. For normal stem cells, the aberrant niche could transform normal stem cells to cancer-initiating cells. In this cancerous niche, exosomes/microvesicles could be a significant factor, which transfer active molecules to recipient normal cells, lead to the malignant transformation of normal stem cells.

Contents

CHAPTER 1

General Introduction

1.1 Cancer stem cells concept and hypothesis

Cancer stem cell (CSC) is a cell within tumor possesses self-renewal capacity, and is responsible to generate the heterogeneous tumor cell population [1]. The CSC hypothesis was presented for the first time about 150 years ago, that cancers arise from ‘stem cells’ or ‘germ cells’ [2]; however, it caused the intensive investigations for the recent decades with research and clinical advancements. It provides the reasonable interpretation to why the cancers are usually therapy resistant and recurrent after removing the tumor focus. The involvement is the CSCs have been proved to be always less sensitive to the chemotherapies and radiotherapies than the bulk tumor cells, which are the progenies of themselves. Although, the primary tumors may be eliminated from the patient, the tumor reappears after a period of time, as a small inconspicuous population of CSCs survived from the treatment either in the primary location or a new position after metastasis (Fig. 1.1). This hypothesis is crucial because it invites the attentions for the clinical treatment to target the CSCs.

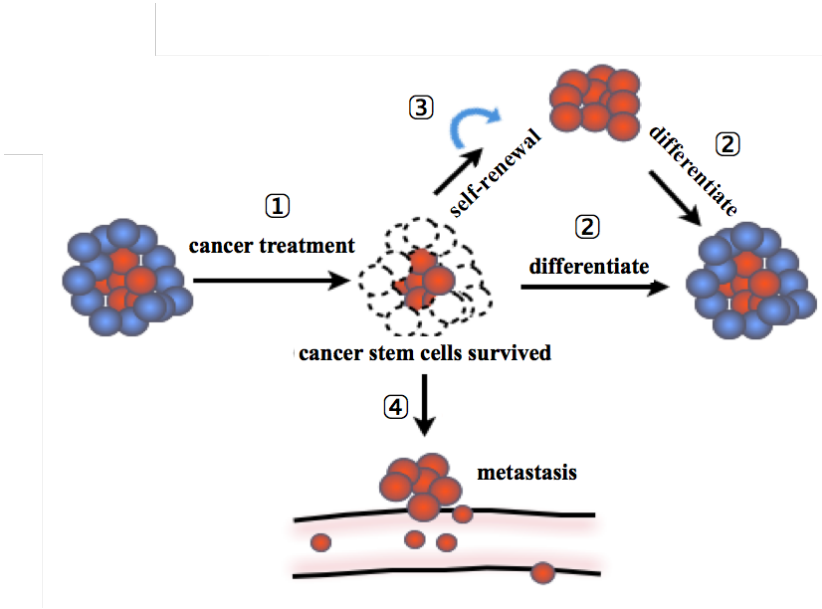


Figure 1.1 Cancer stem cells ① ressist to cancer treatment (such as medicine and radiotherapy), then they can ② differentiate to cancer cells again, or ③ self-renew to more cancer stem cells, worse, they can ④ go into blood vessels and travel to any other place with blood stream. That is why cancer is difficult to eliminate.

The CSC hypothesis concerns the cellular origin that whether the CSCs are originated from normal tissue stem cells. Indeed, the studies so far suggested the stem cells might contribute to tumorigenesis, because the normal stem cells and CSCs are sharing several properties, which include the capacity to self-renew and differentiate, the active telomerase expression, activation of anti-apoptotic pathways, increase of membrane transporter activity and the ability to migrate [2]. The first evidence that showing the existence of normal tissue stem cells is the multi-potent stem cells, which were detected and counted from the hematopoietic system by using a novel method about 50 years ago [3]. Then, it was shown that, only a small part of cells in the tumor mass could generate colonies in the mouse spleen [4], suggested the possible exist of cancer initiating cells. Till about 20 years ago, the first solid evidence of a single progenitor cell that is responsible for the repeatable clonal formation and cancer creation was provided in the study of chronic myelogenous leukemia and acute leukemia [5]. This is the best evidence to prove that the normal tissue stem cells are the target of the mutational accumulation; as a result, the CSCs are developed and afterward start to differentiate to the heterogeneous tumor cell population. However, more recent studies showed another possibility that CSCs arise from progenitor cells, which acquired self-renewal capacity and could generate the more differentiated progenies [6-8].

During last 10 years, CSCs were isolated from various cancers by using special molecular markers or experimental approaches [9-16], and this tumor list is still growing. It allowed the CSC hypothesis accepted increasingly. After challenging the

presence of CSCs, investigators nowadays are focusing on the mechanisms that CSCs drive the tumor growth and maintenance [9, 17, 18]. CSC models were established from embryonic stem cells [19], induced pluripotent stem cells [20] and human fibroblasts [21]. The origin of CSCs is discussed during different method of CSC conversion, either by cancerous niche or by overexpression/silence of special molecules. Additionally, the established CSC models could in turn serve as the candidate cell lines for CSC research and development of therapeutic strategies, overcome the difficulty that isolation of CSCs from tumor mass as they are rare subpopulation.

1.2 Cancer stem cell niche

Normal stem cells in the body are responsible for the organic generation, for their capacity to self-renew as well as differentiate to various lineages, which then constitute and maintain tissues for the human lifetime [22]. A refined balance between these two opposite processes is significant for the proper tissue maintenance and repair [23]. The question arose as whether the activities of stem cells are spontaneous or they need the interactions with other kind of cells. Up-to-date researches showed, that they need the signals from environment to adjust the balance between self-renewal and the switch of differentiation [24]. Giving the similar behavior between CSCs and normal stem cells, researchers are focusing on the mechanism of the maintenance of CSC by the microenvironment “niche” [25-27], in which CSCs reside and that is responsible for the maintenance of unique stem cell properties [28]. The critical factors including soluble molecules, extracellular matrix proteins, stromal support cells [29] and blood vessels [30] make up the unique normal/cancer stem niche. Based on the evolution of stem cell research in the normal tissues, the knowledge and experimental technology were developed to define the CSC niches.

Several groups reported that the CSCs reside adjacent to the tumor blood vessels, which termed the vascular CSC niche [28]. For example, the endothelial cells were proved to reside adjacent to nestin positive cancer cells in glioblastoma, promoted the growth of tumor cell population and self-renewal of the CSCs inside [31], sustained the overcoming of CSCs from radiotherapy [32], suggesting the endothelial cells play a role in the CSC niche. It seems to be regulated by several pathways in the microenvironment that the endothelial cells encourage the maintenance of CSCs, such as Notch signaling pathway [31]. Although, the source and the exact mechanism of this process is remaining unknown and it should be the critical factor for the controlling of CSC maintenance.

A number of cancer-associated fibroblasts (CAFs) are present in the tumor mass with distinct properties, which are different from normal fibroblasts. CAFs are regarded as another kind of tumor niche cells, which are considered to regulate the tumor cell proliferation, invasion and metastasis progression. They are able to influence the CSCs through direct cell-to-cell communication [33], secretion of particular factors [34] or modification of extracellular matrix [35]. Compare to normal fibroblasts, co-injection with CAFs increased the tumorigenesis of normal prostate epithelial cells [36]. A very new study showed, the normal fibroblasts suppressed the epithelial phenotype and metastasis, while CAFs could offer a mesenchymal phenotype and increase the metastasis of the epithelial cells [37]. This kind of 'activated' stromal cells could be converted from several different lineages including normal fibroblasts, smooth muscle cells and other pericytes [38]. And it has been proved the CAFs could act as the tumor niche through secreting several kinds of chemokines, such as CXCL12 and insulin-like growth factor 1 (IGF1) [39]. The functional activities of CAFs suggest the diverse pathways from the heterogeneous population are cooperating in the cancer maintenance and progression, and complicate

the cancer therapies.

However, besides the regulation activities, the CSC induction niche also have gained intensive investigations. Normal stem cells or even differentiated cells reside in an aberrant environment is believed to be the reason for the cancer origination. Continuing expression stromelysin-1/matrix metalloproteinase-3 in the mammary gland, an enzyme that destroys basement membrane, result in epithelial tumorigenesis [40]. Moreover, chronic inflammatory condition has been shown to be an essential inducer of tumorigenesis [41]. For instance, the main inflammatory response molecule, NF-κB can induce epithelial to mesenchymal transition (EMT) directly or through hypoxia-inducible factor (HIF-α). EMT was first observed in the normal embryonic development [42], and governed by the molecules within the niche. Increasing studies suggested the cancer initiation and progression, especially CSC occurrence, are associated with EMT [43], because the acquisition of mesenchymal phenotype is critical for the invasive malignancy, as the loss of molecules of cell-to-cell junction [44]. There seems to be a significant connection between cancer initiation and EMT, especially after the CSC hypothesis has been widely accepted. This connection has been reinforced by the studies on EMT with regard to inflammation and hypoxia environment.

1.3 Extracellular vesicles

As we know, chemokines and cytokines secreted by the cells as well as extracellular matrix in tumor niche are key factors controlling tumor progression. However, recent findings suggested the role of vesicle-based cell-to-cell communication in the tumor niche formation. The extracellular vesicles (EV) including microvesicles and exosomes, are shed from cell membranes of almost all the cell types, and were ignored for long time as a kind of cell debris. They contain

numerous soluble proteins, lipids, membrane proteins and nuclear acids, of which contents are from the releasing cell. These secreted membrane fragments are circulating in the fluid of extracellular space and able to enter to another cell through binding with cell surface ligands, membrane fusion or endocytosis, whereby release the contents to target cells. Thus far, EVs have been found to transfer materials to a number of cell types, such as primary tumor cells [45], endothelial [46], stromal [47] and bone marrow cells [48], and modified the behaviors of these cells.

It is well known that the number of EVs shed from cells is depending on the situation of cells. For example, the cells activated by hypoxia, injury, irradiation or exposure of special molecules will secrete more EVs than that of normal condition [49]. The role of tumor-derived EVs (tEVs) was discussed in increasing studies, in which the tEVs were collected from the conditioned medium of growing tumor cells. There are evidence that tEVs could promote angiogenesis [50] and metastasis [51] to accelerate the tumor progression. Additionally, tEVs play a pivotal role in the suppression of immune response by inactivation of T lymphocytes of natural killer cells [52, 53], preventing differentiation of murine myeloid precursor cells into dendritic cells [54] or aberrant regulation of T lymphocytes and myeloid cells [55]. Based on these facts, tEVs appeared to contribute to the cancer niche to facilitate the tumor progression.

It has been shown that tEV could transfer oncoprotein, EGFR receptor (EGFRvIII) to target cells, acting as the intercellular vehicles of oncogenesis molecules [56]. There is still very few data demonstrating

that the tEVs can convert the normal cells to tumorigenic cells. It would be interesting to test the contribution of tEVs to the oncogenesis process, and analysis the underlying mechanism.

1.4 The Contents of the Dissertation

Chapter 1: The concept of cancer stem cells, cancer stem cell niche, especially the contribution of tumor-derived extracellular vesicles to the tumor environment. The summary previous studies, the study aim and contents of the thesis are also briefly described.

Chapter 2: The properties of Lewis Lung Carcinoma (LLC) cell line derived extracellular vesicles (tEVs), including exosomes and microvesicles, are described. The dependency upon tEVs to colony formation in the miPS culture and the tumorigenicity of resultant cells are investigated.

Chapter 3: In this chapter, the CSC properties were investigated in the tEVs treated miPS cells (miPS-LLCev). I found miPS-LLCev cells showed significant self-renewal capacity in serum-free suspension culture. Most importantly, they could keep the special histophenotype within serial transplantation. Moreover, they could generate a heterogeneous population expressing different patterns of unique markers.

Chapter 4: The CSC model, miPS-LLCcm, which established from miPS cells by culturing with conditioned medium derived from growing LLC cell line, could give rise to endothelial cells, and form vessel like structures in vitro and vivo. And the on-vessel cells showed a heterogeneous of hierarchy. Moreover, the depletion of endothelial-differentiated cells deprived the differentiation capacity of CSCs to endothelial cells.

Chapter 5: The general conclusions were drawn from the present study. The future challenges related on the tumor niche regulation on the normal cells and tumor cells regarded to CSCs also started.

CHAPTER 2

Extracellular vesicles derived from Lewis Lung Carcinoma cell line treated differentiating miPS cells are tumorigenic

Abstract

Several studies have shown that the cancer niche can perform an active role in the regulation of tumor cell maintenance and progression through exosomes/microvesicles-based intercellular communication. However, it has not been reported whether this vesicle-mediated communication affects the malignant transformation of normal stem cells/progenitors. We have previously reported that the conditioned medium derived from the mouse Lewis Lung Carcinoma (LLC) cell line can convert mouse induced pluripotent stem cells (miPSCs) into cancer stem cells (CSCs), indicating that normal stem cells when placed in an aberrant microenvironment can give rise to functionally active CSCs. Here, I focused on the contribution of tumor-derived extracellular vesicles (tEVs), exosomes/microvesicles that are secreted from LLC cells to induce the transformation of miPSCs into CSCs. tEVs were isolated from the conditioned medium of LLC cells, and then the differentiating miPSCs were exposed to tEVs for 4 weeks. The resultant tEVs treated cells (miPS-LLCev) were immortalized. When the miPS-LLCev cells were subcutaneously transplanted into Balb/c nude mice, malignant liposarcomas with extensive angiogenesis developed. Thus, by culturing with tEVs from LLC cell line, the normal stem cells could be transformed to tumorigenic cells.

Key words: extracellular vesicles, niche, mouse iPS, cancer stem cells and cancerous niche

2.1 Introduction

Because cancer stem cell (CSC) shares the self-renewal feature with normal stem cell, it has been proposed that one of the differences between normal stem cells and cancer stem cells is their degree of dependence on the stem cell niche, a specialized microenvironment in which stem cells reside. In other words, the cancer is a disease of stem cells in an aberrant niche [57]. The microenvironment/niche can exert profound genetic and/or epigenetic effects on stem cells through interactions between stem cells and surrounding tissue resident cell populations, or through cell-derived factors originating from the surrounding cells within the niche. Current studies have demonstrated that an aberrant microenvironment with locally derived growth-promoting signals rather than growth-inhibiting signals may contribute to the genesis of CSCs [19, 57]. Despite many studies, the biological nature of CSCs remains largely unclear, especially in the context of the origin of CSCs. Recently, we have reported the successful conversion of mouse induced pluripotent stem cells (miPSCs) into CSCs following exposure to the conditioned medium (CM) of the mouse Lewis Lung Carcinoma (LLC) cells serving as a cancerous niche [20]. The question is that which kind of factor(s) contributed the conversion of normal cell to tumorigenic cells.

Findings pertinent to secreted exosome/microvesicle-based intercellular communication, has provided a potential mechanism involved in niche regulation of cell fate/behavior [58]. In this regard, tumor-derived exosomes and/or microvesicles, termed extracellular vesicles (tEVs) in general, are critical components in the cancerous niche. Pivotal roles of microvesicles secreted by both human and murine lung cancer cell lines have been shown in tumor progression, metastasis and angiogenesis [59]. Exosomes that are derived from highly metastatic melanomas can

also increase the metastatic behavior of primary melanomas through the education of bone marrow progenitor cells to create a pre-metastatic niche [48]. An oncogenic receptor, EGFRvIII, has been shown to be carried by microvesicles from aggressive glioma tumor cells to a non-aggressive tumor cell populations, and the recipient cells exhibited activation of MAPK and Akt signaling pathways, concomitant with a morphological transformation and an increase in anchorage-independent growth [60]. Microvesicles secreted by a human prostate cancer cells can activate normal stromal fibroblasts to cancer-associated fibroblasts, which is well known for contributing to tumorigenicity [47, 61]. In addition, it has been reported that adipose tissue-derived mesenchymal stem cells exhibit tumor-associated myofiblobrastic characteristics after treatment of exosomes that were derived from breast cancer cell lines [62]. These findings clearly indicate that tEVs act as messengers during cell-to-cell communication, which can significantly affect tumor progression and metastasis. To our knowledge, however, no direct report has demonstrated that tEVs can modulate the fate of normal stem cells or progenitor cells to facilitate their conversion or transformation into CSCs.

Since our previous study indicating that secreted factor(s) from various cancer cell lines could promote the formation of CSCs from miPSCs [20], it is highly possible that tEVs could be one mechanism underlying this conversion. In this chapter, I applied tEVs collected from LLC CM to differentiating miPSCs cultures, and characterized the resultant cells both *in vitro* and *in vivo* to assess the contribution of tEVs to induce CSCs from miPSCs. Our results suggested that normal stem cells or differentiating progenitor cells might give rise to CSCs when they are exposed to an abnormal cancerous niche. Understanding the mechanisms and details of this process will hopefully be useful in the development of new therapeutic approaches to target not only CSCs, but also the cancerous niche.

2.2 Materials and Methods

2.2.1 Preparation and detergent treatment of tEVs from LLC cell line

LLC cells at 80% confluence were cultured with serum-free DMEM. Culture supernatants were collected after 48 h, then centrifuged at 300 g for 10 min and 2000 g for 10 min to remove cells and large debris, respectively, followed by centrifugation at 10,000 g for 30 min to remove small debris. tEVs were pelleted by ultracentrifugation (Himac CP70MXX, Hitachi, Japan) at 100,000 g for 2 h, washed twice and suspended in PBS [63]. Particle diameter was measured by dynamic laser scattering (ELS-8000, Otsuka Electronics, Japan). Protein concentration was determined by MicroBCA Protein Assay kit (Pierce). tEVs were stored at -80˚C until use.

To disrupt the tEVs, 0.05 μg/μL tEVs were incubated with Triton X-100 at final concentration of 0.5% in 4˚C on rotator.

2.2.2 Cell culture

Mouse iPSCs [64] that contained a puromycin (puro) resistant gene and green fluorescent protein (GFP) gene (iPS-MEF-Ng-20D-17, Lot No. 012, Riken Cell Bank, Japan) were maintained under the humidified 5% CO_2 atmosphere at 37˚C on feeder layers of mitomycin-C-treated mouse embryonic fibroblasts (MEFs) (Reprocell, Japan) in miPS medium (Dulbecco's Modified Eagle Medium (DMEM) containing 15% fetal bovine serum (FBS), 0.1 mM Non-Essential Amino Acid (NEAA, Life

Technologies), 2 mM L-Glutamine, 0.1 mM 2-mercaptoethanol, 1000 U/mL Leukemia inhibitory factor (LIF, Millipore), 50 U/mL penicillin and 50 U/mL streptomycin). MEFs were removed by culturing in the presence of 1 mg/mL puro. The Lewis Lung Carcinoma cell line (ATCC) was maintained in DMEM supplemented with 10% FBS.

For the treatment of tEVs, miPSCs were first induced to differentiate for 3 days by culturing without LIF. Then, 4×10^5 cells/ 60-mm dish differentiating miPSCs were maintained in conversion medium that consists of miPS medium (without LIF) containing various concentrations of LLC tEVs, and medium was changed daily with fresh tEVs or detergent pre-treated tEVs. When cells reached 80% confluence, they were harvested and seeded in the corresponding medium as the number of 4×10^5 cells/ 60-mm dish. The resultant cells (miPS-LLCev) were maintained with miPS medium without LIF (Fig. 2.3). Cell morphology and GFP fluorescence were monitored and photographed using an Olympus IX81 microscope equipped with a light fluorescence device (Olympus, Japan).

2.2.3 Immunoblotting

Cells were washed twice with ice-cold PBS and lysed by using 50 mM Tris-HCL, pH 7.4, containing 150 mM NaCL, 0.5% triton-X 100, 1mM PMSF, 5mM EDTA and 0.1% protease inhibitor cocktail, followed by sonication with a microprobe setting at level 2 for 30 sec on ice and centrifuged at 13,000 g for 20 min at 4 ˚C. The supernatants were collected and stored at -80 ˚C until use. Protein concentration was determined by MicroBCA Protein Assay kit. Thirty-five micrograms of total cellular protein of each sample was loaded for electrophoresis. Separated proteins were then blotted onto polyvinylidene difluoride (PVDF) membrane (Millipore). After blocking

for 1 h with 5% skim milk, the membranes were incubated overnight at 4˚C with rabbit polyclonal anti-CD63 antibody (1:1000, Santa Cruz). The blots were then incubated with secondary antibody, horseradish peroxidase (HRP)–conjugated anti-rabbit IgG (Cell Signaling). The bound antibodies were detected using Western Lighting plus-ECL reagent (PerkinElmer), and recorded by Light-Capture II cooled CCD camera system (ATTO, Japan). Coomassie blue staining of SDS-PAGE gels were used as controls for equal loading.

