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IV. 研究成果の刊行物・別刷

A Linkage in the Developmental Pathway of Vascular and Hematopoietic Cells

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Summary. Blood vessels consist of at least three kinds of cell: endothelial cells lining the inside of the lumen to form tubes, mural cells (vascular smooth muscle cells and pericytes) supporting the endothelial tubes, and blood cells flowing inside. Blood and vascular cells are closely related to each other in their anatomical locations, origins, and differentiation processes. In addition to a long history of histological analyses, recent progress in stem cell biology using various genetic animal models, especially in vivo cell tracing technologies, and in vitro stem cell differentiation systems are now succeeding in providing molecular and cellular bases of the relation between these two cell populations. Accumulating data suggest that their differentiation processes are more complicated than was previously expected. That is, multiple origins of progenitor cells, multiple pathways of differentiation, and multiple molecular functions regulating cell fates exist and complicatedly interact with each other to complete the functional circulation system with blood and vessels. This chapter summarizes recent advances in the developmental processes and the relation of blood and vascular cells, especially between blood and endothelial cells.

Key words. Hematopoiesis · Endothelial cells · Hemogenic endothelium · Progenitor cells · Stem cells

Introduction

Blood cells and endothelial cells (ECs) directly contact each other and form an essential functional unit to maintain the blood supply for the whole body. These two anatomically and functionally related cells are also closely related in their origin and differentiation process. In the yolk sac of mouse embryos, mesoderm-derived cells form cell aggregates called blood islands [1]. The central cells differentiate into blood cells, and peripheral cells of blood islands develop into ECs and fuse with each other to form the initial vascular network [2]. This phenomenon symbolizes the close association and relation between the blood and EC differentiation process. Nevertheless, the actual differentiation pathway and mechanisms of blood and ECs seem not to be so simple and straightforward. Recent progress of developmental, molecular, and stem cell biology is revealing various novel aspects of their differentiation processes. This chapter aims to review the relation between

blood and ECs in their differentiation and diversification process through recent advances.

Differentiation of Endothelial Cells

The origin of ECs is postulated to be mesoderm cells expressing Flk1 (also designated vascular endothelial growth factor receptor-2, VEGFR2) [2]. $Flk1^+$ cells in the periphery of blood islands and in the aorta-gonad-mesonephros (AGM) region in the embryo proper differentiate into ECs mainly by vascular endothelial growth factor (VEGF) signaling and simultaneously form an endothelial tube network called the primary plexus. Such direct formation of vascular structures from progenitor cells is called vasculogenesis. Vasculogenesis is followed by angiogenesis, in which neovessels are formed from preexisting vessels, followed by vascular remodeling with migration and attachment of mural cells to the vascular wall; mature blood vessels are then gradually formed [2, 3]. Diversification into arterial and venous ECs and formation of arteries and veins occur almost simultaneously with the initiation of vascular development [4]. The existence of arterial- or venous-specific progenitors is suggested but still has not been fully demonstrated [5]. Lymphatic vessels sprout and develop from a specific subset of venous ECs [6] (see the Chapter by P. Carmeliet, this volume). The sequential processes of EC differentiation and diversification (i.e., induction of $Flk1^+$ vascular progenitor cells, differentiation of ECs, and diversification into arterial, venous, and lymphatic ECs) are successfully reproduced in an embryonic stem (ES) cell differentiation system [7]. ES cell-derived $Flk1^+$ cells give rise to ECs by VEGF stimulation and mural cells by platelet-derived growth factor (PDGF) stimulation [8]. Whereas venous ECs are induced from $Flk1^+$ progenitors by VEGF treatment alone, arterial ECs are induced by the combinatory stimulation with VEGF and cyclic adenosine monophosphate (cAMP) [9]. $Prox1^+$ lymphatic ECs are induced from $Flk1^+$ progenitors by coculture with OP9 stroma cells [10] or long-term culture of embryoid bodies [11, 12].

Differentiation of Hematopoietic Cells

Research into the differentiation pathway of blood cells (=hematopoietic cells, or HPCs) has a much longer and complicated history than that of ECs. Until recently, a model of the successive hematopoietic process (primitive and definitive hematopoiesis) has been largely accepted as a major pathway of hematopoietic development [13, 14]. That is, the first transient wave of hematopoiesis (primitive hematopoiesis), which generates primitive erythrocytes, occurs in the yolk sac at E7.0–7.5, but the primitive HPCs carrying the embryonic and fetal-type hemoglobins contribute only in the embryo, not in the adult. A second wave of hematopoiesis that generates the hematopoietic stem cells (HSCs) necessary throughout life (definitive hematopoiesis) originates in the embryo proper. Definitive HSCs arise from the AGM region, including the endothelium and surrounding mesenchyme of the dorsal aorta at E10.5; then the site of blood formation migrates to the fetal liver at E12.5 and finally to the bone marrow (Fig. 1a) [13, 15–17].

This model is currently being challenged and remodeled by various studies. In addition to the AGM region, HSCs appear in umbilical and vitelline arteries [18]. The placenta forms a large HSC pool around E11.5–12.5 through the rapid expansion of

