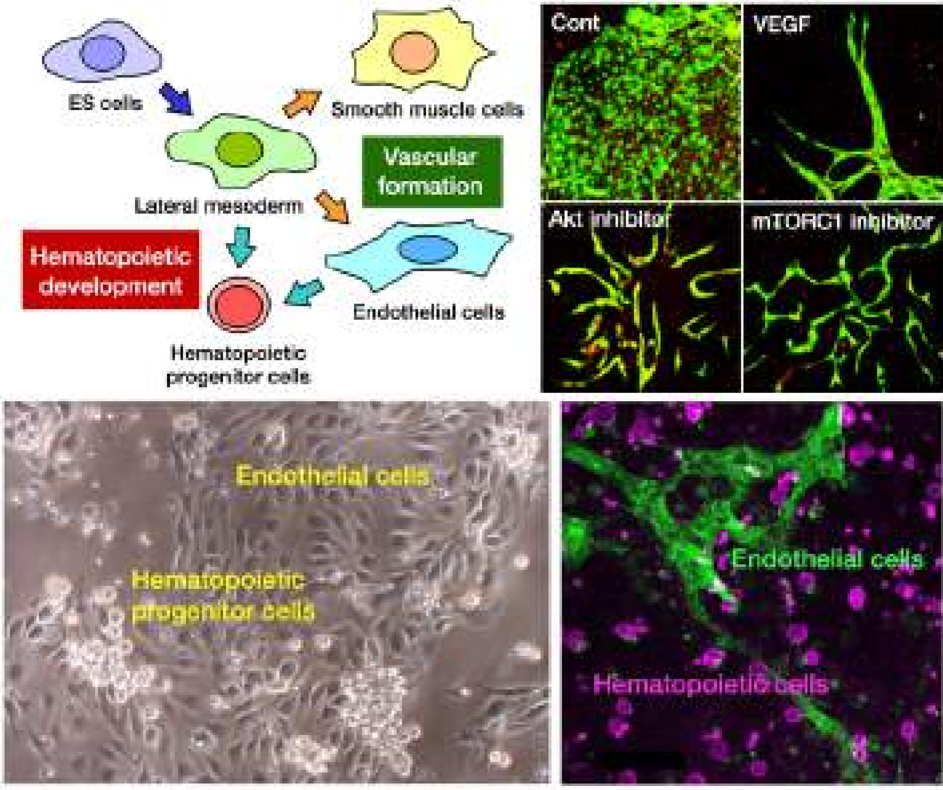
組織幹細胞分野
Department of Cell Differentiation

造血・血管システムが構築される仕組みを分子・細胞学的に解明することを目標として、胚性幹細胞の試験管内分化系を用い、造血幹細胞の自己複製能と多分化能が成立する機構、および血管系が形態的に組織化される機構について解析している。造血幹細胞の発生メカニズムを解明するために、胚性幹細胞から造血幹細胞を分化誘導する培養系の確立を目指している。血管形成に関わる増殖因子と転写因子の細胞生物学的作用を理解することにより、血管形成過程を制御する分子機構の解明を目指している。

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.

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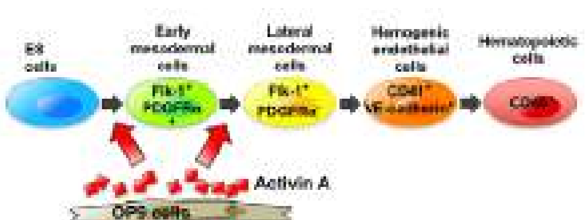
研究概略　Projects

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、最初に卵黄嚢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。卵黄嚢や背側大動脈近傍内臓葉で先ず成体型血液前駆細胞が検出され、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前駆細胞・造血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

