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Generation of robust vascular networks from cardiovascular blast populations derived from human induced pluripotent stem cells *in vivo* and *ex vivo* organ culture system

Tatsuya Kawamoto [1], Yoshifumi Kobayashi [1], Hidenori Nakajima, Yukiko Yamagishi *

Molecular Medicine Research Laboratories, Drug Discovery Research, Astellas Pharma Inc., Tsukuba, Ibaraki, Japan

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ABSTRACT

Vascular network formation is a key therapeutic event in regenerative medicine because it is essential for mitigating or ameliorating ischemic conditions implicated in various diseases and repair of tissues and organs. In this study, we induced human induced pluripotent stem cells (hiPSCs) to differentiate into heterogeneous cell populations which have abilities to form vascular vessel-like structures by recapitulating the embryonic process of vasculogenesis *in vitro*. These cell populations, named cardiovascular blast populations (CBPs) in this report, primarily consisted of $CD31^+$ and $CD90^+$ cells.

By using cell-sheet technology, we observed that CBP with $CD31^+$ cells to $CD90^+$ cells in the ratio of 1:1.5 could reproducibly form robust vascular networks *in vivo* and *ex vivo* organ culture system. To our knowledge, this is the first report demonstrating the generation of vascular network from hiPSCs in *ex vivo* organ culture system that correlates closely with *in vivo* results. Our results suggest that CBP provides a promising approach for studying vasculogenesis and subsequently can be used in regenerative medicine.

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1. Introduction

Blood vessel networks supply oxygen and nutrients to all tissues, therefore, their structural or functional abnormalities causes various ischemic diseases such as myocardial infarction, stroke, and neurodegenerative or obesity-associated disorders [1]. In the case of critical limb ischemia (CLI), wounds can progress to become ulcers, which can result in gangrene and permanent tissue loss. While medical therapies are common, revascularization is typically required to prevent or slow progression to amputation. Although many revascularization approaches with proangiogenic factors or purified stem/progenitor cells have been investigated, these approaches have been mainly resulted in just inducing sprouting angiogenesis and insufficient in *de novo* blood vessel formation in the ischemic tissues [2]. We think one of promising approaches for a sufficient neovascularlization is the optimization of the composition of the transplant.

Ischemic condition is also a problem in tissue engineering. One of the major issues in tissue engineering is the lack of sufficient blood vessels for supplying oxygen and nutrients to tissue [3]. Therefore, efficient blood vessel formation such as vasculogenesis, which is only observed during the embryogenic process, is required to solve the problem.

Vascular vessels primarily consist of two cell types, endothelial cells and mural cells. The majority of vascular cells is considered to differentiate from hemangioblasts derived from mesoderm. Hemagioblasts associate with each other to form the primitive vascular plexus. Thereafter, vascular trees consisting of small and large blood vessels are formed and subsequently undercoated with mural cells, such as smooth muscle cells or pericytes [4,5].

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) established by Takahashi and Yamanaka [6] are excellent tools to recapitulate the embryogenic process. For example, mouse and human ESCs can be differentiated into all cardiovascular cell linages [7,8]. Further, human induced pluripotent stem cells (hiPSCs) can recapitulate multiple steps of the vascular developmental process *in vitro*, such as vascular cell differentiation and diversification from progenitors, endothelial cell maturation and vascular formation [9]. This *in vitro* approach provides a novel possibly for elucidating the cellular and molecular mechanisms of vascular development. However, it was difficult to induce the differentiation of human ESCs or hiPSCs toward a specific organ of interest such as vascular vessels.

* Corresponding author. Address: Molecular Medicine Research Laboratories, Drug Discovery Research, Astellas Pharma Inc., 21 Miyukigaoka, Tsukuba-shi, Ibaraki 305-8585, Japan. Fax: +81 29 852 5444.
E-mail address: yukiko.yamagishi@astellas.com (Y. Yamagishi).
[1] These authors contributed equally to this work.

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Here, we tried to develop a differentiation method for reproducible cardiovascular blast cell populations, which have a ability to form robust vascular vessel-like structures and to recapitulate the process of vasculogenesis *in vitro*.

