Our goal is to unravel molecular and cellular mechanisms underlying development of the hematopoietic and vascular systems. By using an in vitro differentiation system of murine embryonic stem cells, we are trying to identify the genetic program by which the self-renewal capacity as well as multiple potentials of the hematopoietic stem cell is established. Our system also makes it possible to elucidate cell biological functions of angiogenic growth factors and transcription factors, providing a clue to how the morphogenic activity of endothelial cells is regulated by angiogenic stimuli to form a hierarchically organized vascular architecture.

Upper left: Schematic diagram of in vitro differentiation of ES cells into the hematopoietic and vascular lineages. Upper right: Morphological changes of ES cell-derived vascular endothelial cells induced by various angiogenic stimuli. Lower left: Hematopoietic and endothelial differentiation of ES cell-derived lateral mesodermal cells in culture. Lower right: Generation of hematopoietic cells and endothelial cells from an isolated single hemogenic endothelial cell that was derived from ES cells.
# 構成員 Staff (2018.3)

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1. 造血幹細胞の発生メカニズム
哺乳類・鳥類等の個体発生において、最初に卵黄囊で起きる胚型赤血球の産生を胚型造血と呼ぶ。胚型造血よりやや遅れて発生し、胎仔肝を経て最終的に骨髄に定着する造血を成体型造血と呼ぶ。卵黄囊や骨髄の静脈途端の領域で新生性血液幹細胞が検出され、その後、AGM（aorta-gonad-mesonephros）領域で造血幹細胞が検出される。造血幹細胞を含む成体型血液細胞系列は造血性内皮細胞から発生すると考えられている。本研究は、造血性内皮細胞と血液幹細胞の発生機序を明らかにし、ES細胞から造血幹細胞を分化誘導する方法の開発を目指している。

1-1. Activin A による造血性内皮細胞の分化誘導促進
ES細胞から造血幹細胞の分化誘導を実現するためには、造血性内皮細胞の分化誘導効率を高める必要がある。OP9ストロマ細胞との共培養によるES細胞分化系において、血管内皮細胞の造血能を亢進させる増殖因子の探索を行ったところ、Activin Aの存在下に分化誘導した血管内皮細胞の血球分化能は著しく高いことが明らかにされた。Activin A存在下に誘導した血管内皮細胞はCD41陽性細胞（CD41血管内皮細胞）を多く含んでおり、血球系細胞も分認系から分化することが分かった。CD41血管内皮細胞は造血幹細胞の前駆細胞であることが報告されており、ES細胞分化系で同細胞を検出したのは本研究が初めてである。血管内皮細胞は細胞転移葉と側板中胚葉からそれぞれ分化するが、CD41血管内皮細胞は側板中胚葉から主に発生することがわかった。Activin AとのOP9ストロマ細胞の組み合わせは、ES細胞から側板中胚葉の発生を著しく促進するために、側板中胚葉由来のCD41血管内皮細胞を相対的に増加させることが明らかになった（Hirotro & Ogawa, 2015）。

1-2. CXCR4 による造血性内皮細胞の分化調整
ES細胞由来のCD41血管内皮細胞の造血性内皮細胞としての機能を明らかにするために、single cellレベルでの分化能を解析した。CD41血管内皮細胞の20%が内皮細胞コロニー形成能を示し、11%が血球分化能を示した。血球分化能を持つ細胞の20%は内皮細胞コロニー形成能も持つ（二分化能）ことが分かった。CD41血管内皮細胞は、Etv2など血管内皮細胞が発現する転写因子に加えて血球分化に必須の転写因子Runx1も発現しており、造血性内皮細胞として同定することができた。

造血性内皮細胞の二分化能がどのように調節されるのか解析するために、二分化能を持つ細胞をさらに濃縮するマーカーを探索した。
CD41血管内皮細胞のうち、スモノイ受容体CXCR4を発現する一部が、血球分化能を持つ細胞の56%が二分化能を持つことが分かった。二分化能に対するCXCR4シグナルの関与を明らかにするために、CXCR4リガンド（CXCL12）を添加してCXCR4+CD41血管内皮細胞を分化誘導したところ、内皮細胞分化能の抑制による二分化能細胞の減少を観察した。本研究は造血性内皮細胞の分化能力を細胞外シグナルを介して調節し得ることを初めて明らかにした（Ahmed et al., 2016）。

今後、マウス胎仔由来の造血性内皮細胞から確実に造血幹細胞を誘導する培養をまず確立し、この培養法を標準条件としてES細胞由来の造血性内皮細胞の分化能力を評価し、造血幹細胞に分化する能力を持つ造血性内皮細胞を誘導する条件を探索する予定である。