2.2.4 Flow cytometry analysis

To evaluate GFP expression during tEVs conversion, 1×10^6 adherent cells of each sample were dissociated and collected in 100 mL PBS, then analyzed on a FACS Calibur flow cytometer (Becton Dickinson).

2.2.5 Tumorigenicity analysis in immunodeficient mice

4 week-old Balb/c-nu/nu female immunodeficient mice were purchased from Charles River (Japan). 1×10^2 - 1×10^6 cells (shown in Table 2.1) were suspended in sterile PBS and subcutaneously injected into one flank of immunodeficient mice. After 4 weeks, all tumors were resected at autopsy and sectioned for histologic analysis. All animal experiments were reviewed and approved by the ethics committee for animal experiments of Okayama University under the ID OKU-2013252.

2.2.6 Histologic analysis, immunohistochemistry (IHC) and Oil Red O staining

Tumors dissected from mice were fixed for 24 hours in 4% PFA and dehydrated by gradient ethanol, then 4 mm thick sections processed using a routine wax-embedding procedure for histologic examination, followed by staining with hematoxylin and eosin (HE).

IHC staining were performed by using rat monoclonal anti-CD31 antibody (1:200, Santa Cruz), rabbit polyclonal anti-Ki67 antibody (1:200, Abcam), rabbit monoclonal anti-GFP antibody (1:300, Cell Signaling), mouse monoclonal anti-vimentin antibody (1:100, Santa Cruz), or mouse monoclonal anti-PPARg2 antibody (1:200, Santa Cruz). Briefly, 4 mm tissue sections were deparaffinized and antigen retrieved was performed using microwave exposure at 95 ˚C for 5 minutes in a citrate buffer (pH 6.0). After 3% hydrogen peroxide blocking for 10 min and normal serum blocking for 1 h, the sections were then incubated in 4 ˚C for 12 h. The sections were then incubated with biotinylated anti-rabbit, biotinylated anti-rat or biotinylated anti-mouse secondary antibodies (Vector, USA), followed by incubation with the ABC reagent (Vector, USA). Detection was accomplished using 3, 3'-diaminobenzidine tetrahydrochloride (DAB, Vector, USA). Negative controls were performed by incubation of sections with phosphate-buffered saline (PBS) instead of primary antibodies. Counter staining was carried out using hematoxylin.

For Oil Red O staining, cells or 10 mm cryosections were fixed by neutral buffered 10% formalin, 30 min at room temperature. After rinsing in 60% isopropanol, cells were stained with Oil Red O (Sigma) at 60˚C for 5 min.

2.2.7 Invasion assay

The Matrigel invasion assay was performed according to the manufacturer's protocol (Becton Dickinson). The growth factor-reduced Bio-coat Matrigel membrane inserts were rehydrated with serum-free medium and incubated at 37˚C for 2 h. Following aspiration of the medium from the inserts, 2.5×10^4 cells in 500 mL serum-free medium were seeded onto the insert, and 750 mL serum containing medium was added to the bottom well of the insert. After incubation for 30 h in 5% CO_2 atmosphere at 37˚C, cells on the bottom of the membranes were fixed and counted after Gimsa staining.

2.3 Results

2.3.1 Characterization of exosomes/microvesicles derived from LLC cell line

In a previous study, we demonstrated that culturing miPSCs with CM derived from various mouse cancer cell lines could induce miPSCs into CSCs [20]. This suggested that factor(s) secreted from cancer cells might be responsible for the induction of CSC phenotype in miPSCs. Among the CSC lines we established, miPS-LLCcm cells that were induced by the CM of LLC cells developed adenocarcinomas with abundant undifferentiated tumor cells in immunodeficient mice. To more fully define the particular fraction in CM that can enhance CSC generation from miPSCs, I assessed the contribution of tEVs to this process in the current study.

tEVs were prepared from LLC CM by ultra-centrifugation and the particle size was confirmed by dynamic laser scattering with an average size of 100 nm (Fig. 2.1). In addition, the presence of CD63, a common exosomal marker, was identified [65] (Fig.

2.2). Intriguingly, the particle size distribution of the collected fractions indicated two peaks (56.7 nm and 132.0 nm), suggesting that LLC cells secrete two different size populations of particles, presumably exosomes and microvesicles [63]. Although the detailed properties of these particles remain to be defined, I conclude that the precipitated fraction contains tEVs as judged from their size distribution and detection of CD63 [63, 65].

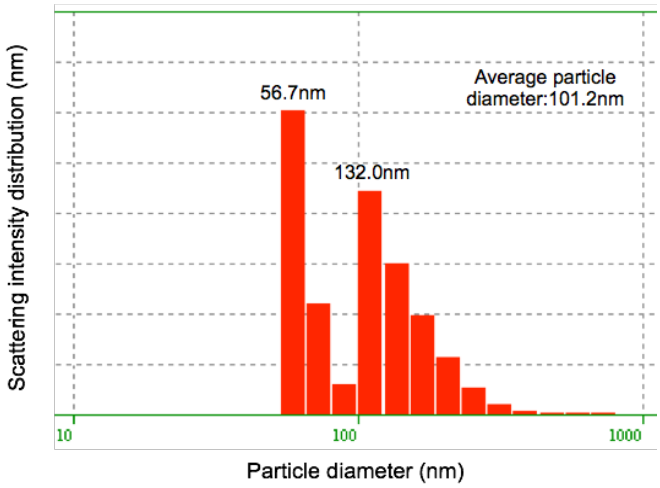


Figure 2.1 Size distributions of tEVs derived from LLC cell line. There are two main peaks, showing the main size of the tEVs, 56.7 nm and 132.0 nm, with an average diameter of 101.2 nm.

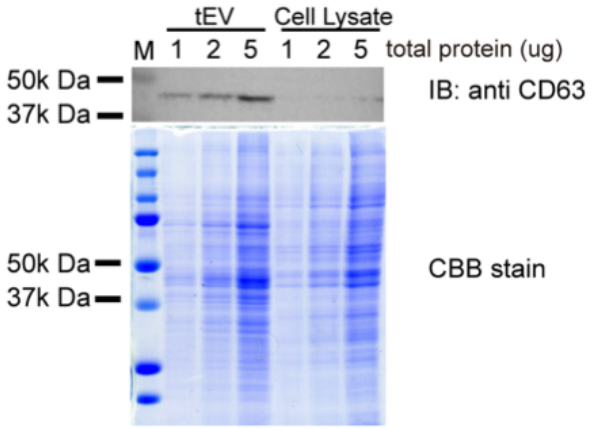


Figure 2.2 Immunoblotting of CD63, a common marker of exosomes, showing the existence of exosomes. Coomassie staining of gel is used as loading control to show the equal loading of protein.

2.3.2 tEVs treatment of differentiating miPSCs gives rise to stem-like population of cells

I then investigated whether tEVs were effective on promoting the conversion of differentiating miPSCs into CSCs. miPSCs cultured without feeder cells in the presence of LIF and puro were allowed to differentiate for 3 days by removing LIF and puro from the miPS medium. The differentiating miPSCs were then cultured in conversion medium containing tEVs for another 1 week. A schematic overview of the conversion procedure is illustrated in Figure 2.3. Since here I used the Nanog-GFP mouse iPS cells, the selection of undifferentiated miPSCs and monitoring of differentiation could be carried out by addition of puro and assessment of GFP fluorescence, respectively, since the expression of the puro resistant gene and GFP gene were under the control of the mouse *Nanog* promoter in the miPSCs used here

[64].

To evaluate the optimal concentration of tEVs that can facilitate GFP positive colony formation, I tested different concentrations of tEVs (0-1000 ng protein of tEVs/mL) in conversion medium. The results indicated that the number of colonies increased in a dose dependent fashion, and was optimal at 100 ng of tEVs/mL. Under this condition, cells grew actively and led to a rapid colony expansion (Fig. 2.4). Therefore, I used the cells treated with tEVs of LLC cells at a concentration of 100 ng/mL for further investigation. To define more clearly the relevance of tEVs to the colony formation, I tested the detergent pre-treated tEVs, whose membrane particle structure were disrupted. I observed that the number and size of colonies were significantly reduced (Fig. 2.5), which suggesting that the factor(s) responsible to the CSC-conversion were delivered from cancer cells as a form of membrane particles, tEVs.

During the 3 days culturing of miPSCs in the absence of LIF, GFP expression, which is an index of *Nanog* expression, gradually decreased, suggesting the cells were differentiated (Fig. 2.6 A&B 3rd day). Then, tEVs were added to the cell culture medium for 1 week. FACS analysis showed the GFP expression appeared after the 1 week-culture by tEVs (Fig. 2.6 A 9th day). In contrast, by the culturing with plain medium, the GFP expression totally lost (Fig. 2.6 B 9th day).

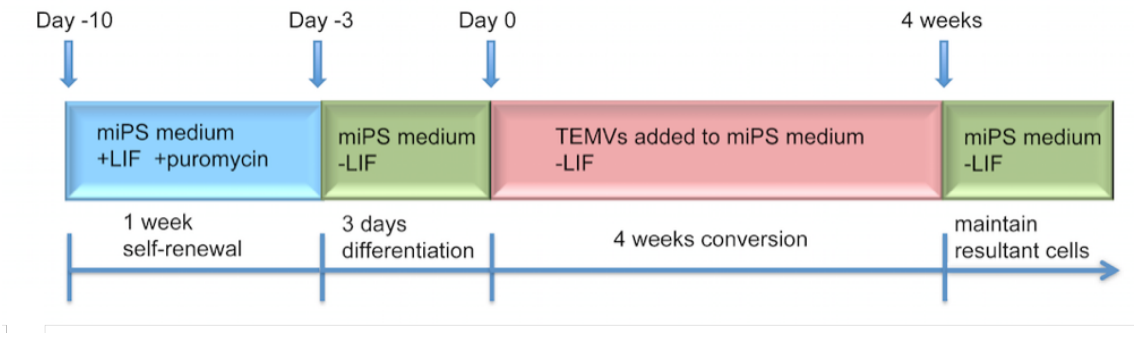


Figure 2.3 Cells are passaged following the conversion schedule. Each color indicates different culture media.

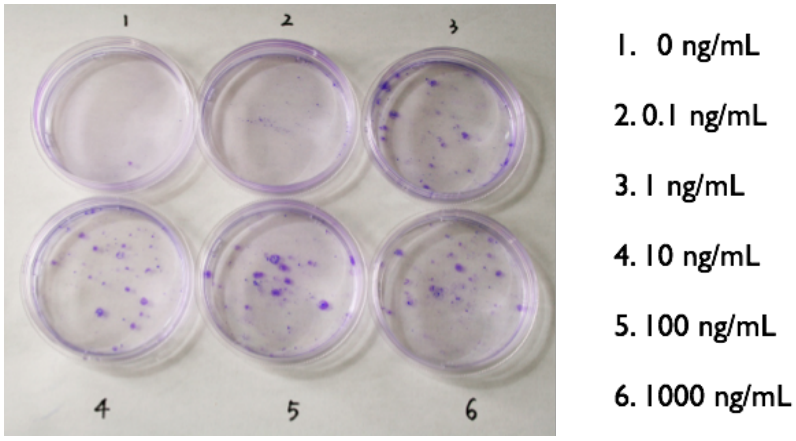


Figure 2.4 By different concentration (0, 0.1, 1, 10, 100 and 1000 ng/mL protein) of tEVs culturing for 2 weeks, from 1ng/mL to 1000 ng/mL, there are colonies appeared, and 100 ng/mL protein of tEVs shows the most active colony expansion.

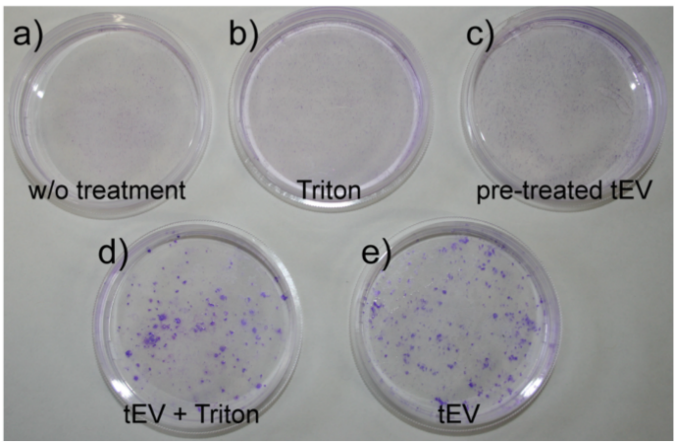
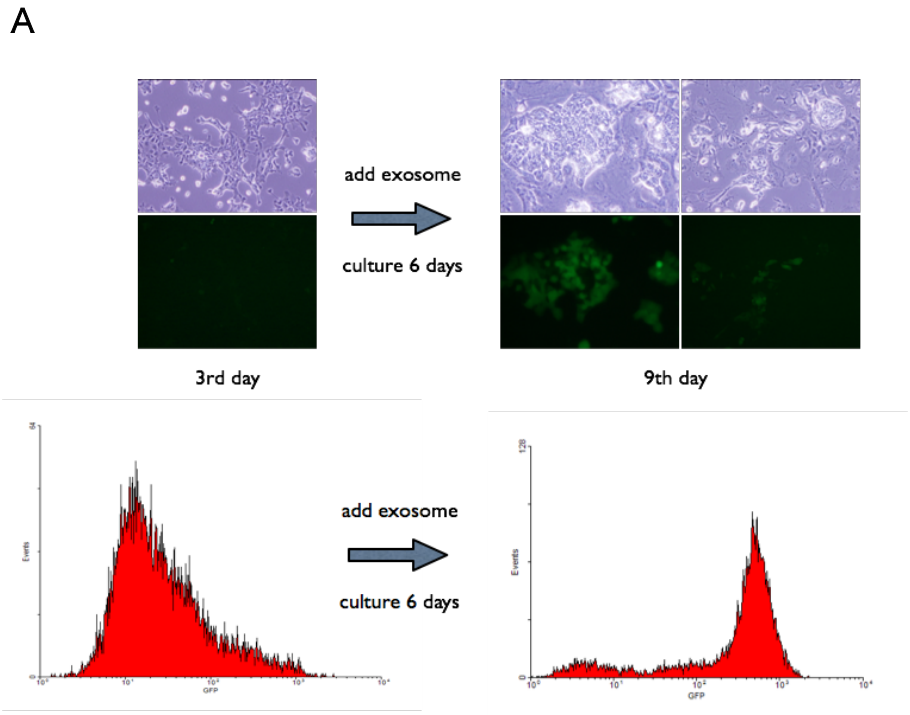


Figure 2.5 Colony formations by using detergent pre-treated tEVs. LLC derived tEVs (0.05 μg/μL) was incubated with 0.5% Triton X-100 for 5 h in 4˚C. Then, we treated cells with detergent pre-treated tEVs (c), that amount was corresponding to untreated tEVs (100ng/mL) (e). As controls, cells were treated with untreated tEVs in the presence of 0.001% Triton X-100 (d) or 0.001% Triton X-100 (b). The detergent pre-treated tEVs are failed to induce the colony growth.



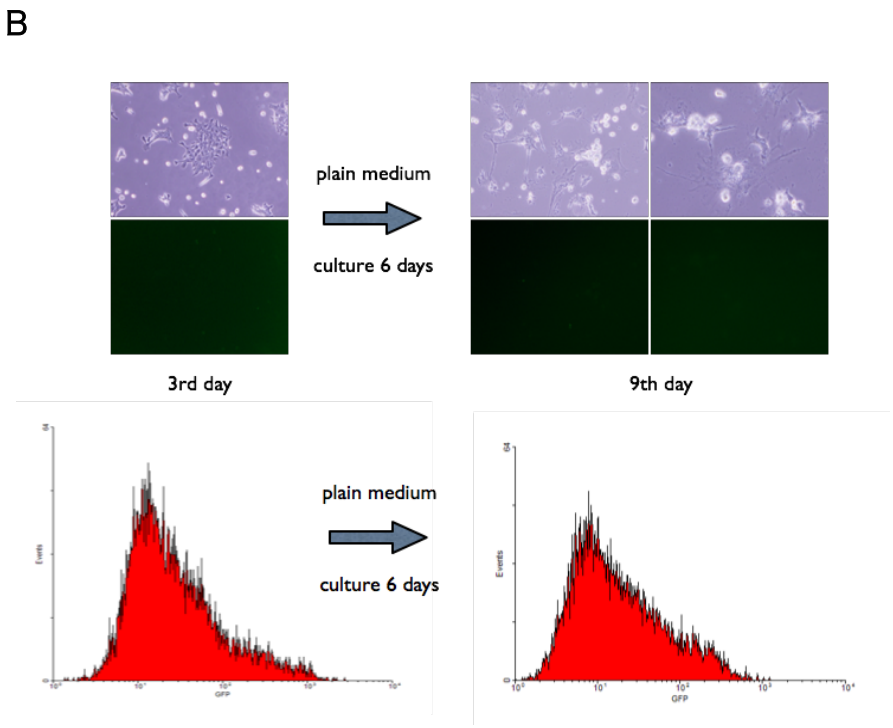


Figure 2.6 FACS analysis showing tEVs treatment of differentiating miPS cells gives rise to stem cells. (A) After 6 days tEVs treatment, GFP negative cells start to express GFP. (B) Instead of tEVs treatment, by plain medium culturing, the GFP expression completely lost.

2.3.3 tEVS culturing enhanced the proliferation of miPS cells

In the previous study, we cultured miPS cells for 4 weeks by LLC CM, whereby the cells transformed to CSC. In order to test whether the tEVs could affect this conversion, I cultured the differentiating miPS cells for 4 weeks by tEVs added

medium. A schematic overview of the conversion procedure is illustrated in Figure 2.3.

In the following two weeks without tEVS treatment, the number of surviving cells decreased in sequential passages, and the cells acquired an enlarged and flattened morphology, indicating normal differentiation of miPSCs (Fig. 2.7, -tEVs). While in the culture with tEVs treatment, GFP positive colonies re-appeared within a week and grew during the conversion period (Fig. 2.7, +tEVs). These colonies were intermixed with GFP negative cells, which were morphologically distinct from the normal differentiated cells. GFP negative cells were found surrounding GFP positive colonies, suggesting that these cells might have differentiated from GFP positive cells. Cell number was counted each passage, and the proliferation curve showed, the tEVs treated cells could proliferate, and cell number increased. The cell number, which cells were cultured in the plain medium, was firstly increased, and then decreased after 1 week (Fig. 2.8), maybe due to the apoptosis of the differentiated cells from miPS cells.

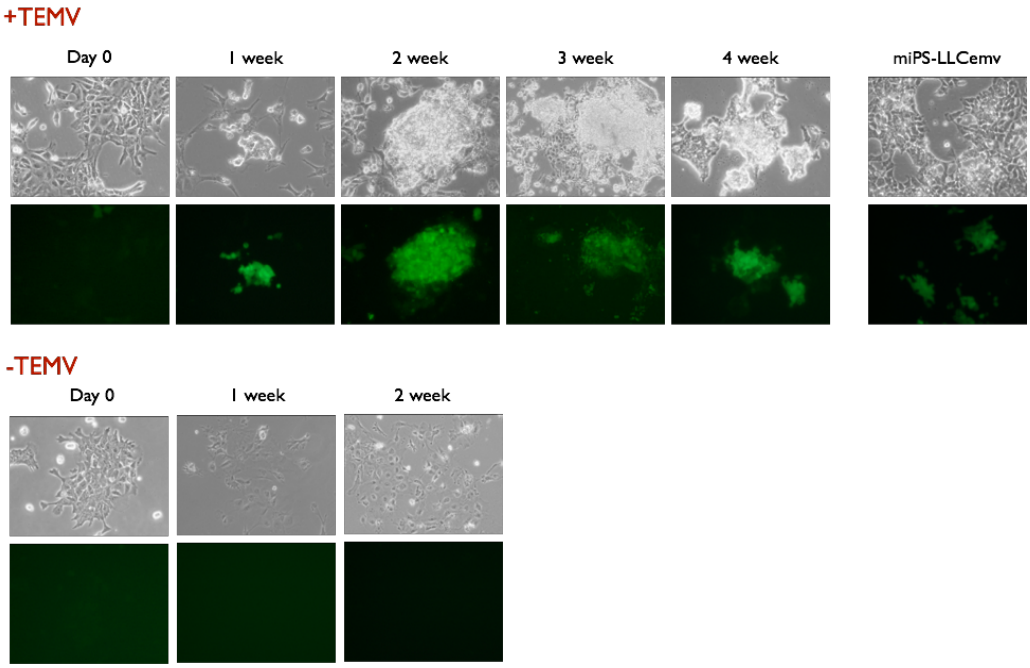


Figure 2.7 Cell images during conversion are shown. Cells passaged in plain medium (-tEVs) are used as control.

