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海 外 特 別 研 究 員 最 終 報 告 書

独立行政法人日本学術振興会 理事長　殿

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受 付 番 号　201960137
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（氏名は必ず自署すること）

海外特別研究員としての派遣期間を終了しましたので、下記のとおり報告いたします。
なお、下記及び別紙記載の内容については相違ありません。

記

1. 用務地（派遣先国名）用務地：ヘルムホルツセンターミュンヘン　（国名：　ドイツ

2. 研究課題名（和文）※研究課題名は申請時のものと違わないように記載すること。
　微小脂肪組織チップを用いた脂肪生成における細胞間コミュニケーションの解明

3. 派遣期間：平成　31 年　4 月　9 日　～　令和　2 年　12 月　16 日

4. 受入機関名及び部局名
　受入機関名：ヘルムホルツセンターミュンヘン
　部局名：Helmholtz Pioneer cumpus

5. 所期の目的の遂行状況及び成果…書式任意　**書式任意（A4 判相当 3 ページ以上、英語で記入も可）**
（研究・調査実施状況及びその成果の発表・関係学会への参加状況等）
（注）「6. 研究発表」以降については様式 10－別紙 1～4 に記入の上、併せて提出すること。

5. 所期の目的の遂行状況及び成果

Introduction

In recent years, there has been a growing interest in cell-cell interaction between heterologous cells in tissues. In particular, vascular endothelial cells are not only important for the formation of vascular structures and the supply of nutrients and oxygen, but also for the early stages organogenesis that precede vascular function.[1] One of the most promising approaches to elucidate the function of endothelial cells in tissues and their interaction with tissue cells is the formation of vascular organoids by tissue engineering methods. Vascular organoids with organized endothelial cells in a three dimensional culture system are possible by culturing HUVECs or stem cell-derived endothelial populations using hydrogels or synthetic scaffolds. Supporting cells, such as mesenchymal cells, are important for endothelial cells to undergo good structure formation and growth in tissues. Recent single-cell RNA sequencing analysis has revealed that mesenchymal cells are also generated simultaneously during the differentiation of endothelial populations from stem cells, and that these cells form vascular structures in hydrogels.[2,3]

Then I have set up a differentiation protocol for pluripotent stem cells into endothelial cells and evaluated the differentiation process of the endothelial cells on the single cell level and imaging. Furthermore, in order to culture vascular organoids in a shear field, I have designed and manufactured the flow-cell chamber.

Results

I. Establishing endothelial differentiation and single cell analysis over endothelial commitment

We established the differentiation procedure. In brief, after making floating aggregates from single cell suspension, cells were treated with BMP4 and CHIR to generate mesoderm for 3 days. From day 3 to 9, cells were treated with VEGF-A and Forskolin. At day 9, we confirmed 40% of cells were CD31/VE-cadherin double positive endothelial cells using flow-cytometry analysis. On the other hand, it has been reported that mesenchymal cells are generated at the same time and they can behave as a perivascular-like cells. [2,3] In order to understand what types of cells are arising in our 3D culturing, single-cell transcriptomic data was obtained from cells across the differentiation protocol using droplet-based microfluidic technology in parallel. UMAP dimensionality reduction clearly showed cells were divided into two divergent populations from day 3 to day 6 (Fig. 1a). The first was characterized by expression of endothelial markers (PECAM1 (CD31), CDH-5), the second by mesenchymal markers such as PDGFRA (Fig. 1b). Mesenchymal population also showed partial increase of perivascular-specific marker such as PDGFRB and ACTA2 indicating it starts to acquire perivascular characteristic (Fig. 1b). In order to identify transcription factors that contribute to endothelial commitment, we obtained putative driver genes systematically detected by characterization of high likelihood models in dynamic models of RNA velocity estimation. Transcription factor enrichment analysis revealed BCL6B, FOXN3 and SOX7 were significantly enriched transcription factors (Fig 1c). It has been noted that BCL6B and SOX7 may contribute to endothelial development, whereas FOXN3 has not been reported. Although overlapped query genes of FOXN3 are mainly related to tissue migration process according to gene ontology enrichment analysis, this velocity and transcription factor enrichment analysis suggested this gene can be a candidate for further experimental validation.

