A comparison of the tube forming potentials of early and late endothelial progenitor cells

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Abstract

The identification of circulating endothelial progenitor cells (EPCs) has revolutionized approaches to cell-based therapy for injured and ischemic tissues. However, the mechanisms by which EPCs promote the formation of new vessels remain unclear. In this study, we obtained early EPCs from human peripheral blood and late EPCs from umbilical cord blood. Human umbilical vascular endothelial cells (HUVECs) were also used. Cells were evaluated for their tube-forming potential using our novel in vitro assay system. Cells were seeded linearly along a 60 μm wide path generated by photolithographic methods. After cells had established a linear pattern on the substrate, they were transferred onto Matrigel. Late EPCs formed tubular structures similar to those of HUVECs, whereas early EPCs randomly migrated and failed to form tubular structures. Moreover, late EPCs participate in tubule formation with HUVECs. Interestingly, late EPCs in Matrigel migrated toward pre-existing tubular structures constructed by HUVECs, after which they were incorporated into the tubules. In contrast, early EPCs promote sprouting of HUVECs from tubular structures. The phenomena were also observed in the in vivo model. These observations suggest that early EPCs cause the disorganization of pre-existing vessels, whereas late EPCs constitute and orchestrate vascular tube formation.

Keywords: Early endothelial progenitor cells Late endothelial progenitor cells Tube-forming activity Endothelial cells Human umbilical endothelial cells Mononuclear cells

Introduction

In healthy individuals, endothelial cells are rarely found in the blood. However, mature endothelial cells can be found in the circulation following detachment from injured vessels. For example, circulating endothelial cells substantially increase in a wide variety of pathological conditions associated with profound vascular insult. Therefore, circulating endothelial cells are a useful marker of vascular damage [1,2]. Endothelial progenitor cells (EPCs), derived from bone marrow, are also found in the circulation and are involved in tumor vasculogenesis and wound healing.
The discovery of EPCs in human blood has led to a new paradigm in which vasculogenesis and angiogenesis occur in adult tissues [5]. These results have suggested a potential new approach to the treatment of cardiovascular and ischemic diseases. Preclinical studies have shown that transplantation of human EPCs to nude mice with hind limb ischemia improved blood flow recovery and capillary density resulting in a significant reduction in the rate of limb loss [5–10]. Clinical trials using autologous EPC transplantation have already been performed and significant improvements have been observed in myocardial function and ischemic diseases [11–15]. The strict roles of EPCs in neovascularization are not well understood because various populations of cells with varying differentiation potentials were transplanted.

EPCs consist of two different subpopulations, termed early and late EPCs [16–18]. Although both EPCs are derived from mononuclear cells (MNC) and express endothelial cell markers, they have different morphologies and growth patterns [18,19]. Early EPCs exhibit a spindle-like morphology and the majority of them are derived from CD14(+) subpopulations [16,19–21]. Late EPCs, named after their late outgrowth potential, exhibit a cobblestone morphology and are derived from CD14(−) fractions [17,19]. These two populations have been characterized for production of vascular endothelial growth factor (VEGF), VEGF receptor expression, cytokine secretion, and tube forming activity in vitro and in vivo [19].

Using photo-catalytic lithography, we have developed a new cell culturing technology for capillary engineering [22]. In this approach, endothelial cells are patterned on a substrate and then transferred to Matrigel. The endothelial cells change their morphology and form tubular vessels as confirmed by electron microscopy and dye microinjection. In the present study, using this novel technology, we focused on the tube-forming activity of early and late EPCs. In addition, we asked whether EPCs were incorporated into preexisting tubular structures.

Materials and methods

Human samples

Human peripheral blood was provided by 7 healthy human volunteers. Human umbilical cords and cord bloods were obtained from 12 healthy newborns. Informed consent was obtained from all donors, and samples were handled according to the tenets of the Declaration of Helsinki, with the approval of university review boards.

Cell isolation

Blood was diluted 1:1 with PBS containing 2 mM EDTA, and overlayed on Lymphoprep (AXIS-SHIELD, Oslo, Norway). Cells were centrifuged at 1100×g for 10 min. The resulting mononuclear cells (MNC) were collected and washed three times in PBS. Human umbilical vein endothelial cells (HUVECs) were isolated enzymatically.

