Development of Vascularized iPSC Derived 3D-Cardiomyocyte Tissues by Filtration Layer-by-Layer Technique and Their Application for Pharmaceutical Assays

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Abstract: *In vitro* development of three-dimensional (3D) human cardiomyocyte (CM) tissues derived from human induced pluripotent stem cells (iPSCs) has long been desired in tissue regeneration and pharmaceutical assays. In particular, *in vitro* construction of 3D-iPSC-CM tissues with blood capillary networks have attracted much attention because blood capillaries are crucial for nutrient and oxygen supplies for CMs. Blood capillaries in 3D-iPSC-CM tissues will also be important for *in vitro* toxicity assay of prodrugs because of the signaling interaction between cardiomyocytes and endothelial cells.

Here, we report construction of vascularized 3D-iPSC-CM tissues by a **newly-discovered filtration-Layer-by-Layer (LbL) technique for cells**, instead of our previous centrifugation-LbL technique. The filtration-LbL allowed us to fabricate nanometer-sized extracellular matrices (ECM), fibronectin and gelatin (FN-G), films onto iPSC-CM surfaces without any damage and with high yield, although centrifugation-LbL induced physical stress and a lower yield. The fabricated FN-G nanofilms interacted with integrin molecules on the cell membrane to construct 3D-tissues. We found that the introduction of normal human cardiac fibroblasts (NHCFs) into the iPSC-CM tissues modulated organization and synchronous beating depending on NHCF ratios. Moreover, co-culture with normal human cardiac microvascular endothelial cells (NHCMECs) successfully provided blood capillary-like networks in 3D-iPSC-CM tissues, depending on NHCF ratios. The vascularized 3D-iPSC-CM tissues indicated significantly different toxicity responses as compared to 2D-iPSC-CM cells by addition of doxorubicin as a model of a toxic drug. The constructed vascularized 3D-iPSC-CM tissues would be a promising tool for tissue regeneration and drug development.

Keywords: iPS cells, cardiomyocyte, layer-by-layer assembly, 3D-tissues, tissue engineering, drug development

Introduction

In a preclinical test of an ordinary drug discovery system, cell monolayer models and animal models have been employed. This system is time-consuming and costly owing to the differences of drug response between two dimensional (2D) cultured monolayer models and living tissues with three-dimensional (3D) structures and species difference [1-3]. Therefore, the use of *in vitro* 3D-tissue models has attracted increasing attention in pharmaceutical assays [4,5]. Among them, since heart tissue is a crucial organ for life support and cardiac diseases remain a leading cause of death [6], and since human CMs are not easily accessible or available, *in vitro* 3D-CM models are of great importance. Accordingly, the recent discovery of human iPSCs [7,8] and methods to differentiate them to CMs including disease specific CMs, such as QT syndrome specific iPSC-derived-CMs (iPSC-CMs) [9,10], and to purify iPSC-CMs [11] have all contributed to significant progress in the development of human normal and disease specific tissue models as a cell source. However, the establishment of normal and disease specific 3D-CM tissue models for not only therapeutic effects tests but also drug toxicity tests on heart tissue, especially the vascularized type, has not yet been achieved due to the lack of CM-specific bottom-up technology.

In the past few years, many researchers have attempted to develop functional 3D-CM tissues by using scaffolds to which CMs could adhere. These scaffolds have been mainly composed with hydrogels [12,13], and oriented fibers [14-16]. Though the oriented fibers acquired aligned cardiac fibers, these tissues contained artificial materials which are not naturally found in the human body. Cell sheet engineering which don't contain artificial materials have been also reported [17-19]. These systems are powerful methods of constructing 3D-CM tissues, but they need complicated devices and procedures and it was difficult to obtain thick and high cell-density 3D-CM tissues with functionality such as synchronous beating. More importantly, *in vitro* vascularization within the human iPSC-CM tissue has not yet been fully achieved, even though the heart is intrinsically hyper-vascularized tissue due to the high consumption of nutrients and oxygen due to its beating. Most recently, Yamashita and co-workers have reported the iPS cell-engineered

cardiac tissue sheet which was composed of CMs, vascular cells, and undifferentiated cells derived from iPSCs spontaneously, but the capillary networks could form only after transplantation [20]. Khademhosseini and co-workers have obtained 3D-neonatal-rat-CM tissues with endothelial cells employing ECM nanofilms and graphene oxide films, but there were no capillary structures of endothelial cells [21].

