Analysis of Tumor Vasculature developed from Cancer Stem Cells.

September 2016

Marta Prieto-Vila

Graduate School of Natural Science and Technology
(Doctor Course)

OKAYAMA UNIVERSITY
Abstract

To grow beyond a size of approximately 1-2 mm³, tumor cells activate many processes to develop blood vasculature. Growing evidences indicate that the formation of the tumor vascular network is very complex, and is not restricted to angiogenesis. Cancer cell-derived tumor vasculatures have been recently described. Among them, endothelial differentiation of tumor cells have been directly related to cancer stem cells, which are cells within a tumor that possess the capacity to self-renew, and to exhibit multipotential heterogeneous lineages of cancer cells. Vasculogenic mimicry has been described to be formed by cancer cells expressing stemness markers. Thus, Cancer Stem Cells have been proposed to contribute to vasculogenic mimicry, though its relation is yet to be clarified. Here, we analyzed the tumor vasculature by using a model of mouse cancer stem cells, miPS-LLCcm cells, which we have previously established from mouse induced pluripotent stem cells and we introduced the DsRed gene in miPS-LLCcm to trace them in vivo. Various features of vasculature were evaluated in ovo, in vitro, and in vivo. The tumors formed in allograft nude mice exhibited angiogenesis in chick chorioallantoic membrane assay. In those tumors, along with penetrated host endothelial vessels, we detected endothelial differentiation from cancer stem cells and formation of vasculogenic mimicry. The angiogenic factors such as VEGF-A and FGF2 were expressed predominantly in the cancer stem cells subpopulation of miPS-LLCcm cells. Our results suggested that cancer stem cells play key roles in not only the recruitment of host endothelial vessels into tumor, but also in maturation of endothelial lineage of cancer stem cell’s progenies. Furthermore, the undifferentiated subpopulation of the miPS-LLCcm participates directly in the vasculogenic mimicry formation. Collectively, we show that miPS-LLCcm cells have advantages to further study tumor vasculature and to develop novel targeting strategies in the future.
# Contents

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Chapter 1. General introduction</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Chapter 2. DsRed-LLCcm is an improved model from miPS-LLCcm cells.</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>Introduction</td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>Materials and methods</td>
<td></td>
<td>18</td>
</tr>
<tr>
<td>Results</td>
<td></td>
<td>19</td>
</tr>
<tr>
<td>Discussion</td>
<td></td>
<td>22</td>
</tr>
<tr>
<td>Chapter 3. miPS-LLCcm and DsRed-LLCcm cells release factors that induce angiogenic response</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>Introduction</td>
<td></td>
<td>27</td>
</tr>
<tr>
<td>Materials and methods</td>
<td></td>
<td>27</td>
</tr>
<tr>
<td>Results</td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>Discussion</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>Chapter 4. miPS-LLCcm cells differentiate into endothelial cells <em>in vitro</em> and <em>in vivo</em>.</td>
<td></td>
<td>41</td>
</tr>
<tr>
<td>Introduction</td>
<td></td>
<td>43</td>
</tr>
<tr>
<td>Materials and methods</td>
<td></td>
<td>43</td>
</tr>
<tr>
<td>Results</td>
<td></td>
<td>45</td>
</tr>
<tr>
<td>Discussion</td>
<td></td>
<td>51</td>
</tr>
<tr>
<td>Chapter 5. DsRed-LLCcm cells participate in the formation of Vasculature Mimicry</td>
<td></td>
<td>57</td>
</tr>
<tr>
<td>Introduction</td>
<td></td>
<td>59</td>
</tr>
<tr>
<td>Materials and methods</td>
<td></td>
<td>59</td>
</tr>
<tr>
<td>Results</td>
<td></td>
<td>60</td>
</tr>
<tr>
<td>Discussion</td>
<td></td>
<td>62</td>
</tr>
</tbody>
</table>
Chapter 6. General conclusions and futures challenges 65

List of publications 69

Acknowledgements 71
CHAPTER 1

General Introduction
1.1 Cancer Stem Cell concept and concern.

Cancer stem cells (CSCs) are cells within a tumor that possess the capacity to self-renew and to exhibit multipotential heterogeneous lineages of cancer cells that comprise the tumor [1].

The CSC hypothesis was firstly postulated more than 150 years ago [2], and cancer cells were thought to arise from germ cells, however this hypothesis was forgotten and not reconsidered and intense researched until in 1997 when John Dick described cancer stem cells in leukemia. [3]

CSC are thought to be responsible for various cancer pathologies, such as metastasis or recurrence due to CSC exhibiting higher drug resistance compared to other tumor cells. And although the primary tumors may be eliminated from the patient with the treatment, the tumor reappears after a period of time, arising from the small subpopulation of CSC that survived, either in the primary location or a new position after metastasis (Figure 1).

**Figure 1. Scheme of tumor relapse due to Cancer stem cells.** After treatment most of tumor cells die, however a small number of cells that are more resistant to the treatment, called cancer stem cells. Those, while self-renewing to maintain the CSC subpopulation (up), are also able to differentiate again (right), generating a tumor on the same place. CSC can also migrate to distant places with blood stream and generate a new tumor there (down).

Normal stem cells and CSC shares several properties, including the capacity of self-renew, differentiation capacity, the active telomerase expression, the activation of
antiapoptotic pathways, the increase of membrane transporter activity and the ability to migrate [2, 4]. However, the origin of cancer stem cells is yet to be clarified. Fialow et al. showed that 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], evidencing that normal tissue stem cells are the target of mutational accumulation resulting in CSC, which afterwards differentiate to heterogeneous tumor cell population. On the other hand recent studies showed that CSC can arise from progenitor cells which acquired self-renewal capacity and generate more differentiate progenies [6, 7].

During the last 20 years, CSCs have been described and isolated from various cancers [8-12] allowing the CSC hypothesis to be increasingly accepted.

### 1.2 Tumor niche.

Normal stem cells in the body are responsible for the organic generation, due to their capacity to self-renew as well as differentiate into various lineages that allows for the preservation of homeostatic pool, necessary for maintenance of a tissue for the life of an organism. Without stem cells the tissue organ would eventually degenerate [13]. A refined balance between these two opposite processes is significant for the proper maintenance and repair [14], therefore the expansion of stem cells pool is restricted to prevent uncontrolled growth [15]. This control is done by the stem cell niche. Niches are specialized microenvironments that regulate adult stem cells fate by providing cues in the form of both cell-cell contacts and secreted factors. They are comprised of fibroblastic cells, immune cells, endothelial and perivascular cells, extracellular matrix (ECM) components and networks of cytokines and growth factors [16, 17].

CSCs, as well as normal stem cells, due to their similarities, are believed to reside in niches [17]. Cells within the CSC niche produce factors that stimulate CSC self-renewal, induce angiogenesis and recruit immune and other stromal cells that, at the same time, those will secrete additional factors to promote tumor cell invasion and metastasis [18, 19].

Among all the elements in tumor niche, tumor vasculature is an essential factor since
vessels are in charge of the supply of nutrients and oxygen to sustain tumor tissues.

1.3 CSCs in tumor vasculature

Considering the tumor vasculature is one element from CSCs niche, it is to assume that CSC would participate either directly or indirectly in tumor vasculature. Several groups reported that the CSCs reside adjacent to the tumor blood vessels, which termed the vascular CSC niche [20]. For example, endothelial cells (EC) were proved to reside adjacent to nestin positive cells in glioblastoma, promoted the growth of tumor cell population and self-renewal of the CSCs inside [20, 21] and sustained the overcoming of CSCs from radiotherapy [22], suggesting that the EC cells play a role in CSC niche. Its seems to be regulated by several pathways in the microenvironment that the EC encourage the maintenance of CSC, such as Notch signaling pathway [21]. 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.

One of the most significant findings regarding tumor vasculogenesis and CSCs is the direct differentiation of glioblastoma CD133+ CSCs into vascular endothelial cells expressing CD31 when these cells were injected into nude mice. Moreover, when CSC were killed, tumor growth was greatly suppressed, indicating that EC directly contribute to blood vessel formation in glioblastoma [23 24]. Additionally, breast and renal CSCs differentiated after 14 days of incubation under hypoxia and with VEGF containing complete medium in vitro [25].