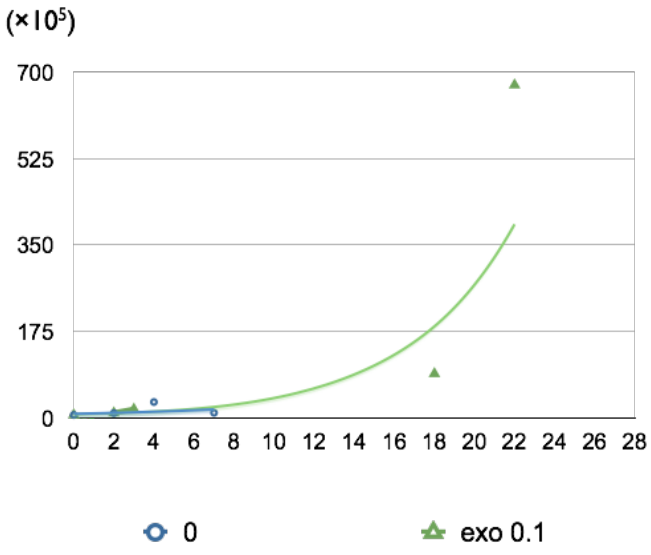


Figure 2.8 tEVs-treatment promotes the proliferation of cells. 5×10^5 cells cultured in plain medium increased in the first two passages, and then decreased after 1 week. By culturing with tEVs added medium, cells could proliferate after 1 week, and after 2 weeks, cells start to rapid increased.

2.3.4 Tumorigenesis of miPS-LLCev cells in immunodeficient mice

Tumorigenic potential is an established property for the assessment of CSC activity [1]. To test this, miPS-LLCev cells were subcutaneously injected into immunodeficient mice (Table 2.1). Tumors that developed in immunodeficient mice after 4 weeks were histologically and immunohistochemically analyzed (Fig. 2.9 A). The results showed that a substantial portion of the tumor expressed a malignant phenotype, such as high nuclear to cytoplasmic ratio, a high mitotic rate and nuclear pleomorphism (Fig. 2.9 B), and elevated expression of Ki-67 (Fig. 2.9 C), which are all properties indicative of a neoplastic state. I found anti-CD31 staining in the tumor, indicating tumor angiogenesis (Fig. 2.9 D). Additionally, during tumor development, I observed some animals with abdominal bleeding (Fig. 2.10 A), and bearing disseminated tumor nodules in the mesentery (Fig. 2.10 B).

Table 2.1 The tumorigenic potential of miPS-LLCev cells within one month.

Samples	No. of cells	Tumor formation	Histologic examination
miPS-LLCev	1 x 10^2	0/4	NA
	1 x 10^3	0/4	NA
	1 x 10^4	0/4	NA
	1 x 10^5	3/4	malignant, angiogenesis
	1 x 10^6	10/10*	malignant, angiogenesis, bleeding

NA: not applicable.

*There are 2 in 10 mice were found to be bleeding with disseminated tumors in the mesentery.

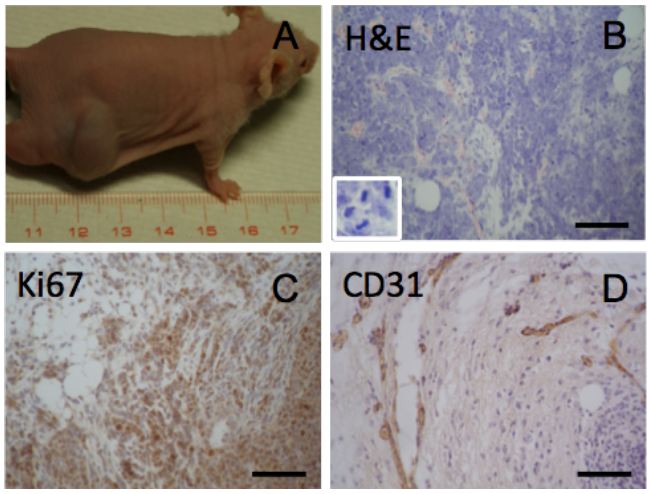


Figure 2.9 (A) Generation of tumors after subcutaneous injection shows rapid growth of tumors. (B) H&E staining of primary tumors dissected from injection site, showing multiple pathologic mitotic figures and hyper-vascularization. (C) IHC staining of Ki67 shows cellular proliferation. (D) IHC staining of CD31 shows angiogenesis in tumors. Scale bars: 100 nm.

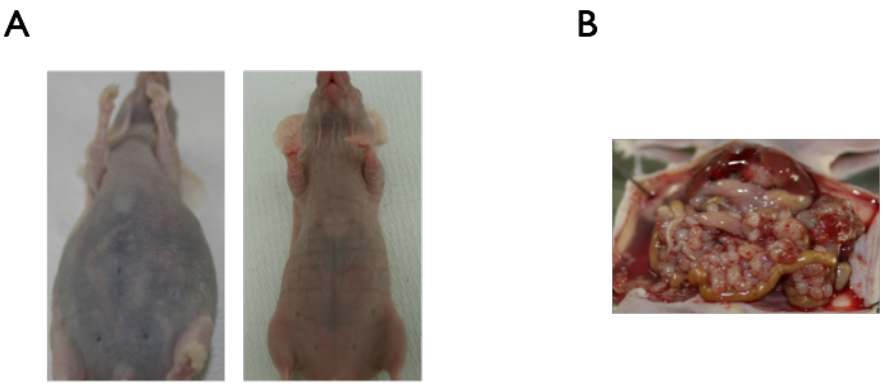


Figure 2.10 Disseminated tumors by injection of miPS-LLCev cells. (A) The disseminated tumors result in abdominal bleeding of mice. (B) The disseminated tumors on mesentery.

2.3.5 miPS-LLCev generated Liposarcoma in immunodeficient mice

I then assessed expression of vimentin and Pan-Cytokeratin for classifying the type of tumor. In the region of tumor where GFP expression was negative, vimentin staining was observed to be expressed in a diffuse pattern. Although a CK-positive area in the tumor was observed (data not shown), I concluded that the tumors were sarcomas. The GFP and vimentin staining patterns indicated the heterogeneity of cells in these sarcomas in the context of mesenchymal differentiation (Fig. 2.11 A&B).

It is worthwhile to note that there was a substantial proportion of adipose tissue in the tumors. I then investigated the expression of PPARγ2, which is a nuclear hormone

receptor that performs a critical role in regulating adipocyte differentiation [66]. Numerous cells were positive for PPARγ2, suggesting differentiation into an adipocyte lineage (Fig. 2.11 C). Furthermore, Oil Red O staining of cryosections revealed the presence of lipid droplets in the tumors (Fig. 2.11 D). Therefore, tumors derived from miPS-LLCev cells are probably liposarcoma [67, 68].

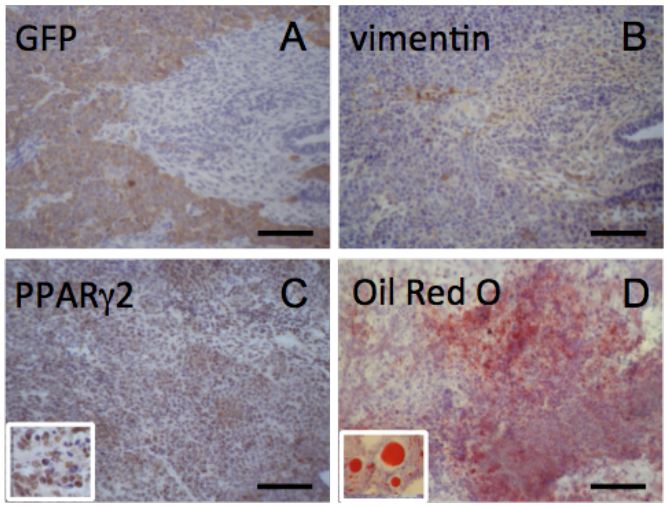


Figure 2.11 Serial sections were stained with vimentin (A) and GFP (B); diffuse staining of vimentin is detected with opposite pattern to GFP in tumor. (C) IHC staining of PPARγ2 shows a large population of PPARγ2 expressing cells. (D) Oil Red O staining of frozen section shows fat droplets in tumors. Scale bars: 100 μm.

2.3.6 The invasive capacity of miPS-LLCev cells

Although I subcutaneously transplanted miPS-LLCev cells into immunodeficient mice, the miPS-LLCevDT cells were established from tumor cells that had disseminated to the abdominal cavity in the mesentery (Fig. 2.10). This suggests that a subpopulation of cells might already exist in the transplanted miPS-LLCev cells that possess a high metastatic ability, which is one characteristic of CSCs [57]. To address this possibility, I examined the invasive capacity of miPS-LLCev, miPS-LLCevPT and miPS-LLCevDT cells *in vitro* (Fig. 2.12). Cells were seeded onto Matrigel-coated transwell membranes and after 30 h, cells that invaded to the lower side of the filter were counted. Compared to the parental miPS-LLCev cells, the invasive capacities of both the miPS-LLCevPT and miPS-LLCevDT cells were significantly higher. This result suggests that cells with higher invasive ability are enriched during *in vivo* tumor development and those cells can metastasize.

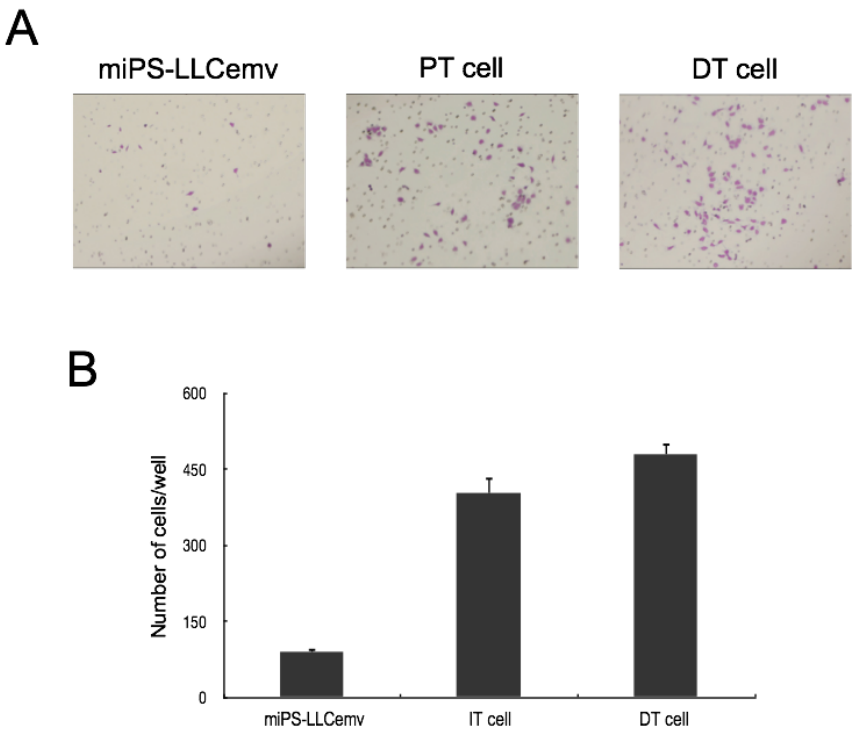


Figure 2.12 Matrigel invasion assay shows significantly higher invasive capacity of both primary cells than resultant cell. DT cell line is slightly more invasive than the PT cell line.

2.4 Discussion

Cells communicate through different mechanisms such as direct contact through cell membrane proteins or by soluble factors. However, evidences are accumulated that tEVs take a part of tumor environment. Recent study showed that colorectal cancer cells derived microvesicles are enriched of cell cycle-related mRNAs, and there is a significant increase in proliferation of endothelial cells treated with these

microvesicles [69]. Recipient cells can translate messages delivered by microvesicles, which derived from glioblastoma tumor cells, and these microvesicles also stimulated proliferation of a human glioma cell line [56]. tEVs are apparently affected differentiating cells in our experiments, since I started the tEVs treatment three days after removing LIF from miPSCs culture when GFP fluorescence were not observed. After 2 weeks culturing in plain medium, miPS cells differentiated and got a enlarged, flatten morphology, indicating the differentiation of normal stem cell. Compare to plain medium, the dose-dependent colony growth suggested tEVs effected in increasing resistance to apoptosis [47]. Fujimori et al. reported that the differentiation of ES cells in newborn bovine serum result in generation of cells with CSC properties [19]. They did not observe such effect in FBS or adult bovine serum. Their results suggest that aberrant conditions overcome 'anti-cancer barrier' such as p53 pathway in differentiating stem cells. Alternatively, 'anti-cancer barrier' should be induced in proper condition. It has been reported that tumor cells could inhibit p53 induction in adjacent fibroblasts [70]. Although I cannot rule out the possibility that tEVs affect directly to a small part of stem cell remains in the culture after three days differentiation, it is possible that tEVs act against 'anti-cancer barrier' [54]. In this study, without tEVs, the differentiated miPS cells could not survive and got the typical morphology of cellular senescence; whereas the tEV treated cells generated the malignant tumors, suggesting the potential role of tEVs by packaging onco-molecules.

I observed the disseminated tumors on the mesentery of intestines in some of the mice, which bearing miPS-LLCev cells, indicating the metastasis capacity of this cell line. There are a number of evidences showing the tEVs can activate cells to a more metastatic phenotype. The exosomes derived from melanomas could locate to sentinel lymph nodes *in vivo*, and recruit melanoma cells to the present positions, which

contribute to melanoma metastasis [51]. The microvesicles secreted by both human and murine lung cancer cells activated and chemoattracted stromal fibroblasts and endothelial cells, and the activated cells, in turn, enhanced the metastatic potential of lung cancer cells [59]. Microvesicles from tumor cells promoted MMP-9 expression and ERK1/2 phosphorylation in stimulated fibroblasts, induced the migration of fibroblasts, and in turn, the activated cells promoted the invasion of cancer cells [47]. All the data suggested the tEVs could act as metastatic niche, educating the non- or less-metastatic cells to a more malignant phenotype. Although this enhance effect of migration has not been observed in the transplantation of miPS-LLCcm cells, which established by culturing with conditioned medium of LLC cells. As we know, there are diverse secreted factors from cancer cells in the conditioned medium besides tEVs, contributed to the cancerous environment force the malignant conversion of miPS cells. It must be a complex cooperation between the different mechanisms of oncogenic progression according to the distinct element. Thus, other major pathways may modify the functional activity of tEVs with respect to the metastatic promotion.

Liposarcomas are the most common type of soft tissue sarcomas and are classified into five major groups, well-differentiated liposarcoma, dedifferentiated liposarcoma, myxoid liposarcoma, pleomorphilic liposarcoma, and mixed-type liposarcoma [67]. Pathological features of the miPS-LLCev tumors suggested that the tumors derived from miPS-LLCev cells were dedifferentiated liposarcomas. The origin of CSCs is not yet elucidated, however, the variety of CSCs may indicate the different manner of the CSCs arise. One of the possibility is that normal stem cell underwent tumorigenic transition could act as the resource of the tumor cells. Another possibility may ascribe to the progenitor cells in the normal tissue. When the partially differentiated cells suffer from oncogenic transformation, they acquire more-stem like features [71]. Once the conversion of CSCs achieved, they can perform self-renewal as well as

differentiate to a heterogeneous hierarchy of cell population and form the bulk of tumor mass. Increasing studies are focusing on the role of epithelial to mesenchymal transition (EMT), which was firstly recognized as a process of morphogenesis during embryonic development. Most of cancers acquired mesenchymal phenotype during the progression, assumed the loss of epithelial marker expression and the increasing of mesenchymal markers, resulting in the dissemble of cell-cell junction and invasion of tumor cells [72]. Recent studies showed the EMT is related to the acquisition of CSC properties [73, 74]. $CD44^{+}/CD24^{-/low}$ cells showed the loss of E-cadherin expression and gain of vimentin expression [75]. Additionally, it was shown that expression of twist or snail, which is transcription repressors of E-cadherin, induced EMT phenotype in the non-tumorigenic, immortalized human mammary epithelial cells. The resultant cells displayed the $CD44^{high}/CD24^{low}$ expression pattern and enhanced the mammoshpere formation as well as tumorigenicity [73].

CHAPTER 3

Tumor derived extracellular vesicles treated differentiating miPS cells are cancer stem cells

Abstract

In light of cancer stem cell hypothesis, a subset of cells, which possesses stem cell properties, has been found in several kinds of tumors. According to this hypothesis, in solid tumor, only the small part of stem-like cells could produce all kind of cells necessary to repopulate a tumor. In the current study, by culturing the differentiating miPS cells with tEVs derived from LLC cell line, the cells could proliferate and, interestingly, started to express GFP, which is a signal of Nanog expression in the iPS cells used here. In order to confirm the cancer initiating capacity, I analyzed the stem cell properties and the tumorigenesis capacity of the tEV treated cells (miPS-LLCev). Immunoblotting analysis showed the cells started to expressed Nanog, Oct3/4 and CD133 proteins during tEV treatment. In suspension culture, both the bulk cells and puromycin selected cells formed spheroids in the serum free medium, indicating the self-renew capacity of the cells. Quantitative RT-PCR analyses showed the embryonic stem cell markers, such as Oct3/4 and c-Myc, are expressed comparably to miPS cells; Sox2 and Klf4 are significantly higher than the parental miPS cells. When the miPS-LLCev cells were subcutaneously allografted into Balb/c nude mice, malignant liposarcomas with extensive angiogenesis developed. Thus, I concluded tEVs derived from LLC cell line could contribute to cancerous niche, where the miPS cells acclimated and got malignant transformation.

Key words: cancer stem cells, self-renewal, heterogeneity and differentiation

3.1 Introduction

Since the cancer stem cell (CSC) hypothesis was raised, it acquired intensive studies and discussions for decades, and the CSCs of special tumor tissues have been identified in most kind of tumors. Increased evidences suggested the existence of the rare cells in the solid tumor, which could initiate tumor mass in the body. In glioblastoma multiforme, which is the most common adult primary brain tumor, CSCs can be identified. These CSCs form neurospheres, possess the capacity for self-renewal, express genes associated with neural stem cells, generate daughter cells of different phenotypes from one mother cell, and differentiate into the phenotypically diverse populations of cells similar to those present in the initial tumor mass [15].

There are several approaches to identify the CSCs from unique tumor; however, various surface markers are the most using one. By CD133 expression, CSCs are identified in human pancreatic tumor [76], Ewing's sarcoma [10], lung cancer [11], liver cancer [13]; they are exclusively tumorigenic and highly resistant to standard chemotherapy. By using the sphere formation, the cells possess self-renewal capacity were enriched from glioblastoma multiforme [15]. Breast cancer stem cells are usually identified as a subpopulation of $CD44^{+}/CD24^{-/low}$ [77]. Combining with CD44 and CD133 expression, the CSC was identified from prostate tumor [78]. Although studies showed CD44v6, a splice variant of CD44, is more reliable to identify a CSC population [79, 80]. Aldehyde dehydrogenase 1(ALDH1), which has a role in early differentiation of stem cell [81], has been used as a useful marker for some CSCs, such as breast cancer [82], colon cancer [83], melanoma [84], and involved in protecting CSCs from chemotherapy [85]. However, there is not a pan-marker for all kinds of CSCs, even by using of combined markers, because normal stem cells and

CSCs are usually sharing signaling pathways for maintaining themselves [86], the more efficient and reliable approaches for CSC identification are required for both the clinical and research fields.

CSCs should be a subpopulation of cancer cells, which can maintain themselves and generate a heterogeneous population of cells to drive the tumor progression [2]. Self-renewal is the key feature of CSC, which is deemed to be the core reason for the cancer spread and relapse. Currently, by transplanting primary tumor cells to immunodeficient mice to generate the similar malignant tumor population with parental tumor is a powerful strategy to identify the self-renewal capacity [87, 88]. However, the experimental approaches limited the investigations of the cellular self-renewal *in vitro*. As the sphere formation in two-dimensional or three-dimensional adherent cultures on matrix and the non-adherent culture condition are typically used for normal stem cells [89, 90], culture conditions for identifying the self-renewing CSCs are frequently reported. A small subset of brain tumor cells is clonogenic in vitro as neurophere [91, 92], and the individual cell from the formed spheres is able to generate a new spheroid. This assay is performed for assessing the self-renewal capacity *in vitro* at a proper cell density, which is very important to evaluate the successful sphere formation or a cell aggregation. The widely accepted proper condition is 0.2 to 20 cells per μL [93], and the high or low concentration will cause fusion of spheres or other problems [90].