In our previous study, we developed a cell-accumulation technique capable of constructing 3D-multilayered tissues of fibroblasts by coating ECM nanofilms onto single cell surfaces using Layer-by-Layer (LbL) assembly [22]. We found that around 10 nm nanofilms with fibronectin and gelatin (FN-G) induced the cell-cell interaction via integrin $\alpha_5\beta_1$ on the cell membrane [23]. We have successfully fabricated various kinds of tissue models such as blood vessel models and a liver model [24,25]. Moreover, the formation of endothelial tubular networks in fibroblast tissues was developed by a sandwich culture using the cell-accumulation technique [26]. However, this technique needed more than 18 times centrifugation ($> 400\text{ g}$) during the process of nanofilm coating to separate cells from solutions. This physical stress may cause damage in sensitive cells like CMs, resulting in cell death. Therefore, a novel CM-specific coating methodology is required for *in vitro* construction of 3D-CM tissues.

Here, we report the construction of vascularized 3D-CM tissue models of iPSC-CMs using a novel coating method with FN-G nanofilms using a filter membrane, termed ‘filtration-LbL’. In energy and environmental fields, the vacuum LbL (VA LbL) has been reported [27]. However, there is no report of filtration-LbL for biomedical application. This filtration-LbL technique can complete the entire process from cell coating to the collection of cells in a trans-well and separate cells from solutions without centrifugation ($< 1.1\text{ g}$), leading to coating individual cells with FN-G nanofilms with high efficiency and viability (Fig. 1). Using this technique, we constructed neonatal rat-CM (rCM) and iPSC-CM tissues and tried to introduce NHCFs into the iPSC-CM tissues to support the tissue organization and synchronous beating. Moreover, vascularization was also

attempted by co-culture with iPSC-CMs, NHCFs, and NHCMECs. This technique appears to be a promising tool for the development of *in vitro* iPSC-CM models for pharmaceutical assays.

Materials and Methods

1.1. Materials

All of the chemicals were used without further purification. Fibronectin (FN) from human plasma ($M_w = 4.6 \times 10^5$ Da) and the monoclonal mouse anti-TroponinT (TnT) antibody were purchased from Sigma-Aldrich (MO, USA). Gelatin (G) ($M_w = 1.0 \times 10^5$ Da), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 10% formalin solution, 4% paraformaldehyde phosphate buffer solution, 25% glutaraldehyde solution, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Wako Pure Chemical Industries (Osaka, Japan). The monoclonal mouse anti-human CD31 antibody was purchased from Dako (Glostrup, Denmark). (MO, USA). Goat anti-mouse Alexa Fluor 488- and 546-conjugated IgG, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), Triton X, fetal bovine serum (FBS), Hank's Balanced Salt Solution, Medium199, and leibovitz L-15 were purchased from Life Technologies (CA, USA). Mouse anti-human VEGF antibody and ELISA assay kit of human VEGF was purchased from R&D systems (MN, USA). 0.22 μ filtered trypsin, trypsin inhibitor, and collagenase were purchased from Worthington (NJ, USA). The 24 well cell culture insert with 0.4 μ m, 6 well cell culture insert with 0.4, 3, and 8 μ m pore size, and cell culture plate were purchased from Corning (NY, USA). 0-1 day-old Sprague-Dawley neonatal rats were purchased from Charles River (Yokohama, Japan). Normal human cardiac fibroblasts (NHCFs), and normal human cardiac microvascular endothelial cells (NHCMECs), FGM-3, and EGM-2MV were purchased from Lonza (NJ, USA). The human iPSCs [3-factor (Oct3/4, Sox2, Klf4), line: 253G1, established by Shinya Yamanaka] were purchased from RIKEN BioResource Center (Ibaraki, Japan). The LDH assay kit was purchased from Cayman Chemical (MI, USA). Doxorubicin was purchased from Carbosynth (Berkshire, UK).

