



Original article

Formation of vascular network structures within cardiac cell sheets from mouse embryonic stem cells



Shinako Masuda, Katsuhisa Matsuura^{*}, Mie Anazawa, Takahiro Iwamiya, Tatsuya Shimizu, Teruo Okano

Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku, Tokyo 162-8666, Japan

ARTICLE INFO

Article history:

Received 5 August 2015
Received in revised form
14 September 2015
Accepted 2 October 2015

Keywords:

Mouse embryonic stem cells
Three-dimensional suspension culture
Endothelial cell differentiation
Cardiac cell sheet
Microvascular network formation
Fibroblast growth factor 1

ABSTRACT

Bioengineered cardiac tissues represent a promising strategy for regenerative medicine. However, methods of vascularization and suitable cell sources for tissue engineering and regenerative medicine have not yet been established. In this study, we developed methods for the induction of vascular endothelial cells from mouse embryonic stem (ES) cells using three-dimensional (3D) suspension culture, and fabricated cardiac cell sheets with a pre-vascularized structure by co-culture of mouse ES cell-derived endothelial cells. After induction, isolated CD31⁺ cells expressed several endothelial cell marker genes and exhibited the ability to form vascular network structures similar to CD31⁺ cells from neonatal mouse heart. Co-culture of ES cell-derived CD31⁺ cells with ES cell-derived cardiomyocytes and dermal fibroblasts resulted in the formation of cardiac cell sheets with microvascular network formation. In contrast, microvascular network formation was reduced in co-cultures without cardiomyocytes, suggesting that cardiomyocytes within the cell sheet might enhance vascular endothelial cell sprouting. Polymerase chain reaction array analysis revealed that the expression levels of several angiogenesis-related genes, including fibroblast growth factor 1 (FGF1), were up-regulated in co-culture with cardiomyocytes compared with cultures without cardiomyocytes. The microvascular network in the cardiac sheets was attenuated by treatment with anti-FGF1 antibody. These results indicate that 3D suspension culture methods may be used to prepare functional vascular endothelial cells from mouse ES cells, and that cardiomyocyte-mediated paracrine effects might be important for fabricating pre-vascularized cardiac cell sheets.

© 2015, The Japanese Society for Regenerative Medicine. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Abbreviations: ES cell, embryonic stem cell; FGF1, fibroblast growth factor 1; 3D, three-dimensional; iPS cell, inducible pluripotent stem cell; Flk1, fetal liver kinase 1; VEGF, vascular endothelial growth factor; MACS, magnetic-activated cell sorting; VE-cadherin, vascular endothelial cadherin; eNOS, endothelial nitric oxide synthase; SMA, smooth muscle actin; Col4a3, collagen type IV alpha 3; EYFP, enhanced yellow fluorescent protein; FBS, fetal bovine serum; NEAA, non-essential amino acids; LIF, leukemia inhibitory factor; PCR, polymerase chain reaction; Gusb, glucuronidase, beta; qRT-PCR, quantitative real-time PCR; Acvr11, activin receptor like 1; CXCR4, chemokine receptor type 4; Dll4, delta-like ligand 4; Efnb2, ephrin-B2; Ephb4, ephrin type-B receptor 4; Flt1, fms-related tyrosine kinase 1; KDR, kinase insert domain receptor; Notch1, Notch homolog 1; Nr2f2, nuclear receptor subfamily 2, group f, member 2; Pecam1, platelet/endothelial adhesion molecule 1; Pou5f1, POU class 5 homeobox 1; Cxcl5, chemokine (C-X-C motif) ligand 5; Egf, epidermal growth factor; Mdk, midkine; Tgfa, transforming growth factor, alpha; Tymp, thymidine phosphorylase; TIMP2, tissue inhibitor of metalloproteinase 2.

^{*} Corresponding author. Tel.: +81 3 5367 9945; fax: +81 3 3359 6046.

E-mail address: matsuura.katsuhisa@twmu.ac.jp (K. Matsuura).

Peer review under responsibility of the Japanese Society for Regenerative Medicine.

1. Introduction

Well-organized, functional tissues are composed of various cell types. Tissue engineering represents a new therapeutic approach for the treatment of congenital defects and functional disorders, and methods for constructing 3D functional tissues are therefore required. We previously developed cell-sheet engineering that allowed the creation of functional 3D tissues by layering two-dimensional confluent cell sheets harvested from temperature-responsive culture surfaces [1–4]. A well-organized vascular network is essential for metabolic exchange throughout the engineered 3D tissues. In the case of neonatal rat cardiac cell sheets, CD31⁺ cells formed a network structure connecting host and graft vessels upon *in vivo* transplantation [5,6]. Furthermore, large numbers of cells are needed to fabricate functional 3D tissues. Embryonic stem (ES) cells and induced pluripotent stem (iPS) cells are considered as likely sources of cells for regenerative medicine.

We developed methods for inducing the differentiation of mouse ES cells in large-scale suspension cultures, and recently reported the creation of cardiac cell sheets by co-culture with cardiomyocytes derived from mouse ES cells and cardiac fibroblasts [7,8]. Large-scale differentiation systems were also applicable for the collection of cardiomyocytes and formation of cardiac cell sheets from human iPS cells [9].

Several studies have reported the induction of cardiovascular cells from pluripotent stem cells [10–12]. Yamashita et al. reported that cardiovascular cells could be differentiated from mouse ES and iPS cells through mesodermal progenitor fetal liver kinase 1 (Flk1)+ cells [13–15], and administration of vascular endothelial growth factor (VEGF) and cAMP resulted in the effective induction of arterial endothelial cells by activation of Notch signaling [16]. Pre-vascularization of cardiac cell sheets is important for prompt microvascular communication between transplanted grafts and host tissue, leading to better engraftment upon transplantation. Although cardiac cell sheets have been prepared from cardiomyocytes, endothelial cells, and mural cells derived from mouse ES or iPS cells [17,18], microvascular network formation *in vitro* has not been clear. Several issues regarding the use of pluripotent stem cell-derived endothelial cells for the fabrication of bioengineered cardiac tissue remain to be resolved, including: (1) genetic and functional differences in endothelial cells derived from heart tissue and pluripotent stem cells; (2) the contribution of pluripotent stem cell-derived endothelial cells to the microvascular network *in vitro*; and (3) the molecular mechanism underlying endothelial cell microvascular network formation in cardiac cell sheets.

With the aim of reconstructing ES cell-derived well-vascularized 3D cardiac tissues, we induced CD31+ cells from mouse ES cells using spinner flask cultures and generated cardiac cell sheets with a microvascular network structure by co-culturing CD31+ cells with mouse ES cell-derived cardiomyocytes and dermal fibroblasts.

2. Methods

2.1. Animals

Wild-type C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). Animal experiments were approved by the Institutional Animal Care and Use Committee of Tokyo Women's Medical University.

2.2. Antibodies

Biotin-conjugated monoclonal antibodies for murine Flk1 (eBioscience, San Diego, CA, USA) and murine CD31 (BD Biosciences, San Jose, CA, USA) were used as primary antibodies for magnetic-activated cell sorting (MACS) separation. Phycoerythrin-conjugated monoclonal antibody for murine vascular endothelial (VE)-cadherin (BioLegend, San Diego, CA, USA) and monoclonal antibodies for cardiac troponin T (Thermo Fisher Scientific, Waltham, MA, USA), murine CD31, endothelial nitric oxide synthase (eNOS; BD Biosciences), murine α -smooth muscle actin (SMA; Sigma–Aldrich, St. Louis, MO) and collagen type IV alpha 3 (Col4a3; kindly provided by Dr. Sado, Shigei Medical Research Institute, Japan) were used as primary antibodies for immunocytochemistry. Antibodies for SM22 α (Abcam, Cambridge, MA, USA) and fibroblast growth factor (FGF) 1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were also used as primary antibodies for immunocytochemistry. Anti-mouse, anti-rat, or anti-rabbit IgG antibodies conjugated with Alexa488, Alexa568 or Cy5 (Molecular Probes, Eugene, OR, USA) were used as secondary antibodies for immunocytochemistry.