Cell culture

Isolated MNC were resuspended in endothelial basal medium-2 (EBM-2) (Clonetics, San Diego, CA) supplemented with the EGM-2 bullet kit (Clonetics), plated on culture dishes precoated with human fibronectin (Sigma, Saint Louis, MO), and maintained in the medium. To obtain early EPCs from peripheral blood MNC and cord blood MNC, medium was changed every 2 days, and after 5 to 7 days of culture, early EPCs were isolated. Late EPCs were obtained from cord blood as previously described by Gulati et al. [17]. Medium was then changed daily for 7 days and on alternate days thereafter. The characteristic colonies of late EPCs were observed under a phase-contrast microscopy (IMT-2; Olympus Optical, Tokyo, Japan) and these cells were cloned by colony isolation. HUVECs were used at passages 1–3.

Cell characterization

Early and late EPCs were seeded onto a chamber slide glass (Naïge Nunc, Naperville, IL) coated with fibronectin (Sigma) and fixed in 4% paraformaldehyde (PFA) next day. Immunocytochemistry was performed using PE-conjugated anti-human CD31 antibody (555446; BD Pharmingen, San Diego, CA; diluted 1:50) and primary antibodies against human VEGFR2, (sc-6251; Santa Cruz Biotechnology, Santa Cruz, CA; 2 μg/ml) or von Willebrand factor (vWF) (M0616; Dako, Glostrup, Denmark; 1.2 μg/ml), followed with Alexa 488-conjugated anti-mouse IgG (A-11029; Molecular Probes, Leiden, Netherlands; 2 μg/ml). Non-immune mouse IgG (sc-3877; Santa Cruz; diluted 1:100) was used as a control. Cell nuclei were stained with 1 μg/ml of To-Pro3 (T3605; Molecular Probe). Samples were observed and photographed under a laser confocal microscope (LSM510META; Carl-Zeiss, Jena, Germany).

Flow cytometric analysis of CD14, CD31, CD45 and VEGFR2 expression in early EPCs and late EPCs

Cells were collected non-enzymatically using Cell Dissociation Buffer (Invitrogen Corp, San Diego, CA) and labeled with FITC-conjugated anti-CD45 monoclonal antibody (Beckman Coulter, Marseilles, France), FITC-conjugated anti-CD31 monoclonal antibody (Pharmingen) and anti-VEGFR2 monoclonal antibody (Santa Cruz Biotechnology) at 4 °C for 30 min. Cells incubated with anti-VEGFR2 monoclonal antibody were subsequently stained with PE-conjugated anti-mouse IgG antibody at 4 °C for 30 min. After washing with PBS containing 1% BSA, flow cytometric analysis was performed with a FACS Calibur (Becton-Dickenson, NJ).

Photo-mask preparation

STK-03 titanium dioxide (TiO₂) photo-catalyst aqueous dispersion (Ishihara Sangyo; Osaka, Japan) was diluted to 33 wt.% with isopropyl alcohol, stirred for 1 h and filtered using a 3.0 μm pore size polytetrafluoroethylene filter (Advantec, Tokyo, Japan). Cr-Quartz photo-masks with slit width 60 μm and slit interval 300 μm were cleaned using a VUM-3184 UV-ozone washing machine (Oak Manufacturing, Tokyo, Japan) to...
decompose low molecular weight adsorbents on the mask surface. The mask was coated with the TiO2 dispersion by spin-coating at ~700 rpm for 15 s and then baking at 150 °C for 10 min.

**Substrate preparation**

NA35 polished glass substrates (NH Techno Glass, Yokohama, Japan) were cleaned using the VUM-3184 for 420 s. Next, 1.5 g of heptadecafluorodecyltrimethoxysilane solution (TSL-8233; GE Toshiba Silicone, Tokyo, Japan), 5.0 g of tetramethoxysilane solution (TSL-8114; GE Toshiba Silicone), and 2.4 g of 0.005 N HCl were mixed and stirred for 24 h at room temperature to make fluoro-alkyl-silane (FAS) mixture solution. The mixture was diluted with 1 wt.% isopropyl alcohol, stirred for 15 min, filtered by Chromato-Disc (filter type 0.45 μm; Kurabo) and then coated onto the glass substrate by spin-coating at ~1000 rpm for 15 s. The FAS-coated substrate was then baked at 150 °C for 10 min.