In addition to the direct differentiation into endothelial cells, the high degree of plasticity of CSCs and the fact that cells lining the channels express stemness-related genes [26, 27] indicate that CSCs might be involved in Vasculogenic mimicry (VM). VM are vascular channels that supply blood to the tumor is lined not by EC, but tumor cells itself, and is rich in ECM. [28, 29] Despite the undifferentiated state of cells lining the VM channels, currently there is no clear evidence that demonstrates the direct relation of CSCs and VM

The processes of tumor vasculogenesis should therefore be investigated with the
consideration of the properties of CSCs. To understand the entire process of tumor vasculogenesis, our laboratory developed a model of CSCs, which formed highly angiogenic tumors in nude mice, and exhibited the capacity of differentiation into endothelial cells *in vitro* [30]. However, the origin of the cells in the vascular structures in the tumor formed by miPS-LLCcm has not been assessed directly. In this study, I evaluated angiogenesis, CSC’s differentiation into ECs, and VM, along with their possible correlations during the vasculature development in the tumors of miPS-LLCcm cells.

1.4 References


[16] Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to


CHAPTER 2

DsRed-LLCcm is an improved model from miPS-LLCcm cells.
2.1 Introduction

We have previously established models of CSCs, that were spontaneously converted from mouse induced pluripotent stem cells (miPS) cultured in the presence of conditioned medium (CM) from various cancer cell lines. We reported previously that miPS-LLCcm, a representative miPS-CSC converted with CM of Lewis lung carcinoma (LLC) cells or LLC-derived microvesicles/exosomes (Figure 1) [1, 2]. We have proposed that these models are very promising to study cancer’s nature from the aspect of CSCs properties, such as the relation between CSCs and differentiated cancer cells [1, 3]. miPS-LLCcm cells generated malignant, highly vascularized tumors when they were transplanted in nude mice. In addition, we could reveal the differentiation capacity of miPS-LLCcm cells into endothelial cells in vitro [2, 3].

![Diagram of miPS-LLCcm generation](image_url)

**Figure 1. Scheme of miPS-LLCcm generation.** miPS cells were cultured without feeder cells and one day without LiF, before the conversion. During the conversion, the CM was daily changed up to one month. After that the resulting cells were injected into nude mouse, where they generated malignant tumor.
In the previous study, however, it remained unclear whether the cells forming the vasculature in the tumor are derived from miPS-LLCcm or not. To confirm whether the cells forming the vasculature in the tumor originated from miPS-LLCcm cells or host mouse cells, a previous student, Yan Ting, established DsRed-LLCcm cells introducing pEF-DsRed into miPS-LLCcm cells. Since miPS-LLCcm cells were derived from Nanog-GFP-iPSCs [4], the undifferentiated state could be monitored by the expression of GFP together with that of DsRed. On the other hand, the cells differentiated from miPS-LLCcm cells would display only DsRed fluorescence.

Once DsRed-LLCcm cells were established, their characteristic had to be analyzed to confirm whether DsRed-LLCcm cells maintained miPS-LLCcm properties.

2. 2 Materials and methods

2.2.1 Construction of DsRed expression vector.

The DsRed2 gene was amplified from pCI-EGFP/DsRed2-puro by PCR with a primer pair; EcoRI-DsRed (5’-CCGGAATTCATGCTCTCTCC-3’) and SalI-DsRed (5’-TCCGGTCGACCTACAGGAACAG-3’) to add EcoRI and SalI sites to the 5’ and 3’ end of Ds-Red gene, respectively. Then, the PCR product was cloned downstream of EF1 constitutive promoter in pEF-EX-HA vector, which was constructed by adding an HA tag to pEF-BOS-EX [5] with the similar construction procedure of Flag tag described in [6], to create pEF-DsRed. For pEF-neo plasmid construction, the G418 resistant gene was amplified from pST-Neo by PCR with a primer pair EcoRI-neo (5’-GCGGGAATTCATGTGAACAGATGGA-3’) and SalI-neo (5’-TGTAGTCGACGCAAGAACTCGTCAAG-3’) to add EcoRI and SalI sites to the 5’ and 3’ sites of the gene. Amplified gene was cloned into pEF-EX-HA.

2.2.2 Cell culture and transfection.

miPS-LLCcm and DsRed-LLCcm cells were cultured in DMEM containing 15% FBS, 0.1 mM MEM Non-Essential amino acids, (100X NEAA, Gibco, NY), 2 mM L-Glutamine (Nacalai Tesque, Japan), 0.1 mM 2-mercaptopethanol, 50 U/ml penicillin/streptomycin and 1000 U/mL and without LIF. All the cells were maintained
at 37 ºC in the atmosphere of 5% CO₂. Medium was changed every two days.

For transfection, 1x10⁷ miPS-LLCcm cells, which had been maintained in suspension culture for 8 days, in 600µL electroporation buffer for ES cells (Millipore, MA) were mixed with 1µg of linearized pEF-neo DNA and 10 µg of linearized pEF-DsRed DNA. Then, the cell suspension was transferred into a 0.4cm gap-cuvette and electroporated by using Gene Pulser II (Bio Rad Labs, VA). The cells were then plated into gelatin-coated dishes without antibiotics for 2 days. Then the DsRed positive transfectants (termed DsRed-LLCcm) were established by culturing for 1 to 2 weeks in the presence of G418 at a concentration of 0.3 mg/mL.

2.2.3 Sphere formation assay.
4x10⁴ cells (1x10⁴ cells/ml) were seeded on 6cm ultra low attachment dishes (Corning incorporated, NY) with the miPS-LLCcm media without FBS, but supplied with Insulin-Transferrin-Selenium-X (ITS-X, Life Technologies, CA). Cells were cultured at 37 ºC under 5% CO₂ for 4 days, and the spheroids with diameters above 100 µm were judged as self-renewing spheroids.

2.2.4 In vitro Tube formation assay
5x10⁴ cells were seeded on growth factors reduced Matrigel (Corning, NY) -coated 96well plate and cultured in 50uL of EBM2 media (EBM-2 Single Quots Kit, Lonza, Switzerland) for 24hr in the presence of angiogenic factors (FBS, hydrocortisone, hFGF-B, VEGF, R3-IGF-1, hEGF, ascorbic acid, GA-1000 and heparin). The experiments were performed in triplicate. Images of formed tubes were captured by Olympus CKX41 microscope, or Keyence BZ-X700.

2.3 Results

2.3.1 DsRed-LLCcm are equivalent to miPS-LLCcm cells.
The newly established DsRed-LLCcm cells are an improved model of CSC derived from miPS-LLCcm. Since miPS-LLCcm cells were derived from Nanog-GFP-iPSCs [4], the undifferentiated state could be monitored by the expression of GFP. DsRed-
LLCcm introduced the DsRed2 gene expressed constitutively, that allowed to trace the cells even when they differentiate (Figure 2.1).

miPS-LLCccm have been described to generate malignant, highly vascularized tumors when they were transplanted in nude mice and, in vitro to present self-renewal and differentiation capacity of miPS-LLCccm cells into endothelial [2,3]. First I evaluated whether DsRed-LLCccm maintained miPS-LLCccm properties.

Self-renewal was assessed by sphere formation assay [7]. DsRed-LLCccm cells were able to form spheres under non-adherent conditions as miPS-LLCccm did (Figure 2.2). All the spheres derived from DsRed-LLCccm were GFP and DsRed positive. Supporting their stemness state, the expression levels of endogenous Oct3/4, Sox2, Klf4 and c-Myc were comparable to those in miPS-LLCccm cells (Figure 2.3). DsRed-LLCccm cells were also able to generate tube-like structures when the cells were seeded on Matrigel (Figure 2.4). Similar to miPS-LLCccm cells, this tube formation was not dependent on the supplement of endothelial cell growth factor such as VEGF or FGF (Chapter 3, Figure 2.5). The entire tubular structures possessed DsRed fluorescence. In contrast, GFP positive cells were found to be concentrated on the nodules of the tubes, where two or more tubular structures are connected (Figure 2.4). As described below in Chapter 3, and as miPS-LLCccm cells do, DsRed-LLCccm cells generated malignant and highly vascularized tumors (Chapter 3, Figure 1).