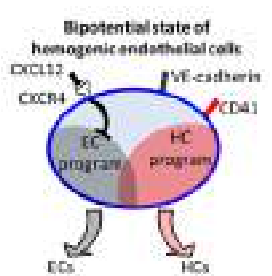
ES 細胞から造血幹細胞の分化誘導を実現するためには、造血性内皮細胞の分化誘導効率を高める必要がある。OP9 ストロマ細胞との共培養による ES 細胞分化系において、血管内皮細胞の造血能を亢進させる増殖因子の探索を行ったところ、Activin A の存在下に分化誘導した血管内皮細胞の血球分化能が著しく高いことを見出した。Activin A 存在下に誘導した血管内皮細胞は CD41 陽性分画（CD41$^+$血管内皮細胞）を多く含んでおり、血液細胞は主にこの分画から分化することが分かった。CD41$^+$血管内皮細胞は造血幹細胞の前駆細胞であることが報告されており、ES 細胞分化系で同細胞を検出したのは本研究が初めてである。血管内皮細胞は沿軸中胚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 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（Hirota & Ogawa, 2015）。



1-2. CXCR4 による造血性内皮細胞の分化調節

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、ケモカイン受容体 CXCR4 を発現する分画では、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始めて明らかにした（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法をまず確立し、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-Myb 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-Myb 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ドン直前に 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に発現する。骨髄細胞の FACS 解析により、各血球系列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 G_0 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た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

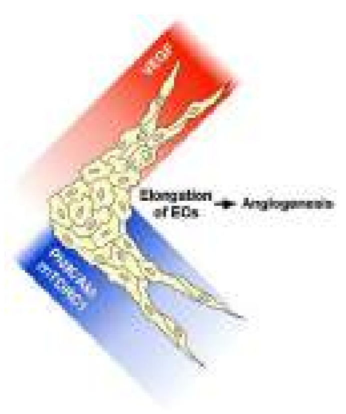
血管の発生は、血管芽細胞から分化した血管内皮細胞が原始的な血管叢を形成する脈管形成過程、既存の血管から新たな血管が出芽して階層性のある血管網へ発達する血管新生過程を経て進行する。ES 細胞から血管内皮細胞を分化誘導してコロニーを形成させる培養系は、細胞間接着・運動・細胞形態を詳細に観察し、血管新生を調節する様々な因子の細胞生物学的な役割を解明する優れたシステムである。本研究は、血管新生因子に応答した血管内皮細胞の細胞伸長のメカニズムを解明し、血管新生を細胞生物学的に制御する方法の開発を目指している。

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

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 に依存しなかった。

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誘導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ことが明らかとなり、VEGF の過剰投与を回避しながら血管新生を誘導する新しい治療法を開発するために有用な知見を与えるものである（Tsuji-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、アクチン・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い不定形な形態

に変化し、三次元培養による管腔形成も阻害された。Everolimus による mTORC1 阻害では、このような効果は観察されない。さらに、Paclitaxel の処理による微小管の脱重合阻害が KU0063794 とよく似た効果（アクチン・フィラメントの集積、微小管ネットワークの乱れ、細胞伸長の阻害）を血管内皮細胞に与えることがわかった。従って、mTORC1 と mTORC2 の同時阻害は微小管を過剰に安定化させることにより細胞骨格に影響を与え細胞伸長を阻害することが示唆された。この結果は、腫瘍血管新生をより効率よく阻害する方法を開発するために重要な知見を与えるものである（Tsuji-Tamura & Ogawa, 2018）。

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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 (bipotential). 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 bipotential 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 bipotential 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 the 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 various angiogenic 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 (Akti-1/2) 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 (Tsuji-Tamura & 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 (Tsuji-Tamura & Ogawa, 2018).