2. Materials and methods

2.1. Cells

Human iPSCs (201B7) [10] were provided by the Center for iPS Cell Research and Application (CiRA), Kyoto University under an agreement with the Super Tokku National Collaboration, Cabinet Office. 201B7 cells were maintained in accordance with the protocol of the supplier. The mouse embryonic cell line, 10T1/2, was cultured in Eagle basal medium (Sigma–Aldrich K.K., Tokyo, Japan) containing 10% fetal bovine serum (FBS; Gibco, Life Technologies Japan Ltd., Tokyo, Japan) and 2 mM L-glutamine (Sigma).

2.2. Animals

Male NOD/SCID mice (NOD.CB17-*Prkdc*^{scid}/J) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Astellas Pharma Inc., which was awarded Accreditation Status by AAALAC International.

2.3. In vitro differentiation of cardiovascular blast populations (CBPs)

10T1/2 cells were treated with 10 μg/ml mitomycin (Wako Pure Chemical Industries Ltd., Osaka, Japan) before use. Mitomycin-treated 10T1/2 cells were seeded onto 60-mm dishes coated with 0.1% gelatin (Sigma) at a density of 2.4×10^5 cells/dish. Small clumps (40–100 μm) of 201B7 cells were recovered using a cell strainer (BD Biosciences, San Jose, CA, USA) and then seeded onto 10T1/2 cells and cultivated in differentiation medium consisted of Iscove modified Dulbecco medium (IMDM; Gibco) supplemented with 15% FBS, 1× Insulin–Transferrin–Selenium-X Supplement (Gibco), 1× Penicillin Streptomycin (Gibco), 2 mM L-glutamine, 0.45 mM α-monothioglycerol (MP Biomedicals, LLC., Santa Ana, CA, USA), 50 μg/ml L-ascorbic acid (Wako), and 20 μg/ml Human VEGF (Wako) for 14 days. After 14 days, these cells were treated with 0.25% trypsin–EDTA (Gibco) at 37 °C for approximately 15–20 min and cell clumps bigger than 40 μm were recovered by cell strainer and centrifuged. The pellets were resuspended in 0.25% trypsin–EDTA and further incubated at 37 °C for 5 min with gentle pipetting. Cells were seeded onto 100-mm dishes coated with 10 μg/ml fibronectin (BD) at a density of $0.6–1.2 \times 10^6$ cells/dish and incubated for 7 days with differentiation medium supplemented with 20 ng/ml bFGF and 10 μM SB431542 (Tocris Bioscience, Bristol, UK) to obtain CBPs. The medium was replaced every 3 days.

2.4. In vitro differentiation of CBPs into various type of cells

CBPs were incubated in differentiation medium supplemented with 20 ng/ml bFGF for 14 days. Cardiomyocyte-like cells were induced by incubating CBPs in differentiation medium supplemented with 20 ng/ml bFGF, 10 μM SB431542, and 10 ng/ml BMP2 (R&D Systems, McKinley Place, MA, USA) for 12 days.

2.5. In vivo formation of vascular networks from CBP

Trypsinized CBPs were seeded on 35 mm RepCell dishes (CellSeed Inc., Tokyo, Japan) with temperature-sensitive surfaces at a density of $1.0–2.0 \times 10^6$ cells/dish, and then incubated at 37 °C for 4–6 days with differentiation medium supplemented with 20 ng/ml bFGF and 10 μM SB431542. After cells reached confluence, CBPs were removed from culture dishes by decreasing the temperature from 37 to 25 °C. Small sections of sheet that naturally floated to the surface were packed by centrifuging. Vessel forming CBPs were transplanted on the center of right-side epididymis adipose tissue in the NOD/SCID mouse.

2.6. Ex vivo organ culture for forming vascular networks from CBP

Vessel forming transplants were replaced on ISOPORE™ membrane filters 0.4 μm HTTP (Millipore, Billerica, MA, USA) and incubated with differentiation medium supplemented with 20 ng/ml FGF for 20–25 days in a floating condition.

2.7. Immunohistochemical study and flow cytometry analysis

CBP derivatives were fixed with 4% paraformaldehyde (Wako) and then labeled with the following monoclonal antibodies: anti-human CD31 (PECAM-1) antibody clone WM-59 (Sigma), anti-human VE-cadherin (R&D), mouse anti-human smooth muscle actin clone 1A4 (Dako, Tokyo, Japan), and anti-cardiac troponin T (Abcam, Tokyo, Japan). Cells were then labeled with Alexa Fluor secondary antibodies (Invitrogen).