Taken all together, I could confirm that DsRed-LLCccm cells remained the characteristics of miPS-LLCccm cells.
Figure 2.1 Morphology of miPS-LLCc m and DsRed-LLCc m cells. Under the control of EF1 promoter, DsRed was expressed constitutively, while GFP was only expressed in undifferentiated cells. Scale bar: 50µm.

Figure 2.2 Sphere formation of DsRed-LLCc m cells. All the spheres are GFP+ and
DsRed⁺, supporting ability to self renewal of undifferentiated cells. Scale bar: 100µm.

Figure 2.3 Expression levels of Sox2, Oct3/4, Klf4, and C-myc in DsRed-LLCcM cells analyzed by RT-qPCR. The expressions of those genes in DsRed-LLCcM cells were equivalent to those in miPS-LLCcM cells (Sox2 p=0.71; Oct3/4 p=0.45; Klf4 = 0.20; c-myc p= 0.73). Expression values are normalized to GAPDH

Figure 2.4 In vitro tube formation of DsRed-LLCcM cells. While all the cells expressed DsRed, the GFP+ cells were found mostly in the nodules. Scale bar: 200µm.
2.4 Discussion

The growing evidences indicate that formation of the tumor vascular network is a complex event in the cancerous niche [8]. Recently two models of tumor vasculogenesis, CSC differentiation into EC and vasculogenic mimicry, emphasize the contribution of CSCs towards tumor vascularization [9,10]. To analyze the relationship of the two models, we adopted our miPS-LLCcm cell, which is a model of CSC that exhibits self-renewal [7], differentiation and ability to generate a malignant tumor in nude mice [1,3]. First of all, we introduced DsRed2 gene into miPS-LLCcm cells, establishing DsRed-LLCcm cells, which besides from the GFP fluorescence under the expression of Nanog, constitutively display red fluorescence. The introduction of DsRed2 gene represents an improvement of miPS-LLCcm model since it allows us to trace miPS-LLCcm throughout the cell differentiation and localization both in vitro and in vivo. Studies in self-renewal and differentiation capacity along with tumorigenicity, indicated that the insertion of DsRed2 gene did not affect miPS-LLCcm properties.

2.5 References


pluripotent stem cells. Nature. 2007; 448:313-317


miPS-LLCcm and DsRed-LLCcm cells release factors that induce angiogenic response
3.1 Introduction

Tumors can only grow to a size of approximately 1-2mm³ before reaching their metabolic demand [1]. To grow beyond this size, tumor cells undergo the process known as angiogenesis [1]. Angiogenesis, firstly described by Folkman in 1971 [2], is a progressive, multistep physiological process by which new blood vessels are developed from preexisting vessels [3]. These vessels are in charge of the supply of nutrients and oxygen to sustain tumor tissues.

Tumor angiogenesis starts with the activation of endothelial cells (EC) by inflammatory cytokines and angiogenic factors released by tumor cells, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and platelet derived growth factor (PDGF), which stimulate a cascade of endothelial changes and promote them to secrete several proteases and plasminogen activators, resulting in the degradation of the vessel basement membrane, allowing EC to invade the surrounding membrane [4, 5]. Then, cells proliferate and eventually migrate to form new vessels. Finally, the endothelial cells deposit a new basement membrane and secret growth factors that attract support cells such as pericytes, ensuring the stability of the new vessels [6].

Many studies showed the importance of CSC in angiogenesis, secreting angiogenic factors such as VEGF [7-10]. In previous studies we observed that miPS-LLCcm exhibited highly vascularized tumors [11]. However, the origin of these vessels still remains unknown. Taking in consideration all the studies, I hypothesized that miPS-LLCcm could actively trigger angiogenesis.

3.2 Materials and methods

3.2.1 Cell culture.

Mouse Lewis Lung Carcinoma (LLC) cells were maintained in Dulbecco’s Modified Eagle’s Medium-high glucose (DMEM, Sigma, MO) containing 10% FBS (Gibco, NY) and 100U/mL penicillin/streptomycin (Wako, Japan).

miPS cells were cultured in DMEM containing 15% FBS, 0.1 mM MEM Non-
Essential amino acids, (100X NEAA, Gibco, NY), 2 mM L-Glutamine (Nacalai Tesque, Japan), 0.1 mM 2-mercaptoethanol, 50 U/ml penicillin/streptomycin and 1000 U/mL Leukemia inhibitory factor (LIF, Millipore, MA).

miPS-LLCcm cells and DsRed-LLCcm cells were cultured in miPS media without LIF.

All the cells were maintained at 37 °C in the atmosphere of 5% CO₂ and medium was changed every two days.

### 3.2.2 Animal experiments

Nude mice (Balb/c-nu/nu, female, 4 weeks old) were purchased from Charlesriver, Japan. For transplantation studies, 1x10⁶ cells were suspended in 200µL in the miPS-media and injected subcutaneously in both flanks of mice. Tumor volumes were measured every 3-4 days and calculated as following formula (0.5 x longer diameter x shorter diameter²). After 5 weeks, tumors were extracted and separated in equal parts that were used for the histological analysis, immunofluorescent analysis, and CAM assay experiment as described below.

The plan of animal experiments was reviewed and approved by the ethics committee for animal experiments of Okayama University under the ID OKU-2013252 and OKU-2016078.

### 3.2.3 Chick embryo chorioallantoic membrane (CAM) Assay

Fertilized eggs were obtained from Yamagishi (Mie, Japan), and sterilized with ethanol 70% to a posteriori be incubated at 37 °C in 60 % of humidity in a incubator P-008(B) (Showa Furani, Japan). After acclimatization for 2 days in the incubator, 3 mL of egg white was extracted using an 18G hypodermic needle and 5 mL syringe generating an air sac directly over the chick embryo chorioallantoic membrane (CAM). On the day 8, an approximately 1 cm² window was opened in the shell of an egg and the 5 mm³ portions of the tumor extracted from the grafted mice was collocated over the CAM with sterilized plastic ring. For the control, only the plastic ring was located on CAM. The window was sealed with transparent tape, then, the eggs were incubated. Images of vasculature were taken on day 12 after injecting 2 mL of 20% Intralipos (Otsuka Pharmaceutical, Japan) under the membrane to increase the contrast.
3.2.4 Histological analysis and Immunohistochemistry.

Extracted tumors were enveloped with paraffin and sectioned at 5 µm of thickness. After deparaffinization, sections were stained with hematoxylin-eosin (Hematoxylin solution, Sigma-Aldrich, MO; 0.5% Eosin Y, Sigma Aldrich, MO) for histological analysis. For immunohistochemistry, antigen retrieval was carried out by boiling in 10mM citrate sodium (pH6) with 0.05 % Tween20 for 15min. After cooling down the samples, the endogenous peroxidase was blocked with 3 % H2O2 for 5 min. Ellite anti-rabbit ABC staining Vectastain kit (Vector, MI, ) and 3, 30-diaminobenzidine tetrahydrochloride (DAB, Vector, MI) were used for detection of Ki67 and DsRed with rabbit monoclonal Ki67 (1:200, #ab66155, Abcam, UK) and rabbit polyclonal anti-DsRed (1:100, #ab62341, Abcam, UK), respectively. Counter staining was carried out using hematoxylin.

3.2.5 Immunofluorescence analysis of tumor tissue.

The freshly extracted tumor tissue samples were embedded with Tissue-Tek OCT compounds (Sakura Finetek, CA), frozen and sectioned into the 10µm thickness. Cryosections were fixed with Paraformaldehyde phosphate buffer solution 4% (PFA, Nacalai Tesque, Japan) for 20 min at room temperature, followed by permeabilization with 0.05 % Tween20 in PBS (PBS-T). After blocking with PBS-T containing 5% BSA, cryosections were incubated with primary antibodies rat monoclonal anti-PECAM1 (1:50, sc-101454, Santa Cruz) and rabbit polyclonal anti-DsRed (1:100, ab62341, Abcam) for overnight at 4ºC. Following washes with PBS-T, sections were incubated with secondary antibodies Texas Red-X conjugated goat anti-rat IgG (1:500, #6392, Invitrogen, CA) and Alexa Fluor 488 donkey anti rabbit IgG (1:500, #A21206, Life Technologies, CA). Nuclei were counterstained with 4’,6-diamidino-3-phenylidole, dihydrochloride (DAPI, Vector, MI).