The multi-potential differentiation capacity is another critical property to distinguish the CSCs from other cells, and it should estimate on the adhesive substrate after the primary sphere formation [90]. Once, the cancer has been deemed to a homogeneous population, and the therapeutic treatment targeted to the rapid proliferating cells [94]. So far, several studies have shown CSCs can differentiate into a large variety of lineage cell types to form the hierarchical heterogeneity [1]. Since

the miPS-LLCcm cells, which converted by LLC conditioned medium, possess all the features of CSCs. And tEVs treated miPS cells, which termed miPS-LLCev cells, generated malignant tumors in the immunodeficient mice. I estimated the characteristics of miPS-LLCev according to the CSC properties, in order to explicit the tEVs can contribute to the cancerous niche to originate CSCs or not.

3.2 Materials and Methods

3.2.1 Cell culture

miPS-LLCev cells were maintained under the humidified 5% CO_2 atmosphere at 37˚C on feeder layers of mitomycin-C-treated mouse embryonic fibroblasts (MEFs) (Reprocell, Japan) in miPS medium (Dulbecco's Modified Eagle Medium (DMEM) containing 15% fetal bovine serum (FBS), 0.1 mM Non-Essential Amino Acid (NEAA, Life Technologies), 2 mM L-Glutamine, 0.1 mM 2-mercaptoethanol, 50 U/mL penicillin and 50 U/mL streptomycin). Differentiated cells were removed by culturing in the presence of 1 μg/mL puromycin (puro).

For suspension culture, 4×10^4 single cells were plated in 60-mm Lipidure®-coated low adhesion dishes (NOF Corporation, Japan) in serum-free miPS medium without LIF, but supplemented with Insulin-Transferrin-Selenium-X (ITS-X, Life Technologies). Spheres with diameters above 100 μm were counted under a fluorescence microscope (Olympus IX81) on day 4. After image capturing on day 7, spheres were collected for further analyses.

3.2.2 Immunoblotting

Thirty-five micrograms of total cellular protein of each sample was loaded for electrophoresis. Separated proteins were then blotted onto polyvinylidene difluoride (PVDF) membrane (Millipore). After blocking for 1 h with 5% skim milk, the membranes were incubated overnight at 4˚C with primary antibodies: rabbit polyclonal anti-Nanog antibody (1:3000, Abcam), mouse monoclonal anti-Oct3/4 antibody (1:3000, Santa Cruz) or rabbit polyclonal anti-CD133 antibody (1:1000, Abnova). The blots were then incubated with secondary antibody, either horseradish peroxidase (HRP)–conjugated anti-rabbit or anti-mouse IgG (Cell Signaling). The bound antibodies were detected using Western Lighting plus-ECL reagent (PerkinElmer), and recorded by Light-Capture II cooled CCD camera system (ATTO, Japan). Coomassie blue staining of SDS-PAGE gels or anti-β-actin (Cell Signaling) immunoblotting were used as controls for equal loading.

3.2.3 Reverse transcription polymerase chain reaction (RT-PCR) and real time PCR

miPS cells, miPS-LLCev bulk cells and miPS-LLCev spheroids were harvested and total RNA was extracted with RNeasy kit (Qiagen, MD). To remove any residual genomic DNA, total RNA was treated with DNase I (Takara, Japan). Then three micrograms of treated RNA were reverse transcribed by SuperScript III Reverse Transcriptase (Invitrogen) primed by oligo-dT18. Real time PCR was performed with LightCycler 480 SYBR Green I Master mix (Roche) in a reaction volume of 20 μL. The sequences of primers used here are listed in Table 3.1. Relative gene expression was normalized to that of Gapdh.

Table 3.1. Primer sequences for qRT-PCR.

No.	Symbol	Accession	Primers	Applications
1	Oct3/4	NM_013633.2	CTG AGG GCC AGG CAG GAG CAC GAG	Total *Oct3/4*
			CTG TAG GGA GGG CTT CGG GCA CTT	
2	Oct3/4	NM_013633.2	TCT TTC CAC CAG GCC CCC GGC TC	Endogenous *Oct3/4*
			TGC GGG CGG ACA TGG GGA GAT CC	
3	Sox2	NM_011443.3	GGT TAC CTC TTC CTC CCA CTC CAG	Total *Sox2*
			TCA CAT GTG CGA CAG GGG CAG	
4	Sox2	NM_011443.3	TAG AGC TAG ACT CCG GGC GAT GA	Endogenous *Sox2*
			TTG CCT TAA ACA AGA CCA CGA AA	
5	Klf4	NM_010637.3	CAC CAT GGA CCC GGG CGT GGC TGC CAG AAA	Total *Klf4*
			TTA GGC TGT TCT TTT CCG GGG CCA CGA	
6	Klf4*	NM_010637.3	GCG AAC TCA CAC AGG CGA GAA ACC	Endogenous *Klf4*
			TCG CTT CCT CTT CCT CCG ACA CA	
7	c-Myc	NM_010849.4	CAG AGG AGG AAC GAG CTG AAG CGC	Total *c-Myc*
			TTA TGC ACC AGA GTT TCG AAG CTG TTC G	
8	c-Myc	NM_010849.4	TGA CCT AAC TCG AGG AGG AGC TGG AAT C	Endogenous *c-Myc*
			AAG TTT GAG GCA GTT AAA ATT ATG GCT GAA GC	
9	Gapdh*	NM_008084.2	AAC GGC ACA GTC AAG GCC GA	*Gapdh*
			ACC CTT TTG GCT CCA CCC TT	
10	Oct3/4		TTG GGC TAG AGA AGG ATG TGG TTC	*Oct3/4* transgene
			TTA TCG TCG ACC ACT GTG CTG CTG	
11	Sox2		GGT TAC CTC TTC CTC CCA CTC CAG	*Sox2* transgene
			TTA TCG TCG ACC ACT GTG CTG CTG	
12	Klf4		GCG AAC TCA CAC AGG CGA GAA ACC	*Klf4* transgene
			TTA TCG TCG ACC ACT GTG CTG CTG	
13	c-Myc		CAG AGG AGG AAC GAG CTG AAG CGC	*c-Myc* transgene
			TTA TCG TCG ACC ACT GTG CTG CTG	

* These two primers were designed in this study; remains are followed Yamanaka's description [64].

3.2.4 Flow cytometry analysis

To evaluate GFP expression during tEVs conversion, 1×10^6 adherent cells of each sample were dissociated and collected in 100 μL PBS, then analyzed on a FACS Calibur flow cytometer (Becton Dickinson).

3.2.5 Tumorigenicity analysis in immunodeficient mice

4 week-old Balb/c-nu/nu female immunodeficient mice were purchased from Charles river (Japan) and housed in specific pathogen free facility at 20˚C with a 12-hour light/12-hour dark cycle. miPS-LLCev cells (shown in Table 3.2) were suspended in sterile PBS and subcutaneously injected into one flank of immunodeficient mice. After 4 weeks, all tumors were resected at autopsy and sectioned for histologic analysis. After experiments, mice were anaesthetized and sacrificed by exposure to diethyl ether vapors. All animal experiments were reviewed and approved by the ethics committee for animal experiments of Okayama University under the ID OKU-2013252.

3.2.6 Histologic analysis and immunohistochemistry (IHC)

Tumors dissected from mice were fixed for 24 hours in 4% PFA and dehydrated by gradient ethanol, then 4 mm thick sections processed using a routine wax-embedding procedure for histologic examination, followed by staining with hematoxylin and eosin (HE).

IHC staining were performed by using rat monoclonal anti-CD31 antibody (1:200, Santa Cruz), rabbit polyclonal anti-Ki67 antibody (1:200, Abcam), rabbit monoclonal anti-GFP antibody (1:300, Cell Signaling), mouse monoclonal anti-vimentin antibody (1:100, Santa Cruz), or mouse monoclonal anti-PPARγ2 antibody (1:200, Santa Cruz). Briefly, 4 mm tissue sections were deparaffinized and antigen retrieved was performed using microwave exposure at 95 ˚C for 5 minutes in a citrate buffer (pH 6.0). After 3% hydrogen peroxide blocking for 10 min and normal serum blocking for 1 h, the sections were then incubated in 4 ˚C for 12 h. The sections were then incubated with biotinylated anti-rabbit, biotinylated anti-rat or biotinylated anti-mouse secondary antibodies (Vector, USA), followed by incubation with the ABC reagent (Vector, USA). Detection was accomplished using 3, 3'-diaminobenzidine tetrahydrochloride (DAB, Vector, USA). Negative controls were performed by ncubation of sections with phosphate-buffered saline (PBS) instead of primary antibodies. Counter staining was carried out using hematoxylin.

3.2.7 Immunofluorescence

For immunofluorescence analysis, cells were deposited on coverslips, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton-X, blocked with 5% BSA, incubated with rabbit polyclonal anti-CD34 antibody (1:200, Santa Cruz), rabbit polyclonal anti-CD36 antibody (1:200, Santa Cruz), mouse monoclonal anti-PPARγ2 antibody and TexasRed-conjugated secondary antibodies and then counterstained with DAPI (Vector Laboratories). Images were captured using a confocal microscope equipped with a light fluorescence device (LSM510META, Carl Zeiss, Germany).

3.2.8 Adipose differentiation and Oil Red O staining

For adipocyte differentiation, spheroids of tumor cells were directly seeded under adherent conditions in miPS medium (without LIF). After growing to 90% confluence, the medium was changed to adipose differentiation medium (miPS medium (without LIF), supplemented with 10 μg/mL insulin and 2.5 μM dexamethasone (AdipoInducer, Reagent Takara, Japan)) for 2 days. Subsequently, the cells were maintained in miPS medium (without LIF) supplemented with 10 μg/mL insulin for another 6 days, followed by Oil Red O staining to confirm the adipogenesis.

For Oil Red O staining, 10 mm cryosections were washed twice with PBS and fixed by neutral buffered 10% formalin for 30 min at room temperature. Then cells were rinsed in 60% isopropanol and stained with Oil Red O (Sigma) at 60˚C for 5 min. After rinse in tap water, images were captured using an Olympus IX81 microscope (Olympus, Japan).

3.2.9 In vitro tube formation assay

Individual cells were suspended in endothelial basal medium supplemented with FBS, hydrocortisone, hFGF-B, VEGF, R^3-IGF-1, ascorbic acid, hEGF, GA-1000 and heparin (EGM-2 Single Quots Kit, Takara) following manufacture's instruction and seeded in triplicate on Matrigel (Becton Dickinson) coated chamber slides (Nunc) [19]. After 24 hours, cells were stained with fluorescence labeled rat anti-CD31 antibody.

3.3 Results

3.3.1 tEV treatment caused the expression of stemness-related proteins

The re-emergence of GFP positive cells in the conversion medium suggested the acquisition of an embryonic stem (ES)-like phenotype in these cells, since the GFP gene was located downstream of the *Nanog* promoter that is activated in ES cells and aggressive tumors [64, 95]. I confirmed the increase in expression of Nanog, Oct3/4 and CD133 by immunoblotting analysis during this transition phase (Fig. 3.1). The protein levels of Oct3/4 first decreased and then gradually recovered to levels that were comparable to the levels in miPSCs following treatment with tEVs. In contrast, the levels of Nanog protein in the tEVs-treated miPSCs (miPS-LLCev) were significantly higher than those in parental miPSCs. These results suggested that treatment with tEVs derived from LLC cells could give rise to a stem-like population from differentiating miPSCs by interfering with a normal differentiation program in these cells. This reprograming process might be important in the re-acquisition of a more embryonic stem-like phenotype. However, we cannot exclude the possibility that tEVs may directly maintain the undifferentiated state of miPSCs even though these cells are exposed to a differentiation competent environment [54].

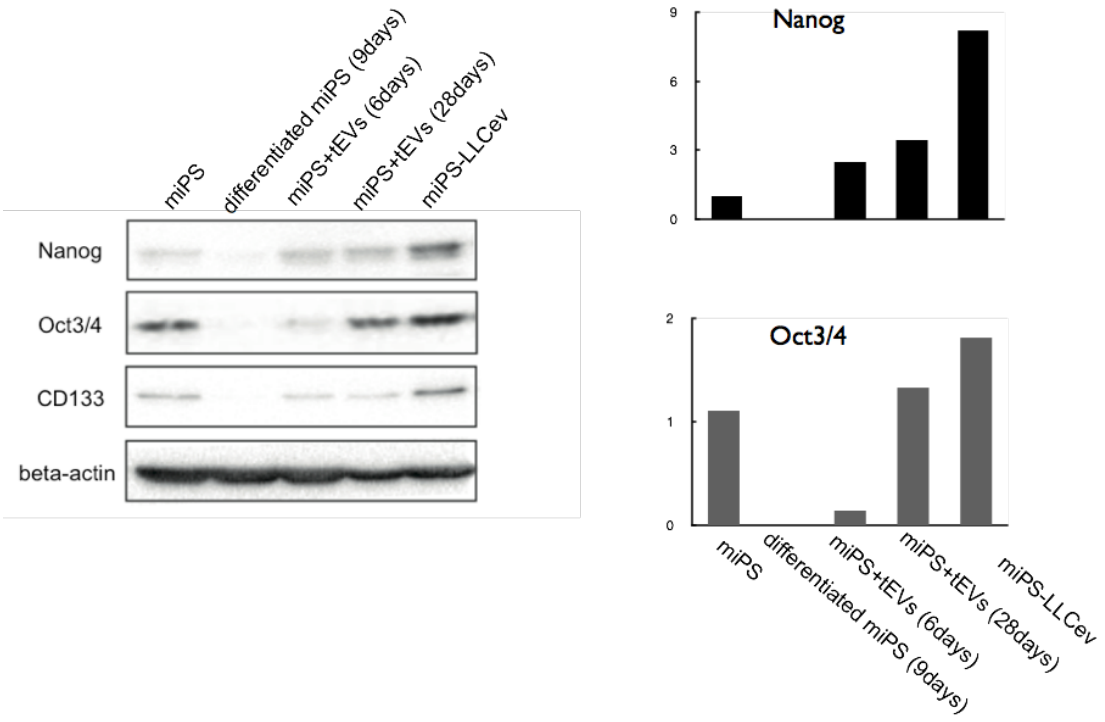


Figure 3.1 Immunoblotting analysis of Nanog, Oct3/4 and CD133 in the total protein from miPSCs (miPS), differentiated miPSCs (differentiated miPS (9days)), differentiated cells by tEVs cultured for 6 days (miPS + tEV (6days)), differentiated cells by tEVs cultured for 28 days (miPS + tEV (28days)) and resultant cells (miPS-LLCev). Relative intensities are normalized to that of β-actin.

3.3.2 miPS-LLCev cells possess self-renewal capacity

Considering the results of our previous study [20], we expected that secreted tEVs was one of the responsible factors that might contribute to the conversion of miPSCs into CSCs. Additionally, by tEV treatment, GFP colonies re-appeared in the

differentiating miPS cells, and the cells started to express stemness related proteins. Thus, I investigated the CSC properties of the miPS-LLCev cells. The self-renewal capacity of miPS-LLCev cells was assessed in the non-adherent condition, since malignant stem-like cells have been shown to form spheroids indicating self-renewal capacity in suspension culture [9]. Approximately 12% of the bulk-unselected populations in miPS-LLCev cells expressed GFP (Fig. 3.2 A). The large population of low GFP expressing miPS-LLCev cells prompted us to concentrate on GFP positive cells as puro resistant cells (Fig. 3.2 B). I assessed the puro resistant GFP positive cells for growth in suspension culture, and found that the GFP positive population could form spheroids. All of the resultant spheroids were GFP positive, demonstrating self-renewal capacity of the GFP positive cells (Fig. 3.3). To our surprise, the bulk population cells of miPS-LLCev that were not selected for puro resistance also formed spheroids, and only 17.8% of spheroids were expressing GFP (Fig. 3.3 and 3.4).

To further evaluate the properties of miPS-LLCev cells as potential CSCs, I established cell lines from both the primary tumors (miPS-LLCevPT) and disseminated tumor nodules (miPS-LLCevDT), respectively (Fig. 3.5). In contrast to the parental miPS-LLCev cells *in vitro*, the populations of GFP positive cells in both the miPS-LLCevPT and miPS-LLCevDT cell lines was significantly higher (Fig. 3.6 A and B). In suspension culture, both cell lines were able to form spheroids indicating self-renewal capacity (Fig. 3.7). Different from the parental cell, miPS-LLCev, all the spheroids from these two cell lines were GFP positive (Fig. 3.8).

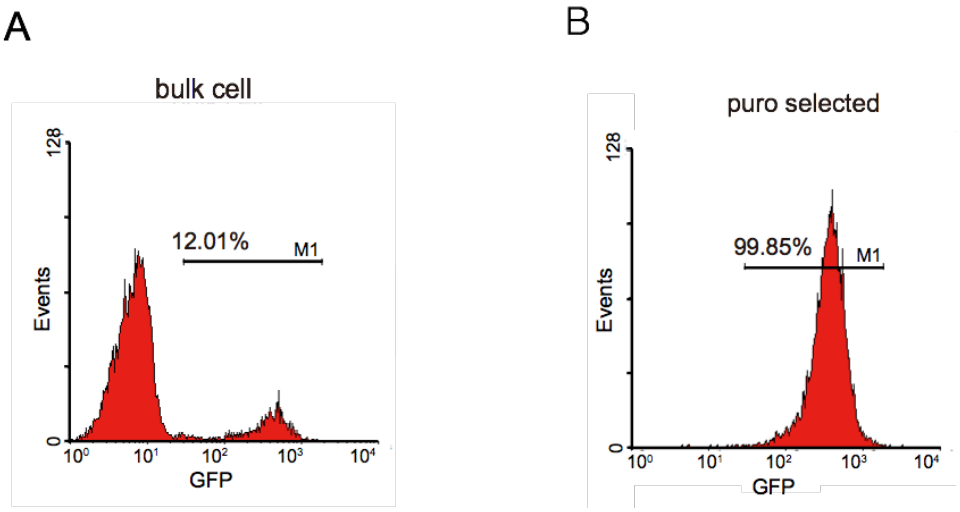


Figure 3.2 FACS analysis is showing GFP population in bulk (A) and puro selected (B) miPS-LLCev cells.

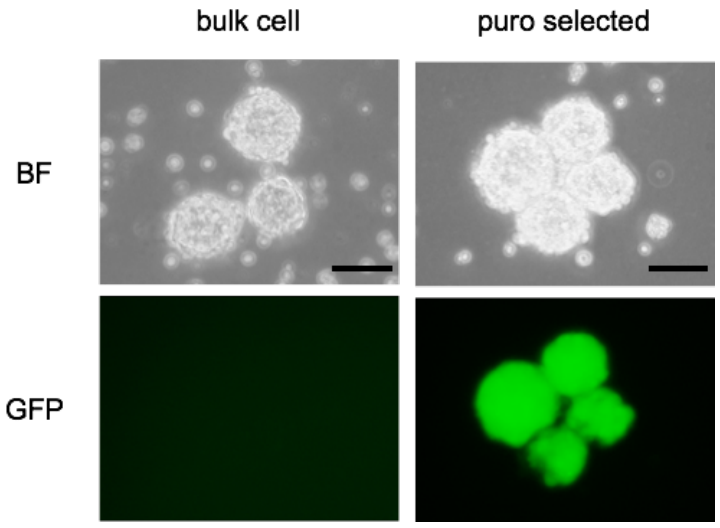


Figure 3.3 Sphere formation assay in serum-free medium shows spherogenic

potential of both GFP negative (bulk cell) and GFP positive (puro selected) miPS-LLCev cells. Scale bars: 100 μm.