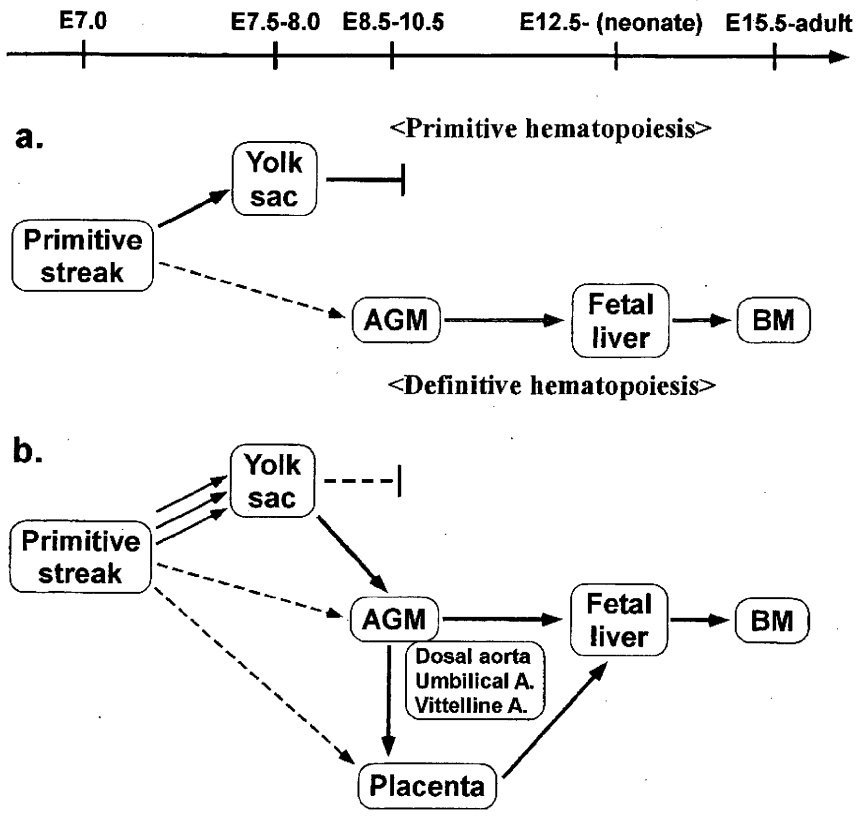

FIG. 1. Models of embryonic and adult hematopoiesis. **a** Conventional model. The first wave of hematopoiesis, called primitive hematopoiesis, occurs in the yolk sac at E7.0–7.5. Primitive hematopoietic cells (HPCs) carrying the embryonic and fetal-type hemoglobins are found only in the embryo, not in the adult. A second wave of hematopoiesis, called definitive hematopoiesis, occurs in the aorta-gonad-mesonephros (AGM) region and generates definitive HSCs that can contribute throughout life. Definitive HSCs colonize the fetal liver and finally the bone marrow (*BM*). **b** A multiorigin, multipathway model. The origin of yolk sac blood islands is polyclonal. Definitive HSCs in the AGM regions at least partly originate from yolk sac precursor cells. Major vessels in the embryo, dorsal aorta, umbilical artery, and vittelline artery as well as the placenta are another source of definitive HSCs

HSCs [19–21]. In the dorsal aorta, a subset of endothelium [15, 16] (hemogenic ECs; discussed later) as well as surrounding mesenchymal cells [22, 23] are postulated to be the origins of definitive HSCs. Recently, Ueno et al. revealed that the origin of yolk sac blood islands in vivo is polyclonal, using a novel direct clonal progenitor analysis in the mouse embryo [24]. Samokhvalov et al. demonstrated that specifically labeled Runx1$^+$ yolk sac precursor cells can develop into fetal lymphatic progenitors and even into adult HSCs [25], clearly indicating that yolk sac hematopoiesis can contribute to the adult. These reports indicate that HSCs develop through multiple origins, locations, and trafficking processes (Fig. 1). In addition, these various hematopoietic organs, yolk sac, AGM, fetal liver, and bone marrow serve as different microenvironments for HSC maturation [17]. Yolk sac-derived HPCs could not reconstitute hematopoiesis in the adult when transplanted into irradiated mouse models [26], even though they have an intrinsic potential of becoming adult HSCs [25]. On the other hand, yolk sac-derived HPCs could give rise to definitive HSCs after being injected

into fetal liver [26], indicating that HSC niches in the embryo proper should serve as an appropriate microenvironment to achieve complete maturation of HSCs for engraftment and self-renewal activity.

Diversification of ECs and HPCs

From many decades ago, the close association of HPCs and ECs in their developmental process in the yolk sac led to the hypothesis that these cells originate from a common precursor, the hemangioblast [27]. Blast colony-forming cells (BL-CFCs), a putative hemangioblast that give rise to both HPCs and ECs, were first reported in differentiating embryonic stem cell culture in vitro [28]. BL-CFCs were enriched in the Flk1$^+$/brachyury$^+$ mesoderm population. Recently, the existence of hemangioblasts in the gastrulating embryo has been demonstrated. Huber et al. revealed that hemangioblast comprises a subpopulation of mesoderm co-expressing Flk1 and brachyury. Highest dual differentiation potential to HPCs and ECs were observed in the posterior primitive streak but not in yolk sac, indicating that the first commitment of mesodermal progenitors to the HPC and EC lineages begins in the posterior primitive streak before the cells migrate into the yolk sac, prior to the formation of the blood islands [29]. The early segregation of HPC and EC lineages is also indicated by the fact that individual Flk1$^+$ precursors rarely contributed to both ECs and HPCs in blood islands by a direct clonal progenitor analysis in the mouse embryo [24] and that Runx1-marked yolk sac precursor cells can contribute to adult HSCs but not to ECs [25].