Images were acquired using an Olympus IX81 microscope equipped with a light fluorescence device (Olympus, Japan).

3.2.6 RNA extraction, cDNA synthesis and quantitative real time PCR.

Total RNA from cells was isolated using RNeasy Mini Kit (QIAGEN, Germany), then treated with DNase I (Takara, Japan). 1µg of RNA was reverse transcribed using SuperScript III First strand kit (Invitrogen, CA).

Quantitative real-time PCR was performed with Cycler 480 SYBR Green I Master
mix (Roche, Switzerland) according to manufacturer’s instructions. Primers used for the real time qPCR were as following (forward and reverse). VEGF-A 5’-ATGAACTTTCTGCTCTCTTGGGTGC-3’ and 5’-CATGGGACTTCTGCTCTCCTTCTG-3’; FGF2 5’-CCTTCCCACCAGGCCACTTCAA-3’and 5’-GGTCCCGTTTTGGATCCGAGTTT-3’. Gene expression level was normalized with that of GAPDH mRNA.

### 3.2.7 In vitro Tube formation assay

5x10⁴ cells were seeded on growth factors reduced Matrigel (Corning, NY) -coated 96well plate and cultured in 50uL of EBM2 media (EBM-2 Single Quots Kit, Lonza, Switzerland) for 24hr in the presence or absence of angiogenic factors (FBS, hydrocortisone, hFGF-B, VEGF, R3-IGF-1, hEGF, ascorbic acid, GA-1000 and heparin). The experiments were performed in triplicate. Images of formed tubes were captured by Olympus CKX41 microscope, or Keyence BZ-X700.

### 3.2.8 Statistical analysis.

All quantitative data is expressed as mean ± SD and statistically analyzed by Student’s t-test. A p-value lower than 0.05 was considered as statistically significant.

### 3.3 Results

#### 3.3.1 DsRed tumorigenicity.

Firstly the tumorigenicity and angiogenic activity of DsRed-LLCcm cells was evaluated and by transplanting 10⁶ DsRed-LLCcm cells subcutaneously into nude mice. miPS-LLCcm cells, miPS cells, and LLC cells were also transplanted for comparison. The tumor derived from LLC cells had the highest growth rate (Figure 1.1). However, on the third week the tumor showed significant necrotic features (Figure 1.2). On the other hand, tumors derived by DsRed-LLCcm and miPS-LLCcm cells, which exhibit similar growth, kept growing without any apparent signatures of necrosis, and host vessels were found to be directed towards the tumor (Figure 1.3).
When LLC tumor and DsRed-LLCcm tumor were histologically analyzed, I found that LLC tumor showed relatively homogenous structure and few and small blood vessels (Figure 1.4 left). On the other hand DsRed-LLCcm tumors constructed unorganized, heterogeneous structures with higher amount of blood vessels (Figure 1.4, right). The vessel structures containing blood cells in the three replicates of tumor of DsRed-LLCcm were significantly abundant when compared to those found in the tumor of LLC cells (Figure 1.5). These could help to explain the observation of necrosis in the LLC derived tumor, but not in the DsRed-LLCcm derived tumor which continuously grew without visible necrosis.

Further analysis of DsRed-LLCcm tumors showed that most of the cells forming the tumor expressed DsRed (Figure 1.6, right), and were stained with Ki67 antibody revealeing that the cells in the DsRed-LLCcm tumors were highly proliferative (Figure 1.6, left), supporting the rapid tumor growth as it has been previously shown in miPS-LLCcm [11].

![Figure 1.1 Growth rates of tumors.](image)

**Figure 1.1 Growth rates of tumors.** LLC (n=2), DsRed (n= 4), miPS (n=3), miPS-LLCcm (n=1). Mice tumors were measured every 3-4 days and the volume was calculated. After 2 weeks, all the cells except for miPS cells, exhibited a tumor that grew rapidly.
Figure 1.2 Images of tumors. miPS was used as a negative control. LLC cells, even they grew very fast, soon showed necrosis region that stop tumor growth. CSC-model cells (DsRed-LLCcm and miPS-LLCcm) generated big tumors that remained non-necrotic.

Figure 1.3 Host blood vessels towards the tumor. Image of tumor extraction in which host-blood vessels we found surrounding the tumor of DsRed-LLCcm.

Figure 1.4 Hematoxylin-Eosin staining images of LLC and DsRed-LLCcm derived
While LLC derived tumor showed a homogeneous structure, the tumor formed by DsRed-LLCcm cells was heterogeneous and highly angiogenic. Scale bar: 100µm.

**Figure 1.5** Blood vessel densities of LLC and DsRed-LLCcm derived tumor. Random camps (n=15) were pictured and all the channels that contained red blood vessels were counted showing that all the replicates of DsRed-LLCcm tumors contained significantly more blood vessels rather than LLC tumor.*:P<0.05.

**Figure 1.6** Immunohistochemistry analysis of DsRed and Ki67 of tumor derived from DsRed-LLCcm cells. Most of the cells were stained against DsRed and Ki67, showing that the cells within the tumor were proliferating. Scale bar: 50µm.

3.3.2 The undifferentiated subpopulation of DsRed-LLCcm secrete proangiogenic factors

Since we had observed host blood vessels surrounding the tumors derived from miPS-LLCcm and DsRed-LLCcm (Figure 1.3) and high amount of vessels within those tumors (Figure 1.4-5), I further confirmed the recruitment of preexisting blood vessels
to the tumors by using CAM assay [12]. In this study, unlike the general protocol that injects cells in a sponge, [13] I implanted a whole tumor portion of 5-mm-cube on CAM that had been freshly extracted from grafted mouse (Figure 2.1).

In CAM assay both tumors of DsRed-LLCcm and LLC promoted chick blood vessels formation when compared with the negative control (Figure 2.2). In addition, a significantly higher number of blood vessels inside the plastic ring and towards the tumor of DsRed-LLCcm cells rather than of LLC cell-derived tumor was observed (8.8±3.3 and 5.6±1.9, respectively), indicating a higher ability of recruitment of blood vessels in DsRed-LLCcm tumor (Figure 2.3).

The expression of VEGF-A and FGF2, angiogenic factors that have been described in CAM assay [14, 15] were analyzed. In accordance with CAM assay results, VEGF-A and FGF2 expression was found to be higher DsRed-LLCcm cells than that in LLC cells (Figure 2.4). Interestingly, the expression level the factors were dramatically higher when the undifferentiated subpopulation of DsRed-LLC cells was concentrated, suggesting that the expression of VEGF-A and FGF2 could be secreted from CSCs predominantly. The expression of angiogenic factors in CSCs could explain the results of the in vitro tube formation assay that were independent of exogenous angiogenic factors (Figure 2.5) [16].

![Figure 2.1 Experimental scheme of CAM assay.](image)

*Figure 2.1 Experimental scheme of CAM assay.* On day 6, a 5-mm-cube piece of freshly isolated tumor was added on the CAM, and on day 10, the pictures were taken and results analyzed.
Figure 2.2 Typical results of CAM assay. Both DsRed-LLCcm and LLC tumors promoted blood vessels formations; however, when compared, DsRed-LLCcm tumor was significantly more angiogenic than LLC tumor.

Figure 2.3 Quantitative evaluation of blood vessel number in CAM assay. Both DsRed-LLCcm and LLC tumors, promoted blood vessels formations in comparison to the negative control. DsRed-LLCcm tumor was significantly more proangiogenic than LLC tumor *: p<0.05.
Figure 2.4 Angiogenic factors expression. Expression of VEGF-A and FGF2 in DsRed-LLCcm and DsRed-LLCcm selected with puromycin in comparison to LLC cells were quantified by RT-qPCR. Expression values are normalized to GAPDH. *:p<0.05. In both factors, the expression was significantly higher in DsRed-LLCcm cells rather than LLC cells and even higher when the CSC-subpopulation was enriched.