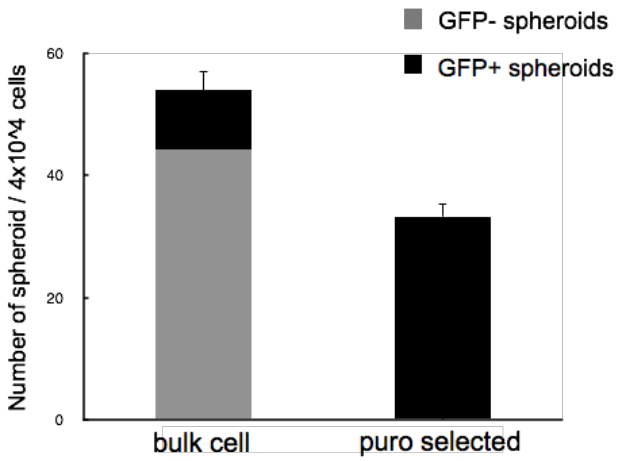


Figure 3.4 4×10^4 bulk or puro selected single cell suspension were applied to serum-free condition, the number of GFP^+ and GFP^- spheroids above 100 μm were scored after 4 days. The assay was performed in triplicate and the error bars indicate the difference in the total number.

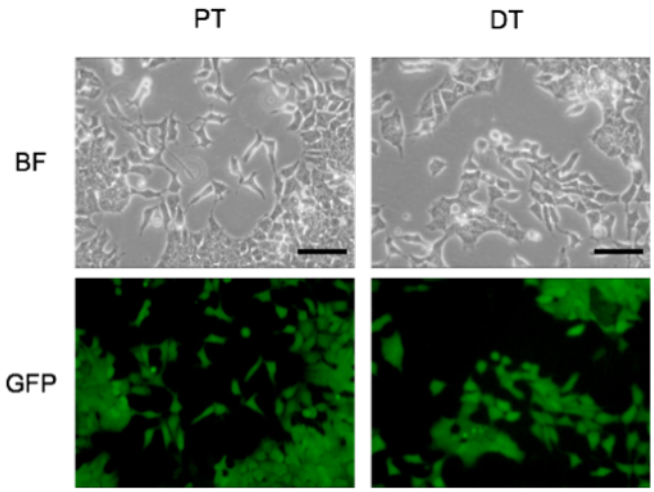


Figure 3.5 Cell morphologies of miPS-LLCevPT and miPS-LLCevDT cell lines, which were established from primary tumor and disseminated tumor nodules, respectively.

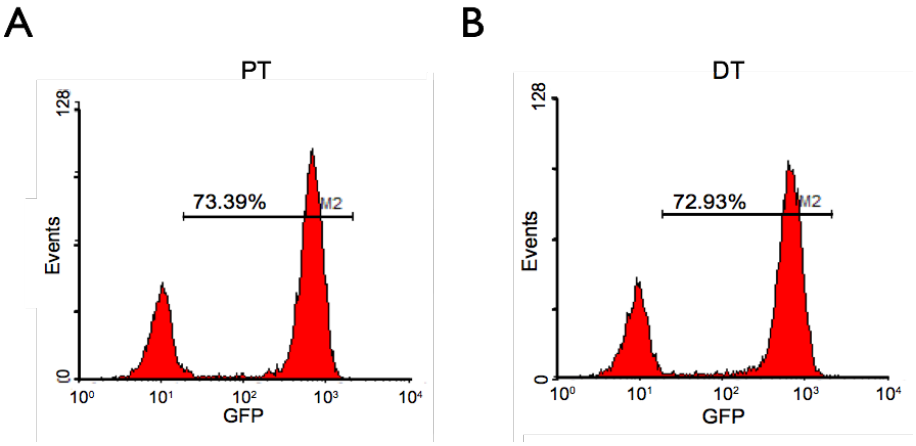


Figure 3.6 FACS analysis of GFP in both primary cell lines shows GFP populations are similar.

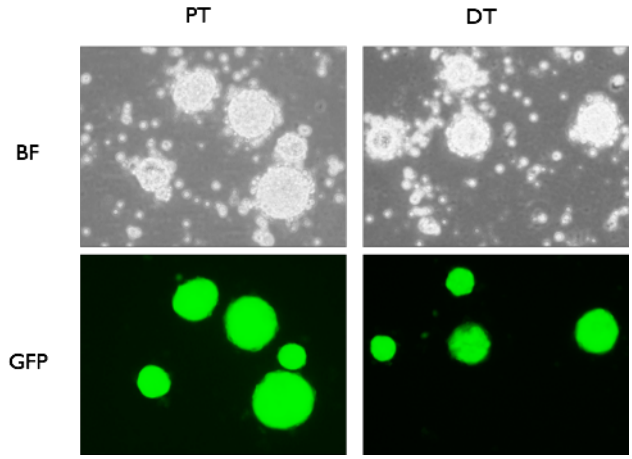


Figure 3.7 Both of the primary bulk cells are able to form spheroids in suspension culture, and all the spheroids are GFP positive.

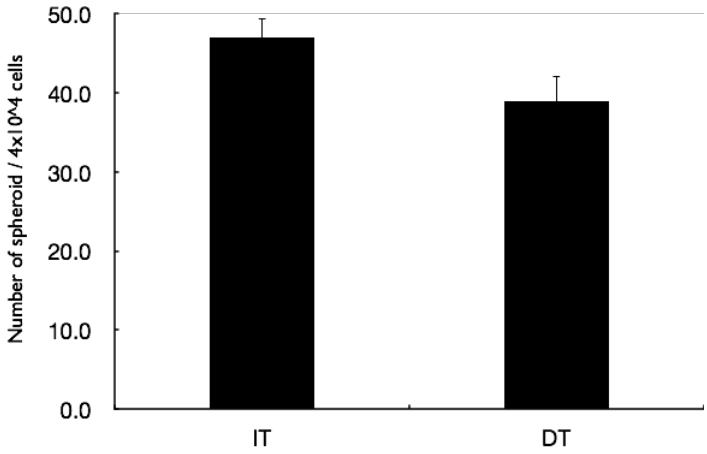


Figure 3.8 The number of spheroids (above 100 μm) from 4×10^4 bulk primary cells. Assays were performed in triplicate and the error bars indicate the difference in the total number.

3.3.3 ES cell marker expression in miPS-LLCev cells

By qRT-PCR analysis, I assessed the expression of genes, such as *Oct3/4*, *Sox2*, *Klf4* and *c-Myc*, which perform a dominant role in ES and iPSC maintenance, self-renewal and reprograming. The level of each endogeous genes and total mRNAs (endogenous + transgene) expression were confirmed by using specific primers [96]. We did not detect any aberrant trensgene activation, pertinent to viral-transduction for the establishment of miPSCs (Fig. 3.9). In spheroids derived from the bulk of miPS-LLCev cells, *Sox2* and *Klf4* expression were significantly upregulated, GFP positive spheroids expressed higher levels of *Sox2* and *Klf4* compared with miPSCs, but less than those of bulk spheroids. *Oct3/4* was found to be highly expressed especially in the GFP positive spheroid cells, implicating a potentially more stem-like state compared with GFP negative spheroids (Fig. 3.10). These results indicate the potential presence of a subpopulation that possesses self-renewal capacity in the total miPS-LLCev cell population. Additionally, the results suggest that the bulk miPS-LLCev cell population contains a hierarchy *in vitro* ranging from $Nanog^{+}(GFP^{+})/Oct3/4^{+}$ stem-like cells to more differentiated cells. The upregulation of *Sox2* and *Klf4* might be related not only to the maintenance of the stem cell-like stage, but also to differentiation stage.

Moreover, RT-PCR analysis showed, the expression levels of *Oct3/4* in miPS-LLCevPT (Fig. 3.11 A&B) and miPS-LLCevDT (Fig. 3.11 C&D), both in adherent and suspension culture were comparable to those in miPSCs. In contrast, significant higher levels of *Sox2* and *Klf4* expression were observed in the spheroids from both cell lines. We confirmed that there was no aberrant expression of the Yamanaka transgenic factors (Fig. 3.9) in either cell line. *c-Myc* was not expressed aberrantly in any of the cells suggesting a negligible contribution of this gene to the

transformation and/or tumorigenicity in these cells. However, endogenous *Nanog*, *Sox2* and *Klf4* might contribute to these properties [95, 97-99]. In a word, the expression pattern of Yamanaka factors in miPS-LLCev, miPS-LLCevPT and miPS-LLCevDT cells are similar, indicating the cells kept the similar characteristics after injection.

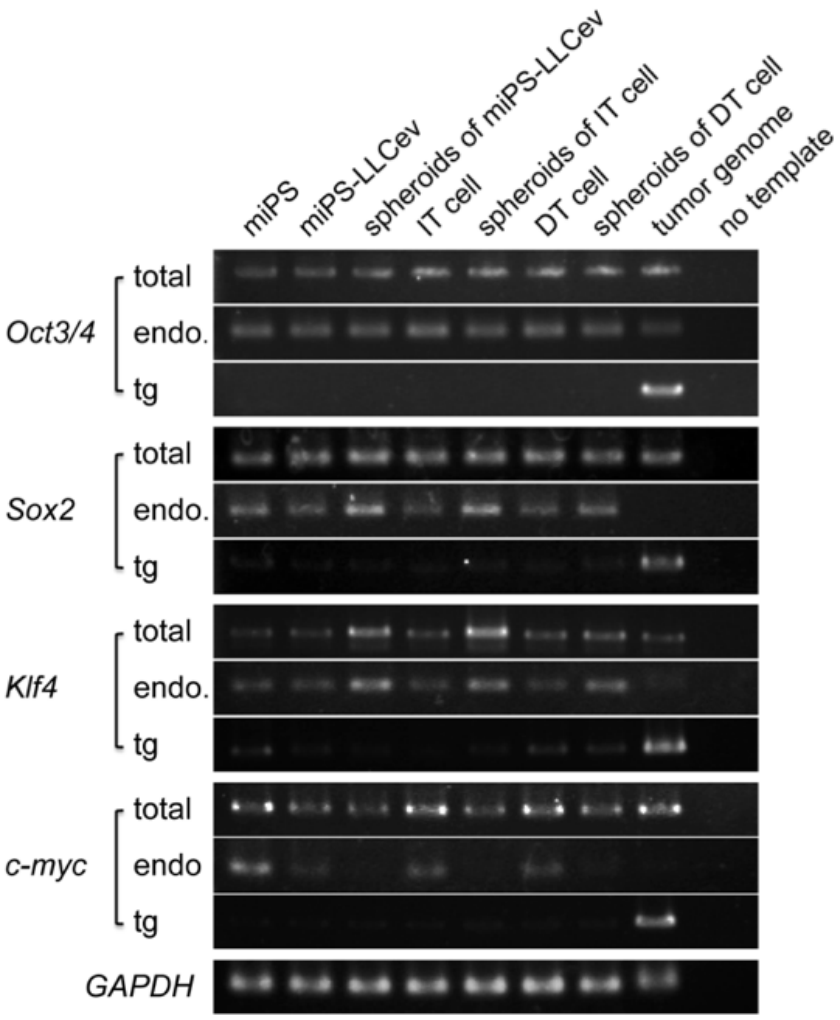


Figure 3.9 Quantitative reverse-transcription PCR analysis of the four transcription factors in indicated samples. The PCR products were the coding regions (Total), endogenous transcripts only (Endo.), and transgene transcripts only (tg). Genome DNA was used as positive control for transgene.

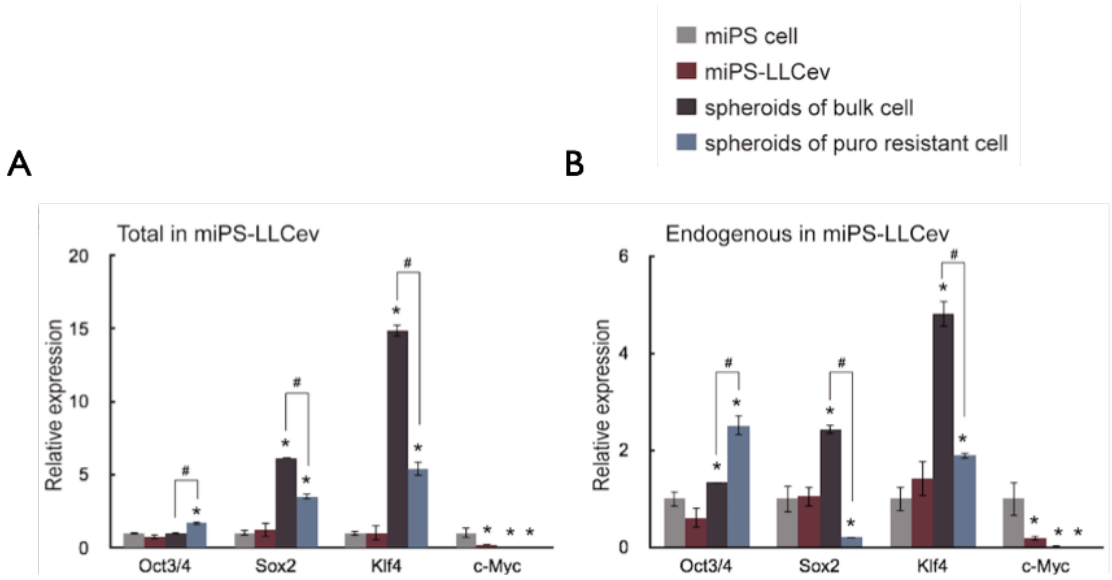


Figure 3.10 qRT-PCR analyses of four transcription factors *Oct3/4*, *Sox2*, *Klf4* and *c-Myc* expression levels in the indicated samples of miPS-LLCev cells. The products of coding regions and endogenous transcripts are regarded as total (A) and endogenous (B). Relative expression values are normalized to *Gapdh* and conducted in triplicate. Student t-test was used to compare the expression level between unique sample and miPSCs. Each asterisk shows the significance of P <0.05. One-way ANOVA followed by post hoc Tukey analysis was used to assess the significance of the genes expression between miPS-LLCev, 'spheroids of bulk cell' and 'spheroids of puro resistant cell'. Each '#' shows the significance of P <0.05.

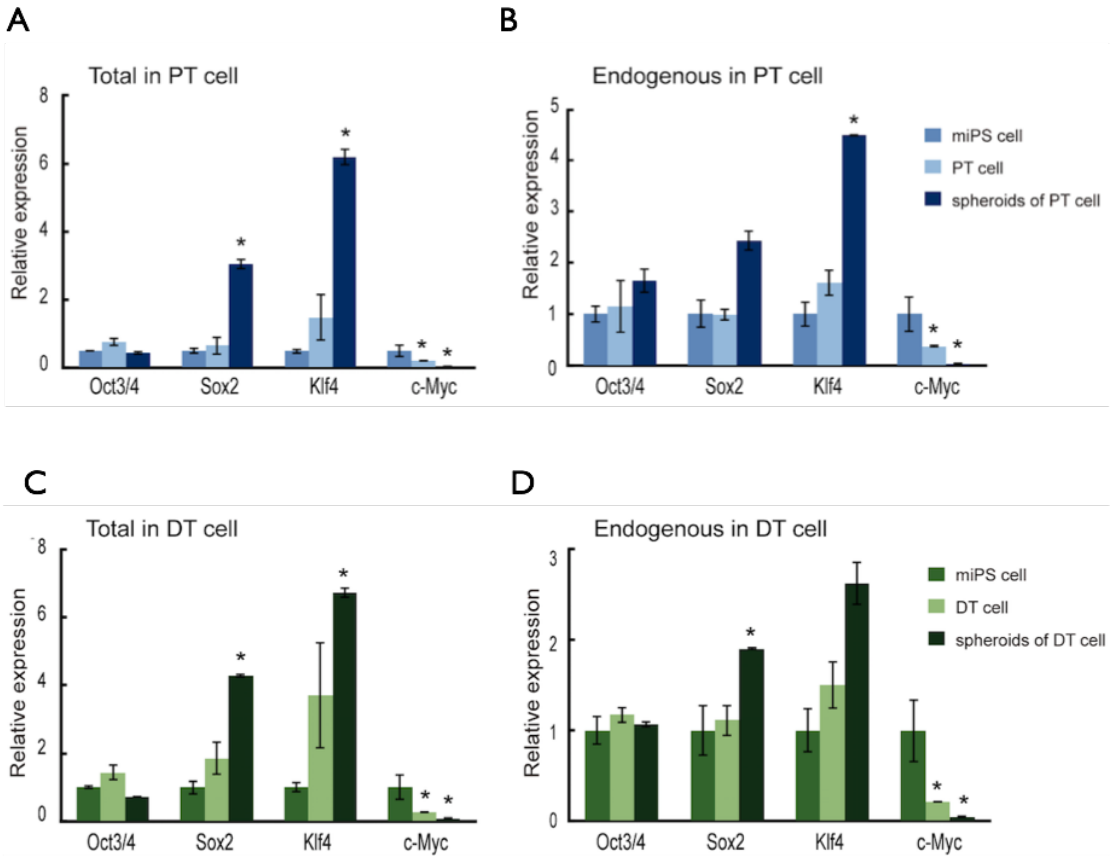


Figure 3.11 qRT-PCR analyses of four transcription factors *Oct3/4*, *Sox2*, *Klf4* and *c-Myc* expression levels in the indicated samples of miPS-LLCev primary culture cells, miPS-LLCevPT (A&B) and miPS-LLCevDT (C&D). The products of coding regions and endogenous transcripts are regarded as total (A&C) and endogenous (B&D). Relative expression values are normalized to *Gapdh* and conducted in triplicate. Each asterisk shows the significance of $P < 0.05$.

3.3.4 The secondary tumors displayed similar histophenotype with the primary tumor

Besides the sphere formation and expression of special markers, the assessment of self-renewal for CSCs requires the similar cancer initiation properties in serial translations [10, 100]. When the miPS-LLCevPT and miPS-LLCevDT cells were injected into immunodeficient mice, malignant liposarcomas again developed (Table 3.2). The pathological and immunohistochemical features of these secondary tumors were similar to the primary tumors indicating the repeatable nature of tumorigenesis in this model (Fig. 3.12). Collectively, I conclude that the miPS-LLCevPT and miPS-LLCevDT cells were able to maintain a self-renewal capacity and the original histotype of the primary tumor.

Table 3.2 The tumorigenic potential of miPS-LLCevPT and miPS-LLCevDT cells within one month.

Samples	No. of cells	Tumor formation	Histologic examination
miPS-LLCevPT	1 x 10^2	0/4	NA
	1 x 10^3	0/4	NA
	1 x 10^4	0/4	NA
	1 x 10^5	5/5	malignant, angiogenesis
	1 x 10^6	5/5	malignant, angiogenesis
miPS-LLCevDT	1 x 10^2	0/4	NA
	1 x 10^3	0/4	NA
	1 x 10^4	0/4	NA
	1 x 10^5	5/5	malignant, angiogenesis
	1 x 10^6	5/5	malignant, angiogenesis

NA: not applicable.

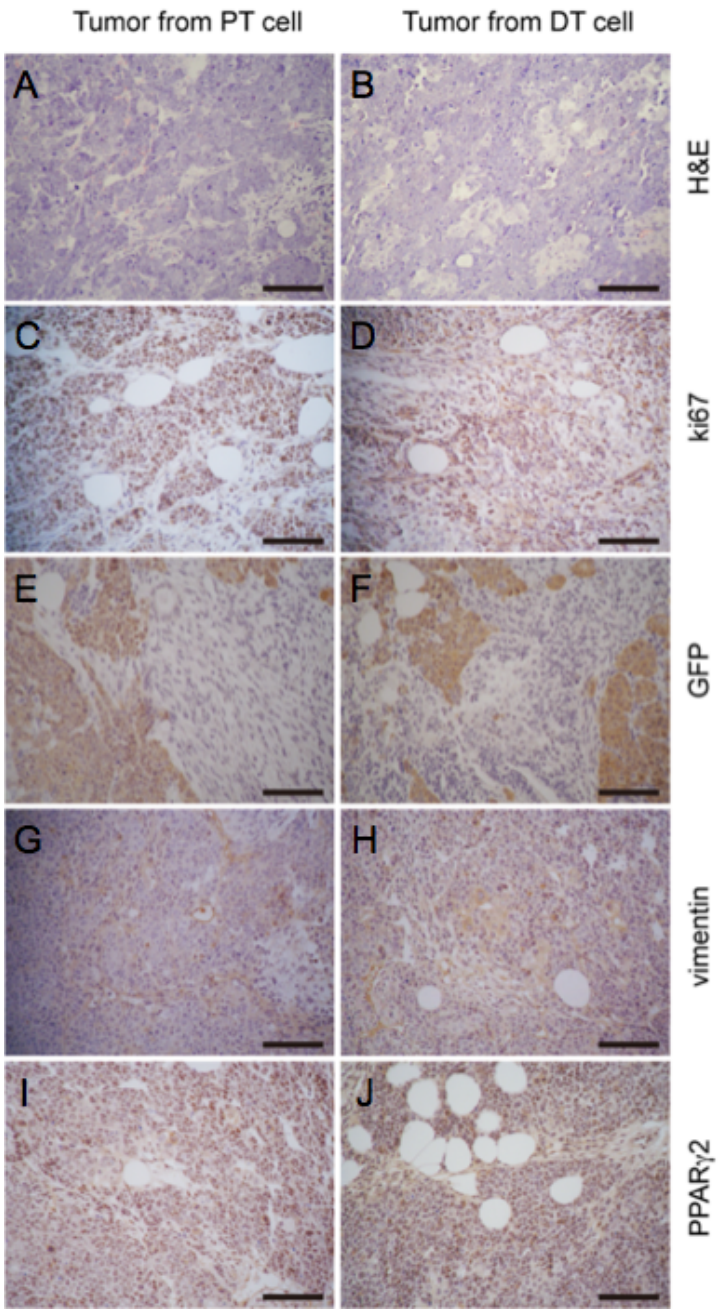


Figure 3.12 Tumor xenografts generated by primary cells display a similar histologic phenotype to the parental tumor. H&E staining of tumor sections to PT cell derived tumors (A) and DT cell derived tumors (B) show similar histologic phenotype.