1-3. c-Mycタンパクの発現量に基づく休止期造血幹細胞の検出と分離
c-Mycは血液前駆細胞の増殖と各血球系列の
分化に関与する転写因子である。c-Myb タンパクの発現量を生細胞で定量するために、c-Myb 遺伝子の翻訳停止コード直前に EGFP の cDNA をインフレームで挿入したノックインマウスを作製した。このレポーターマウスは、c-Myb-EGFP キメラタンパクを、内在性の遺伝子制御領域の支配下で発現する。骨髄細胞の FACS 解析により、各球系の血液前駆細胞だけでなく、造血幹細胞も c-Myb-EGFP を発現していることが分かった。造血幹細胞の中でも c-Myb の発現が低い細胞は大部分が G0 期にあり、c-Myb の発現が高い細胞に比べて骨髄造血再構築能力が高いことを見いだし、造血幹細胞の増殖活性化と c-Myb タンパクの発現量が逆相関することが明らかになった。c-Myb タンパクの発現量に基づいて、休止期にある造血幹細胞を予期的に生きまま分離することができることを初めて示し、造血幹細胞の細胞周期調節機構の解明に貢献するものである（Sakamoto et al., 2015）。

2. 血管形成の細胞生物学的メカニズム

血管の発生は、血管芽細胞から分化した血管内皮細胞が原始的な血管系を形成する管形成過程、既存の血管から新たな血管が出芽して階層性のある血管網へ発達する血管新生過程を経て進行する。ES 細胞から血管内皮細胞を分化誘導してコロニーを形成させる培養系は、細胞接着・運動・細胞形態を詳細に観察し、血管新生を調節する様々な因子の細胞生物学的役割を解明する優れたシステムである。本研究は、血管新生因子に応答した血管内皮細胞の細胞伸長のメカニズムを解明し、血管新生を細胞生物学的に制御する方法の開発を目指している。

2-1. PI3K/Akt 及び mTORC1 の阻害による血管内皮細胞の伸長抑制

VEGF 投与による治療的血管新生では、VEGF の血管透過性亢進作用による浮腫などの副作用が問題となる。過剰な VEGF 刺激に頼らない血管新生誘導法を開発するために、ES 細胞から分化誘導した血管内皮細胞の伸長を促進する薬剤を、阻害剤ライブラリーのスクリーニングにより探索した。

PI3K 阻害剤（LY294002）、Akt 阻害剤（Akti-1/2）、mTORC1 阻害剤（Everolimus、Rapamycin）が、低濃度の VEGF 存在下に血管内皮細胞の伸長を促進することが明らかになった。高濃度の VEGF による細胞伸長と同様に、PI3K/Akt 阻害による細胞伸長は転写因子 Foxo1 に依存していた。一方、mTORC1 阻害による細胞伸長は、低濃度の VEGF 存在下では Foxo1 依存的であったが、高濃度の VEGF 存在下では Foxo1 依存しなかった。

mTORC1 シグナルは mTORC2 を抑制することが知られているが、KU0063794 の処理により mTORC1 と mTORC2 の両方向を阻害すると細胞伸長が抑制されることから、mTORC1 の阻害は mTORC2 シグナルの活性化を介して細胞伸長を抑制することが明らかになった。PI3K/Akt と mTORC1/mTORC2 は異なる機序で血管内皮細胞の形態を調節するが明らかとなり、VEGF の過剰投与を回避しながら血管新生を抑制する新しい治療法を開発するためには有用な知見を与えるものである（Tsujii-Tamura & Ogawa, 2016）。

2-2. mTORC1/mTORC2 の同時阻害による血管内皮細胞の伸長抑制

mTORC1 は腫瘍細胞による VEGF 産生を促進することから、がん治療における標的分子のひとつである。しかし、mTORC1 阻害剤の抗腫瘍効果は必ずしも十分ではない。一方、mTORC1 と mTORC2 の同時阻害は血管内皮細胞の伸長を抑制するため、血管新生を阻害する新たな方法として期待される。細胞伸長抑制のメカニズムを明らかにするために、血管内皮細胞株を用いて KU0063794 処理による細胞骨格への影響を検討した。

mTORC1 と mTORC2 の同時阻害は、アクチノフィラメントの異常な集積と微小管ネットワークの乱れを引き起こし、それに伴って血管内皮細胞は伸長した形態から短縮不定形な形態
1. Mechanism of hematopoietic stem cell development

During the embryogenesis of mammals and birds, the generation of embryonic red blood cells occurring in the yolk sac is called the primitive hematopoiesis. Slightly after the onset of primitive hematopoiesis, first adult-type hematopoietic progenitor cells are detected in the yolk sac and the paraaortic splanchnopleure of the embryos proper. The generation of adult-type hematopoietic cells is referred to as the definitive hematopoiesis. Then, the first hematopoietic stem cells (HSCs) become detectable in the AGM (aorta- gonad-mesonephros) region. The HSCs migrate to the fetal liver and finally settle in the bone marrow. Adult blood cell lineages including the HSCs are believed to be generated from hemogenic endothelial cells. We aim to develop a culture method of inducing differentiation of hemogenic endothelial cells and HSCs from ES cells, thereby elucidating the developmental mechanisms of HSCs.