2.3. ES cell culture

R1 ES cells expressing the neomycin phosphotransferase gene under the control of the α -myosin heavy chain promoter (R1-neo) [19] with the enhanced yellow fluorescent protein (EYFP) gene (R1-neo EYFP) [20] were maintained on 1% gelatin-coated tissue culture dishes in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 0.1 mM non-essential amino acids (NEAA), 1 mM sodium pyruvate, 1% penicillin/streptomycin and 1000 U/ml leukemia inhibitory factor (LIF).

EMG7 ES cells expressing the enhanced green fluorescent protein gene under the control of the α -myosin heavy chain promoter [15] were maintained in Glasgow's minimum essential medium supplemented with 10% fetal bovine serum, 0.1 mM 2-mercaptoethanol, 0.1 mM NEAA, 1 mM sodium pyruvate, 1% penicillin/streptomycin and 1000 U/ml LIF.

2.4. Preparation of cardiac cells

ES cell-derived cardiomyocytes were prepared by neomycin selection of differentiated R1 ES cells, as described previously [7] with a few modifications. To induce differentiation, R1 ES cells were cultured in the presence of noggin (150 ng/ml) (R&D Systems, Minneapolis, MN, USA) from day –3 to day 1 of induction of differentiation. The differentiation medium for R1 ES cells was Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 0.1 mM NEAA, 1 mM sodium pyruvate and 1% penicillin/streptomycin. Trypsinized R1 ES cells were seeded at 5×10^4 cells/ml (total, 200 ml/flask) into spinner flasks and cultured in the absence of LIF until day 18. On day 5, the volume of medium was increased to 400 ml/flask. Granulocyte colony-stimulating factor (1 ng/ml) was added to the differentiation medium from day 5 to day 10, and fetal bovine serum in the differentiation medium was replaced with bovine serum from day 7 to day 18. For selection of ES cell-derived cardiomyocytes, differentiated cells were cultured in the presence of G418 from days 10 to 18. On day 18, undifferentiated cells included in the suspension culture were depleted by MACS using anti SSEA-1 microbeads.

2.5. Preparation of CD31+ cells

CD31+ cells were prepared from differentiated EMG7 or R1-neo EYFP ES cells. To induce differentiation, trypsinized ES cells were seeded at 1×10^5 cells/ml into spinner flasks and cultured in the absence of LIF until day 5. Differentiation medium for EMG7 ES cells consisted of MEM- α supplemented with 10% fetal bovine serum, 0.05 mM 2-mercaptoethanol and 1% penicillin/streptomycin. Differentiation medium for R1-neo EYFP ES cells consisted of Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 0.1 mM NEAA, 1 mM sodium pyruvate and 1% penicillin/streptomycin. On day 5, embryoid bodies were enzymatically dissociated and subjected to MACS to separate Flk1+ cells. Flk1+ cells were re-cultured with both VEGF (50 ng/ml) and 8-bromo-cAMP (0.5 mmol/l) onto collagen IV-coated tissue culture dishes. Three days after re-culture, induced CD31+ cells were isolated from re-cultured Flk1+ cells by MACS.

2.6. Mouse primary cell culture

Dermal fibroblasts were obtained from young adult mice. Dermal tissue specimens were cut into pieces 1 cm² and incubated at 4 °C for 24 h with 500 U/ml dispase. The skin was separated into

dermal and epidermal layers and the dermis were placed on the surface of 100 mm culture dishes and minced into 1 mm² squares. After adherence of the minced dermal tissue, Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin was gently added to the culture dishes and the cells were maintained at 5% CO₂ at 37 °C.

Cardiomyocytes and cardiac fibroblasts were obtained from the heart of neonatal mice (1–2 days old) as described previously [7]. CD31+ cells were prepared from the fraction of cardiomyocytes by MACS using CD31 antibody.

2.7. Immunocytochemistry

Cells were fixed with 5% dimethyl sulfoxide in methanol and blocked with 1% skimmed milk. The fixed cells were stained with primary antibody overnight at 4 °C, followed by incubation with secondary antibody for 3 h at 4 °C. Nuclei were visualized with Hoechst 33342.

2.8. High-throughput image acquisition and data analysis

Images of CD31+ cell network structures were collected using ImageXpress Ultra confocal high content screening system (Molecular Devices, LLC, Sunnyvale, CA, USA). The computer systems

and hardware were operated by MetaXpress software (Molecular Devices, LLC). The 'Z series' acquisition function was used to construct the best-focus images. Twenty sequential images (each 10 μm depth) were acquired along the Z axis. Sequential acquisition of images was performed automatically at each wavelength. The tube length and tube thickness of CD31+ cells were scored using MetaXpress software (Molecular Devices, LLC). The acquired images were analyzed automatically using the 'Journal' function. Images highlighted with Alexa568 (CD31+ cells) were processed with the morphology filter to eliminate the effect of non-specific staining. CD31+ cell network structures were detected using the 'Angiogenesis' and 'Neurite Outgrowth' application modules, and the tube length and tube thickness of CD31+ cells were scored in the overlapping region detected by the above two modules.

2.9. Cell-sheet manipulation

Cell sheets were harvested by simple pipetting, as described by Haraguchi et al. [3]. Briefly, intact cell sheets were detached by transferring confluent cells cultured on a temperature-responsive culture surface to an incubator at 20 °C for 30 min. The detached cell sheet was spread on the surface by aspirating and incubated at 37 °C for 1 h to adhere to the culture surface.