論文目録　Publications

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

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ABSTRACT

Elongation of endothelial cells is an important process in vascular formation and is expected to be a therapeutic target for inhibiting tumor angiogenesis. We have previously demonstrated that inhibition of mTORC1 and mTORC2 impaired endothelial cell elongation, although the mechanism has not been well defined. In this study, we analyzed the effects of the mTORC1-specific inhibitor everolimus and the mTORC1/mTORC2 dual inhibitor KU0063794 on the cytoskeletal organization and morphology of endothelial cell lines. While both inhibitors equally inhibited cell proliferation, KU0063794 specifically caused abnormal accumulation of F-actin and disordered distribution of microtubules, thereby markedly impairing endothelial cell elongation and tube formation. The effects of KU0063794 were phenocopied by paclitaxel treatment, suggesting that KU0063794 might impair endothelial cell morphology through over-stabilization of microtubules. Although mTORC1 is a key signaling molecule in cell proliferation and has been considered a target for preventing angiogenesis, mTORC1 inhibitors have not been sufficient to suppress angiogenesis. Our results suggest that mTORC1/mTORC2 dual inhibition is more effective for anti-angiogenic therapy, as it impairs not only endothelial cell proliferation, but also endothelial cell elongation.

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1. Introduction

During angiogenesis, vascular endothelial cells perform intricate cellular functions, such as elongation, proliferation, migration, adhesion, and lumen formation (for reviews, 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 activation of mTORC1 signaling [4,5] (for a review, see Ref. [6]). Conversely, inhibition of mTORC1 by rapamycin or its derivatives decreases VEGF secretion [7–9]. mTORC1 inhibitors also directly inhibit endothelial cell proliferation under the stimulation of VEGF [7,9–11]. Therefore, inhibition of mTORC1 signaling has drawn much attention as a cancer treatment strategy [5,7] (for a review, see Ref. [12]). However, Xue 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 that the effects of mTORC1 inhibition on tumor angiogenesis might be partial and transient [13].

mTORC1 is positively regulated by RAS homolog enriched in brain (Rheb). Rheb activity is suppressed by tuberous sclerosis 2 (TSC2) via conversion of Rheb 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. [14,15]). mTORC1 phosphorylates rapamycin-insensitive companion of mTOR (Rictor), which is a key component of mTORC2, through the activation of ribosomal protein S6 kinase 1 (S6K1). The phosphorylation of Rictor appears to negatively regulate mTORC2 signaling [16]. Conversely, inhibition of mTORC1 by rapamycin results in compensatory activation of mTORC2 signaling. mTORC2 phosphorylates and activates Akt, thereby counteracting the action of rapamycin [16–18] (for reviews, see Refs. [15,19,20]). Therefore, simultaneous targeting of mTORC1 and mTORC2 might be an effective treatment for tumor angiogenesis.

Elongation of endothelial cells is a crucial step in the process of

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EMBRYONIC STEM CELLS/INDUCED PLURIPOTENT STEM CELLS

CXCR4 Signaling Negatively Modulates the Bipotential State of Hemogenic Endothelial Cells Derived from Embryonic Stem Cells by Attenuating the Endothelial Potential

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Key Words. Mouse embryonic stem cells • Hemogenic endothelial cells • CXCR4 receptor • CXCL12 chemokine • CD41 antigen • VE-cadherin

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ABSTRACT

Hemogenic endothelial cells (HECs) are considered to be the origin of hematopoietic stem cells (HSCs). HECs have been identified in differentiating mouse embryonic stem cells (ESCs) as VE-cadherin^{+} cells with both hematopoietic and endothelial potential in single cells. Although the bipotential state of HECs is a key to cell fate decision toward HSCs, the molecular basis of the regulation of the bipotential state has not been well understood. Here, we report that the CD41^{+} fraction of CD45^{-} CD31^{+} VE-cadherin^{+} endothelial cells (ECs) from mouse ESCs encompasses an enriched HEC population. The CD41^{+} ECs expressed Runx1, Tal1, Etv2, and Sox17, and contained progenitors for both ECs and hematopoietic cells (HCs) at a high frequency. Clonal analyses of cell differentiation confirmed that one out of five HC progenitors in the CD41^{+} ECs possessed the bipotential state that led also to EC colony formation. A phenotypically identical cell population was found in mouse embryos, although the potential was more biased to hematopoietic fate with rare bipotential progenitors. ESC-derived bipotential HECs were further enriched in the CD41^{+} CXCR4^{+} subpopulation. Stimulation with CXCL12 during the generation of VE-cadherin^{+} CXCR4^{+} cells attenuated the EC colony-forming ability, thereby resulted in a decrease of bipotential progenitors in the CD41^{+} CXCR4^{+} subpopulation. Our results suggest that CXCL12/CXCR4 signaling negatively modulates the bipotential state of HECs independently of the hematopoietic fate. Identification of signaling molecules controlling the bipotential state is crucial to modulate the HEC differentiation and to induce HSCs from ESCs. STEM CELLS *2016;34:2814–2824*