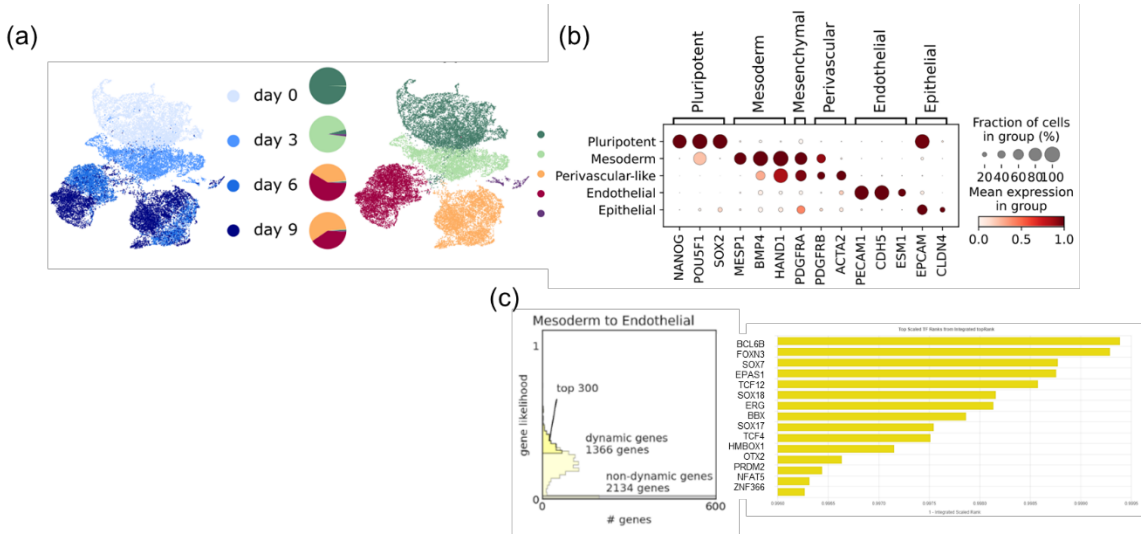

Figure 1. (a) UMAP dimensionality reduction plots, using data from cells sampled at day 0-9 for single-cell RNA sequencing show transition from pluripotent stem cell to mesoderm and separation into endothelial and perivascular-like cell population afterwards. (b) Typical marker expression of respective cell types in (a). (c) We defined 300 putative driver genes according to gene likelihood in the dynamic velocity model from mesoderm population to endothelial population (left). We inferred the transcription factor enriched in putative driver genes defined with velocity analysis using transcription factor enrichment analysis software (ChEA3).

II. Morphological Maturation

In order to see the matrix-assisted morphological development, we dissociated the huge spheroids from shaking culture and re-aggregated in microwell culture camber to form small homogeneous spheroids. And after transferring them into matrigel and culture for a few days. As shown in fig 2a, spheroid expanded outwardly, started to sprout and show network structure. Further, we stained them with both endothelial marker (either VE-Cadherin or CD31) and perivascular marker (either PDGFRß or α–SMA) to see detail structure and localization of two cell types. Just after differentiation, endothelial cells got together inside and tightly attached with each other whereas perivascular-like cells covered them outside. On the other hand, a few days-culture in matrix induced endothelial migration into network structure based on the core of aggregates. We assumed that in floating condition, endothelial cells might be immature and tend to form blood island which is the stage of pre-vessel formation and matrix-assisted maturation might help endothelial cells forming plexus formation which is next stage of blood vessel formation.