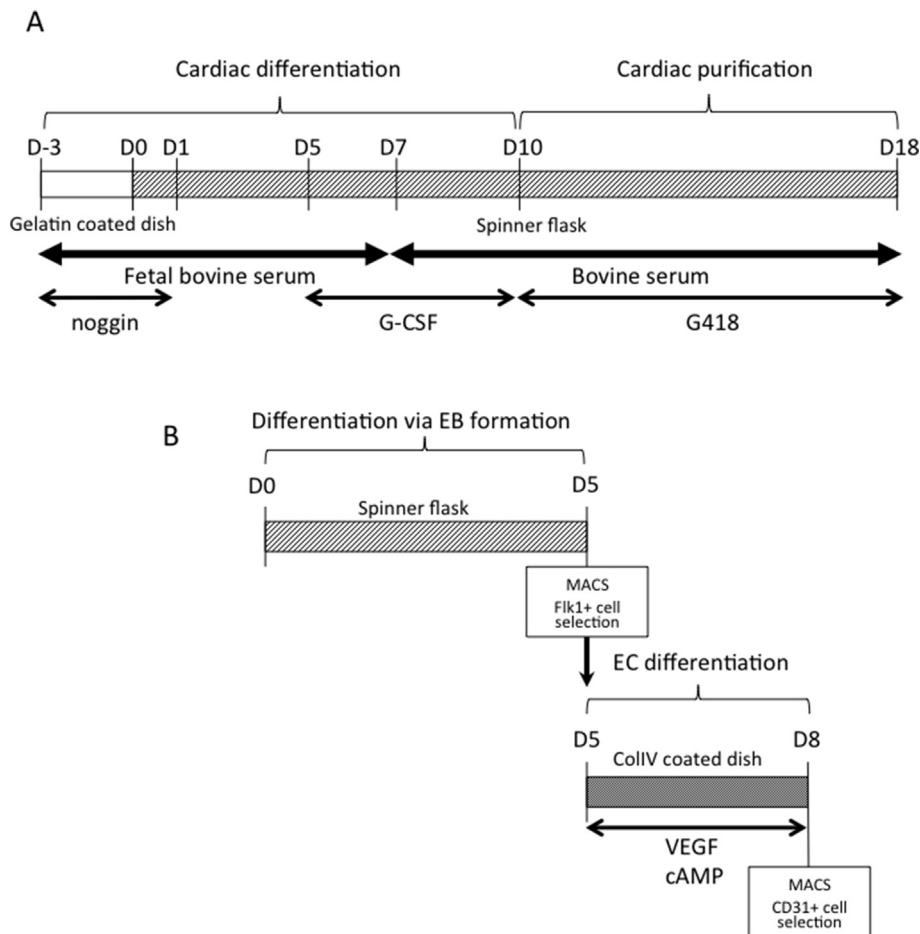


Fig. 1. Scheme for induction of cardiovascular differentiation from mouse ES cells. Mouse ES cells were cultured in spinner flasks for cardiovascular differentiation. (A) For cardiac differentiation, R1-neo ES cells were cultured in the presence of noggin and granulocyte colony-stimulating factor. From day 10 to day 18, differentiated cells were cultured in the presence of G418 to select cardiomyocytes. (B) For CD31+ cell differentiation, embryoid bodies were dissociated enzymatically and subjected to MACS to separate Flk1+ cells on day 5. Flk1+ cells were plated onto collagen IV-coated tissue culture dishes and cultured in the presence of VEGF and 8-bromo-cAMP for further differentiation. After 3 days of re-culture, CD31+ cells were isolated from re-cultured Flk1+ cells by MACS.

2.10. Polymerase chain reaction array analysis

Total RNA was extracted from cells using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions. First-strand synthesis was performed on a T3000 ThermoCycler (Biometra) using a RT² First Strand Kit (Qiagen). The cDNA was mixed with RT² SYBR Green ROX qPCR Mastermix (Qiagen) and added to each well of the Mouse Angiogenesis RT² Profiler PCR Array (Qiagen). Polymerase chain reaction (PCR) was performed using a StepOnePlus system (ABI), following the manufacturer's protocol. Data were analyzed by the comparative CT method with glucuronidase, beta (Gusb) as a housekeeping gene.

2.11. Quantitative real-time PCR

First strand cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (ABI) from purified total RNA isolated using an RNeasy Plus Mini Kit (Qiagen). First-strand synthesis was performed on a T3000 ThermoCycler (Biometra). Quantitative real-time (qRT)-PCR was carried out using a StepOnePlus system (ABI), according to the manufacturer's instructions. The expression levels of genes for activin receptor like 1 (Acvr11), cadherin 5 (CDH5), chemokine receptor type 4 (Cxcr4), delta-like ligand 4 (Dll4), ephrin-B2 (Efnb2), ephrin type-B receptor 4 (Ephb4), fms-related tyrosine kinase 1 (Flt1), kinase insert domain receptor (KDR),

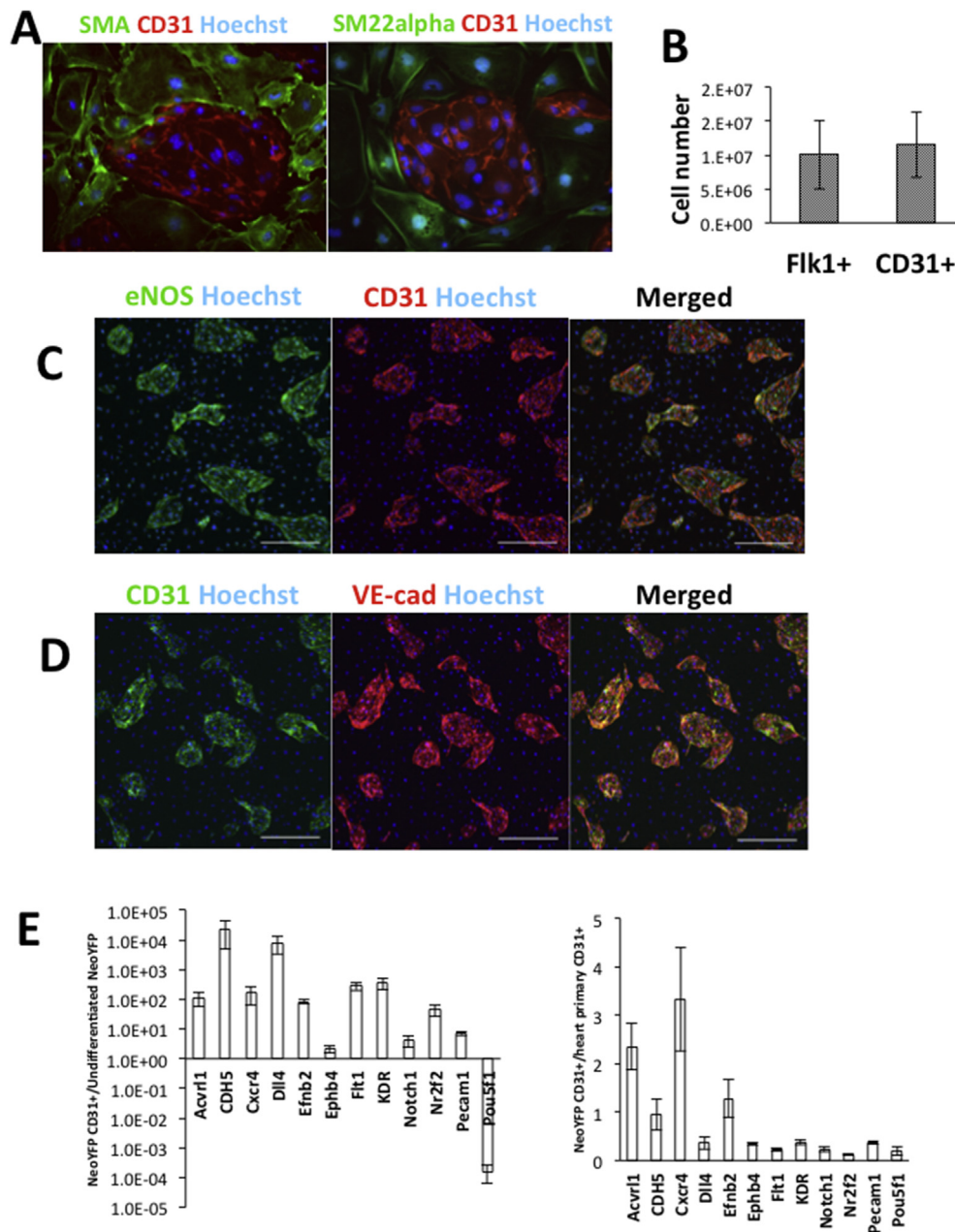
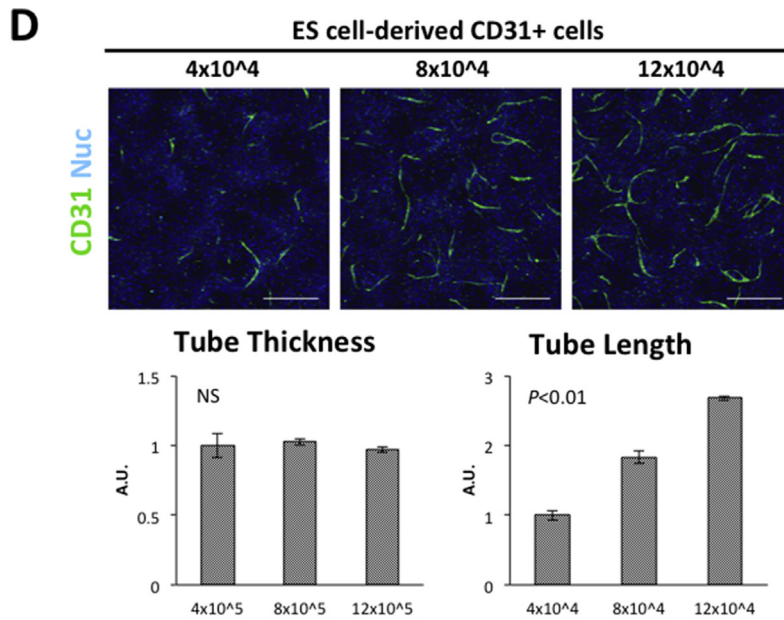
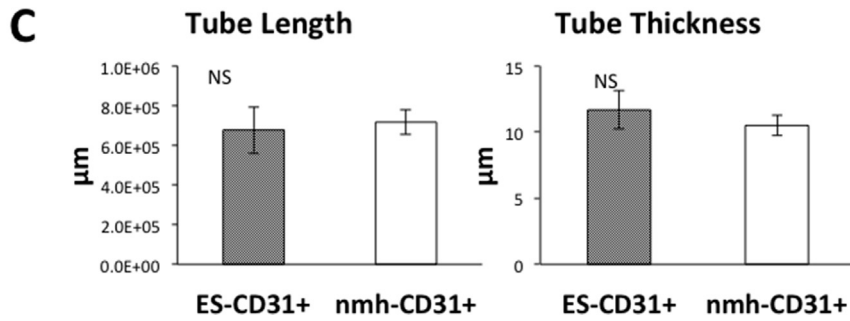
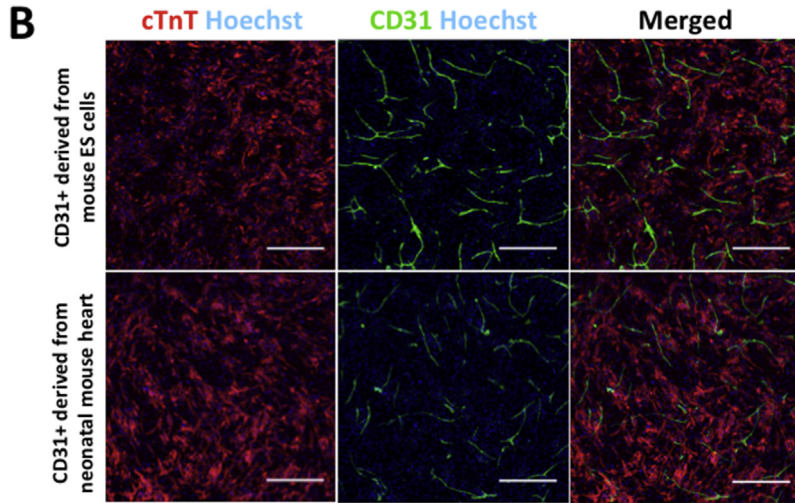
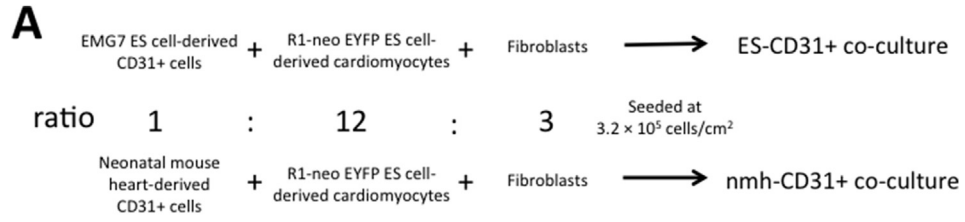