**Surface modification and patterning**

Both TiO2-coated photo-masks and FAS-coated substrates were immersed and sonicated in deionized water for more than 5 min, and then baked at 120 °C for 5 min. The TiO2-coated side of the photo-mask was irradiated with UV for 15 min at irradiation energy of ~30 J/cm2 to rejuvenate the photo-catalytic activity of TiO2. The FAS-coated side of the glass substrate was placed facing and in contact with the TiO2 layer of the photo-mask and UV-irradiated for several minutes through the mask to form hydrophilic regions on the FAS layer.

**Cell patterning**

Prior to cell seeding, glass substrates were placed on cell culture dishes. The cells were trypsinized and suspended, and labeled with PKH26 Red (Sigma) according to the manufacturer’s instructions. Cells were counted, seeded on the substrates, and incubated for 18 h at 37 °C. A total of 2×105 cells were seeded per substrate. During incubation, cells in hydrophobic areas moved to hydrophilic areas.

**Transplant to Matrigel**

The patterned cells on the substrate were turned over onto Matrigel (Becton Dickinson, Bedford, TX) and incubated with the culture medium containing 0.2% FBS for 24 h. When the substrate was removed, the cells were transferred from the substrate to the Matrigel.

**Observation of the tube formation**

Firstly, the pattern of cells on Matrigel was observed by a phase-contrast microscopy (IMT-2; Olympus Optical). Next, live cells which formed tubular structures were labeled with 500 ng/ml Calcein-AM (Molecular Probes) for 30 min in 37 °C, and three-dimensional structures of capillaries were analyzed by a laser confocal microscope. Furthermore, the engineered capillaries on Matrigel were fixed with 4% PFA and stained with 4 μg/ml of anti-human VE-cadherin rabbit polyclonal antibody (210-232-c100; ALEXIS, San Diego, CA), followed by 4 μg/ml of Alexa 488-conjugated anti-rabbit IgG (Molecular Probe). Capillaries were observed by a laser confocal microscope.

**EPC incorporation into tube-like structure**

Early and late EPCs were pre-labeled with PKH26 Red, mixed with unlabeled HUVECs, and seeded on the substrate. Twenty-four hours after transplant to Matrigel, the cells were labeled with Calcein-AM, the fluorescence of EPCs and HUVECs were analyzed by a laser confocal microscope.

To quantify EPC incorporation into tube-like structure, early and late EPCs were pre-labeled with PKH26 Green. These cells were mixed with HUVECs which were pre-labeled with PKH26 Red, and cultured on the substrate. The fluorescence of EPC and HUVECs was analyzed by a fluorescence microscopy (BZ-8000; Keyence, Osaka, Japan). The incorporation ratio of EPCs into the tube structure was calculated by the cellular area of EPC (μm2)/mm of tube structure.

**In vivo angiogenesis assay**

We developed an ear vessel occlusion model for an in vitro angiogenesis assay. After occlusion of the murine auricular vessel, cells were introduced into the subcutaneous pocket below the occlusion point, and newly formed vessels were analyzed. Twelve hours before the cell injection, the mice were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg) and auricular vessels were occluded. Collateral circulation was observed under a stereomicroscope (LEICA Mz9, Leica Microsystems, Wetzlar, Germany). Early or late EPCs were labeled with PKH26 and suspended in growth media, and 5×10⁶ of each EPCs were subcutaneously injected into the occluded pinna within 12 h of vessel occlusion. Forty-eight hours after the cell injection, the mice were given 250 μg of Bandeiraea simplicifolia lectin1 (BS1-lectin) (Vector Laboratories, Burlingame, CA) intravenously and sacrificed 45 min later. Sections (4-μm thickness) of cell-transplanted pinna were made for histological observation. This experiment was performed using 7-week nude mice and all procedures were carried out with the full approval of the ethical committee of Tokyo Medical and Dental University.

**Calculations and statistical analysis**

The statistical significance of differences in the data was evaluated by use of analysis of Welch’s t-test or Student t-test. A value of P<0.05 was accepted as statistically significant.

**Results**

**Characterization of two types of EPCs**

MNC were harvested from human peripheral blood of healthy individuals or from cord blood and seeded on fibronectin-coated dishes. Following 5 to 7 days of cultivation, adherent cells were observed in clusters. The cells exhibited a spindle-
like shape (Fig. 1A, left) and displayed several endothelial cell markers including CD31, vWF and VEGFR2 (Fig. 1B, left). When MNC were isolated from human cord blood, early EPCs were also observed after 3 days of cultivation. Their morphology and expression of endothelial markers used here were almost the same as those of early EPCs derived from peripheral blood (data not shown). They proliferated and thereafter gradually disappeared over the next 1 week.