The next putative diverging point between ECs and HPCs is in the early ECs. During the early developmental stage of the dorsal aorta, attachment of HPC clusters to the EC layer is observed [30], inferring that HPCs including HSCs originate from a subset of ECs, termed hemogenic ECs, that possess the potential to give rise to HPCs. The hemogenic EC hypothesis is supported by several studies in addition to histological evidence. Embryonic as well as ES cell-derived vascular endothelial (VE) cadherin$^+$ ECs give rise to HPCs including T and B cells in vitro [31, 32]. Acetylated low density lipoprotein (Ac-LDL)-labeled ECs in the embryo give rise to CD45$^+$/Ac-LDL$^+$ HPCs in situ in chick and mouse embryos [33, 34]. A diverging point of hemogenic and non-hemogenic ECs was demonstrated using a transgenic embryo and ES cell line carrying the Flk1 promoter/enhancer [Flk(p/e)]-driven GFP gene. VE-cadherin$^+$ ECs first appeared as a GFP$^-$ population, but subsequently all ECs became VE-cadherin$^+$/GFP$^+$. Only GFP$^-$ ECs could give rise to definitive HPCs and were observed in hematopoietic cell clusters in the dorsal aorta, indicating that Flk(p/e)-GFP$^-$ ECs represent hemogenic ECs [35]. α_4 Integrin$^+$/VE-cadherin$^+$ ECs, but not α_4 integrin$^-$ ECs derived from mouse and primate ES cells, were reported to show hemogenic potential [36, 37]. VE-cadherin$^+$/CD45$^+$ cells at E9.5 of the mouse embryo, which give rise to definitive erythroid, myeloid, but not B lymphoid cells, may represent another intermediate cell type of hemogenic ECs [38]. These studies indicate that during the early developmental stage a subset of ECs show a differentiation window to give rise to HPCs. The hemogenic ECs should be at least one of the precursors for the definitive HSCs.

Molecular Machinery of the Fate Determination

Gene knockout technology has largely contributed to uncover the molecular mechanisms in differentiation of ECs and HPCs. In vitro cell differentiation systems using

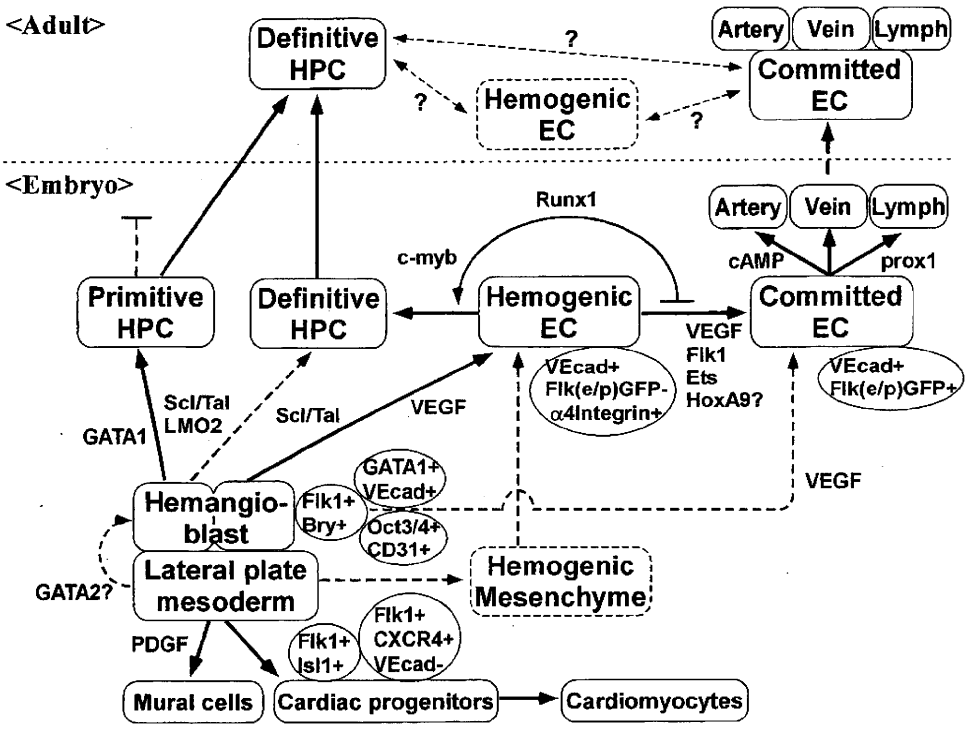

FIG. 2. Possible relation between ECs and HPCs during their differentiation. Cells constituting the circulation—ECs, HPCs, mural cells in the vascular wall, cardiomyocytes—mainly originate from lateral plate mesoderm. The putative relations, differentiation pathways, and regulatory mechanisms of these cells and their progenitors (hemangioblast and hemogenic ECs) are summarized. Cell populations are indicated in *rectangles*. Molecular markers are indicated in *ovals* attached to the corresponding cell populations. Issues not fully demonstrated are indicated by *dotted lines*

ES cells also play an important role in dissecting the molecular machinery for the cell differentiation and diversification process, especially at a single-cell level [39]. Various approaches using both in vitro and in vivo analyses have now demonstrated that many molecules are involved in the differentiation and diversification process of ECs and HPCs (Fig. 2).

Flk1/VEGFR2

Flk1 is one of the VEGF receptors (VEGFR2). As Flk1-deficient mice showed a defect in the development of ECs and HPCs [40], Flk1 is thought to be one of the earliest functional molecules for EC and HPC development and should mark their common progenitor, hemangioblasts. Flk1 is broadly expressed in lateral plate mesoderm and extraembryonic mesoderm [41, 42]. $Flk1^+$ mesoderm cells are demonstrated to give rise to both ECs and HPCs [31]. $Flk1^+$ cells also differentiate into mural cells in the vascular wall and cardiomyocytes [8, 43, 44]. During the differentiation, Flk1 expression is maintained only in ECs and disappears in mature HPCs, mural cells, and cardiomyocytes. Activation of Flk1 by VEGF is required to maintain Flk1 expression in $Flk1^+$ mesoderm cells and drives their differentiation to EC lineage [8, 45]. Flk1 signaling through VEGF is the main inducer of ECs from their progenitors. Recently, phospholipase C-γ activation by VEGF through the autophosphorylation of tyrosine

residue 1173 in Flk1 was revealed to be essential for vasculogenesis in the embryo [46]. Flk1 expression further serves various progenitor populations being combined with other molecular markers. $Flk1^+/Tal1^+$ cells [47] and $Flk1^+/brachyury^+$ cells [29] are reported as hemangioblasts. $Flk1^+/CXCR4^+/VE\text{-}cadherin^-$ cells [43] and $Flk1^+/isl1^+$ cells [48] are shown to be progenitors for ECs and cardiomyocytes.