SIGNIFICANCE STATEMENT

Hematopoietic stem cells originate from hemogenic endothelial cells. A hemogenic endothelial cell is an endothelial cell in which endothelial program and hemogenic program coexist in equilibrium. Modulating the bipotential state of hemogenic endothelial cells is a key to induction of hematopoietic stem cells from ES cells, which has long been a challenging goal. We report for the first time that endothelial and hemogenic programs can be independently regulated by external signals. Especially, CXCR4 signaling specifically attenuates the endothelial potential. Our findings provide a clue to controlling differentiation of ES cell-derived hemogenic endothelial cells toward the induction of hematopoietic stem cells.

INTRODUCTION

Hematopoietic stem cells (HSCs) first arise in the aorta-gonad-mesonephros (AGM) region of the embryo proper at 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 Ly6-A (Sca-1)-expressing cells, which encompass long-term repopulating HSCs [2]. Ly6-A-expressing cells were shown to develop *in situ* from aortic endothelium in an explant culture of embryo slices [3]. Nascent HSCs in embryos express endothelial markers such as VE-cadherin [4]. Cell fate tracing using the Cre/loxP system showed that HSCs in the adult bone marrow originate from VE-cadherin-expressing endothelial cells (ECs) [5, 6]. Targeted deletion of the *Runx1* gene, which is essential for definitive hematopoietic development, in VE-cadherin^{+} cells completely abolished HSC development in the AGM region [6]. More recently, a hemogenic endothelial precursor population (CD45^{-} VE-cadherin^{+} CD41^{+})

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3,5,6,7,8,3',4'-Heptamethoxyflavone reduces interleukin-4 production in the spleen cells of mice

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ABSTRACT

In our previous studies, we reported anti-inflammatory functions of 3,5,6,7,8,3',4'-heptamethoxyflavone (HMF), which is a polymethoxyflavone rich in various citrus fruits. Here, we investigated the immunomodulatory function of HMF in mice. HMF administration (50 mg/kg, i.p., 2 times/week) tended to reduce the production of antigen-specific IgE induced by ovalbumin in combination with aluminum hydroxide gel. Fluorescence-activated cell sorting analysis revealed the reduction of interleukin-$4^+$$CD4^+$ spleen cells and sustained presence of interferon-$\gamma^+$$CD4^+$ spleen cells in mice administered HMF, whereas the ratio of $CD4^+CD8^-$ versus $CD4^-CD8^+$ spleen cells was not affected. Interleukin-4 release from CD3/CD28-stimulated spleen cells of mice administered HMF was reduced, whereas interferon-γ release was not affected. These results suggest that HMF has an immunomodulatory function via reduced interleukin-4 expression.

Gel and Coombs classification classifies hypersensitivity states into four types. Of these, the IgE-mediated hypersensitivity state is called allergy, which is involved in several conditions, such as allergic rhinitis, atopic dermatitis, asthma, and food allergy. Because IgE production is critical for the pathogenesis of these diseases, a treatment to reduce IgE is anticipated. IgE production is promoted by interleukin (IL)-4 and suppressed by interferon (IFN)-γ stimulation. Targeting production of these cytokines might contribute to alleviate the symptoms of diseases mediated by IgE modulation (16).