After early EPCs had disappeared, colonies with different morphology emerged over 12–21 days of cultivation. They exhibited a cobblestone morphology, spindle-like shape (Fig. 1A, right) and displayed several endothelial cell markers such as CD31, vWF and VEGFR2. Also, eNOS expression was observed in both early and late EPCs (data not shown). Thus, these cells were late EPCs as reported by Hur et al. [18]. To characterize two types of EPCs, we carried out a flow...
cytometric analysis of CD31, CD45 and VEGFR2 expression in early EPCs and late EPCs derived from cord blood (Fig. 1C). Similar to the immunocytochemical staining, the expression of CD45 in late EPC was weaker than that in early EPCs, while VEGFR2 expression in late EPCs was stronger than that in early EPCs. CD14 expression was observed only in early EPCs (data not shown).

Tubular structures could be constructed by late EPC, but not by early EPC

To assess the tube-forming activities of early and late EPCs, both cell types were analyzed by a novel method which promoted capillary formation in vitro. Early EPCs, late EPCs and HUVECs were distributed linearly on a specific substrate.

Fig. 2 – Tube forming activities in early and late EPCs compared with HUVECs. Cells (2 x 10^5) were seeded on the glass substrate and cultured for 18 h (A–C, left), then transplanted to Matrigel. Twenty-four hours after transplantation (right), HUVECs formed tubular structures (A). Early EPCs spread on Matrigel and did not form tubular structures (B). Late EPCs formed tubular structures (C).
(Fig. 2, left) and were subsequently transferred to Matrigel (Fig. 2, right). During the transfer of patterned cells to Matrigel, HUVECs changed cellular morphology to form capillary-like structures. Twenty-four hours after cell transfer, the substrate was removed and tubular structures were readily observed by a light microscopy (Fig. 2A). Electron microscopic observation revealed that the luminal structure consisted of four to five HUVECs [22]. In contrast, linearly arrayed early EPCs did not form tubular structures (Fig. 2B). The time course of tube formation in late EPCs was quite similar to that in HUVECs (Fig. 2C).

After HUVECs or late EPC were loaded with calcein-AM, the tubular structures were assessed with a laser confocal microscope. The luminal structure formed by late EPCs was observed as well as that by HUVECs (Figs. 3A, B). When tubes formed by HUVECs and late EPCs were stained for VE-

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**Fig. 3** - Three-dimensional images of tubular structures formed by HUVECs and late EPCs. Tubular structures formed by HUVECs (A) and late EPCs (B) were stained with calcein-AM. The luminal structure was observed with a laser confocal microscope. Scale bar = 20 μm. Immunostaining of HUVEC (C) and late EPCs (D) tubular structures with anti-VE-cadherin. The localization of VE-cadherin was observed at cell-cell junctions.
Fig. 4 – Cooperative tube-forming activity of EPCs and HUVECs. After early or late EPCs were stained with PKH26 Red, EPCs and HUVECs were mixed and co-cultured on the substrate, and then transplanted to Matrigel. The ratio of EPCs to HUVECs was 1:10. (A) Tubular structures formed by these EPCs and HUVECs were then labeled with Calcein-AM. Tubular structure constructed with both early EPCs (yellow) and HUVECs (green). Tubular structures were observed three dimensionally with a laser confocal microscopy. The early EPCs attached to the tube surface and were not incorporated into the tubular structure formed by HUVECs. Scale bar=20 μm. (B) Tubular structure constructed with both late EPCs (yellow) and HUVECs (green). The late EPCs participated in tube formation with HUVECs. Scale bar = 20 μm. (C) The incorporation of EPCs into tube structure was calculated by the area of EPC in the indicated length of tubular structure. Value is the mean and SE (standard error) of 30. **P < 0.01 vs. early EPCs.
cadherin, a specific endothelial cell–cell adhesion molecule, we observed VE-cadherin at cell–cell junctions (Figs. 3C, D).