Scl/Tal1

Scl/Tal1 is a basic helix-loop-helix (bHLH) transcription factor expressed in yolk sac blood progenitors and ECs [49]. The most evident role of Scl/Tal1 is driving the commitment of progenitor cells to hematopoietic lineage. $Scl/Tal1^{-/-}$ mice showed a defect of embryonic hematopoiesis even though the primitive vascular network was normal [50, 51]. $Scl/Tal1^{-/-}$ ES cells did not contribute to hematopoietic tissues in chimeric animal models [52]. On the other hand, induced expression of Scl/Tal1 in the early differentiation stage of $Scl/Tal1^{-/-}$ ES cells restored HPC differentiation [53]. $Flk1^+$ mesoderm cells from $Scl/Tal1^{-/-}$ ES cells gave rise only to mural cell lineage, whereas cell lines expressing Scl/Tal1 in the Flk1 locus could make ECs and HPCs [47]. Phenotypes of Scl/Tal1-deficient mice resemble those of LIM-only protein, LMO2 [54] and GATA-binding protein, GATA-1 [55]. LMO2 is posited to bridge Scl/Tal1 and GATA proteins [56]. The Scl/Tal1-LMO2-GATA protein complex should contribute to specify mesoderm to HPC lineage [57].

GATA

Among GATA-binding transcription factors, GATA1, 2, 3 (considered hematopoietic GATAs) are posited to be involved in early hematopoiesis [58]. Disruption of GATA2, which is expressed in HSCs and progenitors, induces impaired primitive and definitive hematopoiesis in mice, indicating that GATA2 has a role in the proliferation and expansion of early HPCs [59, 60]. Recently, GATA2 induction during an early differentiation stage of ES cell differentiation resulted in an increase in hemangioblasts, erythroid cells, and ECs, suggesting a GATA2 role in the early stage of EC and HPC differentiation [61]. GATA1 is postulated to be involved mainly in erythrocyte differentiation. $GATA1^{-/-}$ mice show loss of erythrocytes [55]. ES cell-derived $GATA1^+/Flk1^-$ cells give rise only to primitive erythrocytes [62]. Recently, $GATA1^+/VE\text{-}cadherin^+$ cells in the early embryo (E7.0–7.5) were demonstrated to be a common precursor for both HPCs and ECs [63]. GATA3, which is expressed in hematopoietic progenitors as well as T cells, is necessary for the production of T-helper 2 cells [64]. GATA3 is also expressed in mesenchymal cells with stromal activity to generate definitive HPCs [65], suggesting that GATA3 may be involved in the formation of the microenvironment to support definitive HPC development.

Runx1

Runx1, also designated AML1 and CBFa2, is a transcription factor showing homology to the *Drosophila* pair-rule gene, *runt*. Runx1 plays pivotal roles in the development of definitive hematopoiesis. $Runx1^{-/-}$ mice showed normal primitive hematopoiesis but completely impaired definitive hematopoiesis with disappearance of budding hematopoietic clusters from ECs in dorsal aorta [66, 67]. In a chimeric animal model,

Runx1$^{-/-}$ ES cells failed to contribute to any hematopoietic tissues, whereas Runx1$^{+/-}$ ES cells could contribute to bone marrow and peripheral blood, among others [66]. Studies using Runx1$^{+/LacZ}$ mice revealed that Runx1 expression marks HSCs [22, 68]. That is, LacZ expression is observed in the dorsal aorta, vitelline, and umbilical arteries where long-term repopulating HSCs reside. Rux1-LacZ cells localize in ECs of the dorsal aorta, HPCs budding from ECs, and in some mesenchymal cells surrounding the aorta. Recently, Runx1-LacZ cells in the yolk sac at E7.5 were demonstrated to contribute to definitive hematopoiesis [25]. These observations suggest that Runx1 is expressed in hemogenic ECs or their precursors and regulates the differentiation of HPCs from ECs. Runx1 directly suppresses Flk1 expression at the transcriptional level during ES cell differentiation [69]. Runx1 should be directly involved in EC-HPC transition by endowing the hematopoietic potential to their precursor cells.

Others

A Myb family transcription factor, c-myb is expressed in hemogenic ECs and is involved in definitive hematopoiesis. Rescue of c-myb expression in c-myb$^{-/-}$ ES cell-derived ECs restores the generation and proliferation of definitive HPCs, indicating that c-myb endows hemogenic properties to early ECs [70]. Ets transcription factors are expressed in hemangioblast, and four of the Ets genes are demonstrated to be essential for early EC specification and differentiation in zebrafish [71]. A homeobox transcription factor, HoxA9, was reported to act as a master switch to regulate the expression of prototypical endothelial-committed genes such as endothelial nitric oxide synthase, VEGFR2, and VE-cadherin in adult EC progenitor cells [72].

Conclusion

Recent studies on EC and HPC development uncovered various cellular and molecular mechanisms in the differentiation processes of these cells. Nevertheless, these findings do not efficiently contribute to the better understanding of EC and HPC development. Current trends in this research area are mainly accumulating evidence of the existence of the "heterogeneity" in the developmental processes. That is, multiple origins (with early segregation), multiple pathways, and multiple molecular functions exist and complicatedly interact with each other to form the functional circulation system. Cell tracing studies in vivo demonstrated multiple origins of and their early segregation to ECs and HPCs [24, 25]. Novel hemangioblastic cell populations are still being reported, such as Oct3/4^{+}/CD31^{+} cells [73] and GATA1^{+}/VE-cadherin^{+} cells [63].