Cryosections were fixed with 4% paraformaldehyde, and mouse CD31 was visualized using rat anti-mouse CD31 (BD Pharmingen) primary antibody, horseradish peroxidase-conjugated secondary antibody, and DAB peroxidase substrate. The nucleus was stained with hematoxyline, and VE-cadherin and smooth muscle actin were visualized by following incubation at 70 °C for 20 min in 1× HistoVT ONE (Nacalai Tesque Inc., Kyoto, Japan), permeabilization with PBS with 0.2% Tween, and blocking with 3% BSA and 1% donkey serum. Samples were then incubated for 1 h in primary antibody blocking solution, washed 3 times in PBS with 0.25% Triton X-100, and labeled with Alexa Fluor conjugated secondary antibodies (Invitrogen) for 1 h. Cells and sections were observed using a BZ-9000 (Keyence, Tokyo, Japan) fluorescence microscope.

Expression of cell surface molecules was analyzed by flow cytometry (FACS Aria; BD). These cells were incubated with fluorescence-conjugated antibodies for 30 min at room temperature, as shown in Table S1.

2.8. In vivo staining by tail-vein injection of UEA-I and GS-IB$_4$ lectin

Twelve days after transplantation, human and murine blood vessels were identified by tail-vein injection method as previously described [11]. Briefly, a mixture of rhodamine-conjugated *Ulex europaeus* agglutinin I (UEA-I; Vector, Burlingame, CA, USA) and Fluorescein isothiocyanate (FITC)-conjugated *Griffonia simplifolia* isolectin B$_4$ (GS-IB$_4$; Vector) (50 μg of each/100 μl/mouse) was injected. The mice received perfusion fixation 10 min after lectin injection, and epididymis adipose tissues were fixed in 4% paraformaldehyde overnight, then further incubated in 30% sucrose overnight, embedded in OCT, frozen, and cryosectioned. Sections were analyzed using a BZ-9000 fluorescence microscope.

3. Results

3.1. In vitro induction of vascular vessel-like structures from hiPSCs

First, we induced progenitor populations that can differentiate endothelial precursor and mesenchymal cells from hiPSCs to recapitulate process of vasculogenesis. We used a modified version of Takayama et al.'s protocol, which enabled hESCs to differentiate into hematopoietic progenitors and then mature megakaryocyte

and release platelets [12]. The overall processes of the differentiation method are shown in Fig. 1A. Human iPSCs were induced to differentiate to lateral plate mesoderm lineages by the culture with 10T1/2 feeder cells and VEGF for 2 weeks (Fig. 1A). The resultant heterogeneous cell population formed networks of cells consisting of CD31 positive ($CD31^+$) cells (Fig. 1B). This heterogeneous cell population was treated with 0.25% trypsin–EDTA and clumps bigger than 40 μm in diameter that were not dissociated with the enzyme treatment were reseeded onto fibronectin-coated dishes. Culture of this population with VEGF, bFGF and SB431542 for approximately 7 days induced to generation of the cardiovascular lineage population. This population had no structural features but contained heterogeneous populations of endothelial precursors ($CD31^+$ $VEGFR2^+$), endothelial cells ($CD31^+$ $VE\text{-}Cad^+$), and some types of mesenchymal cells ($CD90^+$) (Table 1, Fig. 1C). We named this heterogeneous cell population the cardiovascular blast population (CBP). Continuous culturing without SB431542 induced CBPs to form vascular vessel-like structures consisting of VE-cadherin positive vascular endothelial-like cells and alpha smooth muscle actin (α-SMA) positive smooth muscle-like cells (Fig. 1D). To verify that this novel culture method recapitulated a developmental process, we induced CBP to differentiate to other lateral plate mesoderm lineage cells such as cardiac muscle-like cells in combination with growth factors and SB431542 (Fig. 1E). Thus, we succeeded in differentiating CBP originated from hiPSCs to form vascular vessel-like structures by recapitulating the process of vasculogenesis *in vitro*.

Table 1
Sub populations of $CD31^+$ cells in CBPs.