**Late EPCs, but not early EPCs, participated in tube formation with HUVECs**

When EPCs are mixed with HUVECs and incubated on Matrigel, EPCs are incorporated into the tubular structure formed by HUVECs [5]. To confirm this, early EPCs or late EPCs were mixed with HUVECs and they were applied to the same tube formation systems. To distinguish the EPCs from HUVECs, the EPCs were labeled with PKH26 Red. Tubular structures formed by these EPCs and HUVECs were then labeled with Calcein-AM. The fluorescence of EPC and HUVECs was observed with a laser confocal microscopy. The early EPC as well as the late EPCs were observed in the tubular structure. However, by a three-dimensional imaging, we demonstrated that the early EPCs were only attached to the tube surface and were not incorporated into the tube structure formed by HUVECs (Fig. 4A). In contrast, the late EPCs participated in tube formation with HUVECs (Fig. 4B). To quantify the incorporation of EPCs into tube structure was calculated by the area of EPC per the indicated length of tubular structure. The incorporation of late EPCs into tubular structures was over 5-fold larger than that of early EPCs (Fig. 4C).

**Late EPCs integrated into pre-existing tubular structures formed by HUVECs while early EPCs caused tubular sprouting**

In order to investigate whether EPCs could substitute for HUVECs in preformed tubular structures, EPCs and HUVECs were independently cultured. HUVECs labeled with PKH26 Red were seeded onto the substrate and EPCs labeled with PKH26 Green were suspended in Matrigel. The patterned HUVECs on the substrate were transferred to the EPC-containing Matrigel. The late EPCs migrated toward the tubular structures and were observed adjacent after 6 h (Fig. 5A) and attached to the tubes 10 h later (Fig. 5B). Thereafter, they were incorporated into the tubular structure 24 h later (Fig. 5C). In contrast, early EPCs did not migrate toward the tubes and were not

![Image](image_url)

Fig. 5 - Incorporation of EPCs into preformed tubular structures. HUVECs labeled with PKH26 Red were seeded on the substrate and EPCs labeled with PKH26 Green were suspended in Matrigel. The patterned HUVECs on the substrate were transferred to the EPC-containing Matrigel. (A) Six hours after transplantation, late EPCs were migrating toward the tubular structure preformed by HUVECs. (B) Ten hours after transplantation, late EPCs were attached to the tube. (C) Twenty-four hours after transplantation, late EPCs were incorporated into the tubular structure preformed by HUVECs (yellow arrow heads). Scale bar=30 μm. (D) Twenty-four hours after transplantation, early EPCs had not been incorporated into the tubular structure preformed by HUVECs. The HUVECs in the tubular structure were sprouting and migrating. Scale bar=100 μm.
incorporated during the observation period. However, after HUVECs were transferred to Matrigel containing early EPCs, the HUVECs in the tubular structures initiated sprouting and migrated toward early EPCs as evidenced by branching patterns between the tubular structures (Fig. 5D).

**In vivo angiogenesis assay**

To confirm the tube-forming activity of each EPCs in vivo, the vessels derived from implanted EPCs were observed in the ear vessel occlusion model, early or late EPCs labeled with PKH26 Red were subcutaneously injected into the occluded pinna. Forty-eight hours after the cell injection, the mice were given BS1-lectin (green) intravenously and sacrificed. Scale bar = 5 μm. (C) The numbers of blood vessels derived from each EPCs observed in 5 sections were normalized to the number of recipient blood vessels. The data are shown as the mean and SE of three mice and data are mean of 5 fields/mouse. **P<0.01 vs. early EPCs.

**Discussion**

We investigated the tube-forming capacity of early EPCs and late EPCs isolated from human peripheral blood and umbilical cord blood, respectively. In the previous report, it was demonstrated that late EPCs were enriched in umbilical cord blood compared with adult peripheral blood [23]. Therefore, we isolated late EPCs from human umbilical cord blood. We used our novel method for capillary engineering which makes use of photo-catalytic lithography. Traditional assays for assessing characteristics of tube formation in vitro have been carried out by cultivation of endothelial cells in type I collagen and Matrigel. However, these methods could not distinguish between tube formation and morphological changes in the cells. The luminal structure of the vascular tube made in our present method was confirmed by an electron microscopy, a confocal laser microscopy and dye microinjection [22]. This technique is unique because the method allows one to focus on the process of tube formation. In contrast, tube formation in collagen and Matrigel evaluates the total activity of endothelial cells including migration, invasion and tube formation [24]. Using this novel method, we demonstrated that late EPCs participated in the formation of tubular structures with mature endothelial cells, i.e., HUVECs. It is interesting that late EPCs migrated toward and adjacent to pre-existing tubular structures and finally were incorporated
into the structure itself. In contrast, early EPCs could not form tubular structures, and they induced migration and sprouting of HUVECs present in the tubular structure. In a previous study using Matrigel, Hur et al. [18] demonstrated that early EPCs were incorporated into tubules when co-cultured with HUVECs, although the formation was weaker than that of late EPCs. However, that paper demonstrated the incorporation of EPCs into network structures of HUVECs on Matrigel, but could not show the incorporation of EPCs in tubular structures.