Polymethoxyflavones (PMFs) have a broad spectrum of biologic activities (5). Nobiletin (3',4',5,6,7,8-hexamethoxyflavone) is a PMF present in the peel of citrus fruits, and various biologic activities have been demonstrated in *in vitro* and *in vivo* experiments. Nobiletin stimulates the cAMP-dependent protein kinase and extracellular signal-regulated kinase signaling pathways in PC12 cells and cultured hippocampus neurons (7, 8). In *in vivo* experiments, nobiletin has been shown to improve memory deficits in an animal model of Alzheimer's disease (12) as well as ischemia-induced memory deficits (18), and to improve motor and cognitive deficits in an animal model of MPTP-induced Parkinson disease (17). 3,5,6,7,8,3',4'-Heptamethoxyflavone (HMF) is a PMF structurally related to nobiletin (4, 9). HMF is reported to have anti-inflammatory functions in the brain (10, 11), and to suppress inflammatory osteoclastogenesis and alveolar bone resorption (6). However, immunomodulatory functions of HMF have not been addressed.

In the present study, we demonstrate that IL-4 production in mice is reduced by HMF administration and there is a tendency for HMF to reduce antigen-

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RESEARCH ARTICLE

Inhibition of the PI3K–Akt and mTORC1 signaling pathways promotes the elongation of vascular endothelial cells

Kiyomi Tsuji-Tamura* and Minetaro Ogawa*

ABSTRACT

Endothelial cell morphology needs to be properly regulated during angiogenesis. Vascular endothelial growth factor (VEGF) induces endothelial cell elongation, which promotes sprouting of pre-existing vessels. However, therapeutic angiogenesis using VEGF has been hampered by side effects such as elevated vascular permeability. Here, we attempted to induce endothelial cell elongation without an overdose of VEGF. By screening a library of chemical inhibitors, we identified phosphatidylinositol 3-kinase (PI3K)–Akt pathway inhibitors and mammalian target of rapamycin complex 1 (mTORC1) inhibitors as potent inducers of endothelial cell elongation. The elongation required VEGF at a low concentration, which was insufficient to elicit the same effect by itself. The elongation also depended on Foxo1, a transcription factor indispensable for angiogenesis. Interestingly, the Foxo1 dependency of the elongation was overridden by inhibition of mTORC1, but not by PI3K–Akt, under stimulation by a high concentration of VEGF. Dual inhibition of mTORC1 and mTORC2 failed to induce cell elongation, revealing mTORC2 as a positive regulator of elongation. Our findings suggest that the PI3K–Akt–Foxo1 and mTORC1–mTORC2 pathways differentially regulate endothelial cell elongation, depending on the microenvironmental levels of VEGF.

KEY WORDS: Endothelial cell, Embryonic stem cell, PI3K, Akt, mTOR, Foxo1, Cell elongation

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 (Hellström et al., 2007; Leslie et al., 2007; Lobov et al., 2007; Strilić et al., 2009; Suchting et al., 2007; Zeng et al., 2007a). Angiogenesis plays important roles not only in the physiological vascular development, 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 VEGF or overexpression of VEGF by gene transfer has been tested for the purpose of inducing therapeutic vascular growth. However, preclinical studies have shown that VEGF overexpression causes undesirable side effects, such as tissue edema due to elevated vascular permeability, as VEGF is known to be a potent vascular permeability factor (Rissanen et al., 2005; Senger 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; Rajagopalan et al., 2003; Ylä-Herttuala et al., 2007).

In tissue engineering, the establishment of proper vascularization is key to the perfusion and survival of the grafted tissues. VEGF has also been used to achieve neovascularization in the engineered tissues (Jabbarzadeh et al., 2008; Chung and Shum-Tim, 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 hemangiomas when transplanted into muscles (Ozawa et al., 2004).

