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Microvascular Research 105 (2016) 125–131

Contents lists available at ScienceDirect

Microvascular Research

journal homepage: www.elsevier.com/locate/ymvre

Tracing behavior of endothelial cells promotes vascular network formation

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ARTICLE INFO

Article history:
Received 1 July 2015
Revised 8 December 2015
Accepted 8 December 2015
Available online 10 December 2015

Keywords:
Endothelial cells
Vascular network
Cell cycle
Migrate

ABSTRACT

The *in vitro* formation of network structures derived from endothelial cells in grafts before transplantation contributes to earlier engraftment. In a previous study, endothelial cells migrated to form a net-shaped structure in co-culture. However, the specific network formation behavior of endothelial cells during migration remains unclear. In this study, we demonstrated the tracing behavior and cell cycle of endothelial cells using Fucci-labeled (Fluorescent Ubiquitination-based Cell Cycle Indicator) endothelial cells. Here, we observed the co-culture of Fucci-labeled human umbilical vein endothelial cells (HUVECs) together with normal human dermal fibroblasts (NHDFs) using time-lapse imaging and analyzed by multicellular concurrent tracking. In the G0/G1 period, HUVECs migrate faster than in the S/G2/M period, because G0/G1 is the mobile phase and S/G2/M is the proliferation phase in the cell cycle. When HUVECs are co-cultured, they tend to move randomly until they find existing tracks that they then follow to form clusters. Extracellular matrix (ECM) staining showed that collagen IV, laminin and thrombospondin deposited in accordance with endothelial cell networks. Therefore the HUVECs may migrate on the secreted ECM and exhibit tracing behavior, where the HUVECs migrate toward each other. These results suggested that ECM and a cell phase contributed to form a network by accelerating cell migration.

Introduction

Most tissues in the body have vascular networks to supply nutrition and oxygen, which shows how critical appropriate vascularization is for tissue function and survival. Therefore, blood vessel reconstruction is one of the most important goals in the field of tissue engineering research, especially to fabricate thick tissue. Several approaches have been developed to create vascularization, including the incorporation of a growth factor within the material (Ehrbar et al., 2004), the seeding of a vascular precursor or vascular-inducing cells into a scaffold (Black et al., 1998), and the use of microfabrication technologies (Drury and Mooney, 2003).

Previously, we demonstrated endothelial cell co-culture within engineered cardiac tissue to improve neovascularization and cardiac function (Sekine et al., 2008). To ensure survival and speedy engraftment of 3D engineered tissue, early vascularization and perfusion are critical. A previous study demonstrated that endothelial cells migrate to form capillary-like and net-shaped structures in co-culture (Nagamori et al., 2013; Sasagawa et al., 2013; Sekiya et al., 2013). Furthermore, transplanted endothelial cells connected to the host vessels within 1 week after transplantation (Sekine et al., 2013).

Here we demonstrate cell cycle dynamics using Fucci (Sakaue-Sawano et al., 2008; Sugiyama et al., 2009). Fucci is a cell cycle indicator that labels cells to emit red fluorescence at the G1 phase and green fluorescence in S/G2/M phases. Cell cycle, migration and distribution of the extracellular matrix are important for neovascularization (Wood et al., 2011). A recent study has shown that there is a direct relationship between pluripotent stem cell differentiation and the cell cycle (Chetty et al., 2013). Pluripotent stem cells show long S phase in cell-cycle regulation, unlike other cell sources (Singh and Dalton, 2009). The ratio between the cell cycle phases change as stem cells differentiate, such that the G1 phase becomes lengthened and the S phase is shortened, relatively. This shows that cell-cycle control is important for stem cell differentiation (Singh and Dalton, 2009). The Fucci system is widely used in many cell-cycle studies (Sakaue-Sawano et al., 2008; Sakaue-Sawano and Miyawaki, 2014).

On the other hand, a co-culture environment promotes network formation of endothelial cells more rapidly than monoculture, because the co-culture method also provides a matrix for vascularization (Sorrell et al., 2008). The ECM is crucial for supporting cells and tubule formation, and fibroblasts are released from the ECM into the culture medium and induce endothelial tubular formation (Sorrell et al., 2007). Furthermore, endothelial cells secrete fibronectin to form blood vessels during development and angiogenesis (Cseh et al., 2010).