Our results showed that early EPCs were not capable of constructing tubular luminal structures even when they were co-cultured with mature endothelial cells. Instead, they stimulated the migration and sprouting of HUVECs from the tubular structure. These phenomena may be interpreted by the release of some growth factors from early EPCs. In our experiment, the amount of secreted interleukin-8 from early EPCs was significantly higher than that from late EPCs (0.81±0.07 vs. 0.44±0.02, ng/ml, P<0.01). It has been also reported that early EPCs secrete angiogenic factors such as VEGF [18,21], hepatocyte growth factor (HGF), and granulocyte-colony stimulating factor (G-CSF) [21]. These factors are known to stimulate endothelial cell migration and proliferation. In vivo experiments, implanted late EPCs in the occlusion tissues cause to construct new blood vessels by themselves, but early EPC could not. This result may be discussed with a hierarchy of EPCs [23]. It has been also reported that the different types of cells derived from peripheral blood have distinct actions in healing activity [25]. In this study, we demonstrated specific and distinct behaviors of early and late EPCs in tube formation and how they affected mature ECs in tube formation.

Although numerous reports have demonstrate that early EPCs have the potential to construct new blood vessels by themselves [5,6], some studies argued that early EPCs were not actually incorporated into newly formed vessels [26,27]. This discrepancy may be due to marked heterogeneity of EPCs used in their experiments. Numerous studies have fractioned EPC by phenotypic markers such as CD34, CD133, and VEGFR2 and various methods of EPCs isolation have been reported [5,6,28,29]. Among them CD34 has been often used for the marker of EPCs. However, Romagnani et al. showed that CD14 positive MNC-derived EPCs, which had been fractioned as CD34 negative, express very low level of CD34 using an antibody-conjugated magnetofluorescent liposomes (ACMFL) technique [30]. From these results, they suggested that CD14+CD34low cells are the major source of early EPCs obtained from human peripheral blood MNC. This subset exhibited clonogenicity and multipotency to differentiate not only into endothelial cells, but also into osteocytes, or neural cells [30]. This report suggests that it is difficult to sort EPCs into highly defined fractions by ordinary FACS technique. In fact, each EPC population in previous studies is supposed to have a different phenotype even from the same source. To avoid these complexities, we collected and isolated early and late EPCs by focusing on their characteristics such as morphologies and proliferation pattern, and compared their tube-forming activities in short duration in vitro assays.

While the regenerative potential of EPCs has been demonstrated in animal models of myocardial and limb ischemia, the number of EPC available for transplantation is very important for cell-based therapy. Because EPCs are derived from a limited endogenous pool, it is necessary to expand the number of EPCs in vitro or modulate phenotypes of EPCs. Iwaguro et al. reported that VEGF gene transfer in EPCs stimulated neovascularization in an in vivo model [31]. Murasawa et al. demonstrated that gene transfer of human telomerase reverse transcriptase into EPCs enhanced their angiogenic properties, mitogenic and migratory activities, and cell survival [32]. From the standpoint of cellular proliferation and phenotypic stability, late EPCs are superior to early EPCs. Therefore, the use of late EPCs for tissue engineering has been challenged [33–35]. With our method of generating transplantable capillary networks, the formation of tubular structures in vitro is a necessary precondition. The data presented here are thus important since the results demonstrate that late EPCs are a candidate for tissue engineering.

In conclusion, our data provide the first definitive evidence that early EPCs promote angiogenesis through migration and proliferation of mature endothelial cells, whereas late EPCs can form blood vessels. These results suggest that early EPCs and late EPCs have different roles in neovascularization in vivo. Finally, we expect that the novel culture system using a patterned substrate might be useful for future in vitro analyses of neovascularization.

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