Comparable staining pattern of relative markers Ki67 (C and D), GFP (E and F), vimentin (G and H), PPARγ2 (I and J) shows tumors developed from the two cell lines share similar properties. Scale bars: 100 μm.

3.3.5 The multipotency of tumor derived cells

Distinct stages of adipogenesis and immunophenotypic plasticity have demonstrated a cellular hierarchy in human liposarcomas. And it has been previously reported that the expression of both CD34 and CD36 could serve as specific markers indicative of adipose differentiation in liposarcomas [101]. I then assessed the cellular lineage of adipocytes in the miPS-LLCev, miPS-LLCevPT and miPS-LLCevDT cells *in vitro*. Immunostaining against CD34, which is a multipotent progenitor marker, expressed by adipose progenitors and other progenitor cell types [101-103], showed that all of the three kinds of cells contained a population of CD34 expressing cells (Fig. 3.13). There were heterogeneous populations within both cell lines exhibiting $GFP^+/CD34^+$, $GFP^-/CD34^+$ and $GFP^+/CD34^-$. In the case of CD36 (Fig. 3.14) and PPARγ2 (Fig. 3.15) expression, similar to CD34, there were positive and negative cells in both the GFP^+ and GFP^- cells for these additional adipocyte markers.

I then assessed if the heterogeneous cells within the adipocyte lineage were derived by differentiation from the GFP positive cells. After forming spheroids, cells were cultured under adherent conditions and were then induced to adipocyte differentiation by addition of insulin and dexamethasone in the medium. Differentiation into adipocytes was evaluated by Oil Red O staining (Fig. 3.16). I found an accumulation of a significant number of fat droplets in both the miPS-LLCevPT and

miPS-LLCevDT cells. These findings demonstrate the potential for adipocyte differentiation by these cancer stem-like cells, and implicate a cellular hierarchy similar to that comprising the heterogeneity of liposarcomas derived from miPS-LLCev cells.

Several studies have shown that CSCs can give rise to endothelial cells, which contribute to tumor vascularization thereby facilitating tumor growth [104, 105]. Since a population of $CD34^{+}$ cells is considered to be a progenitor population of endothelial cells [106], I assessed in vitro tube formation to evaluate the endothelial differentiation capacity of miPS-LLCev, miPS-LLCevPT and miPS-LLCevDT cells (Fig. 3.17). These results indicate that cells formed vessel-like structures that contained a CD31 positive population of cells, which is a marker of differentiated endothelial cells, demonstrating that miPS-LLCev, miPS-LLCevPT and miPS-LLCevDT cells can differentiate into mature endothelial cells [104]. Along with the adipocyte differentiation, these results validate the multipotency of all the three kinds of cells. Taken together, with tumorigenicity and self-renewal capacity, I conclude that the miPS-LLCev, miPS-LLCevPT and miPS-LLCevDT cells are CSCs capable of developing a liposarcoma that exhibit phenotypic heterogeneity.

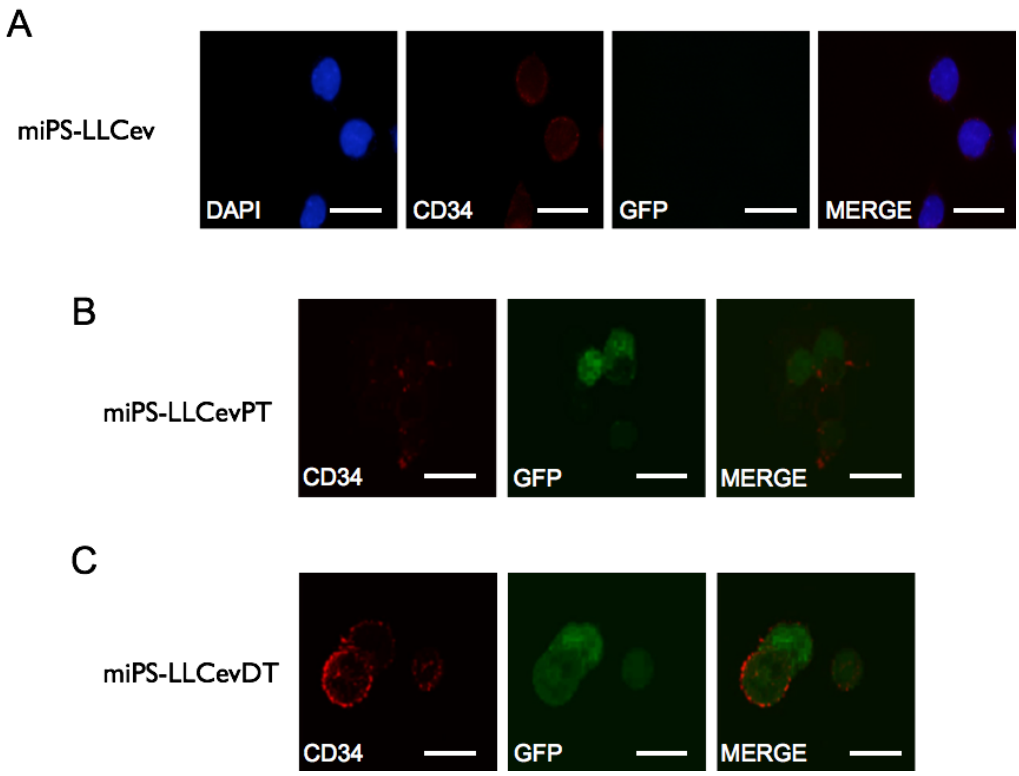


Figure 3.13 CD34 immunofluorescent staining of miPS-LLCev cells (A), miPS-LLCevPT cells (B) and miPS-LLCevDT cells (C) are showing different stages of adipocyte differentiation in the bulk cell culture. Scale bars: 10 μm.

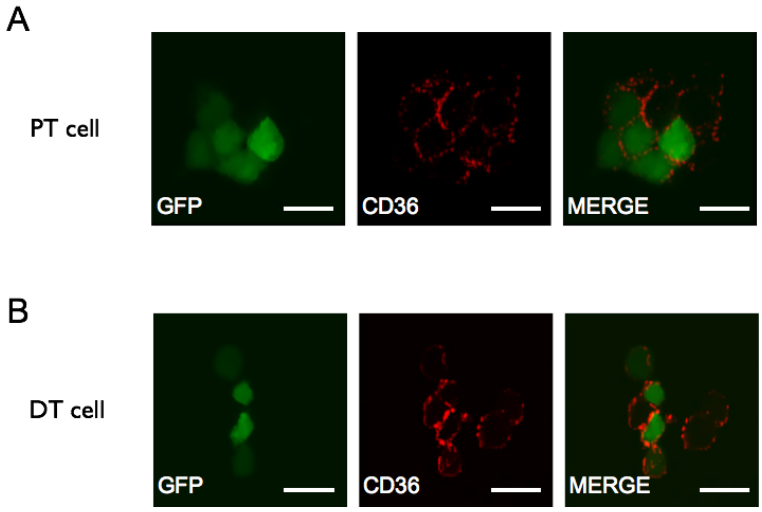


Figure 3.14 CD36 immunofluorescent staining of miPS-LLCevPT cells (A) and miPS-LLCevDT cells (B) are showing different stages of adipocyte differentiation in the bulk cell culture. Scale bars: 10 μm.

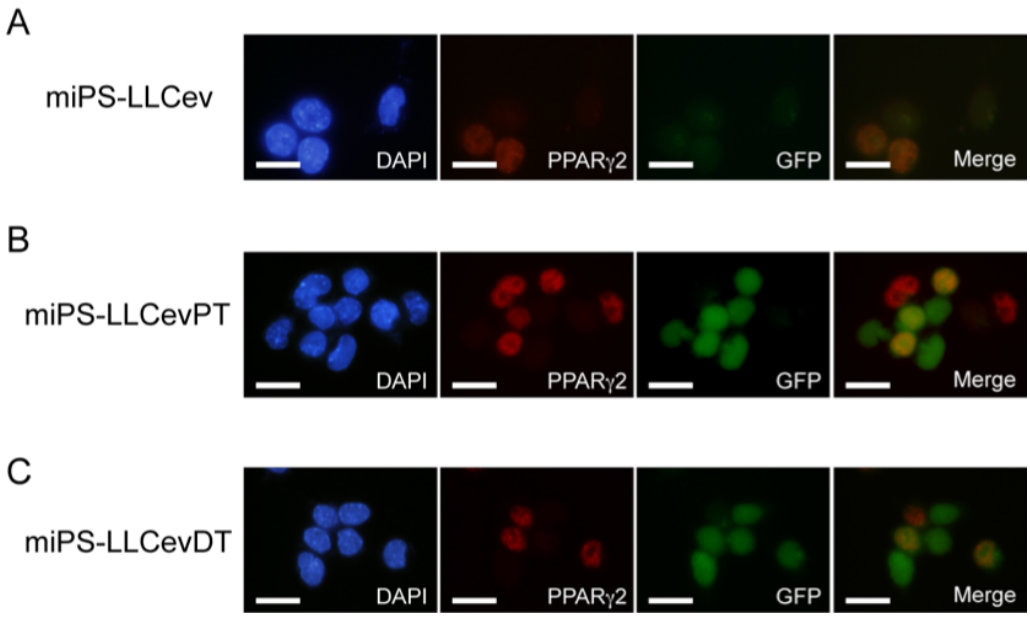


Figure 3.15 PPARγ2 immunofluorescent staining of miPS-LLCev cells (A), miPS-LLCevPT cells (B) and miPS-LLCevDT cells (C) are showing different stages of adipocyte differentiation in the bulk cell culture. Scale bars: 10 μm.

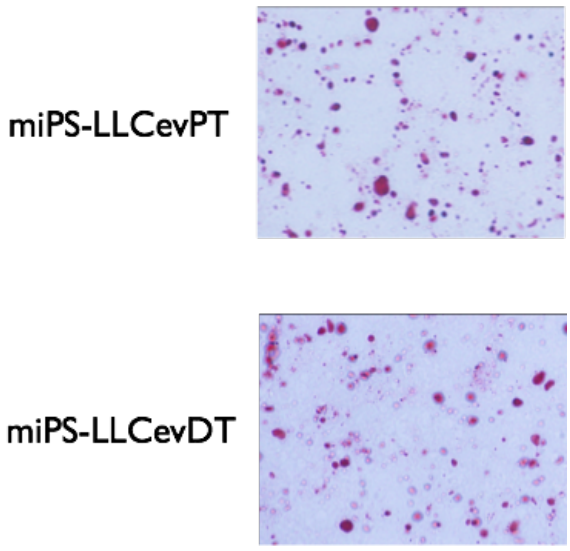


Figure 3.16 Oil Red O staining after *in vitro* differentiation of spheroid cells, showing the two primary cell lines could differentiate into adipocytes.

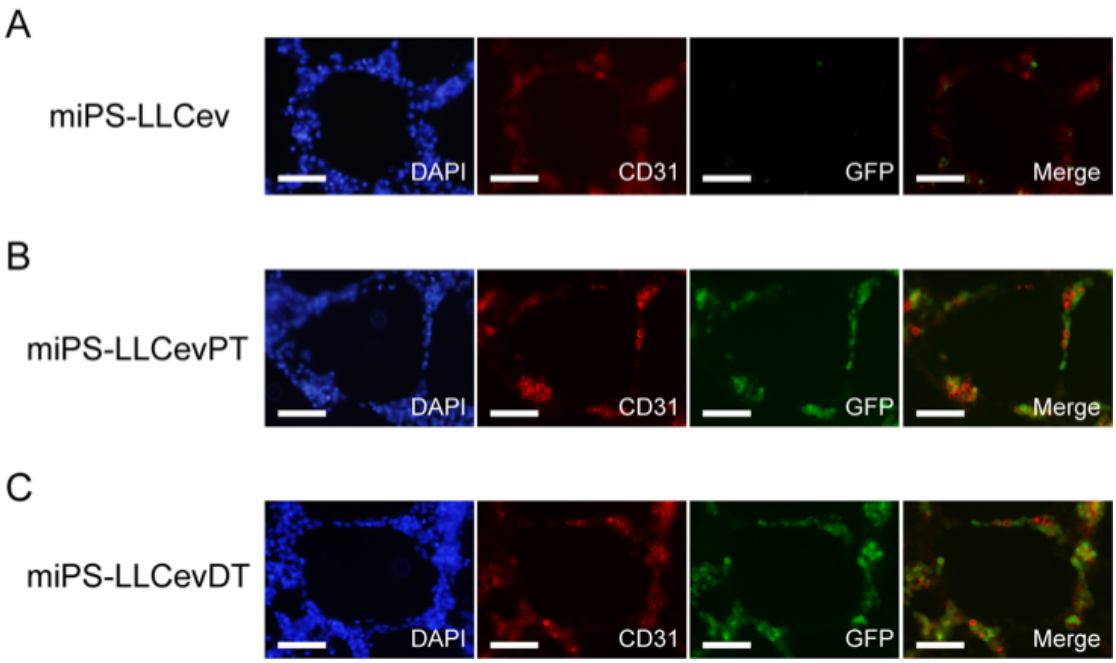


Figure 3.17 In virto tube formation assay shows miPS-LLCev cells (A), miPS-LLCevPT cells (B) and miPS-LLCevDT cells (C) possess tube formation ability. The CD31 positive and negative cells indicate the heterogeneity of primary cells. Scale bars: 100 μm.

3.4 Discussion

CSC is considered as a cell possessing self-renewal capacity can generate heterogeneous tumor cell population [1], and responsible for the tumor maintenance during various cancer therapies for their resistance to chemotherapy [11, 76, 107] or radiotherapy [108, 109]. I already proved the tumorigenic potential of the miPS-LLCev cells in last chapter. To assess whether miPS-LLCev cells are CSC or

not, in this chapter, I studied the characteristics of them according to the CSC properties. I demonstrated that the reprogrammed miPSCs, miPS-LLCev cells and their primary culture cells, miPS-LLCevPT and miPS-LLCevDT exhibited sphere formation in suspension culture. The Yamanaka factors, especially Sox2 and Klf4, were highly expressed in the bulk cells and spheroid cells. I also demonstrated the tumorigenicity of the two cell lines that were established from primary tumors and disseminated tumors, miPS-LLCevPT and miPS-LLCevDT, as well as the histophenotype of the developed tumors. They showed the similar tumorigenic potential to the parental cells, miPS-LLCev, suggesting the cells could retain their features *in vivo*. Moreover, I tested their abilities to differentiate into adipocytes and vascular endothelial cells, and that lead to the development of liposarcomas composed of a heterogeneous cellular population. Thus, I suggest that miPS-LLCev, miPS-LLCevPT and miPS-LLCevDT cells are potential models of liposarcoma CSCs.

I found that the expression of two significant iPS reprogramming genes, *Klf4* and *Sox2*, were upregulated in miPS-LLCev, miPS-LLCevPT and miPS-LLCevDT when these cells were cultured as spheroids. *Klf4* is primarily expressed in postmitotic or differentiated cells of the skin and in the gastrointestinal tract and functions in a variety of roles to control differentiation and proliferation through cell cycle regulators in a content-dependent manner [110]. Recent studies have also reported that *Klf4* is an essential and an early regulator of adipogenesis and stimulates adipogenesis by regulating C/EBPβ [111], and that the expression of *Klf4* can be activated by PPARγ through directly binding to its promotor [112]. Since the miPS-LLCevPT and miPS-LLCevDT cells express PPARγ, the expression of *Klf4* could be, in part, related to adipocyte differentiation that is occurring in these tumor cells. In addition, *Klf4* is also an essential reprograming factor for iPS cells [64], and is essential for the self-renewal of ES cells [113]. According to recent reports, *Klf4*

may also contribute to the self-renewal of some types of CSCs [99]. Although some reports have shown a tumor suppressor role for *Klf4*, our results implicate *Klf4* as an oncogene.

Sox2 is another essential core transcription factor for reprogramming iPSCs and is important in establishing early embryonic cell fate decisions [114]. A very recent report has shown that *Sox2* is important in the lineage determination of osteo-adipo progenitors by induction of PPARγ [115]. For *Sox2*, it has also been shown to be important in maintaining the self-renewal of tumor-initiating cells in breast carcinomas [116], osteosarcomas [98] and glioblastomas [117]. Riggi et al. recently demonstrated that, *Sox2* is a key factor in the determination and regulation of Ewing sarcoma CSCs [97].

Given the fact that miPS-LLCcm cells expressing lower levels of *Sox2* and *Klf4* developed adenocarcinomas in immunodeficient mice [20], CSCs in different tumors may be essentially unique in their self-renewal mechanisms but differ in their capacity to differentiate depending upon whether soluble or vesicle-associated factors are exposed to the iPSCs. Our preliminary analysis indicates that CSCs treated with soluble fractions from LLC CM other than tEVs promote the formation of adenocarcinoma-like CSCs (data not shown). In addition, our recent results indicate that miPS-LLCcm cells and their differentiated progenies, can create a niche *in vitro*, and this niche might regulate the self-renewal and differentiation capacities of miPS-LLCcm. tEVs and/or soluble factor(s) from potential different progenitor cells are in part, responsible for the commitment of differentiation lineages of miPS-LLCcm [105]. Therefore, both tEVs and soluble factor(s) in the CM from tumor cells contain a unique composition or signature of factors that compose the general cancerous niche that can differentially contribute to the genesis/maintenance of CSCs with different lineage commitments. It is also conceivable that different

cancer progenitor cells are derived from a more primitive CSC after exposure to different environmental components.

CHAPTER 4

Characteristics of cancer stem cells (CSCs) derived from mouse induced pluripotent stem cells transformed by conditioned medium of Lewis Lung Carcinoma (LLC) cell line

Abstract

The critical features of cancer stem cells (CSCs) are tumorigenicity, self-renewal and differentiation, which are considered to be responsible for tumor maintenance. It has been proposed that CSCs generate a heterogenietic population in tumor by giving rise to diverse progenies in the apex of differentiation hierarchy. By using miPS-LLCcm cell line, which is the CSC model established from mouse induced pluripotent stem cells (miPSCs), I showed that vascular endothelial cells is one subset of the progeny cells of CSCs. Moreover, the differentiated endothelial cells could form vessel-like structures in the tube formation assay on matrigel and in the developing chick chorioallantoic membrane. Intriguingly, the endothelial differentiation capacity was decreasing followed the depletion of endothelial environment. Thus, I conclude that the CSCs could generate vascular niche by themselves to regulate the differentiation plasticity of CSCs themselves.

Key words: cancer stem cells, endothelial cell, differentiation, tube formation and niche

4.1 Introduction

In previous study, we transformed Nanog-GFP mouse induced pluripotent stem cells (miPSCs) into cancer stem cells (CSCs), which named miPS-LLCcm, by culturing with conditioned medium (CM) of Lewis Lung Carcinoma (LLC) cell line as a cancerous niche. After subcutaneous injection into immunodeficient mice, the cells generated adenocarcinomas with extensive angiogenesis [20].

One of the key features of CSCs is the capacity to generate heterogeneous population that is similar with normal stem cells [100]. Colon cancer cells with stem cell markers can generate adenocarcinoma on xenotransplantation with multi-lineage differentiation [118]. It suggested that, similar with normal stem cells, the CSCs possess the multi-potential to form an entire architecture of cellular population. In line with this scenario, several groups identified that CSCs can differentiate to endothelial cells for tumor angiogenesis in order to fuel the fast tumor growth, such as glioblastoma [104, 119, 120], breast cancer [121] and ovarian cancer [122], for the neovascularization is a master switch of tumor development and progression [123].