Aberrant 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) to Akt signaling pathway (Cross and Claesson-Welsh, 2001; Gerber et al., 1998). PI3K–Akt signaling also indirectly upregulates tumor angiogenesis through activation of the mammalian target of rapamycin complex 1 (mTORC1) (Jiang et al., 2002; Karar and Maity, 2011; Laughner et al., 2001; Okumura et al., 2012; Zhao et al., 2006). mTORC1 increases the level of hypoxia inducible factor 1α (HIF-1α) in tumor cells, thereby promoting VEGF secretion and the formation of leaky tumor vessels. Therefore, the normalization of aberrant vasculature to remedy the blood flow of tumors is now considered as a promising therapeutic strategy for cancer treatment (Carmeliet and Jain, 2011; Falcón et al., 2009; Tong et al., 2004). Based on the above, to therapeutically control angiogenesis, it is necessary to develop a method for promoting normal angiogenesis, without inducing excess 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 cytoskeletal reorganization, and changes in cell morphology, cell–cell adhesion and migration (Arima et al., 2011; Guo et al., 2007; Jakobsson et al., 2010; Lampugnani et al., 2002; Geudens and Gerhardt, 2011). It has been reported that VEGF is a key regulator of endothelial cell morphology (Furuyama et al., 2004; Hirashima et al., 1999). VEGF stimulation induces the elongation of endothelial cells in an *in vitro* differentiation system of murine embryonic stem cells (ESCs). We have also demonstrated that the endothelial cell elongation depends on the function of forkhead box O1 (Foxo1), a forkhead transcription factor, as *Foxo1*-deficient endothelial cells fail to elongate in response to VEGF (Furuyama et al., 2004; Matsukawa et al., 2009). Foxo1 deficiency leads to embryonic lethality caused by impairment of angiogenesis (Furuyama et al., 2004; Hosaka et al., 2004), suggesting that endothelial cell elongation is an indispensable cellular event that drives angiogenesis.

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Activin A in combination with OP9 cells facilitates development of Flk-1$^+$ PDGFRα^- and Flk-1$^+$ PDGFRα^+ hematopoietic mesodermal cells from murine embryonic stem cells

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Lateral mesoderm-derived hemogenic endothelial cells are known to originate the definitive hematopoietic lineage in mouse embryogenesis. The developmental process of the definitive hematopoietic lineage can be recapitulated by inducing differentiation of mouse embryonic stem (ES) cells in a co-culture system with OP9 stromal cells. However, the signaling molecules that can modulate the development of the definitive hematopoietic lineage in the OP9 co-culture system have yet to be identified. Here we report that activin A enhanced the hematopoietic potential of endothelial cells derived from ES cells in the OP9 co-culture system. Activin A in combination with OP9 cells augmented development of Flk-1$^+$ PDGFRα^+ early mesodermal cells and Flk-1$^+$ PDGFRα^- lateral mesodermal cells from ES cells. These Flk-1$^+$ mesodermal cells further differentiated into CD41$^+$ endothelial cells, which preferentially possessed high hematopoietic potential. Furthermore, Flk-1$^+$ PDGFRα^+ cells but not Flk-1$^+$ PDGFRα^- cells produced hematopoietic progenitors with a bimodal pattern when cultured as an aggregate with OP9 cells. Our results suggest that activin A in combination with OP9 cells facilitates differentiation of ES cells to Flk-1$^+$ mesodermal cells, which encompass various precursors that separately contribute to the development of hematopoietic lineages.

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1. Introduction

Hematopoietic stem cells (HSCs) are known to develop in the aorta-gonad-mesonephros (AGM) region of mouse embryos [1]. The dorsal aorta temporally 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 progenies of VE-cadherin$^+$ ECs [4]. CD41 expression marks the onset of HSC development from VE-cadherin$^+$ hemogenic ECs in the AGM region [5]. The origin of hemogenic ECs has been recently traced back to a distinct cell population derived from extraembryonic mesoderm [6]. However, the developmental pathway of hemogenic ECs remains to be fully understood.