1-1. Promotion of development of hemogenic endothelial cells by Activin A

In order to realize the in vitro derivation of HSCs from ES cells, it is necessary to increase the differentiation efficiency of hemogenic endothelial cells from ES cells. An ES cell differentiation system by co-culture with OP9 cells was subjected to search for growth factors to enhance the hematopoietic ability of vascular endothelial cells. It was found that the hematopoietic ability of vascular endothelial cells induced in the presence of Activin A was remarkably high. Vascular endothelial cells induced in the presence of Activin A contained CD41⁺-positive fraction (CD41⁺ vascular endothelial cells) in high frequency. Blood cells were found to be differentiated mainly from this fraction. CD41⁺ vascular endothelial cells have been reported to be progenitor cells of HSCs in the embryos. This is the first time to detect the CD41⁺ vascular endothelial cells in ES cell differentiation system. Although vascular endothelial cells differentiate from both the paraxial mesoderm and the lateral plate mesoderm, CD41⁺ vascular endothelial cells were found to be mainly generated from the lateral plate mesoderm. Combination of Activin A and OP9 cells significantly promotes the generation of the lateral plate mesoderm from ES cells, thereby increasing the CD41⁺ vascular endothelial cells derived from the lateral plate mesoderm (Hirota & Ogawa, 2015).

1-2. Regulation of hemogenic endothelial cell differentiation by CXCR4

To identify the CD41⁺ vascular endothelial cells derived from ES cells as hemogenic endothelial cells, differentiation ability was analyzed at the single cell level. One out of five CD41⁺ vascular endothelial cells showed endothelial cell colony forming ability, and one out of nine CD41⁺ vascular endothelial cells showed hematopoietic ability. It was found that 20% of cells with hematopoietic ability also had endothelial cell colony forming ability (biopotent). CD41⁺ vascular endothelial cells expressed Runx1, an essential transcription factor for generation of definitive hematopoietic cells, in addition to endothelial transcription factors such as Etv2. Thus, CD41⁺ vascular endothelial cells derived from ES cells were identified as hemogenic endothelial cells.

To analyze how the biopotent state of hemogenic endothelial cells is regulated, we searched for a marker to further concentrate the bipotent progenitors in CD41⁺ vascular endothelial cells. In a fraction expressing the chemokine receptor CXCR4, more than one out of two cells with hematopoietic ability showed endothelial cell colony forming ability. In order to clarify the involvement of the CXCR4 signaling to the biopotent state, CXCL12, a CXCR4 ligand, was added during the induction of CXCR4⁺ CD41⁺ vascular endothelial cells from ES cells. CXCR4 signaling was found to suppress the
endothelial potential of CD41+ vascular endothelial cells, thereby reducing the frequency of bipotential progenitors in this population. This study indicates that the differentiation ability of hemogenic endothelial cells can be controlled by extracellular signaling (Ahmed et al., 2016).

In the near future, we expect to establish a reliable culture system for inducing HSCs from hemogenic endothelial cells isolated from mouse embryos. By using the culture system as a standard condition, the differentiation ability of hemogenic endothelial cells derived from ES cells can be evaluated. Based on such studies, we finally expect to explore culture conditions that induce genuine hemogenic endothelial cells with the ability to differentiate into HSCs from ES cells.

1-3. Detection and isolation of dormant HSCs based on the expression level of c-Myb protein
C-Myb is a transcription factor involved in the proliferation of hematopoietic progenitor cells and the differentiation of each hematopoietic cell lineage. In order to quantify the expression level of C-Myb protein in a living cell, a knock-in mouse line, in which EGFP cDNA was inserted in-frame just before the stop codon of the c-Myb gene, was generated. The reporter mouse expresses the c-Myb-EGFP chimeric protein under control of the endogenous transcriptional regulatory domain. By FACS analysis of bone marrow cells, not only progenitor cells of each hematopoietic cell lineages, but also HSCs were found to express the c-Myb-EGFP. HSCs expressing a low level of c-Myb-EGFP were mainly in the G0 phase and found to possess higher capacity of bone marrow reconstructing ability than the cells expressing a high level of c-Myb-EGFP. Therefore, the proliferative activation of HSCs and their expression level of c-Myb protein were suggested to be an inverse correlation. This study contributes to the elucidation of the cell cycle regulating mechanism of HSCs, by demonstrating for the first time that HSCs in the dormant stage can be prospectively identified and isolated based on the expression level of the c-Myb protein. (Sakamoto et al., 2015).