Besides the nutrition provision, vascular endothelial cells can be components of CSC niche to promote CSCs self-renewal. They have been shown to promote stem-like phenotype formation through Hedgehog signaling pathway [124]. The perivascular expression of osteopontin, which is one of the ligand for CD44, can promote the stem cell-like properties and radiation resistance through enhancement of HIF-2α activity [26]. Also, nitric oxide (NO) produced by endothelial cells can activate Notch signaling pathway, thereby reinforce the stem cell like character [125]. Recent studies showed Notch ligands were expressed by endothelial cells and some

tumor cells surrounding the Notch receptor positive CSCs in primary glioblastma tumors [31]. Therefore, vascular endothelial cells are conceivable components of CSC niche to promote the self-renewal of CSCs. However, there is no evidence showing whether the endothelial cells are essential to the differentiation capacity of CSCs.

The vascular endothelium is a dynamic cellular "organ" that controls passage of nutrients into tissues, maintains the flow of blood, and regulates the trafficking of leukocytes [126]. Recently, angiogenesis as a novel feature has been involved in the theory of CSCs. However, beside the formation of blood vessel, I hypothesize that the endothelial cells derived from CSCs themselves could contribute to the CSCs niche to regulate the behaviors of CSCs. In this chapter, I assessed the endothelial differentiation of the established cancer stem cell line, miPS-LLCcm, both *in vitro* and *in vivo*. Moreover, I found the endothelial niche generated by CSCs themselves could regulate the differentiation capacity of CSCs.

4.2 Materials and Methods

4.2.1 Cell culture

miPS-LLCcm cells were maintained under the humidified 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 0.1 mM Non-Essential Amino Acid (NEAA, Life Technologies), 2 mM L-Glutamine, 0.1 mM 2-mercaptoethanol, 50 U/mL penicillin and 50 U/mL streptomycin). For removing the differentiated cells, 1 μg/mL puromycin (puro) was supplemented to the culture medium for 1 week.

For suspension culture, 4×10^4 single cells were plated in 60-mm Lipidure®-coated

low adhesion dishes (NOF Corporation, Japan) in serum-free miPS medium without LIF. After image capturing on day 7, spheres were collected for further analyses.

4.2.2 In vitro tube formation assay and immunofluorescence stain of tube structures

1.4×10^5 individual cells were suspended in endothelial basal medium supplemented with hydrocortisone, ascorbic acid, GA-1000 and heparin (-GF medium) or also added with hEGF, hFGF-B, VEGF, R^3-IGF-1 and FBS (+GF medium) (EGM-2 Single Quots Kit, Takara) following manufacture's instruction and seeded in triplicate on Matrigel (Becton Dickinson) coated imaging chambers (Nunc) [105]. After 24 hours, the cells on Matrigel were fixed with 4% (W/V) paraformaldehyde at room temperature for 20 min and blocked by 5% BSA for 1 h. Then the cells were incubated with rat anti-CD31 antibody (Santa Cruz) at a dilution of 1:200 at 4 °C for 12 h. After wash twice with PBS, the cells were incubated with Texas Red® - X goat anti-rat IgG at room temperature for 1 h. After rinsing in PBS, images were taken using an inverted epifluorescence-light microscope, equipped with a light fluorescence device (IX-80, Olympus, Japan) and confocal laser microscope LSM510-V3.0 (Carl Zeiss, UK).

4.2.3 Reverse transcription polymerase chain reaction (RT-PCR) and real time PCR

miPS-LLCcm cells cultured under various conditions were harvested and Total RNA was prepared with RNeasy kit (Qiagen, MD). To remove any residual genomic

DNA, total RNA was treated with DNase I (Takara, Japan). Then three micrograms of treated RNA were reverse transcribed by SuperScript III Reverse Transcriptase (Invitrogen) primed by oligo-dT18. Real time PCR was performed with LightCycler 480 SYBR Green I Master mix (Roche) in a reaction volume of 20 μL. The sequences of primers used here are following, VEGFR2: 5'-TAGGCGCCTGCACCAAGCCG-3' and 5'-CCTTGCCCTGGCGGAAGCGT-3'; VE-cadherin: 5'-CGCACCAGGTATTCAACGCATC-3' and 5'-GGCATCTTGTGTTTCCACCACG-3'; Gapdh: 5'-CCCTTCATTGACCTCAACTAC-3' and 5'-CCACCTTCTTGATGTCATCAT-3'. Relative gene expression was normalized to that of Gapdh.

4.2.4 Construction of Ds-Red2 expression plasmid vectors

The Ds-Red expression vector was constructed as follows. The Ds-Red2 gene was amplified by PCR with primer pair 5'-EcoRI-DsRed-3' (CCGGAATTCATGGCCTCTCC) and 5'-SalI-DsRed-3' (TCCGGTCGACCTACAGGAACAG) to add EcoR1 and Sal1 sties to the 5'- and 3'-sites of Ds-Red. Then, the product was cloned into pEF-EX-HA vector to create pEF-DsRed. For the pEF-neo plasmid construction, the G418 resistant gene was amplified by PCR with primer pair 5'-EcoR1-neo-3' (GCCGGAATTCATGATTGAACAAGATGGA) and 5'-Sal1-neo-3' (TGTAGTCGA CTCAGAAGAACTCGTCAAG) to add EcoR1 and Sal1 sties to the 5'- and 3'-sites of neo gene. Then the constructions were transfected into Cos-7 cells to confirm the gene expression.

4.2.5 Transfection

About 1×10^7 miPS-LLCcm cells, which maintained in suspension culture for 8 days, were washed with PBS twice and dissociated by trypsin treatment into a single-cell suspension in 600 μL Electroporation Buffer for ES cells (Millipore). Then the cells were mixed with 1 μg linearized G418 resistant gene expression vector (pEF-neo) and 10 μg linearized Ds-Red expression vector (pEF-DsRed). Then, the cell suspension was transferred to a 0.4 cm gap-cuvette and electroporated using Gene Pulser® Ⅱ Electroporation System (Bio-Rad) with condition of 220V, 950 μF following the manufacturer's protocol. The electroporated cells were then plated into gelatin-coated dishes without antibiotics. And the G418 selection was started after 2 days by adding G418 to media at final concentration of 0.3 mg/mL. After 1 week, the Ds-Red positive colonies were picked out.

4.2.6 Chick chorioallantoic membrane (CAM) assay

To test the angiogenesis ability of miPS-LLCcm cells *in vivo*, the cells were applied to CAM assay [127]. Briefly, the fertilized eggs were put in 38˚C incubator after sterilizing with 70% ethanol (day 1). After 4 days incubation, on day 5, the eggs were opened a small hole on the 2 O' clock direction of the sharp side in clean bench, and aspirate 3 mL albumen from the eggs using 25G or 26G hypodermic needle and 1 mL syringe to create a air sac, allowing the dissociation from the shell membrane. Then the holes were covered by tape, and the eggs were incubated for another 1 day. On day 6, the eggs were opened on the round sides by removing the cover in clean bench (about 10 ×10 mm). Then put a sterilized 1 mm^3-gelatin sponge containing 1×10^6 cells on the CAM. After another 2 days incubation, take picture and cut the

membranes out for further analyses.

4.2.7 Immunostaining

For immunofluorescence analysis of the cells on CAM, the membranes were washed twice with PBS, fixed with 4% (w/v) paraformaldehyde for 20 min at room temperature, permeabilized in PBS containing 0.1% Triton-X (PBST), blocked with 5% BSA, incubated with rat monoclonal anti-CD31 antibody for 12 h at 4˚C. Then membranes were washed twice with PBST, and incubated with TexasRed-conjugated secondary antibodies for 1 h at room temperature. After counterstaining with DAPI (Vector Laboratories), images were captured using a confocal microscope equipped with a light fluorescence device (LSM510META, Carl Zeiss, Germany).

4.3 Results

4.3.1 The differentiated cells of miPS-LLCcm give rise to vessel-like structures in vitro

For the extensive angiogenesis of miPS-LLCcm *in vivo*, I tested the endothelial differentiation ability of this cell line. The bulk miPS-LLCcm cells were applied to in vitro tube formation assay. After 24 h incubation, the cells formed vessel-like structures both in complete EGM-2 medium and growth factor absent medium (Fig. 4.1). To confirm the presence of endothelial cells in the vessel-like structures, immunostaining against CD31 which is a specific marker of endothelium [104] was performed directly on the Matrigel. The result showed the vessel-like structures are

positive for CD31 antibody. On the tubes, there are GFP+/CD31-, GFP+/CD31+ and GFP-/CD31+ cells, indicating the heterogeneity of the cell population (Fig. 4.2).

Vascular endothelial growth factor receptors 1-3 are endothelial cell-specific receptor tyrosine kinases which serve as key mediators of the angiogenic responses. While, VEGFR-2 seems to mediate the major growth and permeability actions of VEGF [128]. Vascular endothelial cadherin (VE-cadherin) expression is thought to be a specific marker to investigate the angiogenic phenotype of cancer patient [129]. Thus, I analyzed the mRNA expression levels of VEGFR2 and VE-cadherin in bulk cells to further confirm the endothelial differentiation from miPS-LLCcm. The results showed that, both the expression level of VEGFR-2 and VE-cadherin were similar in the miPSCs and puro selected miPS-LLCcm cells. Whereas, in bulk cells of miPS-LLCcm their expressions were significantly up-regulated, which suggested the presence of endothelial cells (Fig. 4.3).

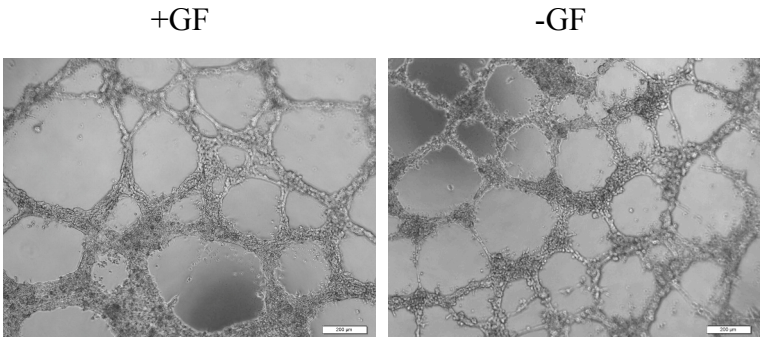


Figure 4.1 In vitro tube formation assay of miPS-LLCcm cells indicating the cells have angiogenic capacity *in vitro*. Scale bar: 200 μm.

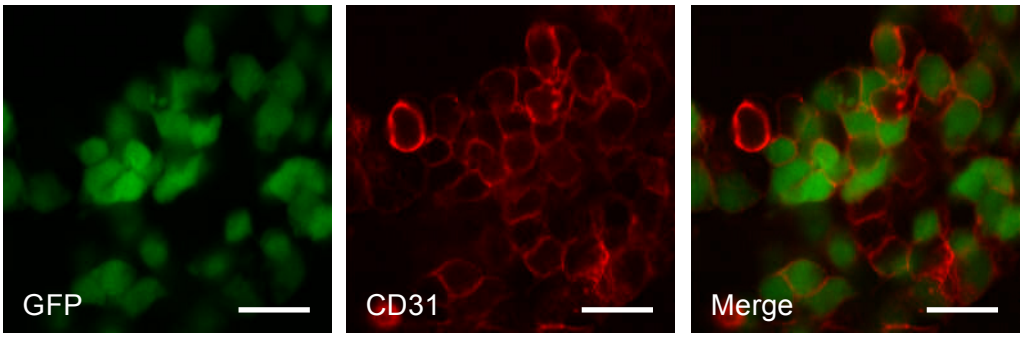


Figure 4.2 Anti-CD31 immunostaining shows the presence of endothelial cells on the vessel-like structures derived from miPS-LLCcm cell. Scale bars: 10 μm.

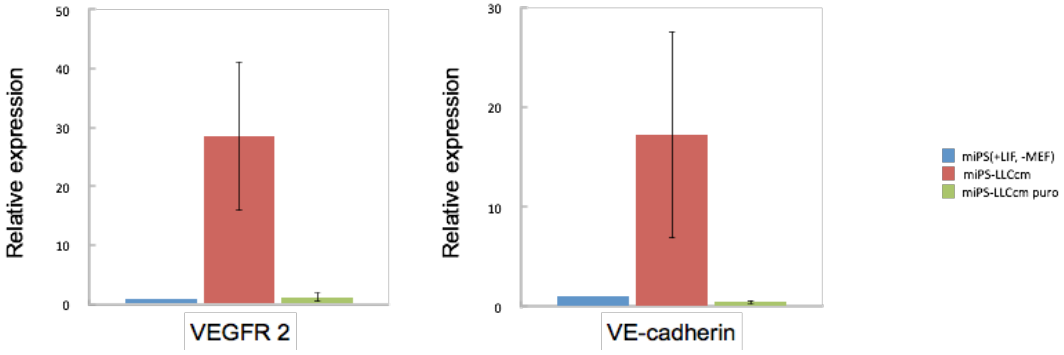


Figure 4.3 Real time PCR analyses of endothelial markers showing the endothelial differentiation of miPS-LLCcm cells.

4.3.2 The differentiated cells of miPS-LLCcm give rise to vessel-like structures in ovo

The observation of tube formation on Matrigel implicated that the miPS-LLCcm could generate vessels by differentiating to endothelial cells. However, the condition of cell culture *in vitro* and *in vivo* maybe different. To assess the angiogenic ability of miPS-LLCcm *in vivo*, I applied bulk miPS-LLCcm cells to the CAM assay, which is a well established model for tumor angiogenesis [130]. Because the differentiated miPS-LLCcm will not express GFP protein any more, it is invisible on the CAM. So, I transfected Ds-Red expressing-plasmid to miPS-LLCcm cells, and pick out the clones which are expressing Ds-Red both in the stem and differentiated stages (Fig. 4.4). Then the cells were implanted to the growing CAM on day 8 of development after confirming the tube formation ability on Matrigel (Fig. 4.5). The result showing that there was a obvious tumor mass on the CAM implanted with cells, and a blood vessel derived from the embryo connected the chick with the tumor mass. There is no such observation in the CAM with vehicle (Fig. 4.6). However, no fluorescence was found on the vessel, which indicated the vessel is from the chick itself (data not shown). By using fluorescent microscope, I found the vessels on the CAM with both GFP and Ds-Red expression, implicating the angiogenesis of miPS-LLCcm *in ovo* (Fig. 4.7). In order to confirm the formation of tube is due to the endothelial differentiation, I applied the normal miPS-LLCcm (non-transfected) to the CAM assay followed by immunostaining against CD31, an endothelial differentiation marker [121] (Fig. 4.8). These results suggested that miPS-LLCcm could differentiate to endothelial cells, and these endothelial cells could form tube-like structures in tube formation assay and *in ovo*.

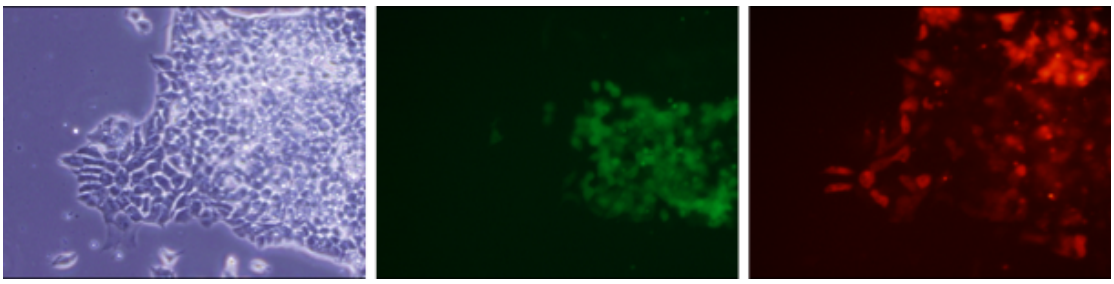


Figure 4.4 The miPS-LLCcm cells which are expressing Ds-Red both in the stem stage and differentiated stage.

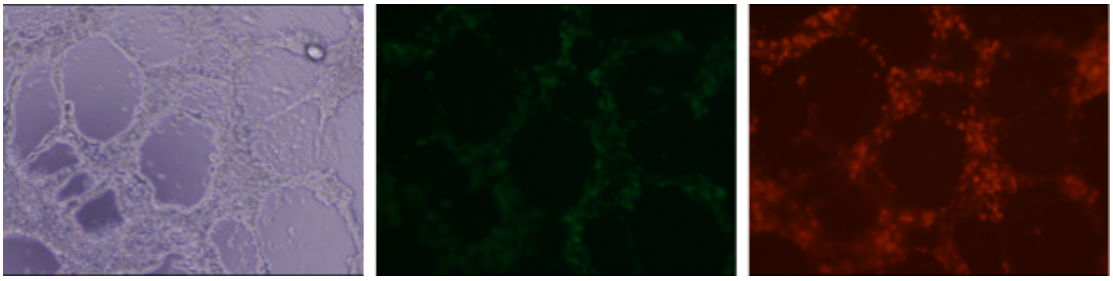
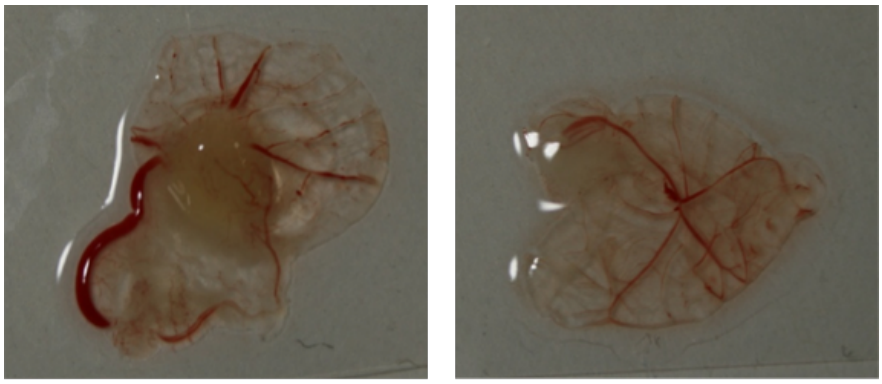


Figure 4.5 The Ds-Red miPS-LLCcm was confirmed tube formation ability *in vitro* on Matrigel before applying to CAM assay.



gelatin sponge with cells with vehicle

Figure 4.6 On the CAM transplanted with cells, there is obvious tumor mass and vessel.

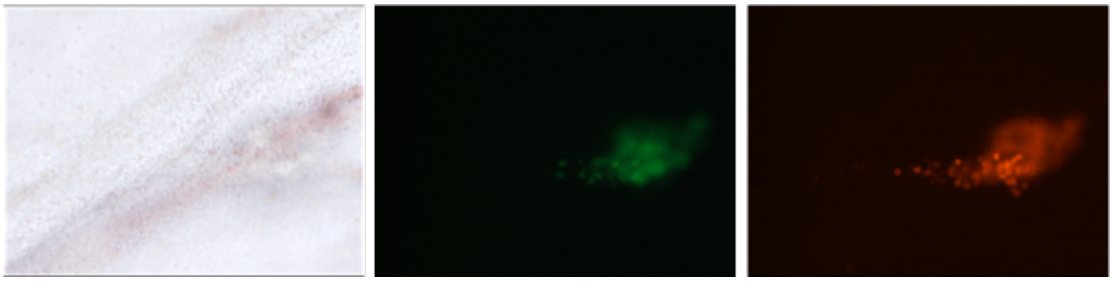


Figure 4.7 The Ds-Red cells can generate vessel-like structures *in ovo*.

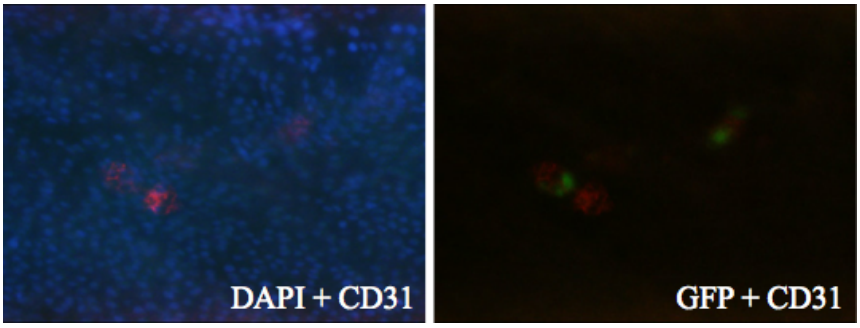


Figure 4.8 Immunostaining of CD31 on the CAM implanted with miPS-LLCcm cells. GFP showing the CSC population; CD31 showing the miPS-LLCcm derived endothelial cells.