Figure 2.5 Tube formation of miPS cells, LLC cells, miPS-LLCcm cells and DsRed-LLCcm. While miPS and LLC cells were not able to form tube formation, miPS-LLCcm and DsRed-LLCcm cells could form tubular structures independent of exogenous angiogenic factors, indicating that the cells are able to secrete these factor by themselves.
2.3.3 DsRed-LLCcm cells capacity of recruit host-derived endothelial cells

Next, I confirmed the existence of penetrated host blood vessels in the tumor generated by DsRed-LLCcm cells. The tumor was stained with anti-CD31 antibody and anti-DsRed antibody. CD31+/DsRed− cells with channel-like structure were found frequently, showing that these blood vessels were originated from host animal (Figure 3.1). Thus, the results suggest that CSCs should contribute to angiogenesis directly by secreting angiogenic factors.

**Figure 3.1** Host-derived endothelial cells within DsRed-LLCcm tumor. Immunofluorescence analysis using CD31 (red)- and DsRed (green)-antibodies in tumors derived from DsRed-LLCcm cells. The tubular structure is CD31+ but DsRed− indicating that these endothelial cells have an origin in the host animal. Scale bar: 25µm.

3.4 Discussion

The histological analysis of the tumor formed by DsRed-LLCcm cells, in comparison to the necrotic tumor formed by LLC cells, showed that their tumors were highly vascularized (Figure 1.4). This, along with the big vessels from the host found towards the tumor, prompted me to hypothesis that DsRed-LLCcm cells present high angiogenic activity (Figure 1.3).

The angiogenic activity of DsRed-LLCcm, which was further confirmed by CAM assay (Figure 2.2-3) and by the presence of host CD31+/DsRed− endothelial vessels derived from the host in the tumor (Figure 3.1), might be corroborated with the fact that miPS-LLCcm cells were able to do tube formation without exogenous angiogenic factors addition (Figure 2.5) and by the high expression of angiogenic factors in CSCs (Figure 2.4). It has been shown that Glioma CSCs, defined as the subpopulation expressing the CSC marker CD133, expressed 10 fold more VEGF than the CD133−
subpopulation in both hypoxia and normoxia, inducing the angiogenesis response [7]. This ability of CSC to secrete higher amount of VEGF has been described in breast cancer and malignant gliomas [7-10]. Although it has not been considered directly related with the secretion from CSCs, FGF2 stimulates survival, proliferation, migration and differentiation of EC. Both VEGF and FGF2 have been described to induce angiogenesis in CAM assay [14,15]. In accordance with these observations, VEGF-A and FGF2 were expressed 6 and 3 folds higher, respectively, in the undifferentiated GFP+ population of DsRed-LLCcm cells compared to the bulk culture of DsRed-LLCm cells (Figure 2.4). Thus the CSC subpopulation in DsRed-LLCm cells presents high angiogenic activity in ovo and in vivo inducing the differentiation and proliferation of ECs by the secretion of angiogenic factors such as VEGF-A or FGF2.

2.5 References


miPS-LLCcm cells differentiate into endothelial cells \textit{in vitro} and \textit{in vivo}. 
4.1 Introduction

Tumor vasculature is mainly developed through angiogenesis sprouting from preexisting vessels and vasculogenesis of tumor cells differentiating into ECs. Both angiogenesis and vasculogenesis are initiated and promoted by angiogenic factors such as VEGF, that even though it has been widely described to be produced by stromal or inflammatory cells [1, 2], in our miPS-LLCcm model is mostly secreted by our CSC subpopulation. As well as CSCs have been described to participate in angiogenesis [3-6] recent studies reported them in other kinds of tumor vasculature.

One of the most significant findings regarding tumor vasculogenesis and CSC is the direct differentiation of those into endothelial cells to fuel the fast tumor growth. For example, glioblastoma stem cells, expressing the stemness marker CD133+ were found to differentiate into vascular endothelial cells expressing CD31 when these cells were injected into nude mice [7, 8]. Furthermore, environmental changes, such us hypoxia or glucose deprivation, induced CSCs to acquire EC markers including CD31, CD34, and vWF [9]. Additionally, breast and renal CSCs differentiated after 14 days of incubation under hypoxia and with VEGF containing complete medium in vitro [10].

In previous studies our laboratory showed that miPS-LLCcm cells were able to form tube formation when cells were seeded on matrigel, and contained CD31positive cells. Thus here, I assessed further this differentiation capacity in vitro and in vivo.

4.2 Materials and methods

4.2.1 Cell culture.

miPS-LLCcm and DsRed-LLCcm cells were cultured in DMEM containing 15% FBS, 0.1 mM MEM Non-Essential amino acids, (100X NEAA, Gibco, NY), 2 mM L-Glutamine (Nacalai Tesque, Japan), 0.1 mM 2-mercaptoethanol, 50 U/ml penicillin/streptomycin and 1000 U/mL and without LIF. All the cells were maintained at 37 ºC in the atmosphere of 5% CO2. Medium was changed every two days.

For selection of GFP-positive undifferentiated cells of miPS-LLCcm and DsRed-LLCcm, cells were cultured in the miPS-LLCcm medium containing 1 mg/mL
puromycin for 7 days, changing the medium daily [11, 12].

**4.2.2 In vitro Tube formation assay.**

5x10^4 cells were seeded on growth factors reduced Matrigel (Corning, NY) -coated 96well plate and cultured in 50uL of EBM2 media (EBM-2 Single Quots Kit, Lonza, Switzerland) for 24hr in the presence or absence of angiogenic factors (FBS, hydrocortisone, hFGF-B, VEGF, R3-IGF-1, hEGF, ascorbic acid, GA-1000 and heparin). The experiments were performed in triplicate. Images of formed tubes were captured by Olympus CKX41 microscope, or Keyence BZ-X700.

**4.2.3 Sphere formation assay.**

4x10^4 cells (1x10^4 cells/ml) were seeded on 6cm ultra low attachment dishes (Corning incorporated, NY) in miPS-LLCcm media without FBS, but supplied with Insulin-Transferrin-Selenium-X (ITS-X, Life Technologies, CA). Cells were cultured at 37 ºC under 5% CO_2 for 4 days, and the spheroids with diameters above 100 μm were judged as self-renewing spheroids.

**4.2.4 Animal experiments.**

Nude mice (Balb/c-nu/nu, female, 4 weeks old) were purchased from Charlesriver, Japan. For transplantation studies, 1x10^6 cells were suspended in 200µL in the miPS-media and injected subcutaneously in both flanks of mice. Tumor volumes were measured every 3-4 days and calculated as following formula 0.5x (longer diameter x shorter diameter^2). After 5 weeks, tumors were extracted and frozen for immunofluorescent analysis, experiment as described below.

The plan of animal experiments was reviewed and approved by the ethics committee for animal experiments of Okayama University under the ID OKU-2013252 and OKU-2016078.

**4.2.5 Immunofluorescence analysis.**

For staining of in vitro formed tubes, 1.5x10^5 cells were seeded on 8-well chamber slides (Corning, NY) coated with matrigel and for the staining of in vitro culture, cells were seeded on gelatin-coated cover glasses. After incubation, cells were fixed, permeabilized, and stained with rabbit polyclonal anti-CD31 antibody (1:100, #ab28364, Abcam, UK) and Alexa Fluor 555 conjugated anti-rabbit IgG (1:500, #A21428, Life
technologies, CA). Nuclei were counterstained with DAPI (Vector, MI).