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http://dx.doi.org/10.1016/j.mvr.2015.12.005
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Therefore, creating a vascular network in engineered tissue is one of the most important tasks for regenerative medicine, and the regulation of the behavior of endothelial cells could provide a more effective cell therapy in tissue engineering. However, there have not been any detailed analyses of the mechanisms or behavior of endothelial cells during culture conditions. In this study, we examined the behavior of endothelial cells in a co-culture environment using Fucci. Accordingly, we searched for factors related to the extracellular matrix that promotes network formation with endothelial cells.

Materials and methods

Generation of Fucci-HUVEC cell line

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Walkersville, MD, USA). Fucci lentivirus vectors were provided by RIKEN BSI. The Fucci indicator was developed to visualize the dynamics of the cell cycle progression. Fucci cells express two fusion proteins: monomeric Kusabira Orange 2 (mKO2) fused to Cdt1 (30/120), and monomeric Azami Green (mAG) fused to Geminin (1/110). Fucci lentiviruses were produced by ViraPower Lentiviral Expression System (Invitrogen, Carlsbad, CA, USA), and the Fucci lentivirus was concentrated using Lenti-X Concentrator (Clontech, Mountain View, CA, USA) according to the manufacturer's suggested protocol. HUVECs were plated on a 100 mm tissue culture plate at 4000 cells/cm^2 with EBM-2 medium (10 ml/well). Twenty-four hours after plating, the medium was replaced with basic medium containing lentiviral vectors (at a multiplicity of infection of 1 [MOIs]). Overnight, after the vectors were introduced, and media was replaced with fresh EBM-2 medium (10 ml/well).

Cell culture

Human skeletal muscle myoblasts (HSMMs) and neonatal normal human dermal fibroblasts (NHDFs) were purchased from Lonza (Walkersville, MD, USA). Fucci-HUVECs up to the 7th passage were seeded onto a 35 mm glass based dish (Iwaki, JP) in EBM-2 medium with EGM-2 at a density of 1.29×10^4 cells/cm^2. Concurrently, NHDFs and HSMMs up to the 7th passage were seeded onto the dish at a density of 2.30×10^5 cells/cm^2. For further analysis of extracellular matrix, normal HUVECs (non Fucci-HUVECs) were seeded onto a 12 well plate (Falcon, New York, USA) in EBM-2 medium with EGM-2 at a density of 5.16×10^4 cells/well. Concurrently, NHDFs were seeded onto the well at a density of 9.20×10^5 cells/well.

Time-lapse imaging of co-culture

Time-lapse images were taken by a LCV110 fluorescence microscope (Olympus, Tokyo, Japan) and MetaMorph observation application (V7.7.6, Molecular Devices Corporation, CA, USA) every 30 min. The cells were maintained in the LCV110 incubator system at 37 °C in a humidified atmosphere with 5% CO_2 for 3 days. All images and movies were processed by the MetaMorph analysis application. HUVEC migrations were evaluated from time-lapse movies. To evaluate the distance between cells within a cluster, we measured nearness of the cells in fixed time; the distance between two cells at the same point in time, and nearness of tracks; the shortest distance between two cells without any consideration of time. Other cell migratory properties [migration speed in each cell cycle] were also assessed by cell tracking system.

Immunofluorescent staining of extracellular matrix components

Three days after co-culture, dishes were fixed with 4% paraformaldehyde for 30 min. For immunostaining, fixed cells were incubated with both a 1/100 dilution of anti-fibronectin antibody (Abcam, Cambridge, UK), a 1/200 dilution of biotinylated anti-collagen I antibody (Chondrex, Redmond, WA, USA), a 1/100 dilution of anti-human collagen IV antibody (Daiichi-fine Chemical, Toyama, JP) and a 1/200 dilution of anti-paxillin antibody (ab32084, Abcam) for 2 h at room temperature. Cells were then treated with both a 1/200 dilution of goat anti-rabbit IgG H&L (Cy5) (Abcam), a 1/200 dilution of Cy5 streptavidin (Jackson Immune Research Laboratories, West Grove, PA, USA), a 1/200 dilution of goat polyclonal secondary antibody to mouse IgG H&L (Cy5) (Abcam) and a 1/200 dilution of goat anti-rabbit IgG H&L (Cy5) (Abcam) for 45 min at room temperature. For further analysis of extracellular matrix, immunostaining of CD31, laminin, thrombospondin and collagen IV were performed. Three days after normal (non-Fucci) HUVEC and NHDF co-culture, dishes were fixed with 4% paraformaldehyde for 5 min. For immunostaining, fixed cells were incubated with a 1/10 dilution of anti CD31 antibody (RB-10333-P, Thermo Fisher Scientific, CA, USA) for overnight at 4 °C. Cells were then treated with either a 1/100 dilution of anti-laminin α4 antibody (ab205568, Abcam), a 1/200 dilution of anti-human collagen IV antibody (ab6586, Abcam), or a 1/500 dilution of anti-thrombospondin antibody (ab85762, Abcam) for overnight at 4 °C. For the detection of ECM, samples were then incubated with both a 1/200 dilution of Alexa-Fluor-568 conjugated anti-rabbit IgG antibodies (A21069, Invitrogen, Carlsbad, CA) to collagen IV and thrombospondin dishes or Alexa-Fluor-568 conjugated anti-mouse IgG antibodies (A11019, Invitrogen) to laminin dish for 1 h at room temperature. Cell nuclei were then counterstained by ProLong-Gold antifade reagent with DAPI (Invitrogen) for 5 min. Sections were finally visualized using confocal laser scanning microscopy (FV1200, IX83; Olympus).