Fig. 2. ES cell-derived CD31⁺ cells were positive for endothelial markers. After 5 days of differentiation, Flk1⁺ cells were isolated by MACS and re-cultured onto collagen IV-coated tissue culture dishes with VEGF and 8-bromo-cAMP for further differentiation. After 3 days of Flk1⁺ cell differentiation, induced cells were immunostained with respective antibodies. (A) Immunostaining for CD31 (red) and mural cell markers (SMA, SM22 α ; green). (B) Numbers of Flk1⁺ and CD31⁺ cells. (C) Immunostaining for CD31 (red) and eNOS (green), and (D) for CD31 (green) and VE-cadherin (red). (E) Relative expression of endothelial marker genes in CD31⁺ cells derived from both mouse ES cells and mouse heart primary cultures. Nuclei were stained with Hoechst 33342 (blue). Scale bars = 400 μ m (C and D). Values are shown as mean \pm standard deviation for three separate experiments.



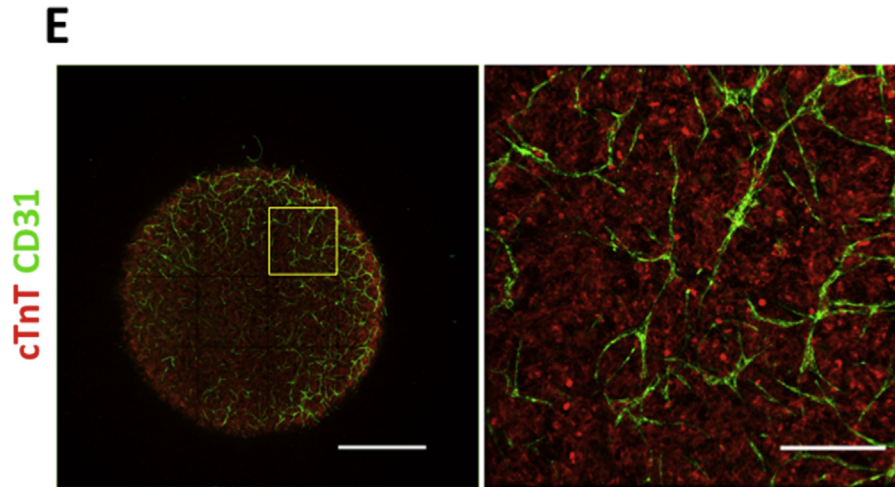


Fig. 3. (continued).

Notch homolog 1 (Notch1), nuclear receptor subfamily 2, group f, member 2 (Nr2f2), platelet/endothelial adhesion molecule 1 (Pecam1) and POU class 5 homeobox 1 (Pou5f1) were analyzed by TaqMan gene expression assay (ABI) and gene expression was normalized to endogenous β -actin. Col4a3, chemokine (C-X-C motif) ligand 5 (Cxcl5), epidermal growth factor (Egf), Fgf1, midkine (Mdk), transforming growth factor- α (Tgfa) and thymidine phosphorylase (Tymp) were also analyzed by TaqMan gene expression assay, and normalized to endogenous Gusb.

2.12. Statistical analysis

Data are presented as means \pm standard deviation. Student's *t*-tests or paired *t*-tests were used to analyze differences between two groups, as appropriate. Data for multiple groups were compared by Tukey–Kramer multiple comparison tests. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Scalable endothelial cell differentiation of mouse ES cells

Differentiation of EMG7 ES cells was induced by seeding at 1×10^5 cells/ml into spinner flasks (Fig. 1). After 5 days of differentiation in spinner flasks, embryoid bodies were dissociated and Flk1+ cells were isolated by MACS. Flk1+ cells were plated onto collagen IV-coated tissue culture dishes with VEGF and 8-bromo-cAMP for further differentiation. After 3 days of re-culture, induced CD31+ cells and SMA+ cells were confirmed by immunocytochemistry (Fig. 2A). The number of isolated Flk1+ cells was about $1.0 \times 10^7 \pm 5.01 \times 10^6$ cells (100% of the initial ES cell number) and $1.16 \times 10^7 \pm 4.86 \times 10^6$ CD31+ cells were obtained from 1×10^7 Flk1+ cells (116% of the initial ES cell number) (Fig. 2B). Nearly all the CD31+ cells were positive for other endothelial markers, including VE-cadherin and eNOS, and negative for smooth muscle

cell markers such as SMA and SM22 α (Fig. 2A, C–E). CD31+ VE-cadherin+ cells were also collected from R-1 neo EYFP mouse ES cells through same procedure (data not shown). qRT-PCR analysis revealed that expression of endothelial marker genes in R-1 neo EYFP ES cell-derived CD31+ cells was higher than in undifferentiated R-1 neo EYFP cells, and similar to that in CD31+ cells from primary heart cells (Fig. 2E). These results suggest that 3D-suspension culture might be useful for collecting large numbers of CD31+ endothelial cells from mouse ES cells.