For tissue samples, freshly extracted tumors were embedded with Tissue-Tek OCT compounds (Sakura Finetek, CA), frozen and sectioned into the 10µm thickness. Cryosections were fixed with Paraformaldehyde phosphate buffer solution 4% (PFA, Nacalai Tesque, Japan) for 20 min at room temperature, followed by permeabilization with 0.05 % Tween20 in PBS (PBS-T). After blocking with PBS-T containing 5% BSA, cryosections were incubated with primary antibodies (rat monoclonal anti-PECAM1 (1:50, sc-101454, Santa Cruz) and rabbit polyclonal anti-DsRed (1:100, ab62341, Abcam) for overnight at 4ºC. Following washes with PBS-T, sections were incubated with secondary antibodies Texas Red-X conjugated goat anti-rat IgG (1:500, #6392, Invitrogen, CA) and Alexa Fluor 488 donkey anti rabbit IgG (1:500, #A21206, Life Technologies, CA). Nuclei were counterstained with DAPI (Vector, MI).

Images were acquired using an Olympus IX81 microscope equipped with a light fluorescence device (Olympus, Japan).

### 4.3 Results

#### 4.3.1 The differentiated subpopulation of miPS-LLCcm is required for tube formation.

As reported in previous studies, miPS-LLCcm cells were able to differentiate into endothelial cells, and were able to form tube-like structures composed by CD31+/GFP- endothelial differentiated cells, CD31-/GFP+ undifferentiated cells and CD31+/GFP+ bipotential cells on Matrigel (Figure 1.1) [11, 13]. The formation of the tubular structure required differentiated population, since GFP+ cells concentrated by puromycin in adherent culture, which contains CD31-/GFP+ and CD31+/GFP+ cells (Figure 1.2) were able to form the structure when seeded on Matrigel (Figure 1.3). GFP+ self-renewing spheres cultured in non-adherent dishes were not able to do tube formation neither (Figure 1.4) [11]. In contrast, when spheres were seeded into adherent culture for two days to give rise to GFP+ differentiated cells; an seeded *a posteriori* on Matrigel, these cells were able to generate tube structures (Figure 1.5).
Figure 1.1 Staining of CD31 and GFP in tube formation of miPS-LLCcm cells. Staining of CD31 (red) and GFP (green) showed 4 kinds of cells: CD31⁺/GFP⁻ endothelial differentiated cells, CD31⁻/GFP⁻ differentiated cells, CD31⁻/GFP⁺ undifferentiated cells and CD31⁺/GFP⁺ bipotential cells. Original magnification x20.

Figure 1.2 Analysis of cells present in puromycin-selected miPS-LLCcm culture. The Immunofluorescence staining shows a population mostly CD31⁻/GFP⁺ undifferentiated with some bipotential cells (CD31⁺/GFP⁺). Original magnification x60.

Figure 1.3 Incomplete tube formation of puromycin-selected miPS-LLCcm cells. Puromycin selected cells were seeded on Matrigel and stained with CD31 antibody (red). Cells were no able to differentiate and to form tubular structures. Original magnification x20.
Sphere forming self-renewing miPS-LLCcm cells were not able to do tube formation. When the cells were allowed to differentiate under adherent conditions, the cells could do tube formation. Scale bar: Day 0: 200µm; Day 2: 50µm; Tube formation Original magnification x2.

**Figure 1.4 Tube formation ability of GFP+ spheroids.**

4.3.2 miPS-LLCcm are able to differentiate into endothelial cells *in vitro*

The bulk culture of miPS-LLCcm cells consists of 2 distinct kinds of morphologies: cells forming GFP⁺ embryonic stem (ES)-like colonies and GFP⁻ differentiated cells (Figure 2.1) [14].

When culture of miPS-LLCcm cells was stained with anti-CD31 antibody, CD31⁺ cells were detected (Figure 2.2). As found in the tube formation, four kind of cells were found: CD31⁺/GFP⁻ differentiated cells, CD31⁺/GFP⁺ differentiated endothelial cells, CD31⁻/GFP⁺ undifferentiated cells, and CD31⁺/GFP⁺ bipotential cells. Outside the colonies, most the cells were CD31⁻/GFP⁻ differentiated cells (Figure 2.3), while all the kinds of cells inside forming ES-like colonies could be find. CD31⁺/GFP⁻ differentiated cells were predominantly located at the bottom of the ES-like colonies (Figure 2.2).
This observation implied that miPS-LLCcm formed a structure that resembles an endothelial niche \textit{in vitro} (Figure 2.4).

Even though I could find some CD31\(^+\) cells in the bulk culture, it was not until the cells were seeded on Matrigel that most of them differentiated (Figure 2.5). It is highly possible that some of the CD31\(^+\)/GFP\(-\) cells acted as endothelial precursor cells, such as VEGFR2\(^+\) cells, mature into endothelial cells as CD31\(^+\)/GFP\(^-\) cells during the culture on Matrigel.

\textbf{Figure 2.1 miPS-LLCcm bulk culture.} miPS-LLCcm bulk culture present two kind of subpopulations: GFP\(^+\) embryonic stem (ES)-like colonies and GFP\(^-\) differentiated cells. Scale bar: 100 µm.

\textbf{Figure 2.2 Kinds of cells in the colony within the bulk culture.} Immunofluorescence analysis of CD31 (red) and GFP (green) in bulk culture of miPS-LLCcm cells. In the lower section, a 3D image of the colony cut in three different height sections, showing the predisposition of differentiated cells in the bottom and undifferentiated cells in the top of the colony. Original magnification x100.
Figure 2.3 Kinds of cells outside the colony in the bulk culture. Immunofluorescence analysis of CD31 (red) and GFP (green) in bulk culture of miPS-LLCcm cells shows that most of the cells are CD31⁻/GFP⁻ differentiated cells with few CD31⁺/GFP⁻ endothelial cells. Original magnification x100.

Figure 2.4 Scheme of the position of the cells in the bulk culture, resembling a niche in vitro. Outside the colony were differentiated cells (CD31⁻/GFP⁻). CD31⁺/GFP⁻ endothelial cells are concentrated at the bottom of the colony, while undifferentiated cells are situated at the edges and top of the colony. Inside the colony bipotential CD31⁺/GFP⁺ can be found.
Figure 2.5 Time course analysis of in vitro tube formation. Cells were stained with CD31 (red) antibody at indicated time. Even though the tubular structures could be identified after 6hr, it was not until 24hr that CD31\(^+\) cells were mostly found. Original magnification x20.

4.3.3 DsRed-LLCc\text{m} cells differentiate into endothelial cells in vivo

The observation of endothelial cell differentiation of miPS-LLCc\text{m} in vitro prompted me to investigate whether CSC originated blood vessels in vivo. I looked for tubular structures that were CD31\(^+\)/DsRed\(^+\) double positive in the tumor derived from DsRed-LLCc\text{m} cells by immunofluorescence staining. I could find not only these structures (Figure 3.1), but also mosaic vasculatures, in which blood vessels were mostly formed by CD31\(^+\)/DsRed\(^+\) cells, which should be derived from host, and CD31\(^+\)/DsRed\(^-\) cells, which should be derived from DsRed-LLCc\text{m} cells (Figure 3.2). The physical interaction between host-derived ECs and tumor-derived ECs, explains the functionality of CSC-derived blood vessel.
**Figure 3.1** CSC differentiate into EC *in vivo*. Tumors derived from DsRed-LLCcm cells were stained with CD31 (red) and DsRed (green) antibodies. A typical CD31* endothelial vessel comprised of DsRed* cells indicate the differentiation of CSC into EC *in vivo*. Scale bar: 25µm.

**Figure 3.2** Mosaic vessels *in vivo*. Tumors derived from DsRed-LLCcm cells were stained with CD31 (red) and DsRed (green) antibodies. A blood vessel comprised of CD31*/DsRed* cells and CD31*/DsRed* explains the functionality of CSC-derived vessels. Scale bar: 58µm.

### 4.4 Discussion
The angiogenic factors secreted predominantly by the CSC subpopulation, as described in chapter 3, should contribute not only in the recruitment of host-EC but also into the differentiation of CSCs into ECs. CSC differentiation into EC was first reported in glioblastoma, in which some EC were found contained glioma-specific chromosomal aberration, indicating that the endothelial cells were derived from tumor cells and not from the host [15]. Brossa et al. described the differentiation of breast-CSC into endothelial cells but this required of addition of external VEGF or hypoxia stimulation [10]. We have also reported that our miPS-LLCcm differentiated into endothelial cells *in vitro* (Figure 1.1) [11]. Contrasting to breast-CSCs, EC differentiation from miPS-LLCcm cells occurred spontaneously on gelatin-coated dish exhibiting CD31 positive cells, which were readily detectable in the bulk culture (Figure 2.2), resembling an *in vitro* niche (Figure 2.4). This finding is consistent with the reports describing that ECs secrete factors that will promote survival and self-renewal of CSCs [11, 16, 17].
When miPS-LLCcm cells were seeded on matrigel, endothelial CD31\(^+\) cells appeared, however we could not determine which was the origin of CD31\(^+\)/GFP ECs in vitro.