	%
$CD31^+$ $VECad^+$	9.5
$CD31^+$ $VEGFR2^+$	19.6

3.2. Vascular vessel networks formation from the particular population of CBP in immunodeficient mice

CBPs exhibited a high degree of population diversity in the ratio of $CD31^+$ and $CD90^+$ cells, which varied from 1:0.4 to 1:10 by FACS analysis. To investigate the potentials of CBPs with different ratios of $CD31^+$ to $CD90^+$ cells in vascular vessel network formation, we transplanted CBPs into mice. First, cultured CBPs in the temperature-sensitive dishes were harvested from the dishes without enzyme treatment by decreasing the temperature from 37 to 25 °C, which allowed CBPs to retain an intact basal membrane composed of extra cellular matrix. Then, the resultant cell sheets were packed to make transplants by centrifuging (Fig. 2A). Since the basement membranes were kept intact, the transplants retain the inherent adhesiveness that can be transplanted without any means of artificial scaffolds. We prepared transplant from CBPs with different ratios of $CD31^+$ to $CD90^+$ cells. These transplants were transplanted on the right side of epididymis fat tissue in NOD/SCID mice, and no visual conformation of vascular vessel formation was obtained in the left side of the same mouse (negative control) (Fig. 2B). After

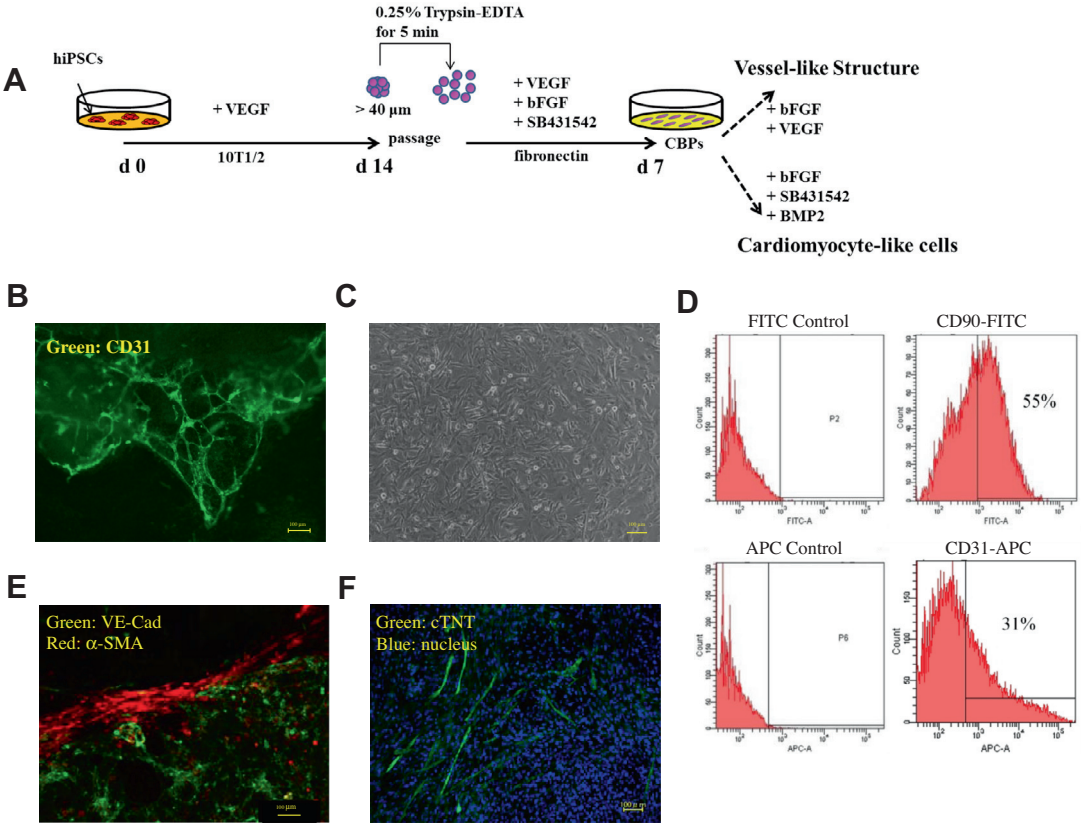

Fig. 1. Isolation and characterization of CBPs induced from human iPSCs. (A) Overall strategy to induce CBPs. (B) Cells differentiated from human iPSCs were positive for $CD31^+$ (green) at 10 days. (C, D) CBPs were obtained after 14 days by dissociation from iPSCs in differentiation medium and further incubated with differentiation medium in the presence of bFGF and SB431542 for 7 days. Phase-contrast image of CBPs (C), expression ratio of CD90-FITC and CD31-APC of CBPs (D). (E) CBPs incubated in the presence of bFGF for 20 days were stained for both VE-cad (green) and α-SMA (red). (F) CBPs cultivated in the absence of both bFGF and SB431542 for 5 days were stained for cardiac-troponin T (green) and nucleus (blue). Scale bars, 100 μm.