Attempts have been made to emulate the developmental processes of HSCs 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]. We reported that the OP9 co-culture system recapitulates stepwise development of the HC 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 modulate 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, hereafter referred to as activin, has been generally used for mesoderm induction in EBs. For example, activin was shown to be necessary for induction of hematopoietic mesoderm from primitive streak in EBs under serum-free conditions [11]. In contrast, activin induced organizer-like mesendoderm, which lacked hematopoietic potential, from ES cells cultured on type IV collagen matrix [12]. Therefore, the function of activin appears to depend on culture conditions. Recently, the OP9 co-culture system in the presence of activin was reported to induce ES cells to definitive endoderm [13].

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TISSUE-SPECIFIC STEM CELLS

Determining c-Myb Protein Levels Can Isolate Functional Hematopoietic Stem Cell Subtypes

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ABSTRACT

The transcription factor c-Myb was originally identified as a transforming oncoprotein encoded by two avian leukemia viruses. Subsequently, through the generation of mouse models that affect its expression, c-Myb has been shown to be a key regulator of hematopoiesis, including having critical roles in hematopoietic stem cells (HSCs). The precise function of c-Myb in HSCs although remains unclear. We have generated a novel *c-myb* allele in mice that allows direct observation of c-Myb protein levels in single cells. Using this reporter line we demonstrate that subtypes of HSCs can be isolated based upon their respective c-Myb protein expression levels. HSCs expressing low levels of c-Myb protein (c-Myb^{low}HSC) appear to represent the most immature, dormant HSCs and they are a predominant component of HSCs that retain bromodeoxyuridine labeling. Hematopoietic stress, induced by 5-fluorouracil ablation, revealed that in this circumstance c-Myb-expressing cells become critical for multilineage repopulation. The discrimination of HSC subpopulations based on c-Myb protein levels is not reflected in the levels of *c-myb* mRNA, there being no more than a 1.3-fold difference comparing c-Myb^{low} and c-Myb-^{high}HSCs. This illustrates how essential it is to include protein studies when aiming to understand the regulatory networks that control stem cell behavior. STEM CELLS *2015;33:479–490*

INTRODUCTION

Hematopoietic stem cells (HSCs) are defined as cells having both self-renewal and multilineage differentiation capability. On the basis of the duration of reconstitution in a transplantation 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 subtype between LT-HSCs and ST-HSCs [3, 4]. Similarly, the LT-HSC population has been subdivided into dormant and activated HSCs [5, 6]. Dormant HSCs (~30% of LT-HSCs) have been estimated to enter the cell cycle only once every 145 days or more, meaning that they divide only five to six times during the lifetime of a mouse. Conversely, the counterpart-activated HSCs contribute to the daily production of hematopoietic cells by dividing every 28–36 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 cells [7–10]. Myeloid-biased and lymphoid-biased HSCs predominantly generate myeloid progeny and lymphoid progeny, respectively, whereas balanced HSCs give rise to myeloid and lymphoid cells in the same ratio. Most recently, platelet-biased HSCs have been identified and characterized through use of a Vwf-eGFP reporter mouse line [11]. Interestingly, myeloid-biased HSCs exhibit the most potent repopulation potential and CD150^{high} cells in the CD34$^-$LSK population have been prospectively verified to exhibit both long-term repopulating activity and myeloid-biased differentiation [12, 13].