In the future, dual directions of studies will be required for a complete understanding of EC and HPC development. One direction is to extend the studies in various ways to accumulate novel results. In vivo animal models for cell tracing, in vitro analyses for the cell differentiation process, various large-scale biology, called "omics", for gene and protein expressions, epigenetic analyses, and so on—all of these strategies would provide novel outcomes from various points of view. The other direction is to make them relevant to each other and converge them to reconstitute a novel concept and understanding (although it is not easy). By repeating these processes, we should be able to approach a better or true "stereoscopic" understanding of the developmental processes.

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4 ES細胞、iPS細胞を用いた血管再生医療技術

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1 はじめに

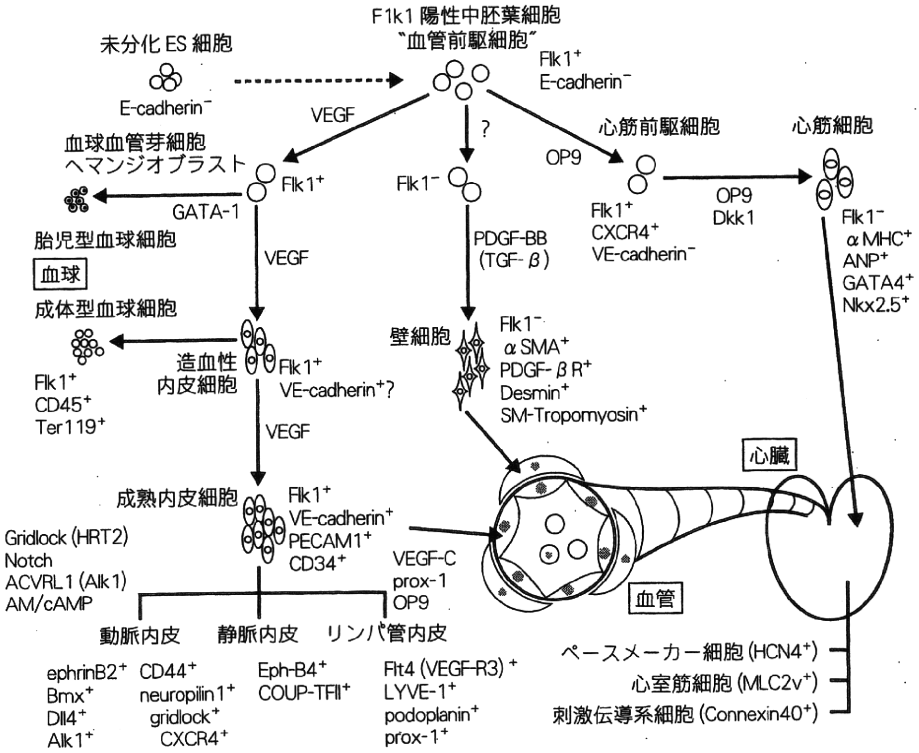
未分化性と分化能を維持したまま無尽蔵に増殖することができるES細胞(胚性幹細胞：Embryonic Stem cells)は、再生医療への応用がその大きな使命であり研究ターゲットとされているが、日本ではまだヒトES細胞の臨床応用は認められていない。世界的にもES細胞由来細胞をヒトに用いた治療の報告はない。また、最近の体性幹細胞(成体内に存在する幹細胞)研究は、細胞移植に至るハードルが倫理面、安全面等でES細胞よりも低いため、再生医療応用において注目されている。にもかかわらず、ヒトを含めたES細胞研究は国内および世界的広がりを見せている。ES細胞研究は、直接的な細胞治療応用のみならず、細胞分化機構に関する基礎研究をもとにした新たな遺伝子治療、創薬、薬品の効果や毒性等のドラッグスクリーニングへの応用等、基礎面臨床面に果たせる貢献は非常に大きい。2006年、2007年のマウスおよびヒトiPS細胞(人工多能性幹細胞)[1)~3)]の樹立は、ヒトES細胞における倫理的問題および細胞移植における技術的問題の一部をクリアすることにより、ES細胞が有していたポテンシャルをさまざまな形で実現できる可能性を示し、幹細胞を用いた再生医学研究に大きなインパクトを与えた。本稿では、ES細胞およびiPS細胞を用いた血管再生分野の現況と将来における可能性等について概説する。

2 ES細胞からの血管細胞の分化多様化

血管壁の内側を一層に覆い血管腔を形成している血管内皮細胞と、血管内皮による管腔を外

側から取り巻き、収縮弛緩や血管構造の維持に寄与している血管壁細胞(動静脈における血管平滑筋細胞および毛細血管におけるペリサイト)の2種類の細胞は血管を構成する主要な細胞である。これら血管構成細胞は、胚様体を用いて分化誘導できることが以前より報告されている。未分化ES細胞は、フィーダー細胞やLIF(Leukemia Inhibitory Factor)などの非存在下で浮遊培養することにより、ES細胞が寄り集まった球形の胚様体(embryoid body)と呼ばれる構造を形成する。胚様体内では、さまざまな細胞間の相互作用が文字通り擬似胎仔のように働き、さまざまな細胞が分化誘導されてくる。胚様体法を用いてES細胞を分化誘導することにより、血管前駆細胞および中胚葉の分子マーカであるFlk1(2型VEGF(血管内皮増殖因子)受容体；VEGFR2)陽性の細胞、さらには内皮細胞のマーカであるCD31、Tie2、VE(Vascular Endothelial)-カドヘリン陽性細胞が順次出現し、内皮細胞の分化と網状に張り巡らされた原始的な血管構造の形成が起こることが観察されている。また、レチノイン酸とdibutyryl-cyclic AMPを添加することにより、胚様体中に血管平滑筋細胞が誘導されることも以前に報告されている。筆者らは、胚様体を用いない新しいES細胞分化誘導法により、ES細胞由来Flk1陽性細胞が、血管を構成する血管内皮細胞と血管壁細胞の共通の前駆細胞であり、Flk1陽性細胞から内皮細胞および壁細胞の双方が分化誘導でき、毛細血管様の高次構造を培養下に形成できることを示した[4]。Flk1陽性の血管前駆細胞は、VEGFの刺激により内皮細胞に、主にPDGF-BB(血小板由来増殖因子)により壁細胞に分化すると考えられる。また、血流による物理的刺激であるshearストレスや拍動性進展刺激がFlk1陽性細胞からの内皮細胞分化や壁細胞分化を誘導することも明らかにされている。筆者らはさらに、Flk1陽性細胞から心筋細胞を分化誘導することにも成功し[5]、Flk1陽性細胞を共通の前駆細胞として、血球細胞、内皮細胞、血管壁細胞、心筋細胞といった循環器系の細胞を系統的に分化誘導できることを明らかにしてきた(**図1**)。最近になり、Flk1を含む種々のES細胞由来前駆細胞が心筋細胞や内皮細胞、血管平滑筋細胞に分化することが相次いで報告され[6]、ES細胞の心血管分化研究は世界的広がりを見せている。