Studies in glioblastoma have shown a bipotential CD31\(^+\)/CD133\(^+\) subpopulation that gave rise to EC via differentiation [7]. These bipotential cells in glioblastoma seems to be equivalents to our CD31\(^+\)/GFP\(^+\) cells found in the bulk (Figure 2.2, 2.4) since both cells expressed stem cell marker and endothelial marker. However, as shown in our previous study [11], concentrated GFP\(^+\) miPS-LLCcm cells, which contain GFP\(^+\)/CD31\(^-\) and GFP\(^+\)/CD31\(^+\) cells (Figure 1.2), alone were not able to generate tube-like structures on Matrigel (Figure 1.3). Similarly, self-renewing spheroids of miPS-LLCcm did not form tube-like structures directly (Figure 1.4, left). On the other hand, these cells gained the ability to form tube-like structures when the spheroids are allowed to differentiate into adhesive GFP\(^-\) cells, which contain GFP/CD31\(^-\) and GFP/CD31\(^+\) cells (Figure 1.4, right). This suggests that the dominant cells responsible to form tubes were GFP\(^-\) cells, which were also found to be mostly CD31\(^-\) before tube formation and resided outside of ES-like colonies in bulk culture of miPS-LLCcm cells (Figure 2.3-4). We have previously reported the presence of VEGFR2\(^+\) cells, which are considered to be EC precursors, in the GFP\(^-\) subpopulation [11]. Taking our previous results together, it is likely that ECs were maturated from CD31\(^+\)/GFP\(^-\) with VEGFR2 expressing cells as precursors of ECs derived from ES cells were maturated in VEGF dependent manner [18]. Considering the significant expression of VEGF-A and FGF2 from GFP\(^+\) cells (Chapter 3, Figure 2.4), these angiogenic factors should be responsible for the maturation of miPS-LLCcm progenies as ECs in paracrine manner.

The CD31\(^+\)/GFP\(^+\) bipotential cells in miPS-LLCcm cells might be corresponding to the stem cell/progenitor-like cells, which were considered to differentiate into endothelial cells, present in vascular structures [19]. Recently, stem cell/progenitors excluding Hoechst with CD31 and other endothelial markers were isolated from mouse normal lung [20]. These cells were found to be involved in the tumor angiogenesis by supplying ECs. Hoechst excluding side population has been recognized as stem cell enriched population in both normal and cancer tissues [21]. Collectively, CD31\(^+\)/GFP\(^+\) bipotential cells in miPS-LLCcm cells could be one of the origins of CSC-derived ECs in vivo since the differentiation of DsRed-LLCcm into ECs was confirmed in vivo as CD31\(^+\)/DsRed\(^+\) vessels were found (Figure 3.1-2).

However, further investigation is required to identify the exact pathway of CSC differentiation into ECs since the differentiation process is not clear. Also the CSCs
properties, such as the ability to form malignant tumors, of CD31⁺/GFP⁺ bipotential cells should be assessed.

4.5 References


DsRed-LLCcm cells participate in the formation of Vasculature Mimicry.
5.1 Introduction

Recent therapies have been focusing in tumor vasculature. With targeting EC specific molecules, many therapeutic strategies have been developed to interfere with the formation of tumor blood vessels. For example, bevacizumab, an antibody against VEGF receptors, has been tested in many types of solid tumor [1, 2]. Unfortunately, despite the efforts, most of these treatments failed to reveal the expected results [3]. Accumulating knowledge regarding the complexity of tumor vasculogenesis might explain, at least in part, the failure of the classical anti-angiogenic drugs targeting ECs.

Vasculogenic mimicry (VM), a concept which was first described in 1999 by Maniotis et al., is a process for tumor vasculature generation found in malignant and poorly differentiated tumors, in which tumor cells themselves generate organized networks of vessels. The VMs channels are rich in extracellular matrix (ECM), but are typically without CD31 positive endothelial cells [4, 5]; therefore, VM should not rely on typical essential angiogenic markers [6].

EphA2-VE cadherin connection, Tie1, Slpi and Serpine2 have been described as important factors for the structure of VM [7-10]. VM has been found in many kinds of tumors such as glioblastoma [4,11], hepatoma [6], melanoma [5] and colon cancer [12]. It has been described [5] that cells forming VM forming cells express stemness markers, and it has proposed that CSC could be responsible for VM. However there are not clear evidences.

5.2 Materials and methods

5.2.1 Animal experiments.

Nude mice (Balb/c-nu/nu, female, 4 weeks old) were purchased from Charlesriver, Japan. For transplantation studies, 1x10^6 cells were suspended in 200µL in the miPS-media and injected subcutaneously in both flanks of mice. Tumor volumes were measured every 3-4 days and calculated as following formula (0.5 x longer diameter x shorter diameter^2). After 5 weeks, tumors were fixed for the histological analysis.

The plan of animal experiments was reviewed and approved by the ethics committee for animal experiments of Okayama University under the ID OKU-2013252 and OKU-2016078.
5.2.2 Immunohistochemistry analysis.

For CD31-PAS double staining, extracted tumors were enveloped with paraffin and sectioned at 5 µm of thickness. After deparaffinization, antigen retrieval was carried out by boiling in 10mM citrate sodium (pH6) with 0.05 % Tween20 for 15min. After cooling down the samples, the endogenous peroxidase was blocked with 3 % H2O2 for 5 min. Then, Ellite anti-rabbit ABC Vectastain kit was used following manufactures protocol with rabbit polyclonal anti CD31 (1:100, #ab28364, Abcam) incubation of hr at room temperature. Immunoreactivity was detected by using DAB. Afterwards, tissues were stained with PAS solution by following manufactures protocol, omitting hematoxylin counterstaining to reduce visual noise. These sections were viewed under light microscopy (FSX100, Olympus, Japan).

For GFP staining, rabbit monoclonal anti-GFP antibody (1:400, #2956, Cell Signaling, MA) and Ellite anti-rabbit ABC staining Vectastain kit (Vector, MI) were used folloing manufactures protocol. DAB (Vector, MI) were used for detection. Counter staining was carried out using hematoxylin. Then, the following section was stained with PAS solution as described previously.

5.3 Results

5.3.1DsRed-LLCcm cells are able to generate Vasculogenic mimicry

While blood vessels composed of ECs were observed in the tumor of miPS-LLCcm cells and DsRed-LLCcm cells, I also looked into the presence of VM in the tumors. WM are blood channels rich in ECM which are lined by tumor cells instead of vascular endothelial cells. These VM-forming cells have been described as very malignant and poorly differentiated [4].

I stained paraffin-embedded sections of the tumors with anti-CD31 antibody and PAS, to investigate ECM rich structures to find VM, which is defined as CD31+/PAS+ patterns. As the result, I found abundant PAS+ patterns and tubular blood vessels-like outlines. In this context, approximately 7.6% of all the channels were judged VM while most parts of the structure were not VM as CD31+. Blood cells were frequently observed in the
cavities surrounding PAS\textsuperscript{+} structures (Figure 1.1). This indicates that VM was developed in the tumor of DsRed-LLCc\textsubscript{m} cells. In contrast, tumor derived from LLC cells exhibited little PAS\textsuperscript{+} staining (data not shown).