Data analysis

All data are expressed as mean ± SD. All analyses were performed using SPSS software. An unpaired Student's *t*-test was performed to compare two groups. One-way ANOVA was used for multiple group comparisons. A P-value of less than 0.05 was considered significant.

Results

Efficiency of lentiviral transduction of HUVECs

The viral titer was calculated using a Lenti-X p24 rapid titer kit (Clontech). The calculated viral titer was 2.28×10^7 IFU/ml of Cdt1, and 2.55×10^7 IFU/ml of Geminin. To count the transduction rate, the HUVECs were immunostained by Hoechst. The numbers of Fucci positive cells or Hoechst positive cells were counted. The Fucci vector induced toxicity to the HUVECs at a MOI of 2 or higher. Therefore, in this study we decided to examine the Fucci system at a MOI of 1, which showed that 59% ± 1% (n = 3) of the HUVECs were transduced (Fig. 1B, C). Increasing the MOI up to 20 did not elevate the number of Fucci-positive cells.

Relationship of cell migration and cell cycle

Time-lapse movies were made of HUVECs co-cultured with NHDFs or HSMMs. Fig. 2A–C and J–L demonstrates that Fucci-HUVECs spread randomly and then migrate toward each other to form a network (Supplementary Material Movies 1 and 2) as shown in the schematic illustration of Fig. 2S. In addition, during the G0/G1 period the HUVECs migrated very quickly to form vascular networks; whereas, in the S/G2/M period HUVECs migrated very slowly and did not form any vascular networks (Fig. 2D–F, M–O, Supplementary Material Movies 1 and 2). In contrast, during the G0/G1 period, the HUVECs migrated much faster than in the S/G2/M period, then the HUVECs gathered around each other to form a network structure (Fig. 2G–I, P–R, Supplementary Material Movies 1 and 2). To determine the migration properties of HUVECs in each cell cycle, G0/G1 and S/G2/M periods, we analyzed the migration speed of HUVECs. During the G0/G1 period, HUVECs

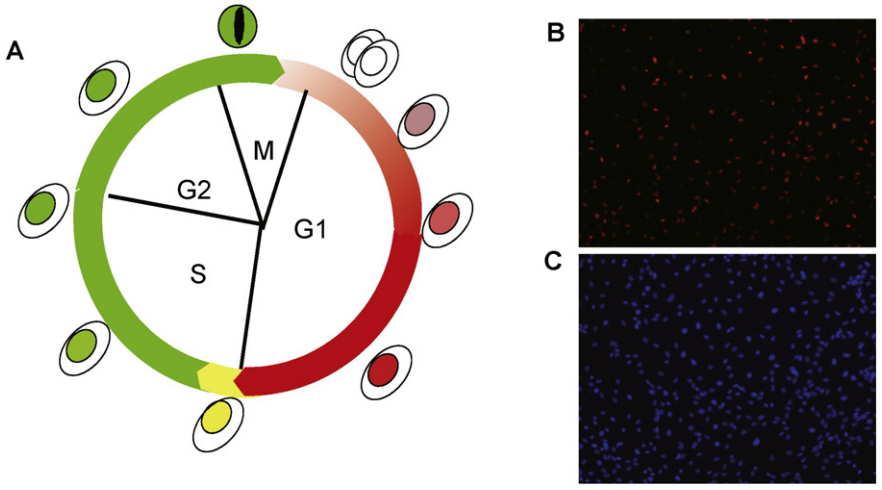

Fig. 1. Confirmation of the characteristics and transduction rate of lentivirus-transduced human umbilical vein endothelial cells (HUVECs). (A) Schemes illustrating the Fucci. A fluorescent probe that labels G0/G1 phase nuclei in red and S/G2/M phase nuclei in green. (B, C) Fluorescent microscope images of Fucci transduced HUVECs. Microscopy images of Fucci-HUVECs (B), and Fucci-HUVECs dyed by Hoechst (C). The experiments were repeated three times with similar results.

had greater migration ability than during the S/G2/M period, in both NHDF and HSMM co-culture conditions (Fig. 2T).