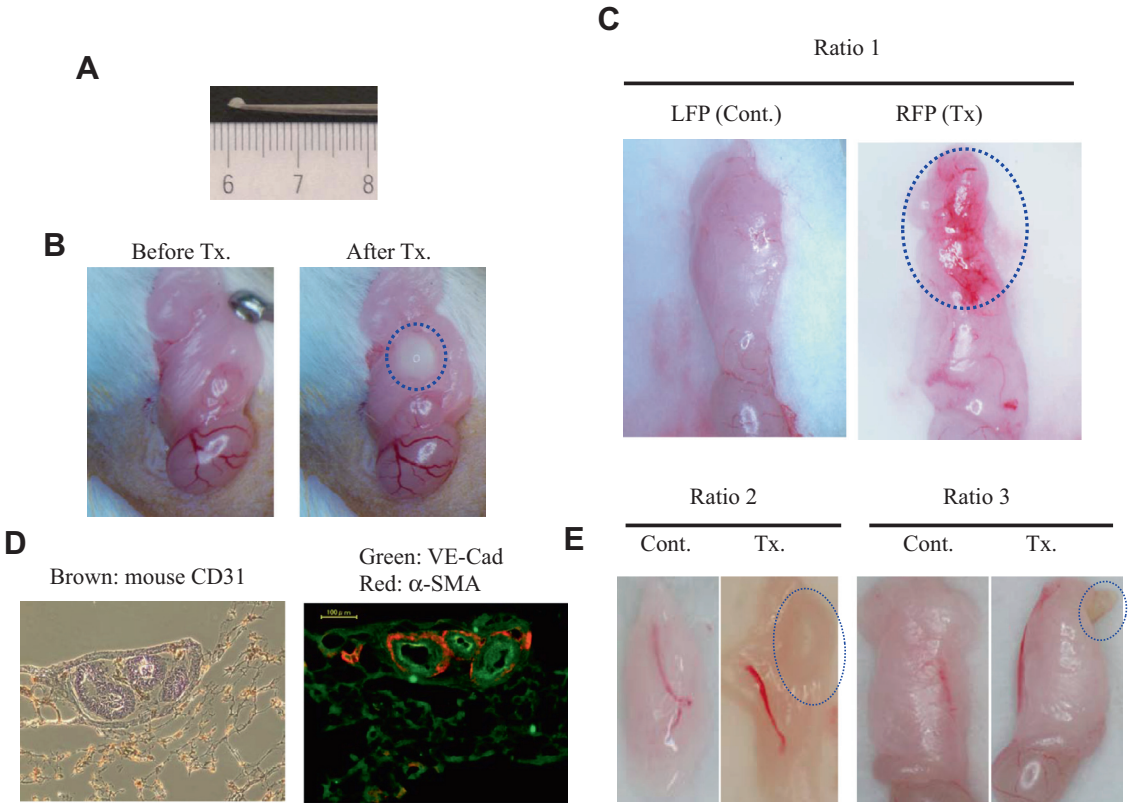

Fig. 2. *In vivo* blood vessel formation capability of CBPs. (A) Vessel forming transplants from CBPs obtained using RepCell dishes. CBPs were removed from culture dishes, keeping niche structures intact by decreasing the temperature from 37 to 25 °C. Resultant small niche sheets were packed to transplants by centrifuging at 5000 rpm for 2 min. (B) Vessel-forming CBP transplants were implanted on the center of right-side of the epididymis fat tissue (lower; dashed circle). Left-side epididymis fat tissue from the same mouse was used as a negative control. (C) Blood vessels transporting blood were observed 12 days after transplantation (right, dashed circle). (D) Representative phase-contrast and fluorescent images of transplants with immunostaining. Left: mouse CD31 (brown) and right: both human VE-Cad (green) and human α-SMA (red). Vessel structure composed of VE-Cad stained cells surrounded by α-SMA were observed at day 12. (E) Epididymis adipose tissue after transplantation of CBPs at Ratio 2 (at day 22) and CBPs at Ratio 3 (at day 14). Unknown clots were observed after transplantation of both types of niche (dashed circle). Cont., control; Tx., transplantation. Scale bars, 100 μm.

2 weeks, we checked the vascular network formation in transplant recipients. In replicated experiments, we obtained visual confirmation that transplants from some CBPs underwent *de novo* formation of vascular networks, whereas other CBPs formed lumps instead.

We observed that the CBPs formed vascular networks had $CD31^+$ to $CD90^+$ cell ratio of 1:1.5 prior to transplantation. To evaluate the potential of CBPs with different $CD31^+$ to $CD90^+$ cell ratio, we selected three typical CBP ratios (Ratio 1, Ratio 2 and Ratio 3) and performed repeated experiments. Ratio 1 was the optimum ratio ($CD31^+$:$CD90^+$ = 1:1.5), Ratio 2 had more than twice as many $CD31^+$ cells as $CD90^+$ cells ($CD31^+$:$CD90^+$ = 1:0.4) and Ratio 3 had 12 times as many $CD90^+$ cells ($CD31^+$:$CD90^+$ = 1:12) (Table 2). Fourteen days after transplantation of CBPs, the *de novo* formation of vascular networks was visually confirmed in mouse transplanted with