2. Mechanism of cell biological regulation of vascular development
Development of the blood vessels progresses through two major steps. The first step is the vasculogenesis, in which vascular endothelial cells differentiate from angioblasts and form a primitive vascular plexus. The second step is the angiogenesis, in which new blood vessels are generated from the existing vessels to develop into a hierarchically ordered vascular network. Vascular endothelial cell colonies generated from ES cells in culture are suitable to observe cell-cell adhesion, cell movement and cell morphology in detail, thereby providing an excellent system to elucidate the cell biological function of variousangiogenic factors. The purpose of this study is to elucidate the mechanism of elongation of vascular endothelial cells in response to angiogenic factors, and to develop a method of cell biological control of pathological and therapeutic angiogenesis.

2-1. Induction of vascular endothelial cell elongation by inhibition of PI3K/Akt and mTORC1
In therapeutic angiogenesis by VEGF administration, side effects such as edema, which is due to the vascular permeability enhancing action of VEGF, cause a problem. In order to develop a method of inducing angiogenesis that does not rely on an excessive VEGF stimulation, a drug that promotes the elongation of ES cell-derived vascular endothelial cells was searched by screening a library of chemical inhibitors.

PI3K inhibitors (LY294002), Akt inhibitors (Akt1-2) and mTORC1 inhibitors (Everolimus, Rapamycin) were found to promote elongation of vascular endothelial cells even in the presence of low concentrations of VEGF. As in the case of cell elongation induced by high concentrations of VEGF, cell elongation induced by PI3K/Akt inhibition was dependent on a transcription factor Foxo1. On the other hand, while cell elongation induced by mTORC1 inhibition was dependent on Foxo1 in the presence of low concentrations of VEGF, it was Foxo1 independent in the presence of high concentrations of VEGF.

The mTORC1 signaling is known to suppress the activity of mTORC2. As endothelial cell elongation was suppressed when both mTORC1 and mTORC2 were inhibited by a dual inhibitor KU0063794, mTORC1 inhibition was suggested to induce cell elongation through the activation of mTORC2 signaling. PI3K/Akt and mTORC1/mTORC2 were suggested to regulate the morphology of vascular endothelial cells via different mechanisms. These findings provide an insight into a new therapeutic approach to induce angiogenesis without an excessive administration of VEGF (Tsujitamura & Ogawa, 2016).

2-2. Suppression of vascular endothelial cell elongation by dual inhibition of mTORC1/mTORC2
Since mTORC1 promotes VEGF production by tumor cells, mTORC1 is one of the target molecules in cancer treatment. However, the anti-tumor effect of the mTORC1 inhibitors is not necessarily sufficient enough to suppress tumor growth. As simultaneous inhibition of mTORC1 and mTORC2 suppresses elongation of vascular endothelial cells, mTORC1/mTORC2 dual inhibition is expected as a new method of inhibiting angiogenesis. In order to clarify the mechanism of inhibition of cell elongation, the effect of KU0063794 treatment on the cytoskeleton of vascular endothelial cell lines was investigated.

Simultaneous inhibition of mTORC1 and mTORC2 by KU0063794 caused an abnormal accumulation of actin filaments and a disturbance of microtubule network, thereby resulted in morphological changes of vascular endothelial cells from an elongated form to a short irregular form. Tube formation in a three-dimensional culture was also inhibited. Inhibition of mTORC1 by Everolimus did not show such an effect. Furthermore, the inhibition of tubulin depolymerization by Paclitaxel treatment exerted effects similar to KU0063794 treatment (accumulation of actin filaments, disturbance of microtubule network, and inhibition of cell elongation). Therefore, simultaneous inhibition of mTORC1 and mTORC2 was suggested to inhibit endothelial cell elongation by affecting the cytoskeleton through an overstabilization of the microtubules. This result gives an important knowledge to develop an efficient method of inhibiting tumor angiogenesis (Tsui-Tamura & Ogawa, 2018).
論文目録  Publications


著書・総説目録  Publications


学会・研究集会 発表目録  Meeting Presentations

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アウトリーチ活動 Outreach activity

Dual inhibition of mTORC1 and mTORC2 perturbs cytoskeletal organization and impairs endothelial cell elongation