Furthermore, the co-cultured HUVECs showed regional differences in migration. The neighboring HUVECs in the G0/G1 period congregated to form clusters, suggesting that this behavior contributes to the formation of vascular networks (Supplementary Material Movies 1 and 2). Then we evaluated HUVECs under specific conditions: HUVEC groups that migrate along the same track (cluster) and HUVECs that migrate as isolated cells (isolated) (Fig. 3A–B). We analyzed five clusters and 10 isolated cells in each movie; then we analyzed the distance between each HUVEC, and extracted five HUVECs from each cluster. The HUVEC clusters had greater migration ability than isolated cells; they also contained G0/G1 period cells, as well as a few S/G2/M period cells (Fig. 3C).

To determine whether the HUVECs within a cluster followed each other in a systematic pattern, the distance between each cell position and between each of the tracks was measured. Five HUVEC clusters were selected and the distance between each of the cells within each cluster was measured every 30 min over a period of 72 h. Within each cluster, the tracks were identified and at each time point, the nearest distance to every other track was measured over a total period of 72 h. The distances between HUVECs and between tracks tended to become narrower over time, which indicates that the HUVECs did follow each other in a systematic manner (Fig. 3D, E).

To compare the motion of HUVECs with NHDFs, the NHDFs were stained with YOYO-1, which is a dye for nucleic acid whose fluorescence excitation spectrum is Ar488. Then the stained NHDFs and non-stained NHDFs were mixed at a ratio of 1:9 and cultured on a glass bottom dish then observed with a LCV110 time-lapse microscope. The NHDFs did not migrate as the HUVECs did; however, they did continue to proliferate naturally (Supplementary Material Movie 3).

Expression of extracellular matrix in co-culture

Three days after co-culture, many extracellular matrixes were observed in the dish. HUVECs formed networks and existed in accordance with the deposition of collagen IV. In contrast, NHDF monoculture did not show network deposition of collagen IV. Compared with collagen IV, fibronectin and collagen type I had no correlation with the HUVEC distribution. Fibronectin and collagen type I were uniformly distributed on the dish. Furthermore, we examined expression of paxillin, which is focal adhesion-associated protein and regulates cell motility. Paxillin was mainly observed within endothelial cell networks (Fig. 4A). For additional ECM evaluation, we examined distribution of laminin and thrombospondin in normal HUVEC and NHDF co-culture. HUVECs existed in accordance with the deposition of laminin and thrombospondin as well as collagen IV (Fig. 4B).

Discussion

The survival of transplanted grafts commonly used in regenerative medicine requires a constant supply of blood. This critical blood supply requires construction of a stable vascular network (Koike et al., 2004), especially when transplanting thick tissue (Sekiya et al., 2006), because oxygen and nutrients supplied by the blood is required immediately. Recently, various natural and synthetic biomaterials have been used to prefabricate blood vessel substitutes or tissue-engineered vascular scaffolds such as using a co-culture of mesenchymal precursor cells (Auxenfans et al., 2012); collagen and fibrin gel (Drury and Mooney, 2003; Rao et al., 2012); an acellular matrix (Xi-xun et al., 2010); or patterning and overlay by a stamp technique (Muraoka et al., 2012; Sasagawa et al., 2013). However, the most effective environment and appropriate cell cycle phase for endothelial cells to stimulate the formation of a vascular network remains unclear.

In the present study, we demonstrate the co-culture of Fucci-HUVECs to evaluate cell migration and the cell cycle by time-lapse scanning microscopy with image processing. We also present new findings indicating the most appropriate endothelial cell cycle phase to promote a vascular network, critical for engineering thick tissue.