Ratio 1 and the transplants disappeared at the site of transplantation. We observed no formation of vessel at the left side in the same mouse (Fig. 2C). In the fat tissues containing *de novo* vascular network, we detected the mRNA of human specific β-actin, CD31 and α-SMA by real time PCR (data not shown). Further, visible vessels (>100 μm diameter) was stained by selective immuno-staining with human VE-cadherin and human α-SMA antibodies but not mouse CD31 antibodies (Fig. 2D).

In addition, to examine whether or not the *de novo* vascular network is connected with mouse endogenous vascular vessels, we intravenously injected the mixture of lectins composed of rhodamine-conjugated UEA-1 specific to human vascular endothelial cells and FITC-conjugated GS-IB_4 specific to murine vascular endothelial cells before sacrifice. The human capillary vessels were selectively stained and clearly distinguished from mouse vessels (Fig. 3). These results indicate that the *de novo* vascular network is derived from transplanted human cells and that the vascular network is functional, as it was connected with endogenous murine vascular vessels and transported bloods. In contrast, in mice that received CBP transplants with Ratio 2 or Ratio 3, we were unable to detect *de novo* vascular vessel formation. Instead, CBP transplants at these ratios formed unknown lumps (Fig. 2E). These results were reproducible in 3 independent experiments, with 86% (6/7) of Ratio 1 recipient mice producing *de novo* visible vascular network structures compared to 0% (0/9) of Ratio 2 or 3 recipients. These results indicate that a robust and functional vascular network can be induced from CBPs at $CD31^+$ to $CD90^+$ ratio of 1:1.5 *in vivo*, and that the vascular forming ability of CBP can be predicted in advance by measuring the ratio of $CD31^+$ to $CD90^+$ cells.

Table 2
CBP ratios of transplants.

	Ratio 1		Ratio 2		Ratio 3	
	%	Ratio	%	Ratio	%	Ratio
$CD90^+$	51.2	1.5	14	0.4	72.2	12
$CD31^+$	33.3	1	37	1	6.1	1

CBPs were seeded on RepCell 3.5 cm dish and cultivated in differentiation medium in the presence of bFGF and SB431542 for 4–6 days. Resulting transplants were analyzed by FACS analysis.