4.3.3 Differentiated cells regulated the differentiation ability of stem cells

To investigate whether the differentiated cells could regulate the fate of stem cells, I performed puromycin selection cycle and tested the tube formation ability during the cycles (Fig. 4.9). Since the puromycin (puro) resistant gene is under the control of *Nanog* promotor [64], I applied puromycin in the culture medium of miPS-LLCcm to remove the differentiated cells (puro selection) for 1 week (Fig. 4.10 b, d, f), followed by removing the puromycin from culture medium, cells were allowed to differentiate (puro release) for another 1 week (Fig. 4.10 c, e, g). After every time of puro release, the bulk cells were applied to tube formation assay, in order to confirm the endothelial differentiation of the CSCs. I performed this process as one cycle and uninterruptedly accomplished 3 cycles (Fig. 4.9). The results showed, after puro selection, all the cells remained are GFP positive (Fig. 4.10 b-2, d-2, f-2), indicating the stem stage of cells. When the cells applied to tube formation assay, these stem cells failed to form the tube structures (Fig. 4.10 b-3, d-3, f-3), suggesting that stem cells themselves don't

have the endothelial cells. After removing puro from culture medium, the GFP negative cells (differentiated cells) were allowed to grow with stem cells for one week (Fig. 4.10 c-2, e-2, g-2), then cells could form tube structures in verying degrees (Fig. 4.10 c-3, e-3, g-3). The puro selected GFP positive cells in the third round still differentiated to the GFP negative cells after removing puro from cell culture (Fig. 4.10 g-1, 2), indicating the differentiation capacity was not affected by the puromycin selection cycle. However, the tube formation ability was decreasing after each time releasing, indicating the endothelial differentiation was getting less after puro selection. To confirm it, I checked the mRNA expression level of VEGFR2, which is a special marker of endothelial cells [131], in the third round released bulk cells. The result showed that the expression level of VEGFR2 was significant lower than the initial miPS-LLCcm.

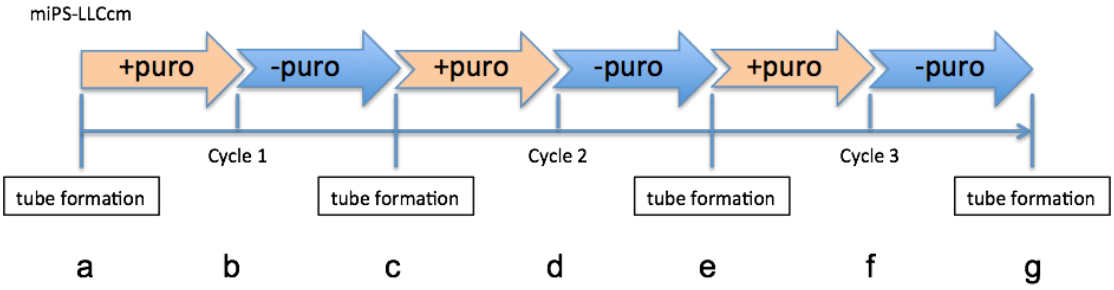


Figure 4.9 miPS-LLCcm cells are performed puromycin selection for 3 cycles following this procedure during 6 weeks. The tube formation ability was confirmed after finishing each cycle.

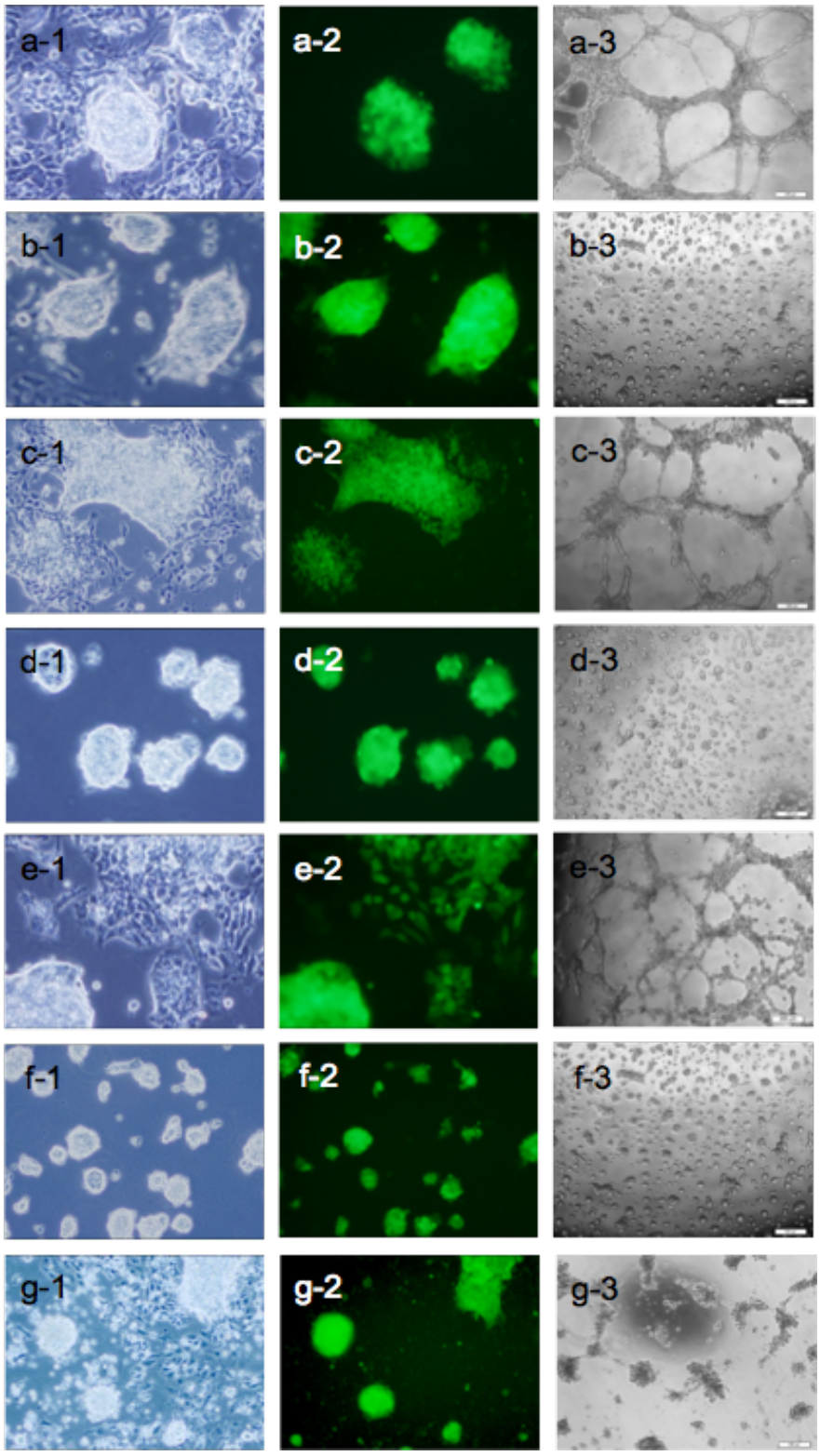


Figure 4.10 Tube formation assay along with puromycin selection cycles. The cells

of initial miPS-LLCcm cells (a), after 1 week-puromycin selection (b), followed by another 1 week-release from puromycin (c), cycle 2 (d and e) and cycle 3 (f and g) were applied to the tube formation. The ability to form tube like structures was decreased.

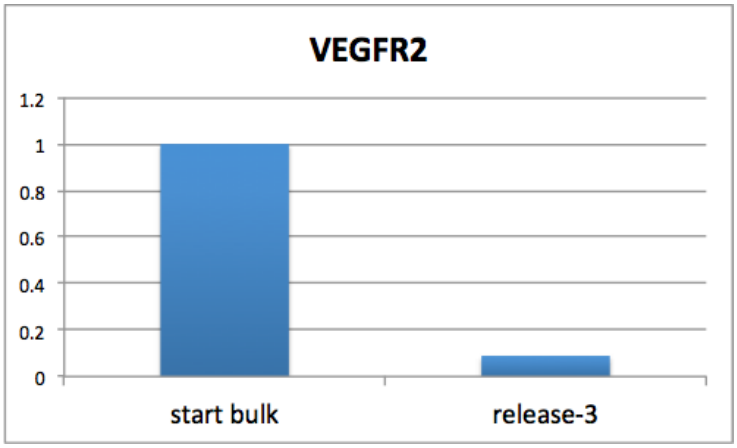


Figure 4.11 qRT-PCR analysis of VEGFR2 expression level in the start bluk miPS-LLCcm cells and the puro release cells of the third round. Relative expression values are normalized to Gapdh.

4.4 Discussion

One key feature of all the cancers is the cellular heterogeneity in a single tumor, and it complicates the clinical treatment of cancer therapies. However, there are two main opinions for arise of tumor heterogeneity, CSC hypothesis and clonal evolution [132]. It is well accepted nowadays that CSCs could generate the heterogeneous

lineage of cancer cells in tumor by giving rise to diverse progenies in the apex of differentiation hierarchy [1]. As showing by CD31 immunostaining, the CSCs could differentiate to CD31 expressing cells, which included both GFP positive and negative cells. Also, there are GFP positive and negative cells in the CD31 negative cells (Fig. 4.2). Moreover, after puro selection, the remained GFP positive cells again differentiated to endothelial cells by removing puro from the culture medium, indicating that the GFP positive cells could differentiate to a hierarchical population of endothelial cells. These data suggested that a subset of cells is responsible for heterogeneity of hierarchy [2]. Our results clearly support the CSC hypothesis, by which the tumor heterogeneity was induced.

The progenies of CSCs containing multi-types of differentiated cells. Similar with normal stem cells, the progeny cells display diverse phenotypes and functions, indicating the differentiated cells of CSCs can in turn contribute to the CSC niche as normal stem cells [133]. Giving the similar behavior between CSCs and normal stem cells, many researchers are focusing on the mechanism of the maintenance of CSC by the microenvironment “niche” [25, 26], where CSCs reside and which is necessary for the maintenance of unique properties of CSCs [28]. Cancer associated fibroblast cells are proved to promote tumor progression [34, 134]; also, tumor endothelial cells could serve as the self-renewal supporter through Hedgehog signaling pathway [124], enhancement of HIF-2α activity [26], Notch signaling pathway [125, 133]. However, our research opened a new field of view that the progenies of the CSCs could regulate their differentiation capacity.

The classical model of tumor angiogenesis was widely accepted that the blood vessels formed from the pre-existing vessels by sprouting controlled by various growth factors, which were released by either the host cells or the tumor cells. However, investigators from our laboratory and others recently provided evidences

that vascular endothelial cells are the members of the cells differentiated from the CSCs. As the result showing, the tube formation ability was lost after the third round of depletion of differentiated cells, suggesting the differentiation capacity towards endothelial cells was faded away. Because the differentiated endothelial cells from CSCs could also promote the self-renewal of CSCs, I defined it as vascular niche for the regulative activity to the CSCs [133]. It was showed that the differentiation of neural stem/progenitor cells was promoted when they were co-cultured with endothelial cells [135], and a large part of dividing cells are associated with the endothelial cells during neurogenesis [136]. This vascular niche should be a key factor to influence the differentiation plasticity of CSCs. Although, the mechanism under this plasticity regulation by vascular niche is not yet defined, it is worth paying attention to understand the differentiation lineage of CSCs. As it is well accepted, the CSCs are often resistant to clinical treatments for which the cancer relapse was attributed [137]. Thus, thorough differentiation of CSCs may be a novel strategy for cancer therapy in the future.

CHAPTER 5

General conclusion and future challenges

5.1 Summaries of important findings

1) Tumor-derived extracellular vesicles, including exosomes and microvesicles, can contribute to the cancerous niche to convert the differentiating mouse iPS cells into tumorigenic cells, named miPS-LLCev. Moreover, the cells developed malignant liposarcomas in immunodeficient mice, suggesting the mouse iPS cells can be transformed to diverse tumor cell depending on the different cancerous niche. Additionally, some of the transplanted tumors have shown to disseminated to mesentery, indicating the metastatic potential of the miPS-LLCev.

2) miPS-LLCev cells, which established by culturing with tumor-derived extracellular vesicles, could form the spheroids in serum-free suspension culture. Oct3/4, Sox2, Klf4 and c-Myc were showed highly expression in miPS-LLCev cells, especially spheroids of the cells. More importantly, the primary culture cells could generate similar liposarcomas with parental tumors by secondary transplantation. All the above indicated the self-renewal capacity of the miPS-LLCev cells. And using immunostaining against to diverse markers of different differentiation stages showed the heterogeneous of hierarchical population with regard to the adipocyte lineage, suggesting the differentiation capacity of miPS-LLCev cells. Thus, the miPS-LLCev was proved to be a tumor cell line with cancer stem cell (CSC) properties. In general, tumor-derived extracellular vesicles could convert normal stem/progenitor cells to CSCs.

3) miPS-LLCcm cells, which established by culturing with conditioned medium of Lewis Lung Carcinoma cell line, could give rise to endothelial cells with highly expression of endothelial markers. And the differentiated cells could form vessel-like structures *in vitro* and *in vivo*. The depletion of endothelial cells result in the loss of the differentiation potential into endothelial cells, suggesting the endothelial cells could serve as a tumor niche to regulate the differentiation capacity of CSCs.

5.2 Future challenges

CSC niche concerns two problems, CSC origination and regulation. Our new findings revealed that the tumor-derived extracellular vesicles converted mouse iPS cells to CSCs. Future studies will focus on the critical components in EVs, which contribute to the oncogenesis, such as onco-proteins and microRNAs. And the mechanism of CSC behaviors regulated by self-generated diverse CSCs niche will acquire more attention, especially the differentiation induction of CSCs, which will be a novel strategy of cancer therapy against CSCs.

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List of publications

Papers

1. Characterization of Cancer Stem-Like Cells Derived from Mouse Induced Pluripotent Stem Cells Transformed by Tumor-Derived Extracellular Vesicles.
   Ting Yan, Akifumi Mizutani, Ling Chen, Mai Takaki, Yuki Hiramoto, Shuichi Matsuda, Tsukasa Shigehiro, Tomonari Kasai, Takayuki Kudoh, Hiroshi Murakami, Junko Masuda, Mary J. C. Hendrix, Luigi Strizzi, David S. Salomon, Li Fu, Masaharu Seno. *J Cancer* 2014; 5(7):572-584. doi:10.7150/jca.8865.

2. Mutual dependence between cancer stem cells and their progenies: the niche created by the progenies is sustaining cancer stem cells.
   Ting Yan, Akifumi Mizutani, Shuichi Matsuda, Hiroshi Murakami, Tomonari Kasai, Masaharu Seno. *Cancer Cell & Microenvironment* 2014; 1(4).

3. Cancer stem cells maintain a hierarchy of differentiation by creating their niche.
   Shuichi Matsuda*, Ting Yan*, Akifumi Mizutani, Tatsuyuki Sota, Yuki Hiramoto, Marta Prieto-Vila, Ling Chen, Ayano Satoh, Takayuki Kudoh, Tomonari Kasai, Hiroshi Murakami, Li Fu, David S. Salomon, Masaharu Seno. *Int J Cancer* 2014; 135: 27-36.

4. Mouse induced pluripotent stem cell microenvironment generates epithelial-mesenchymal transition in mouse Lewis lung cancer cells.
   Ling Chen, Akifumi Mizutani, Tomonari Kasai, Ting Yan, Guoliang Jin, Arun Vaidyanath, Bishoy YA El-Aarag, Yixin Liu, Takayuki Kudoh, David S Salomon, Li Fu, Masaharu Seno. *Am J Cancer Res.* 2014; 4(1): 80–88.

5. Eosinophil cationic protein enhances stabilization of b-catenin during cardiomyocyte differentiation in P19CL6 embryonal carcinoma cells.
   Guoliang Jin, Akifumi Mizutani, Takayuki Fukuda, Takayuki Otani, Ting Yan, Marta Prieto-Vila, Hiroshi Murakami, Takayuki Kudoh, Satoshi Hirohata, Tomonari Kasai, David S. Salomon, Masaharu Seno. *Molecular*

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6. Eosinophil cationic protein enhances cardiomyocyte differentiation of P19CL6 Embryonal carcinoma cells by stimulating the FGF receptor signaling pathway.
   Guoliang Jin, Akifumi Mizutani, Takayuki Fukuda, Ling Chen, Keisuke Nakanishi, Ting Yan, Takayuki Kudoh, Satoshi Hirohata, Tomonari Kasai, Hiroshi Murakami, David S. Salomon, Masaharu Seno. *Growth Factors,* 2012; 30(5): 344-355.

Oral presentations

1. がん由来エクソソーム/微小小胞で iPS 細胞から誘導したがん幹細胞様細胞の解析。
   Ting Yan, Akifumi Mizutani, Hiroshi Murakami, Masaharu Seno.
   日本癌学会，第 73 回日本癌学会学術総会，2014.09.

2. Tumor derived exosomes/microvesicles convert mouse induced pluripotent stem cells to cancer stem cells.
   Ting Yan, Akifumi Mizutani, Ling Chen, Hiroshi Murakami, Masaharu Seno.
   Select Biosciences, The 4th Exosomes and Single Cell Analysis Summit, 2014.09.

3. Tumor derived exosomes/microvesicles convert mouse iPS cells to cancer stem-like cells.
   Ting Yan, Akifumi Mizutani, Hiroshi Murakami, Masaharu Seno.
   Institute of Complex Medical Engineering, The 2014 ICME International Conference on Complex Medical Engineering, 2014.06.27.

4. Characterization of cancer stem-like cells derived from mouse induced pluripotent stem cells transformed by tumor-derived exosomes/microvesicles.
   Ting Yan, Junko Masuda, Akifumi Mizutani, Ling Chen, Tsukasa Shigehiro, Shuichi Matsuda, Tomonari Kasai, Takayuki Kudoh, Hiroshi Murakami, Mary J.C. Hendrix, Luigi Strizzi, David S. Salomon, Li Fu, Masaharu Seno.
   American Association for Cancer Research, Annual meeting of American Association for Cancer Research, 2014.04.08.

5. Cancer stem cells maintain a hierarchy of differentiation by creating their niche.
   Akifumi Mizutani, Shuichi Matsuda, Ting Yan, Marta Prieto-Vila, Ling

Chen, Ayano Satoh, Tomonari Kasai, Junko Masuda, Takyuki Kudoh, Hiroshi Murakami, Li Fu, David S. Salomon, Masaharu Seno.
American Association for Cancer Research, Annual meeting of American Association for Cancer Research, 2014.04.08.

6. Characterization of cancer stem-like cell converted from mouse iPS cell by tumor derived exosome/microvesicle.
Ting Yan, Akifumi Mizutani, Ling Chen, Yuki Hiramoto, Mai Takaki, Hiroshi Murakami, Masaharu Seno.
The Molecular Biology Society of Japan, The 36th Annual Meeting of the Molecular Biology Society of Japan, 2013.12.03.

7. Characterization of cancer stem-like cells converted from mouse iPS cells by tumor derived exosomes/microvesicles.
Ting Yan, Akifumi Mizutani, Hiroshi Murakami, Masaharu Seno.
The Society of Biocatalysis Japan, 第17回生体触媒化学シンポジウム, 2013.12.21.

8. In vitro niche created by cancer stem-like cells derived from mouse induced pluripotent stem cell.
Akifumi Mizutani, Shuichi Matsuda, Ting Yan, Tatsuyuki Sota, Tomonari Kasai, Takayuki Kudoh, Hiroshi Murakami, Masaharu Seno.
American Association for Cancer Research, Annual meeting of American Association for Cancer Research, 2013.04.09.

9. miPS-CSC maintains self-renewal and differentiation capacity in vitro by creating a niche.
Ting Yan, Shuichi Matsuda, Akifumi Mizutani, Hiroshi Murakami, Masaharu Seno.
The 5th International Symposium for Future Technology Creating Better Human Health and Society. 2013.02.07.

10. Cancer stem-like cells derived from mouse induced pluripotent stem cell create a niche to promote self-renewal by themselves.
Ting Yan, Shuichi Matsuda , Marta Prieto Vila, Akifumi Mizutani, Hiroshi Murakami, Masaharu Seno.
The Molecular Biology Society of Japan, The 35th Annual Meeting of the Molecular Biology Society of Japan, 2012.12.12.

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