This study shows that HUVECs in the G0/G1 period migrate faster than the S/G2/M period in co-culture with NHDF or HSMM. Previous studies showed that cardiomyocyte proliferation and migration are important for regeneration of heart injury models (Choi et al., 2013; Itou et al., 2012; Porrello et al., 2011); and this result suggests that cells divide and increase first in the G2 phase, and then they migrate to reproduce the organism in the G1 phase (Itou et al., 2012). Other studies using cancer cells, epithelial cells or endothelial cells also demonstrated the link between cell motility and cell cycle progression (Walmod et al., 2004; Kagawa et al., 2013; Madri et al., 1988). Here we demonstrated the same relationship between co-cultured endothelial cell network formation and their cell cycle. Vascular network formation should require the migration of cells in the G1/G0 phase.

Previous results have shown that HUVECs in engineered tissue migrate and form capillary-like tubular structures (Black et al., 1998). In addition, *in vitro* co-culture of endothelial cells and fibroblasts showed that they produced a more capillary-like network compared

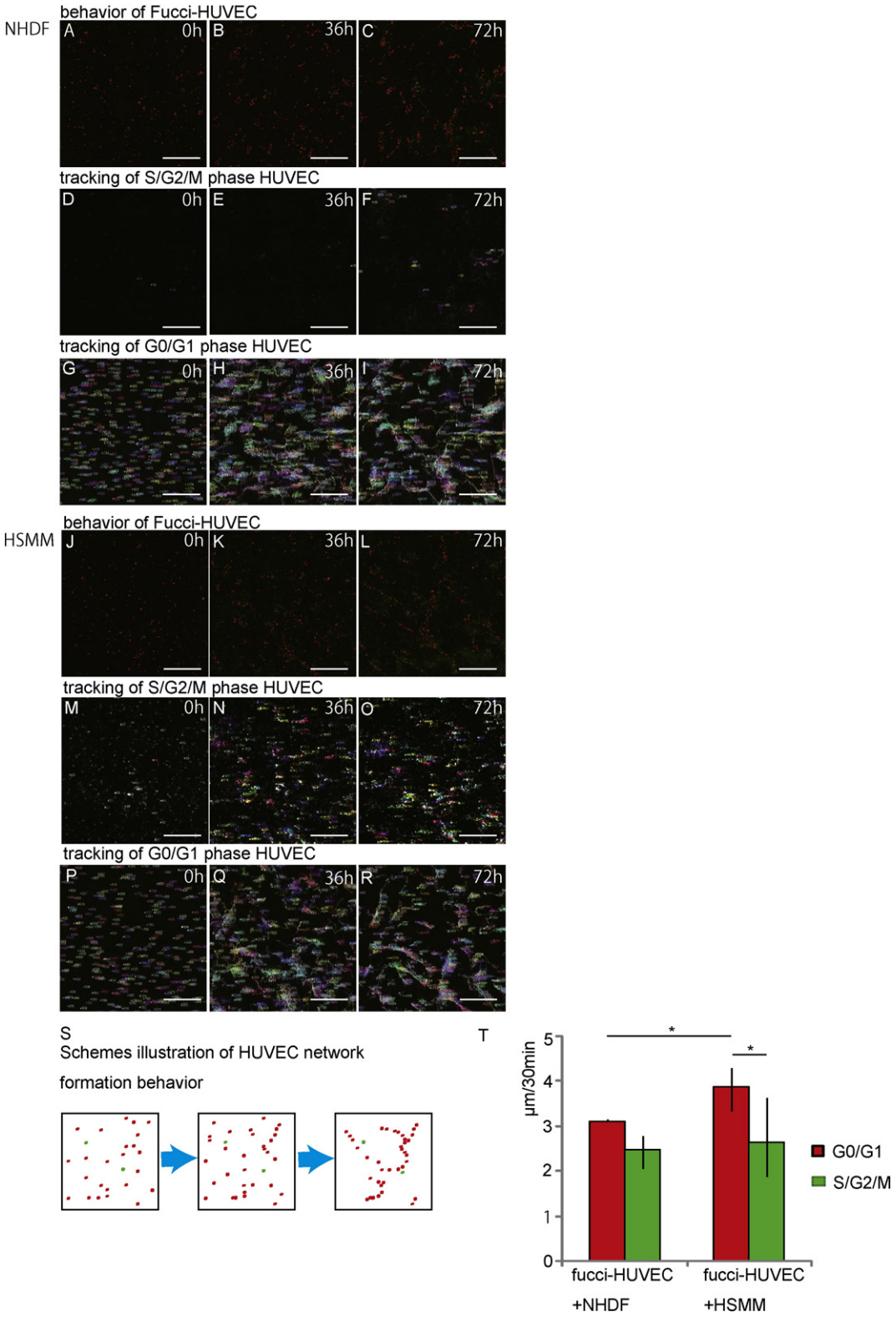

Fig. 2. Migration and cell cycle of HUVECs during co-culture with fibroblasts and myoblasts. (A–R) Horizontal images of Fucci-HUVEC morphology. HUVECs evenly distributed at 0 h after start, then migrated to form a network by 72 h. Fluorescent microscope images of Fucci-HUVECs and NHDF in co-culture (A–C), HSMM coculture (J–L). Time-lapse images were taken every 30 min over 3 days. Migration pathways of HUVECs used by multicellular synchronous tracking, the courses of several frames of HUVECs are shown as lines colored for every HUVEC. Tracking of S/G2/M period HUVECs co-cultured with NHDF (D–F) and HSMM (M–O). Tracking of G0/G1 period HUVECs co-cultured with NHDF (G–I) and HSMM (P–R). Tracking of G0/G1 HUVECs showed a longer migration distance than S/G2/M HUVECs. (S) Schemes illustrating the HUVEC network formation behavior during co-culture. (T) Migration profiles of HUVECs at each cell cycle. G0/G1 HUVECs migrate faster than S/G2/M HUVECs. Error bars indicate ±SD (*$P < 0.05$).

with endothelial cell monoculture (Kunz-Schughart et al., 2006; Liu et al., 2013; Sorrell et al., 2008). In this study, we demonstrated that HUVECs approached and then followed each other, migrated along a fixed course, and accelerated their movement in co-culture. In contrast, NHDFs proliferated, but were dispersed, did not exhibit any tracking behavior, and did not accelerate (Supplementary Material Movie 3).