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1. Introduction

During angiogenesis, vascular endothelial cells perform intricate cellular interactions, such as elongation, proliferation, migration, adhesion, andocument formation for (review, see Refs. [1,2]). Vascular endothelial growth factor (VEGF) is a potent inducer of not only physiological angiogenesis, but also tumor angiogenesis (for a review, see Ref. [3]). VEGF secretion is upregulated in tumor cells by inhibition of mTORC1 signaling [4,5] (for a review, see Ref. [6]). Conversely, inhibition of mTORC1 by rapamycin or its derivatives increases VEGF secretion. VEGF inhibitors also directly inhibit endothelial cell proliferation under the stimulation of VEGF [7,8]. Therefore, inhibition of mTORC1 signaling has drawn much attention as a cancer treatment strategy [9] (for a review, see Ref. [10]). However, Xu et al. reported that rapamycin inhibited early stages of angiogenesis but failed to affect late stages of vascular malformations in an in vivo model of angiogenesis, suggesting mTORC1 inhibition on tumor angiogenesis might be partial and transient [11]. mTORC1 is positively regulated by RAS homolog enriched in brain (RhoB). RhoB activity is suppressed by tuberin mesenchymal 2 (TSC2) via conversion of RhoB from the GTP-bound active form to the GDP-bound inactive form. When TSC2 is phosphorylated and inhibited by Akt, activation of the PI3K/Akt signaling increases mTORC1 activity (for reviews, see Refs. [12,13]). mTORC1 phosphorylates rapamycin-insensitive companion of tuberin (Rictor), which is a key component of mTORC1, through the activation of ribosomal protein S6 kinase 1 (S6K1). The phosphorylation of Rictor appears to negatively regulate mTORC2 signaling [14]. Conversely, inhibition of mTORC2 by rapamycin results in compensatory activation of mTORC2 signaling. mTORC2 phosphorylates and activates Akt, thereby counteracting the action of rapamycin [15–17] (for reviews, see Refs. [18,19]). Therefore, simultaneous targeting of mTORC1 and mTORC2 might be an effective treatment for tumor angiogenesis.

1. Introduction

Hematopoietic stem cells (HSCs) first arise in the aorta-gonad-mesonephros (AGM) region of the embryo prior around embryonic day 10.5 (E10.5) [1]. Many studies have suggested that the origin of HSCs is the aortic endothelium. The endothelial lining of the dorsal aorta harbors Ly-6A (E-cadherin)-expressing cells, which encompass long-term repopulating HSCs [2]. Ly-6A-expressing cells were shown to develop in vivo from aortic endothelium in an elegant culture of embryo slices [3]. Nascent HSCs in embryos express endothelial markers such as VECadherin [4]. Cell fate tracking using the Cre/LoxP system showed that HSCs in the adult bone marrow originate from VECadherin-expressing endothelial cells (VEC) [5,6]. "Ar-"

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1. Introduction

Hematopoietic stem cells (HSCs) first arise in the aorta-gonad-mesonephros (AGM) region of the embryo prior around embryonic day 10.5 (E10.5) [1]. Many studies have suggested that the origin of HSCs is the aortic endothelium. The endothelial lining of the dorsal aorta harbors Ly-6A (E-cadherin)-expressing cells, which encompass long-term repopulating HSCs [2]. Ly-6A-expressing cells were shown to develop in vivo from aortic endothelium in an elegant culture of embryo slices [3]. Nascent HSCs in embryos express endothelial markers such as VECadherin [4]. Cell fate tracking using the Cre/LoxP system showed that HSCs in the adult bone marrow originate from VECadherin-expressing endothelial cells (VEC) [5,6]. "Ar-"
**ABSTRACT**

In our previous studies, we reported anti-inflammatory functions of 3,5,6,7,8,3',4'-heptamethoxyflavone (HMIF), which is a polymethoxyflavone rich in various citrus fruits. Here, we investigated the immunomodulatory function of HMIF in mice. HMIF administration (50 mg/kg, i.p., 2 times/week) tended to reduce the production of antigen-specific IgG induced by ovalbumin in combination with aluminum hydroxide gel. Fluorescence-activated cell sorting analysis revealed the reduction of interleukin-4 (IL-4) splenocyte cells and sustained presence of interferon-γ (IFN-γ) splenocyte cells in mice administered HMIF, whereas the ratio of CD4+CD8- versus CD4-CDF8 splenocyte cells was not affected. Interleukin-4 release from CD4+CD8-activated stimulated spleen cells of mice administered HMIF was reduced, whereas interferon-γ release was not affected. These results suggest that HMIF has an immunomodulatory function via reduced interleukin-4 expression.