最近、動脈、静脈、リンパ管それぞれの内皮細胞特異的に発現している分子が数々報告され、内皮細胞の多様性に分子的根拠が与えられるようになってきた。それにより、生体内の位置情報がない培養細胞においても、動静脈リンパ管分化が解析できるようになった。筆者らは最近、Flk1陽性細胞からの血管分化系を用い、ephrinB2陽性(動脈)内皮、ephrinB2陰性(静脈)内皮、およびprox-1陽性(リンパ管)内皮細胞と考えられる細胞の誘導と純化にそれぞれ成功した[7),8)]。すなわち、Flk1陽性細胞をVEGFおよび血清存在下に内皮細胞に誘導するとほとんど(＞90～95%)の内皮細胞がephrinB2陰性の静脈内皮細胞となる。VEGFに加えて、cAMPアナログである8bromo-cAMPまたは細胞内cAMPを上昇させる液性因子の一つであるアドレノメデュリン(AM)を加えcAMP経路を活性化することにより、内皮細胞においてNotch

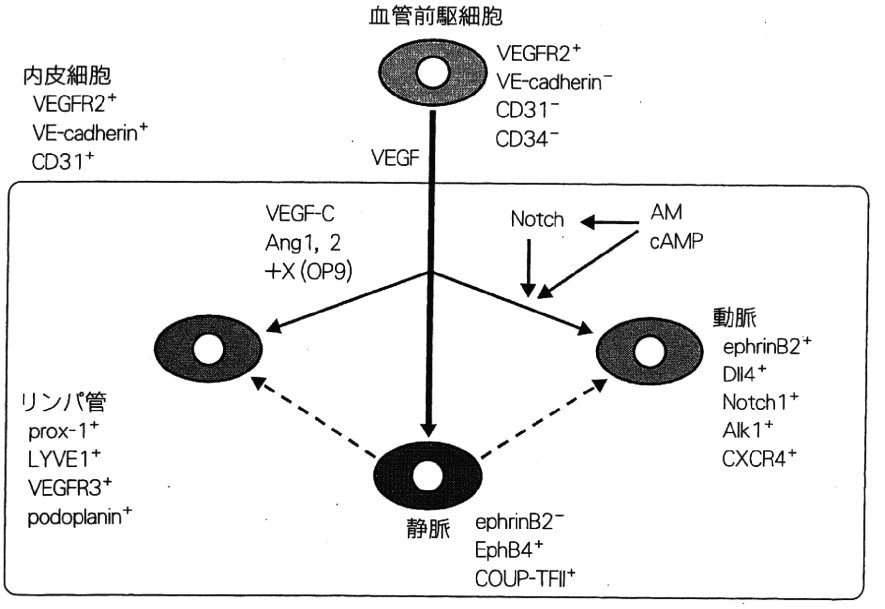


ES 細胞由来 Flk1 陽性細胞は、そこから全ての心血管構成細胞(内皮細胞、壁細胞、血球細胞、心筋細胞)が分化することのできる共通の前駆細胞と考えられる。Flk1 陽性細胞から分化した内皮細胞は、一時的に血球形成能を有する時期があるが、その後内皮細胞が成熟するにしたがって血球形成能は失われる。内皮細胞は成熟するとともに多様化も同時に行い、動脈、静脈、リンパ管内皮をはじめとしたさまざまな内皮細胞に分化すると考えられる。VEGF が Flk1 に結合することによるシグナルが不十分であると Flk1 の発現は失われ、主に PDGF-BB の作用により壁細胞が誘導される。これら誘導された血管構成細胞は、培養下および生体内において血管構造を形成できる。誘導された内皮細胞は、培養条件によりさらに動静脈リンパ管に分化する。また Flk1 陽性細胞を OP9 ストローマ細胞上で培養することにより、心筋前駆細胞を経て心筋細胞が分化誘導される。誘導心筋細胞には、ペースメーカー細胞、心室筋細胞、刺激伝導系細胞に類似した多様な細胞が含まれる。

図１　Flk1 陽性細胞からの心血管細胞の分化

シグナルの活性化が誘導され、ephrinB2 陽性の動脈内皮細胞が誘導されることを明らかにした[7]。また一方、Flk1 陽性細胞を OP9 ストローマ細胞上で培養して内皮細胞を誘導したところ、prox-1 陽性リンパ管内皮細胞が出現した。この OP9 によるリンパ管誘導作用は、VEGF-C および angiopoietin の作用をブロックすることによりほぼ完全に阻害されたが[8]、VEGF-C と angiopoietin のみではリンパ管内皮は誘導されず、OP9 細胞由来の何某かの因子が必要であると考えられた。これらの結果により、ES 細胞を用いて、動脈、静脈、リンパ管内皮細胞の全てを系統的に分化誘導することが可能になるとともにその新たな分化メカニズムが明らかになった[9]。現在、それぞれの分化誘導因子や分化機構のさらなる検討を行っている(投稿中)。