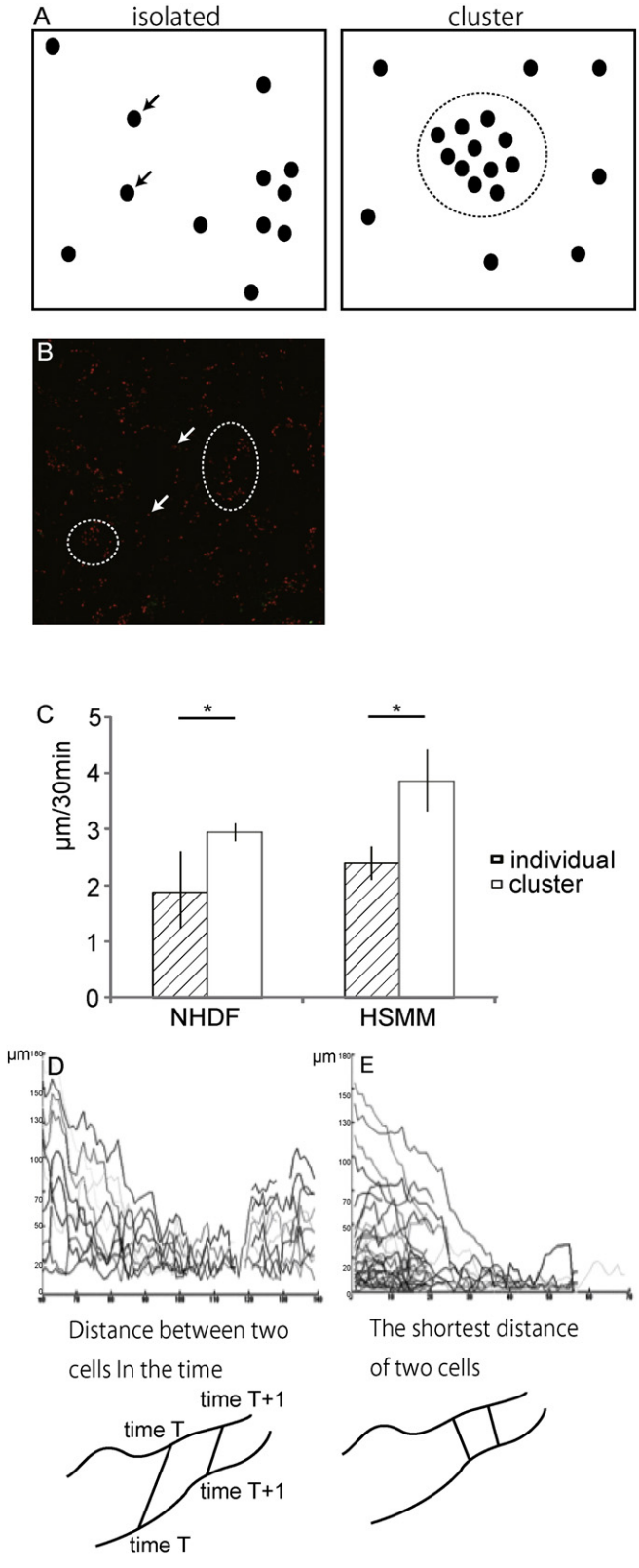

Fig. 3. Migration speed of HUVECs classified by cell density, relationship of migration, and cell–cell distance. (A, B) We classified HUVECs into individuals (arrow) and clusters (circle). (A) Schemes illustrate individuals and clusters. (B) Fluorescent microscope image contains individuals and clusters of HUVECs. Clusters of HUVECs were determined by the surrounding cell density. (C) Migration profiles of the individual movement of HUVECs and cluster HUVECs. Individual HUVECs migrated more slowly than cluster HUVECs (*$P < 0.05$). (D, E) Relationship of cell–cell distance and track distance for cluster HUVECs. To evaluate the distance between cells within a cluster, we measured the distance between two cells at the same point in time, and the shortest distance between two cells without any consideration of time. (Left) Two cells migrate apart at the same time. (Right) The cell approaches the tracks of other cells in the cluster as time progresses. This result indicates that the one cell did not trace the other cell, but rather the track of the cell.

Therefore, this result suggests that HUVECs form network structures by accelerating migration, whereas other cell sources do not have the ability to accelerate migration. Time-lapse imaging showed that HUVECs migrated along a fixed course, much like previous studies have shown blood vessels migrating into a collagen scaffold (Yahyouche et al., 2013). To verify the HUVEC footprint, we examined the immunostaining of several extracellular matrixes. Although HUVECs were distributed along the tracks of collagen type IV, HUVECs did not distribute along with collagen type I or fibronectin. In a previous study, collagen type IV was secreted by fibroblasts and supported endothelial cell structures, which allowed endothelial cells to form 3D structures (Sorrell et al., 2007). Another study has also shown that fibroblasts secrete collagen type IV and fibronectin for vascular formation (Soucy and Romer, 2009). This suggests that HUVEC do not move randomly, but migrate along the path of collagen