**INTRODUCTION**

The process of forming new blood vessels through the sprouting of preexisting vessels is referred to as angiogenesis. Vascular endothelial growth factor (VEGF) promotes angiogenesis by driving widespread events in the involved endothelial cells. The cellular events involving VEGF include the tip cell selection for the initiation of sprouting, the proliferation of sprouting endothelial cells and the lumen formation in the elongated new vessels (Hellstrom et al., 2002; Apte et al., 2007; Sendi et al., 2009; Scutt et al., 2007; Zeng et al., 2007a). Angiogenesis plays important roles not only in the physiological developmental process but also in the therapeutic vascular formation in pathological settings.

The major objective in treating ischemic disorders is the reconstruction of damaged blood vessels. Administration of recombinant human endothelial cell-derived VEGF by gene transfer has been tested for the purpose of inducing therapeutic vascular growth. However, preclinical studies have shown that VEGF-mediated neovascularization causes undesirable side effects, such as tissue edema due to increased vascular permeability, as VEGF is known to be a potent vascular permeability factor (Riseman et al., 2005; Sniger et al., 1983). Although the tissue edema appeared to remain at the level of a temporal effect in clinical trials of VEGF gene therapy, this potential problem should be eliminated (Lee et al., 2000; Ruggenegger et al., 2003; Vila-Hermida et al., 2007).

In tissue engineering, the establishment of proper vascularization is key to the perfusion and survival of the grafted tissue. VEGF has also been used to achieve neovascularization in engineered tissues (Jahanbakhsh et al., 2008; Chung and Shum-Shum, 2012). However, the microenvironmental level of VEGF has been shown to be crucial, as myoblasts secreting VEGF at a concentration higher than a certain threshold induce hemorrhages when transplanted into muscles (O’brien et al., 2004). Abnormal angiogenesis induced by high microenvironmental levels of VEGF is one of the major therapeutic targets in cancer treatment. VEGF mediates tumor angiogenesis by activating the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway (Cross and Clash-McKee, 2001; Gerber et al., 1998). PI3K-Akt signaling also indirectly suppresses tumor angiogenesis through activation of the mammalian target of rapamycin complex 1 (mTORC1) (Irie et al., 2002; Kurihara et al., 2011; Kurihara et al., 2003; Suzuki et al., 2010; Hata et al., 2006). mTORC1 increases the level of microenvironmental factors (e.g., VEGF) in tumor cells, thereby promoting VEGF secretion and the formation of leaky tumor vessels. Therefore, normalization of aberrant vascularization is remaying the blood flow of tumors is now considered as a promising therapeutic strategy for cancer treatment (Carmeliet and Jain, 2011; Falcò et al., 2009; Teng et al., 2004). Based on the above, therapeutically controlling angiogenesis is necessary to develop a method for normalizing angiogenesis, without causing excessive proliferation of endothelial cells or hyper-permeability.

The cell biological functions of endothelial cells are dynamically regulated during the process of angiogenesis. These functions include cytokine production, and changes in cell shape, morphology, cell-cell adhesion and cell-matrix interaction (Arina et al., 2011; Guo et al., 2007; Jahnson et al., 2010; Lampugnani et al., 2002; Gooden and Gherardi, 2011). It has been reported that VEGF is a key regulator of endothelial cell morphology (Furuyama et al., 2005; Hirakawa et al., 1990). VEGF stimulation induces the reorganization of endothelial cell morphology by modification of microtubulin embryonic stem cells (ESCs). We have also demonstrated that the endothelial cell elongation is dependent on the function of forkhead box O (FoxO), a forkhead transcription factor, as FoxO-deficient endothelial cells fail to elongate in response to VEGF (Ishida et al., 2006; Masuda et al., 2009). FoxO deficiency leads to embryonic lethality caused by impotency of angiogenesis (Furuyama et al., 2004; Hosaka et al., 2004), suggesting that the endothelial cell elongation is an indispensable cellular event that drives angiogenesis.
Activin A in combination with OP9 cells facilitates development of Flk-1<sup>+</sup> PDGF-Rα<sup>-</sup> and Flk-1<sup>+</sup> PDGF-Rα<sup>+</sup> hematopoietic mesodermal cells from murine embryonic stem cells

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1. Introduction

Hematopoietic stem cells (HSCs) are known to develop in the aorta-vasculogenesis (AGM) region of mouse embryos [1]. The dorsal aorta temporarily harbors hematopoietic cell (HC) clusters attached to the luminal face of endothelium [2]; HC clusters, which contain nascent HSCs, are proposed to originate from endothelial cells (ECs) [3]. Lineage tracing analysis has shown that HSCs are indeed progenitors of V<sup>21</sup>-cathernins<sup>+</sup> ECs [4]. CD41 expression marks the onset of HSC development from V<sup>21</sup>-cathernins<sup>+</sup> hematopoietic ECs in the AGM region [5]. The origin of hematopoietic ECs has been recently traced back to a distinct cell population derived from extramedullary mesoderm [6]. However, the developmental pathway of hematopoietic ECs remains to be fully understood.