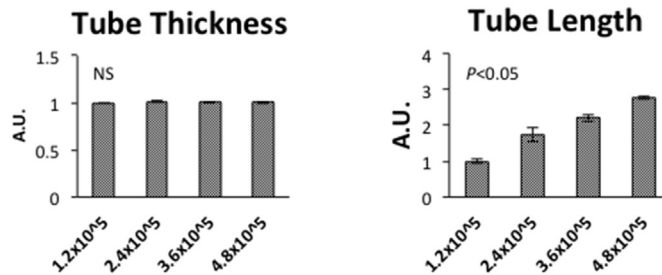
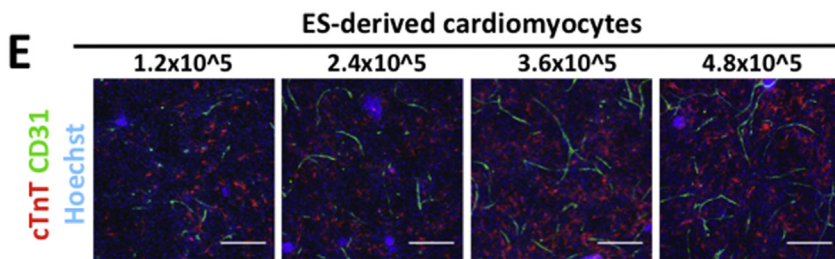
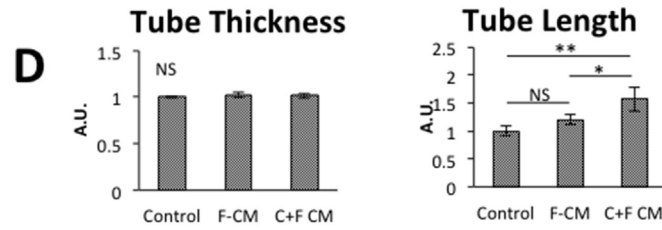
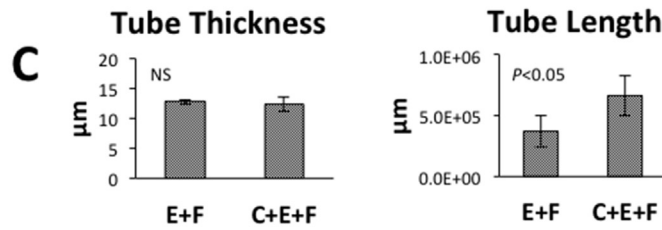
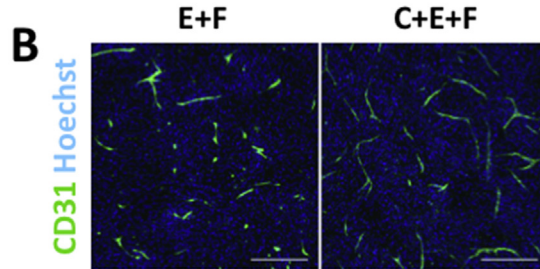
3.2. ES cell-derived CD31+ cells form vascular network structures

We compared the abilities of ES cell-derived CD31+ cells (ES-CD31+) and CD31+ cells from neonatal mouse heart (nmh-CD31+) to form network structures when mixed with ES cell-derived cardiomyocytes and mouse dermal fibroblasts at ratios of 1:12:3 and seeded at 3.2×10^5 cells/cm² (Fig. 3A). After 4 days of culture, the formation of a CD31+ endothelial cell network structure was observed (Fig. 3B). Tube length and tube thickness of the endothelial cell network in ES-CD31+ co-cultures were similar to those in neonatal mouse heart CD31+ co-cultures (tube length, ES-CD31+ co-culture: $6.02 \pm 0.87 \times 10^5$ μ m; nmh-CD31+ co-culture: $7.56 \pm 0.86 \times 10^5$ μ m; tube thickness, ES-CD31+ co-culture: 11.67 ± 1.44 μ m; nmh-CD31+ co-culture: 10.50 ± 0.76 μ m) (Fig. 3C). These results indicate that ES cell-derived endothelial cells had the ability to form vascular network structures in co-culture with cardiac cells, and there was no notable difference between endothelial cells derived from ES cells and those from neonatal mouse heart.

We examined the effect of the number of endothelial cells on vascular network formation. When R1-neo EYFP ES cell-derived endothelial cells were co-cultured with R1-neo ES cell-derived cardiomyocytes and mouse dermal fibroblasts, the tube length in the resulting endothelial network structure increased with increasing endothelial cell number (Fig. 3D). In contrast, there were

Fig. 3. ES cell-derived CD31+ cells formed vascular network structures in co-culture with ES cell-derived cardiomyocytes and fibroblasts. (A) Schematic diagram of co-culture. (B) CD31+ network structure derived from mouse EMG7 ES cells (ES-CD31+) and neonatal mouse heart (nmh-CD31+) after 4 days in co-culture with R1-neo EYFP ES cell-derived cardiomyocytes and mouse dermal fibroblasts. Cardiac troponin T (cTnT) (red) and CD31 (green). (C) Analysis of CD31+ cell network structures within co-cultured cells at day 4. (D) R1-neo EYFP ES cell-derived CD31+ cell network structure in co-culture with R1-neo ES cell-derived cardiomyocytes and mouse dermal fibroblasts. CD31 (green). Nuclei were stained with Hoechst 33342 (blue). Immunostaining for CD31+ cells in (B) and (D) was analyzed using MetaXpress software. Tube length and tube thickness are indicated. Scale bars = 400 μ m in (B) and (D). Values are shown as mean \pm standard deviation for three separate experiments in (C) and six separate experiments in (D). NS, not significant. (E) Immunostaining of cardiac cell sheet with CD31+ cell network structure. After non-invasive harvesting of cell sheets using temperature-responsive culture dishes, CD31+ cells formed well-established network structures throughout the cardiac cell sheet at day 4. CD31+ cells were derived from R1-neo EYFP ES cells. cTnT (red), CD31 (green). Square in left panel is magnified as right panel. Scale bars = 2 mm in left panel and 400 μ m in right panel.

A	C:	E:	F:
	R1-neo ES cell-derived cardiomyocytes 2.4×10^5 cells/cm ²	R1-neo EYFP ES cell-derived CD31+ cells 2×10^4 cells/cm ²	Fibroblasts 6×10^4 cells/cm ²
C + E + F	○	○	○
E + F	-	○	○
C + F	○	-	○