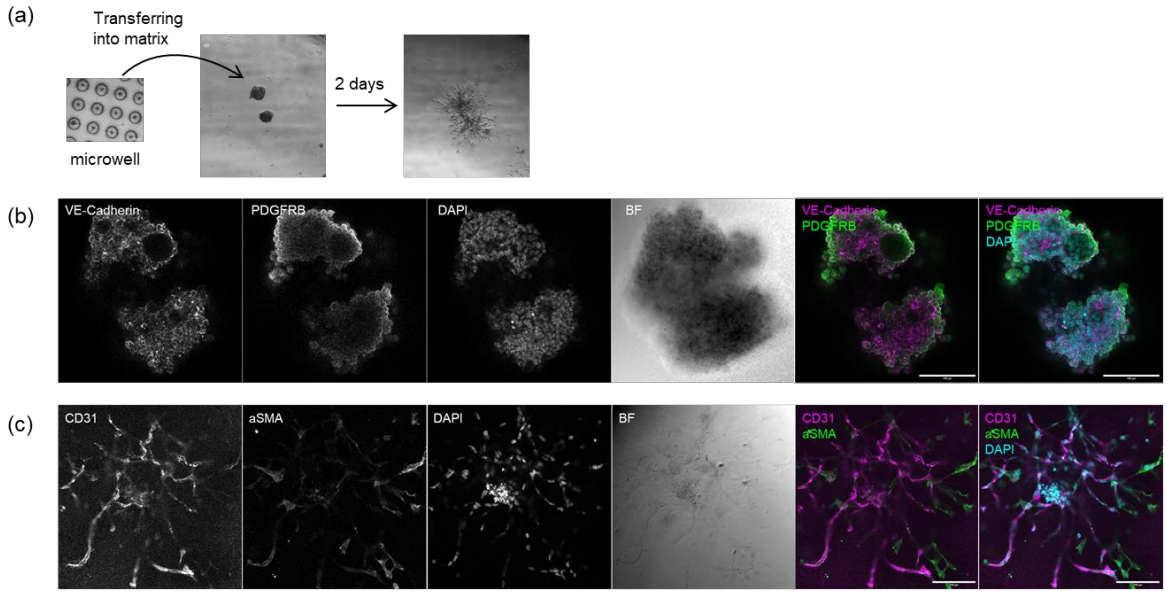

Figure 2. (a) Bright field image of organoids. After transfer from suspension culture to matrix, the spheroids begin to sprout outward and form a network structure. (b) Immunohistochemistry of differentiated floating organoids, in which two types of cells (endothelial cells (VE-Cadherin) and perivascular-like cells (PDGFRB)) formed two layers. Endothelial cells were particularly aggregated and showed blood island-like morphology. (c) Two days after culturing in matrix, endothelial cells stained with CD31 showed network structure.

III. Lumen formation

One of important achievements of vascular organoid is development of perfusable vascular lumen which can be connected with other tissue organoids. We first tried to develop fluidic device in which endothelial cells form lumen structure in the gel (Fig. 3a). By using microtubing, we prepared the lumen structure made by collagen gels and culture endothelial cells in the device. As a control experiment, we first cultured human

umbilical vein endothelial cells (HUVECs) in lumen for 24 hr. HUVECs showed sheet-like structure tightly connecting each other. On the other hands, when we cultured iPSC derived endothelial cells in the lumen without flow, they did not cover whole lumen completely and migrated into several separated areas. However, when we cultured them under the flow, cells showed sheet–like structure like HUVECs. This suggests that shear stress might have impact on the migration pattern of endothelial cells. In addition, when we performed tube formation assay on matrigel, HUVECs formed thin and tightly connecting tube whereas iPSC-derived endothelial cells formed sheet-like structure instead of tube formation (data not shown). We assumed that iPSC-derived endothelial cells are not matured enough and flow shear stress might play an important role on maturation, migration and ability of lumen formation. We are attempting to evaluate maturation degree and establish a protocol for culturing vascular lumen. Furthermore, immature endothelial cells might be used for developing tissue-specific endothelial cells.

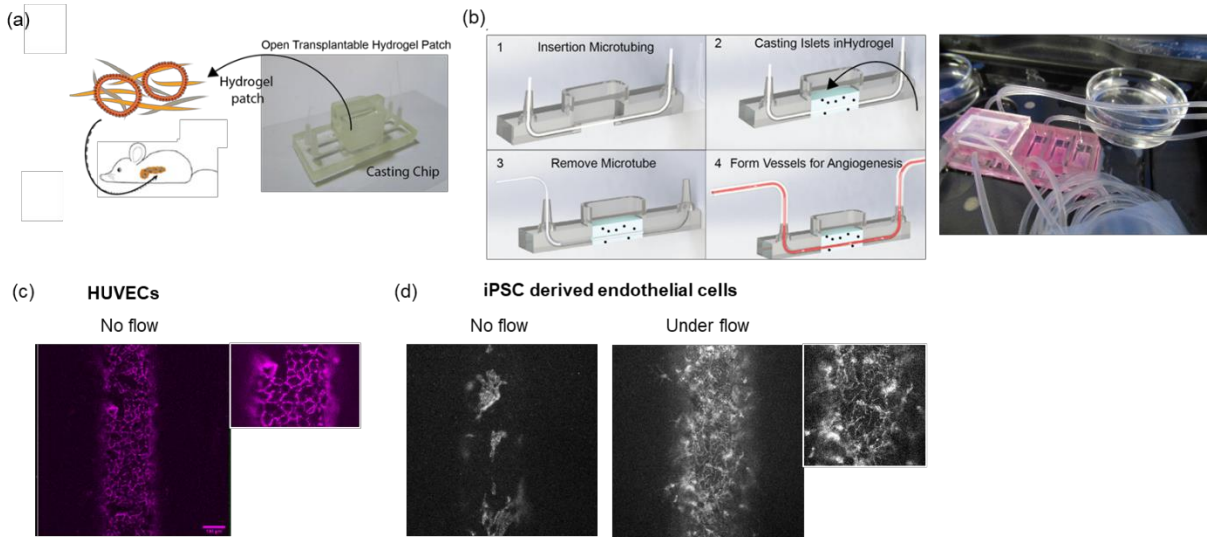

Figure 3. (a) Possible application of lumen chip. (b) Design principle to create lumen formation in casting gel using microtube. (c,d) We cultured HUVECS (c) and iPSC-derived endothelial cells (d) in the lumen and cells were stained with VE-cadherin antibody.

[References]

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