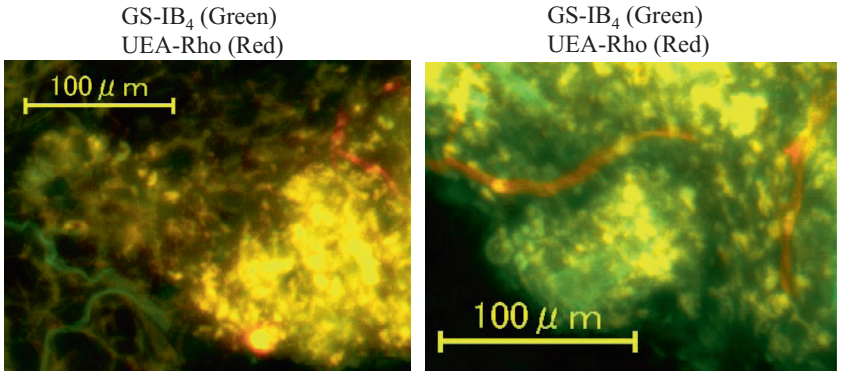

Fig. 3. Detection of perfused human and murine vessels *in vivo*. Human and murine vessels were identified by tail-vein injection of a mixture of rhodamine (red)-conjugated UEA-I and FITC (green)-conjugated GS-IB_4 at day 12 (left). Vessels stained by only UEA-I were observed at day 12 (right). Scale bars, 100 μm.

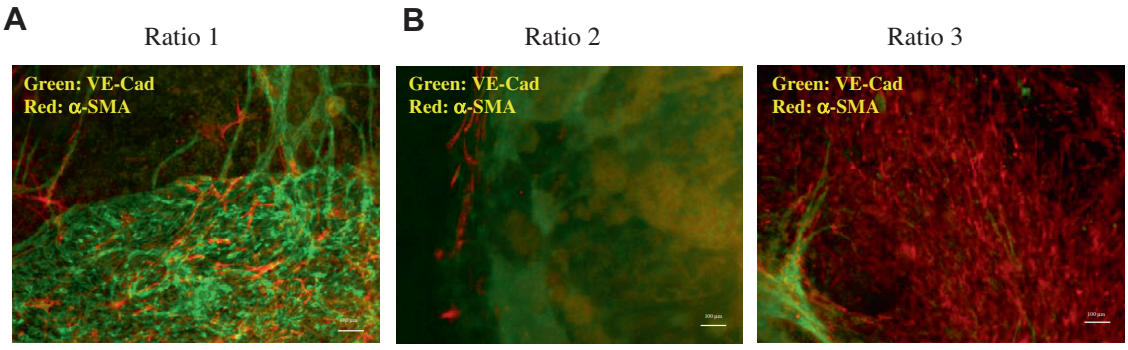

Fig. 4. *Ex vivo* blood vessel formation of CBPs depending on the ratio of $CD31^+$ cells to $CD90^+$ cells. (A) CBPs with Ratio 1 formed a vessel-like structure composed of endothelial-like cells stained with VE-cadherin and smooth muscle-like cells stained with α-SMA at day 23. (B) CBPs with Ratio 2 (at day 17) or 3 (at day 24) did not form a vessel-like structure. Scale bars, 100 μm.

3.3. Correlation between vascular network formation ex vivo organ culture system and in vivo

We successfully established a novel differentiation method for the generation of CBP, that could form vascular vessels *in vitro* and vascular network *in vivo*. The ability of some populations to induce organ-like structures in an *ex vivo* organ culture system has been reported to correlate well with that *in vivo* for organs such as the kidney [13]. However, no reports have yet been published concerning vascular vessels. We therefore examined whether or not CBPs could form vascular vessels in the *ex vivo* organ culture system.

CBPs with Ratio 1–3 were prepared in accordance with the *in vivo* method using cell-sheet technology, and then placed on hydrophobic filters floating in medium and cultured. The resultant from CBP with Ratio 1 was able to recapitulate the dynamics of the three-dimensional (3D) vasculogenesis process characterized by network formation of VE-cadherin positive endothelial-like cells and α-SMA positive smooth muscle-like cells (Fig. 4A). In contrast, neither resultant from CBPs with Ratio 2 nor 3 formed any dynamic 3D structure but resulted in uncontrolled proliferation of endothelial- or smooth muscle-like cells (Fig. 4B). These results indicate that vascular networks can be induced from the CBPs with Ratio 1 in the *ex vivo* organ culture system and the vascular forming potentials of CBPs are well correlated with those observed in *in vivo* experiments.