type IV that is secreted from other HUVECs and co-cultured cells. On the other hand, some reports demonstrated that migrating endothelial cells deposit laminin and thrombospondin, and endothelial cells migrating along these ECMs (Madri et al., 1988; Madri and Stenn, 1982). We also confirmed these two proteins deposited along endothelial cell network in co-culture system, so several ECMs in addition to collagen IV may construct the track for endothelial cell migration.

In this study, we demonstrated that HUVECs in the G0/G1 phase have a high capability for migration, and can quickly migrate by moving along specific ECMs as if they were following the ECM track. Moreover, HUVECs migrate in search of other nearby HUVECs to build clusters, which can connect to form larger vascular networks. Therefore, high cell density contributes to the rapid formation of cell tracks, which accelerates the speed of cell migration (Udan et al., 2013).

Recently regulation of endothelial cell migration and proliferation depending hydrogel compliance and composition has been reported (Wood et al., 2011). The present study also indicates the possibility that ECM deposition might control endothelial migration. Therefore biomaterial modification should contribute to the formation of functional vascular network in tissue engineering research field.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mvr.2015.12.005.

Author contributions

N.Y., H.S., T.S. and T.O. were involved in the design of experiments. N.Y., H.S., R.B. and T.S. conducted experiments and analyzed data. N.Y., H.S. and T.S. wrote the manuscript. T.S. and T.O. initiated the project. All authors discussed the results and commented on the paper.

Acknowledgments

This work was supported by a grant from the Japan Society for the Promotion of Science (JSPS) through the "Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program)," initiated by the Council for Science and Technology Policy (CSTP). Ryoma Bise is a staff of Dai Nippon Printing Co., outside of submitted work; we report grant from Dai Nippon Printing Co. We thank Atsushi