以上の結果をまとめるとES細胞からの血管の分化多様化機構は**図2**のようになる。すなわち、Flk1陽性前駆細胞からの血管内皮全般の分化にはVEGFが必須であり、VEGFに加えてcAMPおよびNotchシグナルが働くと動脈内皮が、OP9ストローマ細胞からの誘導作用によりリンパ管内皮細胞が出現すると考えられる。このようにES細胞を用いて構成的系統的に血管細胞を分化誘導する新たなアプローチは、ノックアウトマウスなどでは困難であった血管の分化・多様化メカニズムの細胞レベル分子レベルでの解析を可能にする新たな手法と考えられる[10]。こうした新たなアプローチで血管分化多様化機構を解析することにより、種々の血管特異的な血管新生やリンパ管特異的新生抑制による抗ガン治療などの開発も期待される。さらに詳細に臓器特異的・病態特異的な血管の多様性を解析し理解することは、血管を介した臓器機能や病態の理解とそれに基づくさまざまな新しい治療戦略の開拓に結びつくと考えられる。



VEGFR2(Flk1)陽性血管前駆細胞は、主にVEGFのシグナルによりVE-カドヘリン陽性内皮細胞に分化する。静脈内皮細胞と考えられる細胞はVEGFおよび血清のみで誘導されるが、動脈内皮分化にはそれに加えてNotchおよびcAMPシグナルが、リンパ管内皮にはVEGF-C、angiopoietinとOP9細胞由来因子がそれぞれ必要である。動脈内皮およびリンパ管内皮は、静脈内皮(または厳密にはどれにも当てはまらないプロトタイプ内皮細胞?)からそれぞれ分化する可能性もある。

図2　血管前駆細胞からの動静脈リンパ管分化機構

3　ES細胞による血管再生

筆者らはES細胞由来Flk1陽性細胞が生体内においても血管細胞に分化し、血管再生に寄与しうるかを検討するため、純化Flk1陽性細胞のニワトリ胎仔への移植実験を行った。心腔内注入により経血管的に移植されたFlk1陽性細胞は、内皮細胞および壁細胞に分化するとともにニワトリ胎仔発生にともなって形成された新生血管に寄与した[4]。筆者らはさらに、ES細胞由来細胞の血管再生治療応用における可能性を検討するため、ES細胞由来血管細胞の成体に対する移植効果を検討した[11]。すなわち、ES細胞由来血管細胞をヌードマウスに移植した腫瘍周囲に注入し、移植細胞の新生血管への寄与を検討したところ、ES細胞由来Flk1陽性細胞は、内皮細胞および壁細胞として新生血管へ寄与した。次に、成体への移植に適切な細胞の分化段階を検討するため、分化段階の異なる血管細胞、すなわち、ソート直後のFlk1陽性血管前駆細胞と、Flk1陽性細胞をさらに3日間培養して初期内皮細胞に分化した細胞（VE-カドヘリン陽性)の移植を比較した。Flk1陽性細胞を移植した群では、血管内皮細胞として寄与しているものの他に、それ以外の細胞として組織内に存在するものが多数(約60%)認められた。一方、初期内皮を移植した群では、ほとんど全ての細胞(95%以上)が内皮細胞として血管に寄与していた。また、Flk1陽性細胞移植群では、細胞移植した腫瘍における血流増加は認められなかったが、分化させた血管細胞を移植した群では、有意な血流増加が認められた。これらの結果より、ES細胞由来血管細胞の移植により、血管新生促進効果が認められるが、成体における血管新生をターゲットとした細胞移植においては、血管前駆細胞のレベルの細胞よりも、やや血管に分化した初期内皮細胞のステージがより有効かつ特異的であると考えられた。このように、ES細胞由来細胞の移植においては、むやみに未分化細胞を移植すればよいわけではなく、ドナー細胞の分化段階とレシピエント側の状況を対応させた至適な分化段階の細胞、—おそらくは標的細胞への分化が運命づけられた直近の前駆細胞—、を選択する必要があると考えられた。また同時に、移植をされる側においても標的細胞の分化を効率的に促進できる微小環境ができるだけ再現されていることが、有効な再生の実現には重要であると考えられる。

4　ヒトES細胞からの血管分化再生

ヒトES細胞を用いた血管細胞分化としては、胚様体を用いてCD31やVE-カドヘリン陽性内皮細胞の誘導と、フローサイトメトリーを用いての純化・再培養、培養下および免疫不全マウスに移植したゲル内における血管構造の形成が報告されている。京都大学のグループは、マウスES細胞と同様にサルES細胞においても2型VEGF受容体陽性細胞からの内皮細胞・壁細胞の分化[12]、培養下における血管構造形成に成功している。さらに同グループは、2002