Attempts have been made to emulate the developmental processes of ECs via in vitro differentiation of embryonic stem (ES) cells [7]. Co-culture with OP9 stromal cells is one of the most efficient methods to induce lymphohematopoietic differentiation from ES cells [8]. However, we reported that the OP9 co-culture system recapitulates stepwise development of the EC lineage, i.e., mesoderm induction for the first step, EC differentiation from mesoderm for the second step, and EC to HC transition for the final step [9,10]. However, signaling molecules that mediate the hematopoietic development in the OP9 co-culture system have not been well documented.

Embryoid body (EB) formation is another commonly used method for induction of ES cell differentiation. Activin A, a hematopoietic inducer referred to as a hematopoietic inducing factor (HIF), has been generally used for induction in EBs. For example, activin A is shown to be necessary for induction of hematopoietic mesoderm from primitive streaks in ES cells under serum-free conditions [11]. In contrast, activin A-induced organogenesis-like mesendoderm, which lacks hematopoietic potential, from ES cells cultured on type IV collagen matrix [12]. Therefore, the function of activin A appears to depend on culture conditions. Recently, the OP9 co-culture system in the presence of activin A was reported to induce ES cells to definitive endoderm [13].

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2. Methods

Hematopoietic stem cells (HSCs) are defined as cells which both self-renew and multi-lineage differentiation capability. On the basis of the duration of reconstitution in a transplant assay, HSC populations have generally been classified as long-term HSCs (LT-HSCS) and short-term HSCs (ST-HSCS) [1, 2]. Recently, intermediate-term HSCs (IT-HSCS) have been proposed as a subset of LT-HSCS and ST-HSCS [3, 4]. Similarly, the LT-HSC population has been subdivided into common and active HSCs [5, 6]. Dendritic HSCs (~30% of LT-HSCs) have been estimated to enter the cell cycle only once every 5-6 days or more, meaning that they divide only five to six times during the lifetime of a mouse. Conversely, the counterparts of HIV-infected HSCs contribute to the daily production of hematopoietic cells by dividing every 24-36 days.

In addition to LT-HSC heterogeneity with respect to repopulating potential, several subtypes of LT-HSCs have been identified based on their potential to generate mature lymphoid and myeloid cell lineages [7-10]. Myeloid- and lymphoid-biased LT-HSCs predominantly generate myeloid progeny and lymphoid progeny, respectively, whereas balanced LT-HSCs give rise to myeloid and lymphoid cells in the same ratio. Most recently, platelet-biased LT-HSCs have been identified and characterized through use of a Wt4<sup>-GFP</sup> reporter mouse line [11]. Interestingly, myeloid-biased LT-HSCs exhibit the most potent reconstitution potential and CDS<sup>+</sup> HSCs in the CD34<sup>+</sup>CD19<sup>+</sup> population have been prospectively enriched for HSCs by long-term repopulating activity and myeloid-biased differentiation [12, 13].

We have previously shown that appropriate levels of c-Myc expression are required at distinct differentiation stages of each cell lineage in hematopoietic progenitor cells (HPCs) [14, 15]. Recently, HIC5 has been reported to regulate differentiation in megakaryocyte-erythrocyte progenitors (MEPs) via the control of c-Myc protein levels [16] in contrast to HSCs, its roles in the correct functioning of HSCs remains to be elucidated. HIC5 is a transmembrane receptor that is encoded by the HIC5 (HIC5) gene, which is 50% of the transactivation activity compared to wild-type.
Redefining the In Vivo Origin of Metanephrine Nephron Progenitors Enables Generation of Complex Kidney Structures from Pluripotent Stem Cells

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SUMMARY

Recapitulating three-dimensional (3D) structures of complex organs, such as the kidney, from pluripotent stem cells (PSCs) is a major challenge. Here, we define developmental origins and gene expression patterns of various kidney structures from PSCs. Mouse or human embryonic stem cells (hESC) were differentiated into mesenchyme in a culture system for the development of the mesonephros. The mesenchyme formed a mesonephroblast in the ectoderm, which differentiated into the mesonephros. The mesonephros was implanted into the kidney of ES/iPS cells, and it differentiated into various kidney structures in vitro. This system allows for the development of various kidney structures from PSCs, which can be used to study kidney development.