We have previously shown that appropriate levels of c-Myb expression are required at distinct differentiation stages of each cell lineage in hematopoietic progenitor cells (HPCs) [14, 15]. Recently, miR150 has been reported to regulate differentiation in megakaryocyte-erythrocyte progenitors (MEP) via the control of c-Myb protein levels [16]. In contrast to HPCs, its roles in the correct functioning of HSCs remain to be elucidated. Hypomorphic and conditional knockout alleles of murine *c-myb* exhibit a decrease in the number of HSCs in the bone marrow (BM) [17, 18]. Mice homozygous for the expression of the c-Myb^{M303V} mutant, which has 50% of the transactivation activity compared to wild-type

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Redefining the In Vivo Origin of Metanephric 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 the developmental origins of the metanephric mesenchyme (MM), which generates most kidney components. Unexpectedly, we find that posteriorly located T⁺ MM precursors are developmentally distinct from Osr1⁺ ureteric bud progenitors during the postgastrulation stage, and we identify phasic Wnt stimulation and stage-specific growth factor addition as molecular cues that promote their development into the MM. We then use this information to derive MM from PSCs. These progenitors reconstitute the 3D structures of the kidney in vitro, including glomeruli with podocytes and renal tubules with proximal and distal regions and clear lumina. Furthermore, the glomeruli are efficiently vascularized upon transplantation. Thus, by reevaluating the developmental origins of metanephric progenitors, we have provided key insights into kidney specification in vivo and taken important steps toward kidney organogenesis in vitro.

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 (Murry and Keller, 2008; Williams et al., 2012). This is partly because the specification of the kidney lineage in vivo has not been well clarified, owing to its complex processes involving the formation of three consecutive primordia (pronephros, mesonephros, and metanephros) during embryogenesis (Saxen, 1987). The pronephros and mesonephros eventually degenerate in females, whereas a portion of the mesonephros in males contributes to a drainage system for the future testis, including the epididymis. The kidney derives from the embryonic metanephros, which develops at the most posterior part of the body trunk. The metanephros is formed by reciprocally 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 appears around embryonic day 8.5 (E8.5) and expresses the transcription factor *Osr1* (Mugford et al., 2008b). However, the mechanisms underlying how the nascent mesoderm becomes committed to the intermediate mesoderm and how the MM and ureteric bud lineage segregate from one another have not been clarified, despite many reports showing the importance of various growth factors during kidney development (Costantini and Kopan, 2010; Fleming et al., 2013; Kim and Dressler, 2005; Moriya et al., 1993; Poladia et al., 2006). Moreover, it remains to be elucidated how the anteroposterior axis is formed along the intermediate mesoderm and gives rise to the posteriorly located metanephros. In the present study, we addressed these questions by in vivo lineage-tracing experiments together with in-vitro-directed differentiation systems utilizing sorted embryonic kidney precursors at each developmental stage. Importantly, by establishing conditions for the later stages first, and then moving backward to the earlier stages, we were able to optimize the multistep culture conditions from embryonic precursors toward nephron progenitors. Finally, the protocol established by these strategies was successfully applied to the induction of metanephric nephron progenitors from mouse embryonic stem cells (ESCs) and human induced pluripotent stem cells (iPSCs), thereby advocating a model for kidney lineage specification.

RESULTS

The Osr1⁺/Integrina8⁺/Pdgfra⁻ Population Represents Colony-Forming Nephron Progenitors

The MM gives rise to the epithelia of glomeruli (i.e., podocytes) and renal tubules, which constitute the major parts of the nephrons, as shown by cell fate analyses involving labeling of

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

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Abstract Assembly of complex vascular networks occurs in numerous biological systems through morphogenetic processes such as vasculogenesis, angiogenesis and vascular remodeling. Pluripotent stem cells such as embryonic stem (ES) and induced pluripotent stem (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 morphogenic 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

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Introduction

Diverse pathways give rise to the vertebrate 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 vascularization (vasculogenesis) [1]. Arterially-fated angioblasts adjacent to the endoderm form the first artery (the dorsal aorta) ventrally, whereas venous-fated angioblasts migrate dorsally and form the first vein (the cardinal vein) adjacent to the neural tube [2]. The angioblast develops to ultimately form 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 vascular smooth muscle cells. Veins are thinner-walled vessels with little smooth muscle and exhibit valves to prevent backflow of blood (Fig. 1).