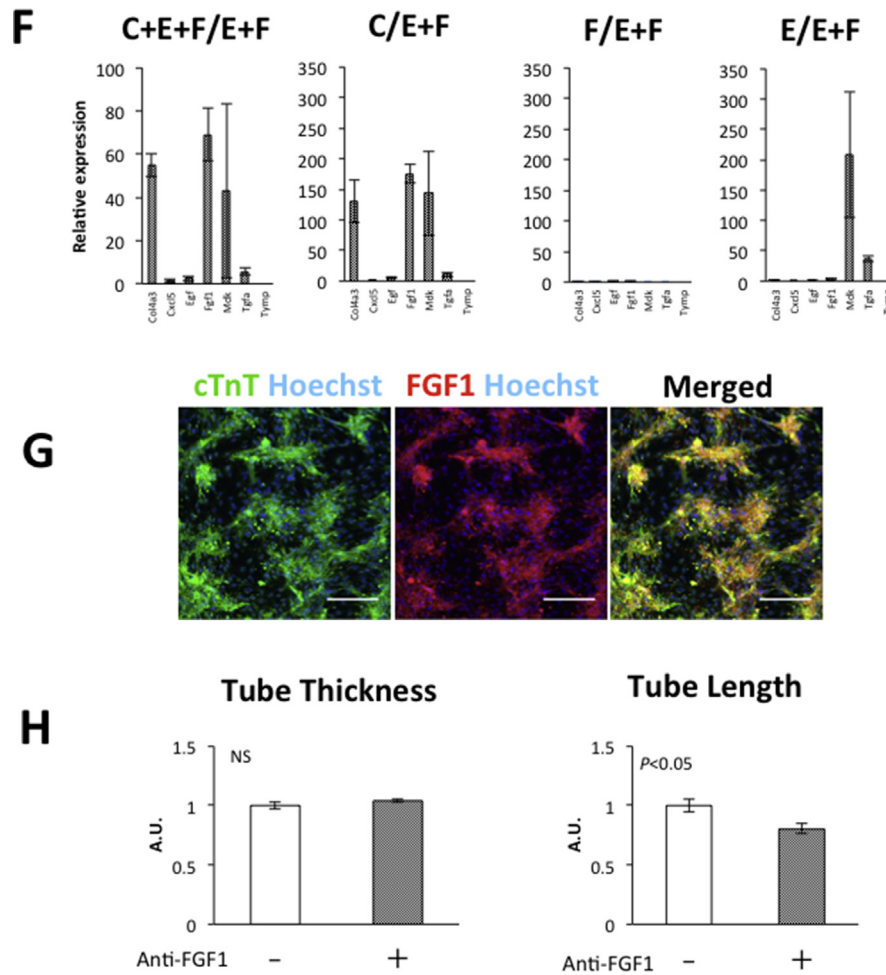


Fig. 4. (continued).

no significant associations between tube thickness and other conditions independent of endothelial cell density in the co-culture.

Cardiac cell sheets with an endothelial cell network structure were fabricated by co-culturing R1-neo ES cell-derived cardiomyocytes, mouse dermal fibroblasts, and R1-neo EYFP ES cell-derived endothelial cells on fetal bovine serum-coated 24-well temperature-responsive culture plates. After 4 days of co-culture, the co-cultured cells were detached as intact cell sheets at 20 °C within 1 h. Endothelial cell network structures were maintained throughout the cardiac cell sheet (Fig. 3E).

3.3. Involvement of cardiomyocyte-derived FGF1 in the formation of endothelial cell network structure

Endothelial cell network structures were formed by co-culture of R1-neo ES cell-derived cardiomyocytes (C), R1-neo EYFP ES

cell-derived endothelial cells (E), and mouse dermal fibroblasts (F) (Fig. 4A; C + E + F, panels B, C). In contrast, endothelial cell network structures were reduced in the absence of R1-neo ES cell-derived cardiomyocytes (Fig. 4A; E + F, panels B, C). The endothelial cell network structure was mostly rescued when ES cell-derived endothelial cells and fibroblasts were co-cultured with conditioned medium from C + F (Fig. 4A; C + F, panel D). Tube lengths in the endothelial cell network structure increased with increasing density of ES cell-derived cardiomyocytes within the co-culture, though tube thickness remained largely unchanged (Fig. 4E). These results imply that soluble factor(s) derived from ES cell-derived cardiomyocytes might contribute to the formation of the CD31+ cell network structure in co-culture. To identify the key factor(s), the expression levels of 84 key angiogenesis-related genes were examined in C + E + F and E + F by RT² Profiler PCR Array (Qiagen). The gene expression levels of Col4a3, Cxcl5, Egf, Fgf1,

Fig. 4. ES cell-derived cardiomyocytes had a positive effect on the formation of CD31+ cell network structure. (A) Schematic diagram of co-culture. (B) CD31+ cell network structure formed in co-culture with R1-neo ES cell-derived cardiomyocytes, R1-neo EYFP ES cell-derived CD31+ cells, and mouse dermal fibroblasts (C + E + F). Little CD31+ cell network structure was observed when R1-neo ES cell-derived cardiomyocytes were depleted from the co-culture (E + F). CD31 (green), nuclei were stained with Hoechst 33342 (blue). (C) Comparison of CD31+ cell network structure formation between C + E + F and E + F. Tube length and tube thickness are indicated. (D) CD31+ cell network structure formed in E + F with conditioned medium from co-cultures containing ES cell-derived cardiomyocytes (C + F). Tube length and tube thickness are indicated. (E) Effect of ES cell-derived cardiomyocyte density on formation of CD31+ cell network. Tube length and tube thickness are indicated. (F) Real-time PCR analyses of six genes demonstrated higher expression (>5-fold) in C + E + F compared with E + F by RT² Profiler PCR Array. Gene expression levels in C + E + F, E + F, R1-neo ES cell-derived cardiomyocytes (C), mouse dermal fibroblasts (D), and R1-neo EYFP ES cell-derived CD31+ cells (E) were analyzed and the gene expression ratios for C + E + F, C, E, and F relative to E + F are indicated. (G) After 4 days of co-culture (C + E + F), cells were immunostained with respective antibodies for cTnT (green) and FGF1 (red). Nuclei were stained with Hoechst 33342 (blue). (H) Inhibition of CD31+ cell network formation by neutralizing FGF1 antibody. Nuclei were stained with Hoechst 33342 (blue). Tube length and tube thickness were calculated from CD31+ cell staining. Scale bars = 400 μm in (B) and (E) and 200 μm in (G). Values are shown as mean ± standard deviation for eight separate experiments in (C), four separate experiments in (D), three separate experiments in (E) and (F), and six separate experiments in (H). *P < 0.05, **P < 0.01. NS, not significant.

Mdk, Tgfa and Tymp in C + E + F was >5-fold that in E + F (Table 1). qRT-PCR confirmed that Col4a3, Fgf1 and Mdk gene expression levels were significantly higher in C + E + F compared with E + F, while differences in Cxcl5, Egf, Tgfa and Tymp were not significant. Col4a3 and Fgf1 were predominantly expressed in cardiomyocytes, while Mdk was expressed in cardiomyocytes and endothelial cells. Furthermore, Mdk expression in endothelial cells was higher than in cardiomyocytes. Consistent with the modest endothelial network formation in E + F, Mdk might not be important for cardiomyocyte-mediated endothelial network formation.

We also examined the protein expression levels of FGF1 and Col4a3 in cardiomyocytes. As shown in Fig. 4G, FGF1, but not Col4a3 (data not shown), was expressed in ES cell-derived cardiomyocytes. We finally elucidated the contribution of FGF1 to endothelial network formation in C + E + F cultures using anti-FGF1 neutralizing antibody (Fig. 4H). Tube length, but not tube thickness, was significantly reduced in endothelial cells co-cultured with cardiomyocytes and fibroblasts in the presence of anti-FGF1 antibody. These findings suggest that cardiomyocytes might promote endothelial network formation in bioengineered cardiac tissue via secreted FGF-1.

4. Discussion

The results of the present study provide the first evidence to support the use of a 3D-suspension culture system for collecting Flk1+ mesodermal progenitor cells and CD31+ endothelial cells from mouse ES cells. ES cell-derived CD31+ endothelial cells were identical to CD31+ endothelial cells from neonatal mouse heart in terms of gene expression and network formation. The potential of 3D tissue for regenerative medicine has been extensively reported. We previously reported on the fabrication of 3D cardiac tissue with microvascular networks by layering cardiac cell sheets from neonatal rat hearts containing endothelial cells on various types of vascular beds [21–23]. Furthermore, several reports have indicated the importance of endothelial cells for fabricating 3D tissues, including liver and cartilage [24,25]. However, the generation of sufficient numbers of endothelial cells is a prerequisite for further advances in functional regenerative medicine. Cardiovascular cells including cardiomyocytes, endothelial cells, and mural cells have been shown to differentiate from mesodermal progenitor Flk1+ cells in two-dimensional culture [14,15]. The addition of 8-bromo-cAMP and VEGF promoted the differentiation of CXCR4+ arterial endothelial cells from Flk1+ cells via activation of Notch signaling [16]. We recently reported the importance of 3D-suspension culture for collecting sufficient numbers of cardiomyocytes from mouse ES cells and human iPS cells for the fabrication of bioengineered cardiac tissue. In the current study, mouse ES cells were cultured in spinner flasks to induce Flk1+ cells. Around 1×10^7 Flk1+ cells were isolated after 5 days in culture, and the cell number was identical to the starting cell number. After a further 3

days culture on collagen-coated dishes, we were able to collect around 1×10^7 CD31+ endothelial cells. These findings suggest that scalable cultures using 3D-suspension systems may produce large numbers of endothelial cells.