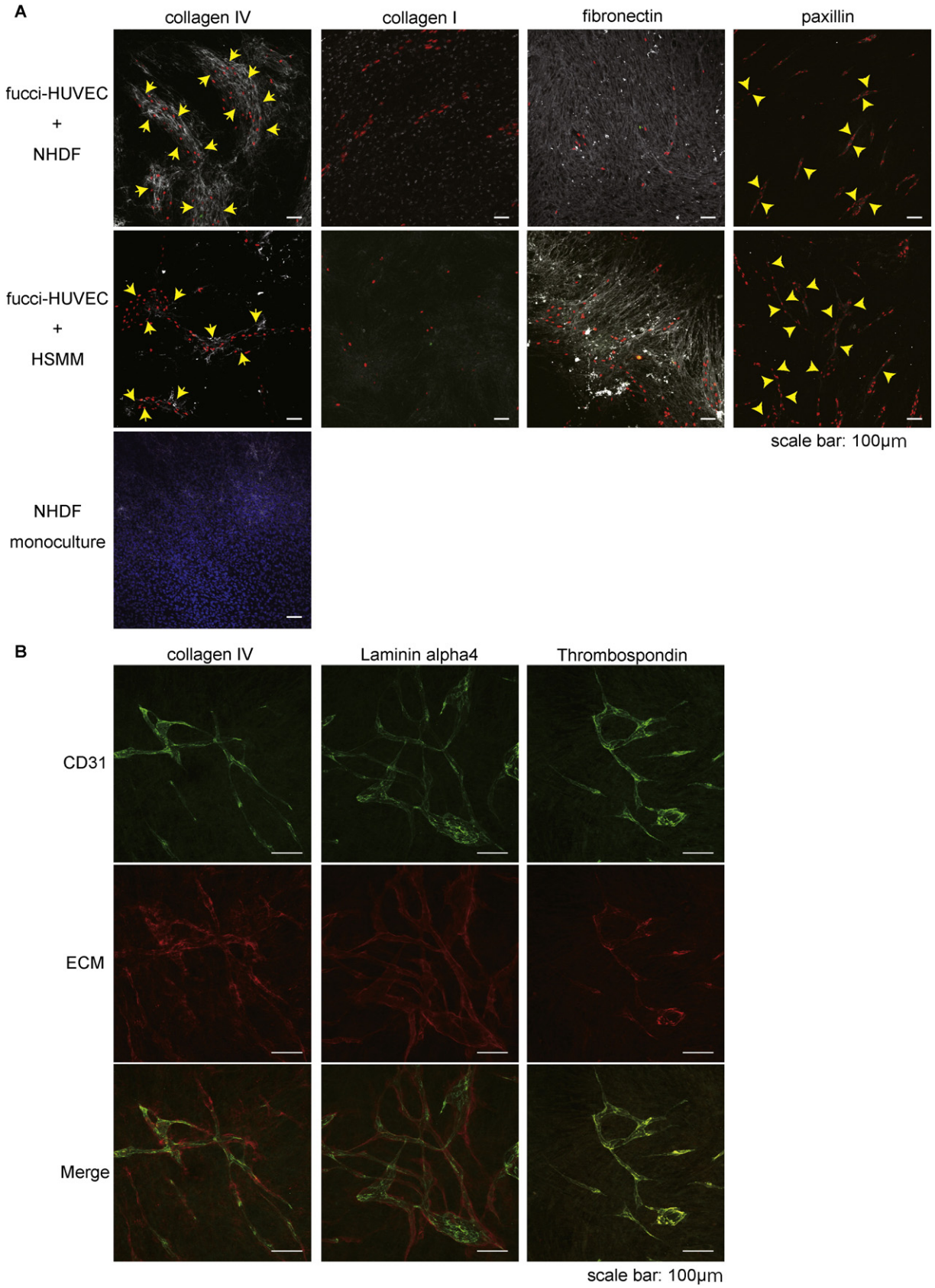

Fig. 4. Histological analysis and secretion assay of extracellular matrix and HUVECs. (A) Distribution of ECM in Fucci-HUVEC and NHDF co-culture. Immunostaining of the extracellular matrix shows that HUVECs are concentrated along the collagen type IV tracks (arrows). Collagen type I and fibronectin are spread uniformly in the co-culture dish. Furthermore, we examined paxillin, focal adhesion-associated, phosphotyrosine-containing protein, which regulates cell spreading and motility. Paxillin is observed within the HUVECs (arrowheads). (B) Distribution of ECM in normal HUVEC and NHDF co-culture. Immunostaining shows that HUVECs are concentrated along the depositions of the laminin alpha4 and thrombospondins as well as collagen type IV.

Miyawaki and Asako Sakaue-Sawano (Laboratory for Cell Function and Dynamics, Brain Science Institute, RIKEN) for providing Fucci plasmid. We thank Atsushi Miyawaki, Asako Sakaue-Sawano and Allan Nisbet for his useful comments and editing of the manuscript.

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