4. Discussion

In this study, we established a novel culture method for hiPSCs to generate CBPs, which can form vascular vessels *in vitro* and vascular networks *in vivo*. We also established a novel *ex vivo* organ culture system which well reflects an *in vivo* system in the vascular forming potential.

Endothelial precursor and mesenchymal cells, which are derived from lateral plate mesoderm, are thought to be the principal players in vasculogenesis. Our CBPs primarily consist of $CD31^+$ and $CD90^+$ cells. CD31 is reported to be a marker throughout the development of endothelial cell lineages, which are especially important as they are thought to play fundamental roles in the formation of the primary vascular plexus at the beginning of vasculogenesis. FACS analysis showed that the $CD31^+$ population contained few VE-cadherin positive cells in CBPs, suggesting that this $CD31^+$ population includes endothelial lineage cells at various developmental stages such as mature endothelial cells or endothelial progenitor cells (EPCs). EPCs has been applied in treating ischemic diseases since Asahara et al. discovered and isolated them from the peripheral blood of adult donors [14]. Although EPCs have been shown to facilitate successful limb salvage in patients with CLI, the clinical use of EPCs derived from autologous vascular tissue is limited due to the difficulty of obtaining sufficient quantities of cells with minimal donor site morbidity [15]. Ii et al. also reported that the majority of EPCs do not become components of newborn blood vessels but instead reside outside of vascular vessels [16]. Further, EPCs have been reported incapable of forming blood vessel-like structures alone *in vivo* and require co-implantation with perivascular cells or mesenchymal cells to produce high-density, stable vascular networks, which is consistent with our findings [17]. However, our findings provide new insights regarding the induction of CBPs and their transplantation at $CD31^+$ to $CD90^+$ cells ratio of 1:1.5 to form robust vascular networks. Although both $CD31^+$ and $CD90^+$ cells have been studied extensively in vasculogenesis,

our optimum ratio of 1:1.5 appeared to be promising as it can be reproduced in repeated experiments *in vivo*. However, it remains necessary to determine the acceptable levels of deviation from the optimum cell ratio and number of $CD31^+$ and $CD90^+$ cells, and evaluate the involvement of other populations. Nevertheless our results suggest that the presence of essential cell populations and their collaboration with each other in adequate cell numbers are required to ensure high-density, stable vascular network formation *in vivo*. CBPs including adequate cell numbers of essential cell populations may be useful in understanding of the mechanism of vascular network formation and improving the efficiency of vascular vessel formation in current cell therapies.

Investigation into how hiPSCs undergo proper organ formation may also help to solve issues regarding their safety. In the case of *in vivo* organogenesis using hiPSCs, minor populations resistant to differentiation are thought to contribute to the formation of unknown lumps, preventing other primed cells from proper organ formation. Our results suggest that sufficient blood vessels were formed without any unknown cell lumps as long as CBP transplants were made at optimum ratio. Further, we also developed a novel *ex vivo* organ culture system that provided a means to assess the ability of CBPs to form vascular networks in real time. Our *ex vivo* organ culture system provided a more accurate representation of the dynamics of 3D vascular networks in the living body than a common two-dimensional (2D) plate culture, and could possibly provide a method of drug screening which can mimic *in vivo* phenomenon.

In this report, we successfully differentiated CBPs to form vascular networks both in *in vivo* and in the *ex vivo* organ culture systems by recapitulating the process of vasculogenesis from hiPSCs. In the developing organs, there are tissue-specific microenvironments named niche composed of matrices and growth factors, which maintain stem and progenitor cells quiescence and regulate their proliferation [18]. We were able to differentiate CBPs without disrupting these niches by using cell-sheet technology and prepare optimal CBPs by setting the markers of quality control such as $CD31^+$ to $CD90^+$ cell ratio. This concept of essential key populations and adequate cell numbers may be applicable to more complex organs as well, such as liver and kidney.

In conclusion, this reproducible neovascularization method with our CBPs and *ex vivo* organ culture system have a potential to be a powerful tool in the elucidation of the vasculogenesis code and in drug discovery, and furthermore will open the way for organ regeneration and regenerative medicine [19].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.035.

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