Understanding the differences in gene expression and function between endothelial cells derived from ES cells and hearts is a prerequisite for fabricating bioengineered vascularized tissue. In this study, CD31+ cells derived from both EMG7 and R1-neo EYFP ES cells were positive for both VE-cadherin and eNOS and demonstrated the ability to form CD31+ cell network structures. qRT-PCR analysis found no notable difference between CD31+ cells derived from ES cells and CD31+ cells from heart tissue in the expression patterns of vascular endothelial genes. In addition to artery genes such as Acvrl1, CXCR4, Dll4, and ephrin B2, the venous endothelial cell marker Nr2f2 was highly expressed in ES cell-derived CD31+ cells. Although Notch signaling and Nr2f2 are crucial for specifying arterial and venous differentiation, cell fate in terms of artery versus vein is relatively plastic in immature endothelial cells [26] and the fate of ES cell-derived CD31+ cells might not be decided because of their immaturity. Both CD31+ cells derived from ES cells and CD31+ cells from heart tissues formed microvascular network when co-cultured with ES cell-derived cardiomyocytes and dermal fibroblasts. Images of network structures of CD31+ cells were collected and calculated the length and thickness of tube structures. ‘Tube length’ is an index of the length of tube structure formed of CD31+ cells, and the value of CD31+ cells derived from ES cells was almost equal to that of CD31+ cells from heart tissues. It is thought that sprouting activity of these CD31+ cells in co-culture were identical. In addition, both of the value of ‘tube thickness’, an index of the diameter of tube structure formed of CD31+ cells, were similar to that of capillary, approximately 10 μ m, and characteristic endothelial cobblestone structure in 2D culture was not observed in both CD31+ cells in co-culture. These findings suggest that ES cell-derived endothelial cells display a similar phenotype to endothelial cells from heart tissue, and may thus be useful for fabricating vascularized bioengineered cardiac tissue.

We fabricated cardiac cell sheets using dermal fibroblasts as stromal cells and produced endothelial microvascular networks. In contrast however, Masumoto et al. fabricated cardiac cell sheets in ES cell-derived cardiomyocyte, endothelial cell, and mural cell co-cultures, but endothelial cells were spottily distributed and little network formation was observed after 4 days in culture [17]. This apparent discrepancy in terms of microvascular network formation might be associated with differences in cellular components. One possible mechanism for the inhibitory effect of mural cells on endothelial cell network formation might be their ability to stabilize vascular structures. Sphingosine-1-phosphate-induced release of tissue inhibitor of metalloproteinase 2 (TIMP2) from vascular smooth muscle cells inhibits angiogenesis [27]. In contrast, fibroblasts play important roles in promoting angiogenesis through the secretion of growth factors and production of extracellular matrix molecules [28,29]. Furthermore, we previously reported that the pre-vascular structure of human aortic endothelial cells was created by sandwiching them between human dermal fibroblast sheets [30]. These observations suggest that the behavior of CD31+ cells might depend on the types of co-cultured cells, and fibroblasts might have supportive effects on the formation of endothelial cell network structures.

Cardiomyocytes are indispensable for endothelial cell microvascular network formation. Accordingly, co-culture of endothelial cells with dermal fibroblasts without cardiomyocytes resulted in little microvascular network formation. Cardiomyocytes secrete abundant FGF1, and treatment with anti-FGF1 antibody inhibited microvascular network formation, suggesting that cardiomyocytes

Table 1
Angiogenesis-related gene expression calculated by PCR array analysis. The genes, which expressed in C + E + F co-culture higher more than 5 fold than that in E + F co-culture, were indicated.

Gene symbol	Fold
Fgf1	93.5
Col4a3	24.62
Mdk	10.81
Tgfa	8.61
Tymp	6.37
Cxcl5	5.57
Egf	5.55

might promote endothelial cell network formation in cardiac cell sheets through paracrine effects. FGF1 is a well-known growth factor involved in many biological processes, including cell proliferation, differentiation, migration, and angiogenesis [31]. In this study, treatment with anti-FGF1 antibody resulted in the reduction of the value of tube length, but not tube thickness, suggesting that cardiomyocyte-derived FGF1 might promote endothelial cell sprouting in the co-culture. Among several signaling modules related to FGF1, the Akt signaling pathway is implicated in FGF1-induced angiogenesis *in vivo* [32]. Transgenic FGF1 overexpression in myocardial tissue resulted in increased density and branching of coronary arteries [33], and new vessels were formed in ischemic human heart after injection of human recombinant FGF1 [34]. Cardiomyocytes have recently been reported to function as angiogenic cells in development and diseases. Cardiomyocyte-derived angiopoietin 1 plays a crucial role in coronary vein formation in the developing heart [35]. Cyclic mechanical stretching promotes VEGF expression in adult cardiomyocytes through NF- κ B activation [36] with increasing capillary density during the early stage of heart hypertrophy, via HIF-1-mediated VEGF up-regulation [37]. Indeed, PCR array analysis indicated up-regulation of several angiogenic factors, such as VEGF and Ang-1, in C + E + F co-cultures. The present study focused on genes that were up-regulated ≥ 5 -fold in C + E + F compared with E + F, but we cannot exclude the possibility that VEGF and Ang-1 may also contribute to cardiomyocyte-mediated endothelial cell network formation.

In conclusion, we developed a scalable suspension culture method for the differentiation of endothelial cells from mouse ES cells. ES cell-derived endothelial cells formed network structures within cardiac cell sheets, and ES cell-derived cardiomyocytes contributed to the formation of these networks via FGF1 expression. The development of a scalable strategy for generating and collecting human endothelial cells, and a better understanding of the molecular mechanisms of pre-vascularization in human bioengineered cardiac tissue, will enable us to fabricate functional 3D tissues for regenerative medicine and tissue models.

Conflict of interest

Teruo Okano, Ph.D. is a founder and a member of the board of CellSeed Inc., which has licenses for certain cell sheet-related technologies and patents from Tokyo Women's Medical University. Teruo Okano and Tatsuya Shimizu are stakeholders in CellSeed Inc. Tokyo Women's Medical University receives research funding from CellSeed Inc.

Acknowledgments

This work was funded by a grant from the Japan Society for the Promotion of Science 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, and a grant from Projects for Technological Development in Research Center Network for Realization of Regenerative Medicine of the Japan Science and Technology Agency.