![Figure 1.1 Vasculogenic mimicry in DsRed-LLCc\textsubscript{m} tumor. Tumor sections were stained with CD31 antibody (brown) and PAS (pink). EC lined vessels (CD31\textsuperscript{+}/PAS\textsuperscript{-}) and tumor cell lined VMs (CD31\textsuperscript{-}/PAS\textsuperscript{+}) were observed. Arrows show VM. Scale bar: 50\mu m (left), 25\mu m (right).](image)

5.3.2 Vasculogenic mimicry is formed by the undifferentiated subpopulation of DsRed-LLCc\textsubscript{m} cells

GFP\textsuperscript{+} cells lining vasculatures were also found (Figure 2.1, up). Since GFP was used as a reporter of Nanog expression [13, 14], this indicates that the channel was structured by undifferentiated cells. Moreover, these vessels were also PAS\textsuperscript{+} (Figure 2.1, down). Both undifferentiated cells and PAS\textsuperscript{+} channels should illustrate VM, and suggests that undifferentiated subpopulation of the CSC model, DsRed-LLCc\textsubscript{m} cells, participate in the VM formation.
Figure 2.1 The undifferentiated subpopulation of DsRed-LLCcm cells form VM. Immunohistochemistry of GFP (brown) and PAS (Pink) staining in serial sections of tumor derived from DsRed-LLCcm cells showing GFP+/PAS⁺ channels. Scale bar: 50µm.

5.4 Discussion

Despite its clinical importance [5], the biological features of the tumor cells that form VM, as well as the cellular and molecular events underlying its formation remain largely unknown. Maniotis et al. suggested that melanoma cells lining VM could have been reverted to an embryonic-like phenotype [9] since they were found to express multiple stemness markers. We observed that 7.6% of all the tumor vasculature in DsRed-LLCcm tumor could be defined as VM, which were ECM-rich vessels lacking of ECs (Figure 1.1). Furthermore, we detected the presence of GFP⁺ undifferentiated cells, reflecting the stemness gene Nanog expression, in PAS⁺ channels (Figure 2.1).
These channels were frequently found in the region where most of stromal cells were GFP+. Although we cannot state that the CSC ability remains in all of the GFP+ cells including those forming the PAS+ channel, GFP+ cells are indeed the ones with the ability of self-renewal (Chapter 2, Figure 2.2) and differentiation (Chapter 2, Figure 2.4) giving new insights in the direct relation between CSC and VM.

5.5 References


[7] Alameddine RS, Hamieh L, Shamseddine A. From sprouting angiogenesis to


CHAPTER 6

General conclusions and futures challenges
6.1 Conclusion and Important findings

In this research I found that miPS-LLCcm and DsRed-LLCcm cells simultaneously exhibited different three types of tumor vasculature in a tumor tissue as angiogenesis, endothelial differentiation from CSCs and formation of VM in vivo. As far as I know, this is the first model of CSC cells that exhibit these three kinds of vasculature together. It is highly possible that miPS-LLCcm also present other kinds of vasculature. Tumor vasculature is essential for tumor growth; however current therapeutic strategies targeting only angiogenesis are leading to insufficient outcomes. Taking these under consideration, miPS-LLCcm could be an appropriate model to understand entire tumor vascularization and to develop novel drugs and therapeutic strategies.

The CSC subpopulation of miPS-LLCcm cells predominantly donates angiogenic factors that not only attract host endothelial vessels into tumor, but also to promote maturation of endothelial linage of CSC’s, probably VEGFR2 expressing GFP/CD31⁻ progenies. Moreover, GFP expressing cells, and CD31⁻ cells were found lining VM (Figure 1). Thus, it seems that all the vasculatures types are interconnected.

Figure 1. Scheme of the cells found in bulk culture and its relation with the different kinds of vasculature.
We have shown that CSCs can differentiate into ECs which are able form vessels as well as contribute to VM formation in vivo. At the same time, early stages of in vitro tube formation showed that these were formed mostly by CD31+ cells (Figure 2.5). Taking these observations into consideration, I hypothesized that VM could be an intermediate stage of CSC differentiation. Zhang et al. discussed the presence of three kinds of vessels in melanoma, which were VM, mosaic vessels and endothelium patterned vessels. VM was described as the dominant in the early stage of tumor growth, following EC differentiation and proliferation and mosaic vessels would appear as a transitional pattern [16, 43]. Other authors proposed that VM might be CSCs that remain incomplete in differentiation towards the endothelial lineage [19] these resembles the cells that remained GFP expression within the tubular structures (Figure 2.5).

6.2 References


List of publications

Papers.
1. iPSC-derived Cancer Stem Cells Provide a Model of Tumor Vasculature.

2. Cancer stem cells maintain a hierarchy of differentiation by creating their niche.

Oral presentations.
1. Studies of the mechanism inducing Cancer Stem Cells from normal Stem Cells
   Marta Prieto-Vila.
   平成26高校生・大学院生による研究紹介と交流の会(Okayama, 2014.07)

2. Acquisition of immortalization prior to malignancy during the miPS-CSC generation.
   Marta Prieto-Vila, Tsukasa Shigehiro, Anna Sanchez Calle, Tomonari Kasai, Hiroshi Murakami, Akifumi Mizutani, Masaharu Seno.
   The 74th Japanese Cancer Association meeting (Nagoya 2015.10).

3. iPSC-derived Cancer Stem Cells Provide a Model of Tumor Vasculature
   Marta Prieto-Vila, Anna Sanchez Calle, Neha Nair, Akifumi Mizutani, Masaharu Seno.
   5th Mouse Resource Workshop 2016 (Tokyo, 2016.07).

4. The significance of c-kit proto-oncogene in CSC derived PDAC
   Anna Sanchez Calle, Kenta Hoshikawa, Neha Nair, Marta Prieto-Vila, Arun Vaidyanath, Tomonari Kasai, Masaharu Seno.
5. Generation of Mammary ductal carcinoma associated carcinoma fibroblast from cancer stem cells.
Neha Nair, Tomonari Kasai, Kenta Hoshikawa, Anna Sanchez Calle, Marta Prieto-Vila, Arun Vaidyanath, Masaharu Seno.
平成27 高校生・大学院生による研究紹介と交流の会 (Okayama, Japan 2015.07)

6. The significance of c-kit proto-oncogene in CSC-derived PDAC model
Anna Sanchez Calle, Kenta Hoshikawa, Neha Nair, Marta Prieto-Vila, Arun Vaidyanath, Tomonari Kasai, Masaharu Seno.

7. The significance of c-kit proto-oncogene in CSC-derived PDAC model
Anna Sanchez Calle, Kenta Hoshikawa, Neha Nair, Marta Prieto-Vila, Arun Vaidyanath, Tomonari Kasai, Masaharu Seno.
CIRA/ISSCR 2016 International symposium (Kyoto, Japan 2016.03).

8. Intracellular localization of Nanog in cells within cancer stem cell niche.
Molecular Biology Society of Japan (Kobe, 2015.12)
Acknowledgements

I would like to thank all the people who made this thesis possible, specially to my supervisor, Professor Masaharu Seno in the Laboratory of Nanobiotechnology, Graduate school of Natural Science and Technology, Okayama University, who gave me the opportunity to do my doctoral course under his guidance and for all his help along these three years, without him, I could not complete my study of doctor course and this dissertation.

I would like to express my sincere appreciation to Assistant Professor Akifumi Mizutani, for his valuables advices and who helped and encouraged me daily, reviewing and correction of the manuscript.

I additional extend my great thanks to Dr. Tomonari Kasai, who helped me, not only giving me instructions of CAM assay, but many helpful suggestions and humorous words.

I would like to thank Associate Professor Hiroshi Murakami, Assistant Professor Junko Masuda, Professor Hiroki Kakuta, Research Assistant Professor Arun Vaidyanath, Research Assistant Professor Akimasa Seno, Ms Mami Asakura and Ms Kaoru Furuse for their kindly help. Without their cooperation it would had been very difficult for my lab time.

Special thanks should go to Anna Sanchez Calle, Neha Nair, Ting Yan, Kenta Hoshikawa and all the students from the laboratory for their valuable suggestion, encouragement and friendliness giving me a wonderful memory of my doctoral course. Finally, I want to thank my parents, my brother and my boyfriend who supported me in every possible way and always encouraged me.

I gratefully acknowledge the funding received towards my PhD from MEXT (Ministry of Education, Culture, Sports, Science and Technology).

Marta Prieto
Okayama University
September 2016