INTRODUCTION

Despite the successful achievement of various types of tissues from pluripotent stem cells (PSCs), kidney generation in vitro has remained a challenge for developmental biology and regenerative medicine (Murty and Kohler, 2008; Williams et al., 2013). This is partly because the specification of the kidney lineage in vivo has not been well characterized, owing to its complex processes involving the formation of several consecutive primordial (nephric, mesonephric, and metanephric) structures during embryogenesis (Glater, 1987). The proximities of mesonephros and metanephros eventually degenerate in females, whereas a portion of the mesonephros in males contributes to a drainage system for the testis, including the epididymis. The kidney derives from the embryonic mesoderm, which develops at the most caudal part of the body trunk. The mesonephros is formed by reciprocal inductive interactions between two precursor tissues, namely the metanephric mesenchyme (MM) and the ureteric bud. Cell fate analyses have shown that both the MM and ureteric bud derive from the intermediate mesoderm, which arises around embryonic day 8.5 (E8.5) and expresses the transcription factor Osr1 (Miyamoto et al., 2008). However, the mechanisms underlying how the metanephric mesenchyme becomes committed to the intermediate mesoderm and how the MM and ureteric bud lineages segregate from one another have not been elucidated, despite many reports showing the importance of various growth factors during kidney development (Costantini and Karsenty, 2000; Finning et al., 2001; Kim and Dzierzak, 2006; Morrisey et al., 1993; Fuchs et al., 2006). Moreover, it remains to be elucidated how the apical ectodermal ridge is formed along the intermediate mesoderm and gives rise to the posteriorly located mesonephros. In the present study, we address these questions by using a mouse and human embryonic kidney organoid culture system together with in-vitro-differentiated systems utilizing adherent embryonic kidney organoids. The results indicate that the intermediate mesoderm gives rise to the mesonephros, which forms the kidney structures from PSCs, thereby allowing for the development of various kidney structures from PSCs.

RESULTS

The Osr1+/Eya1-/-/Pdgfrα Population Represents Colony-Forming Nephron Progenitors

The MM gives rise to the nephrogenic blastema (a, primitive, and renal tubules, which constitute the major parts of the nephrons, as shown by cell fate analyses involving labeling of in vivo studies. The in vivo vascular morphogenesis process. Through sequential steps of maturation, ECs derived from ES/iPS cells can be further differentiated into arterial, venous, capillary and lymphatic ECs, as well as smooth muscle cells. Here, we review EC development from ES/iPS cells with special attention to molecular pathways functioning in EC specification.

Keywords: Endothelial cells - Differentiation - Molecular pathways - Embryonic stem cells - Induced pluripotent stem cells

Molecular Pathways Governing Development of Vascular Endothelial Cells from ES/iPS Cells

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Acoustivo Assembly of complex vascular networks occurs in numerous biological systems through morphogenetic processes such as vasculogenesis, angiogenesis and vascular remodelling. Pluripotent stem cells such as embryonic stem cells (ES) and induced pluripotent stem cells (iPS) cells can differentiate into any cell type, including endothelial cells (ECs), and have been extensively used as in vitro models to analyze molecular mechanisms underlying EC generation and differentiation. The emergence of these promising new approaches suggests that ECs could be used in clinical therapy. Much evidence suggests that ES/iPS cell differentiation into ECs in vitro mimics the in vivo vascular morphogenetic process. Through sequential steps of maturation, ECs derived from ES/iPS cells can be further differentiated into arterial, venous, capillary and lymphatic ECs, as well as smooth muscle cells. Here, we review EC development from ES/iPS cells with special attention to molecular pathways functioning in EC specification.

Introduction

Diverse pathways give rise to the vascularize circulatory system, which forms early in embryogenesis. Vascular ECs initially differentiate from mesoderm-derived angioblasts, which form a primitive vascular plexus at or near their sites of origin during the onset of vasculogenesis (angiogenesis) (1). Arteriole-lined angioblasts adjacent to the endoderm (the first arteries (the dorsal aorta) ventrally, whereas venous-lined angioblasts migrate down the first vein (the cardinal vein) adjacent to the neural tube (2). The angioblast develops into a mature arterial and venous ECs, depending on activation of specific molecular pathways and expression of other factors. Through a sequential maturation process, arterial and venous ECs become functionally specified, giving rise to functional blood vessels such as arteries, veins and capillaries (3). Arteries transport oxygen-rich blood to all tissues, and veins bring oxygen-depleted blood back to the heart. Arteries are thicker-walled vessels surrounded by multiple layers of smooth muscle cells. Veins are thinner-walled vessels with little smooth muscle and exhibit valves to prevent backflow of blood (Fig. 1).