References

- [1] Okano T, Yamada N, Okuhara M, Sakai H, Sakurai Y. Mechanism of cell detachment from temperature-modulated, hydrophilic-hydrophobic polymer surfaces. *Biomaterials* 1995;16:297–303.
- [2] Masuda S, Shimizu T, Yamato M, Okano T. Cell sheet engineering for heart tissue repair. *Adv Drug Deliv Rev* 2008;60:277–85.
- [3] Haraguchi Y, Shimizu T, Sasagawa T, Sekine H, Sakaguchi K, Kikuchi T, et al. Fabrication of functional three-dimensional tissues by stacking cell sheets *in vitro*. *Nat Protoc* 2012;7:850–8.
- [4] Okano T, Yamada N, Sakai H, Sakurai Y. A novel recovery system for cultured cells using plasma-treated polystyrene dishes grafted with poly(N-isopropylacrylamide). *J Biomed Mater Res* 1993;27:1243–51.
- [5] Sekine H, Shimizu T, Hobo K, Sekiya S, Yang J, Yamato M, et al. Endothelial cell coculture within tissue-engineered cardiomyocyte sheets enhances neovascularization and improves cardiac function of ischemic hearts. *Circulation* 2008;118:S145–52.
- [6] Sekiya S, Shimizu T, Yamato M, Kikuchi A, Okano T. Bioengineered cardiac cell sheet grafts have intrinsic angiogenic potential. *Biochem Biophys Res Commun* 2006;341:573–82.
- [7] Matsuura K, Masuda S, Haraguchi Y, Yasuda N, Shimizu T, Hagiwara N, et al. Creation of mouse embryonic stem cell-derived cardiac cell sheets. *Biomaterials* 2011;32:7355–62.
- [8] Matsuura K, Wada M, Konishi K, Sato M, Iwamoto U, Sato Y, et al. Fabrication of mouse embryonic stem cell-derived layered cardiac cell sheets using a bioreactor culture system. *PLoS One* 2012;7:e52176.
- [9] Matsuura K, Wada M, Shimizu T, Haraguchi Y, Sato F, Sugiyama K, et al. Creation of human cardiac cell sheets using pluripotent stem cells. *Biochem Biophys Res Commun* 2012;425:321–7.
- [10] White MP, Rufaihah AJ, Liu L, Ghebremariam YT, Ivey KN, Cooke JP, et al. Limited gene expression variation in human embryonic stem cell and induced pluripotent stem cell-derived endothelial cells. *Stem Cells* 2013;31:92–103.
- [11] Yang L, Soonpaa MH, Adler ED, Roepke TK, Kattman SJ, Kennedy M, et al. Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. *Nature* 2008;453:524–8.
- [12] Taura D, Sone M, Homma K, Oyama N, Takahashi K, Tamura N, et al. Induction and isolation of vascular cells from human induced pluripotent stem cells—brief report. *Arterioscler Thromb Vasc Biol* 2009;29:1100–3.
- [13] Narazaki G, Uosaki H, Teranishi M, Okita K, Kim B, Matsuoka S, et al. Directed and systematic differentiation of cardiovascular cells from mouse induced pluripotent stem cells. *Circulation* 2008;118:498–506.
- [14] Yamashita J, Itoh H, Hirashima M, Ogawa M, Nishikawa S, Yurugi T, et al. Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature* 2000;408:92–6.
- [15] Yamashita JK, Takano M, Hiraoka-Kanie M, Shimazu C, Peishi Y, Yanagi K, et al. Prospective identification of cardiac progenitors by a novel single cell-based cardiomyocyte induction. *FASEB J* 2005;19:1534–6.
- [16] Yurugi-Kobayashi T, Itoh H, Schroeder T, Nakano A, Narazaki G, Kita F, et al. Adrenomedullin/cyclic AMP pathway induces Notch activation and differentiation of arterial endothelial cells from vascular progenitors. *Arterioscler Thromb Vasc Biol* 2006;26:1977–84.
- [17] Masumoto H, Matsuo T, Yamamizu K, Uosaki H, Narazaki G, Katayama S, et al. Pluripotent stem cell-engineered cell sheets reassembled with defined cardiovascular populations ameliorate reduction in infarct heart function through cardiomyocyte-mediated neovascularization. *Stem Cells* 2012;30:1196–205.
- [18] Masumoto H, Ikuno T, Takeda M, Fukushima H, Marui A, Katayama S, et al. Human iPS cell-engineered cardiac tissue sheets with cardiomyocytes and vascular cells for cardiac regeneration. *Sci Rep* 2014;4:6716.
- [19] Zandstra PW, Bauwens C, Yin T, Liu Q, Schiller H, Zweigerdt R, et al. Scalable production of embryonic stem cell-derived cardiomyocytes. *Tissue Eng* 2003;9:767–78.
- [20] Song H, Yoon C, Kattman SJ, Dengler J, Masse S, Thavaratnam T, et al. Interrogating functional integration between injected pluripotent stem cell-derived cells and surrogate cardiac tissue. *Proc Natl Acad Sci U. S. A* 2010;107:3329–34.
- [21] Shimizu T, Sekine H, Yang J, Isoi Y, Yamato M, Kikuchi A, et al. Polysurgery of cell sheet grafts overcomes diffusion limits to produce thick, vascularized myocardial tissues. *FASEB J* 2006;20:708–10.
- [22] Sekine H, Shimizu T, Sakaguchi K, Dobashi I, Wada M, Yamato M, et al. *In vitro* fabrication of functional three-dimensional tissues with perfusable blood vessels. *Nat Commun* 2013;4:1399.
- [23] Sakaguchi K, Shimizu T, Haraguchi S, Sekine H, Yamato M, Umezumi M, et al. *In vitro* engineering of vascularized tissue surrogates. *Sci Rep* 2013;3:1316.
- [24] Takebe T, Sekine K, Enomura M, Koike H, Kimura M, Ogaeri T, et al. Vascularized and functional human liver from an iPSC-derived organ bud transplant. *Nature* 2013;499:481–4.
- [25] Takebe T, Kobayashi S, Suzuki H, Mizuno M, Chang YM, Yoshizawa E, et al. Transient vascularization of transplanted human adult-derived progenitors promotes self-organizing cartilage. *J Clin Invest* 2014;124:4325–34.
- [26] Oliver G, Srinivasan RS. Endothelial cell plasticity: how to become and remain a lymphatic endothelial cell. *Development* 2010;137:363–72.
- [27] Mascall KS, Small GR, Gibson G, Nixon GF. Sphingosine-1-phosphate-induced release of TIMP-2 from vascular smooth muscle cells inhibits angiogenesis. *J Cell Sci* 2012;125:2267–75.
- [28] Hudon V, Berthod F, Black AF, Damour O, Germain L, Auger FA. A tissue-engineered endothelialized dermis to study the modulation of angiogenic and angiostatic molecules on capillary-like tube formation *in vitro*. *Br J Dermatol* 2003;148:1094–104.
- [29] Newman AC, Nakatsu MN, Chou W, Gershon PD, Hughes CCW. The requirement for fibroblasts in angiogenesis: fibroblast-derived matrix proteins are essential for endothelial cell lumen formation. *Mol Biol Cell* 2011;22:3791–800.
- [30] Sasagawa T, Shimizu T, Yamato M, Okano T. Expression profiles of angiogenesis-related proteins in prevascular three-dimensional tissues using cell-sheet engineering. *Biomaterials* 2014;35:206–13.

- [31] Raju R, Palapetta SM, Sandhya VK, Sahu A, Alipoor A, Balakrishnan L, et al. A network Map of FGF-1/FGFR signaling system. *J Signal Transduct* 2014;2014:962962.
- [32] Forough R, Weylie B, Patel C, Ambrus S, Singh US, Zhu J. Role of AKT/PKB signaling in fibroblast growth factor-1 (FGF-1)-induced angiogenesis in the chicken chorioallantoic membrane (CAM). *J Cell Biochem* 2005;94:109–16.
- [33] Fernandez B, Buehler A, Wolfram S, Kostin S, Espanion G, Franz WM, et al. Transgenic myocardial overexpression of fibroblast growth factor-1 increases coronary artery density and branching. *Circ Res* 2000;87:207–13.
- [34] Schumacher B, Pecher P, von Specht BU, Stegmann T. Induction of neo-angiogenesis in ischemic myocardium by human growth factors: first clinical results of a new treatment of coronary heart disease. *Circulation* 1998;97:645–50.
- [35] Arita Y, Nakaoka Y, Matsunaga T, Kidoya H, Yamamizu K, Arima Y, et al. Myocardium-derived angiopoietin-1 is essential for coronary vein formation in the developing heart. *Nat Commun* 2014;5:4552.
- [36] Leychenko A, Konorev E, Jijiwa M, Matter ML. Stretch-induced hypertrophy activates NFkB-mediated VEGF secretion in adult cardiomyocytes. *PLoS One* 2011;6:e29055.
- [37] Sano M, Minamino T, Toko H, Miyauchi H, Orimo M, Qin Y, et al. p53-induced inhibition of Hif-1 causes cardiac dysfunction during pressure overload. *Nature* 2007;446:444–8.