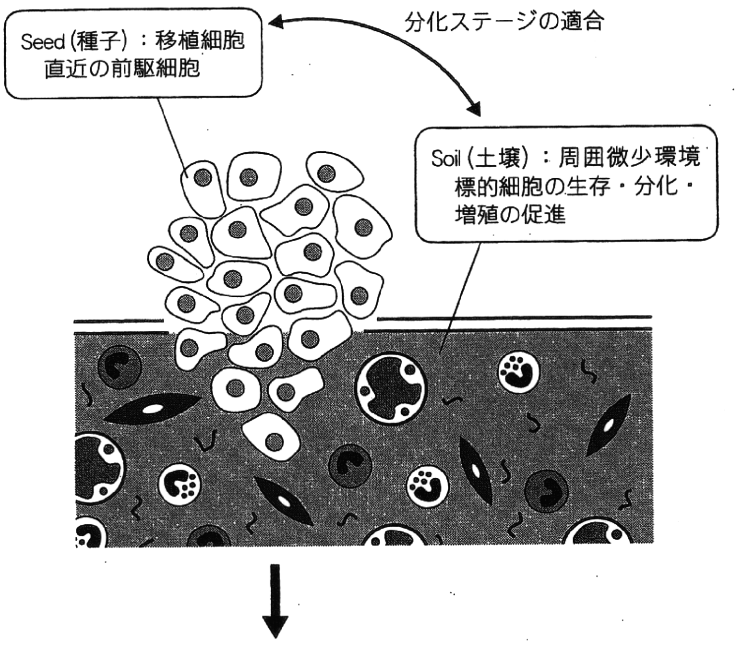
年より日本最初のヒトES細胞分化研究を輸入ヒトES細胞を用いて開始し、ヒトES細胞においても血管構成細胞の分化誘導と*in vitro*における管腔構造形成、さらにはマウス血管新生モデルにおける新生血管への移植細胞の寄与と血流改善効果を認めることを明らかにした[13),14)]。ヒトES細胞由来血管細胞の移植においては、純化した内皮細胞だけの移植よりも、血管壁細胞と混合した細胞群の方が血管新生作用が強かった[14)]。類似の現象は、心筋や神経に関する細胞移植においても報告されている。すなわち、純粋な心筋細胞や神経細胞のみならず、心臓間質細胞やグリア細胞などが共存する形の移植の方が心筋や神経再生効果が高い可能性が示唆されており、純粋に必要とされる細胞だけを移植するのではなく、移植細胞と周囲環境との相互作用、効果的に標的組織の再生を促進する局所微小環境を考慮した細胞移植戦略が新たな再生治療法の開発に有効であると考えられる。

5 iPS細胞からの血管・リンパ管分化

iPS細胞は、線維芽細胞等の成体由来分化細胞にOct4、Sox2、Klf4、c-mycの4因子(または3因子)を導入することにより誘導される新しい多能性幹細胞である[1)~3)]。筆者らは、マウスおよびヒトiPS細胞を用いた心血管分化研究にもいち早く取り組んでいる。[1]項に述べたマウスES細胞の血管分化誘導法をマウスiPS細胞に適用することにより、マウスES細胞と同様に、iPS細胞からの血管内皮細胞、壁細胞、動静脈リンパ管内皮細胞の分化誘導に成功した。内皮細胞および壁細胞からなる血管構造の3次元的形成にも成功した[15)]。また、担がんヌードマウスへの細胞移植実験により、内皮および壁細胞として生体内血管新生に寄与し得ることも確認した。マウスiPS細胞3クローンを用いて検討したが、クローン間で多少の分化能、増殖能に差異を認めたが、ES細胞においても認められるクローン間の差異と同程度かそれ以下のものであり、マウスiPS細胞はマウスES細胞とほぼ同様の分化特性を有していると考えられた。ただし、1～2ヵ月以上の長期分化誘導培養中にc-mycをはじめとするiPS細胞誘導時の導入遺伝子群の再発現を認める例があり、iPS細胞における特性の一つとして注意する必要があると考えられる。現在ヒトiPS細胞の心血管系への分化誘導も行っているが、マウスiPS細胞はマウスES細胞と、ヒトiPS細胞はヒトES細胞とほぼ同様の性質を持っていると考えられる。iPS細胞は、さまざまな病態モデル動物やヒト症例から比較的簡便に多能性幹細胞が誘導できるため、薬剤の安全性試験や新たなドラッグスクリーニングなど、直接的な細胞移植以外にも種々の応用が可能である。実際筆者らは、マウスiPS細胞からの3次元的血管形成モデルを用いて海洋生物由来HDAC阻害物質Ageladineの血管新生抑制作用を示すことに成功している[16)]。このようにiPS細胞を用いることにより、病態や疾患と幹細胞およびケミカルバイオロジーを結びつけた新しい再生医学や創薬研究が可能になると考えられる。

6　おわりに

このように、血管の発生・分化・再生機構に関してさまざまな知見が蓄積されてきているが、いまだ血管再生治療が明らかに有用な形で臨床応用されたといえるレベルには至っていない。臓器を構成する細胞を誘導して移植するあるいは前駆細胞を移植するというだけで臓器の再生が進むというほど単純ではないことがようやく学習されてきたというのが実情に近いであろう。今後は、細胞そのものの分化メカニズムの解析―細胞外シグナルから細胞内環境の変化と安定化の過程をエピジェネティックな視点も含めて解明する―に加えて、細胞間および細胞―細胞外マトリックス相互作用や臓器・組織間相互作用等臓器としての機能を果たし得る機能ユニットを形成するために必要な要素全てに関して理解を深め、それらを生体内でできるだけ再構成することが重要であろう。[３][４]の項でも述べたように、有効に分化し得る幹・前駆細胞(Seed：種子)と、分化と機能発現を可能にする周囲環境(Soil：土壌)の双方を整えた治療(Seed & Soil Therapy)(**図３**)を目指すことにより、再生医療はより実効性が期待されるものに近づ



細胞移植による臓器再生を行うためには、移植細胞(Seed：種子)とレシピエント側の微少環境(Soil：土壌)の分化ステージを適合させるとともに、双方の要因を整える必要がある。Seed としては、標的細胞の直近の前駆細胞が好ましいと考えられる。Soil は、移植細胞の生着・生存、分化、増殖を促進できる微少環境が存在することが必要である。これら Seed & Soil を最適化することにより、効率的臓器再生が実現されると考えられる。

図３　Seed & Soil therapy