1.2. Isolation of neonatal rat cardiomyocytes.

The rat-cardiomyocytes (rCMs) were prepared from 0-1 day-old neonatal rats according to the neonatal cardiomyocyte isolation system described in Worthington. Briefly, beating hearts were surgically removed and minced to less than 1 mm³ pieces keeping tissue at 4 °C in HBSS solution. The fragments of hearts were digested in HBSS containing trypsin at 4 °C overnight following inhibition by a trypsin inhibitor. Then, the collagenase was added and the dish was shaken at 100 min⁻¹ for 30 min at 37 °C. Isolated cells were suspended in a culture medium composed of Medium199 containing 10% FBS and 1% antibiotics. The study was carried out under the supervision of the Animal Research Committee of Osaka University and in accordance with the Japanese Act on Welfare and Management of Animals.

1.3. Human iPSCs culture and differentiation of iPSCs to CMs in a bioreactor system

The preparation of iPSC-CMs has previously been reported [29]. Briefly, the human iPSCs (253G1) purchased from RIKEN (Tsukuba, Japan) were cultured in ES Cell Medium (ReproCELL, Japan) supplemented with 5 ng / ml basic fibroblast growth factor (bFGF; ReproCELL). Cells were passaged as small clumps every 3–4 days using CTK solution (ReproCELL). The iPSC-aggregates after CTL solution treatment were re-suspended in 100 ml mTeSR1 (STEMCELL Technologies Inc., Canada) containing 10 µM Y27632 (Wako, Japan) and seeded into a 250 ml stirred bioreactor (Bio Jr. 8; ABLE Co., Japan). After three days, EBs were cultured in StemPro34 medium containing 50 µg / ml ascorbic acid (Sigma–Aldrich), 2 mM L-glutamine and 400 µM 1-thioglycerol (Sigma-Aldrich). The following growth factors and small molecules were used at the corresponding days: days 3-4, 0.5 ng / ml BMP4 (R&D systems, Minneapolis, MN); days 4-7, 10 ng / ml BMP4, 5 ng / ml bFGF, 3 ng / ml activin A (R&D Systems); days 7-9, 4 µM IWR-1 (Wako); after day 9, 5 ng / ml VEGF (R&D Systems) and 10 ng / ml bFGF. At days 4, 7, 9, 11 and 14, the culture medium was exchanged. The differentiation rate of iPSC-CMs we employed in this paper was between 35 – 70% according to flow cytometry analysis with anti-cardiac TnT antibody.

1.4 Nanofilm coating by centrifugation-LbL

Isolated rCMs and iPSC-CMs were coated with FN-G nanofilms according to our previously published paper [22], with modification. Briefly, rCMs and iPSC-CMs were suspended in 0.04 mg / ml of FN ($M_w = 4.6 \times 10^5$) and G ($M_w = 1.0 \times 10^5$) / Tris-HCl solution (50 mM, pH = 7.4) and alternately incubated for 1 min using a Microtube Rotater (MTR-103, AS ONE, Japan) through washing steps. The centrifugation was performed at 800 g for 1 min at each step. The relative centrifugal force (RCF) was calculated by follow equation: $RCF = 1.1118 \times 10^{-5} \times r \times rpm^2$, where r is the rotor radius in centimeters.

1.5 Nanofilm coating by Filtration-LbL

To prepare for the filtration-LbL, 2.5 ml of 0.2 mg / ml FN and G / Tris-HCl solution and Tris-HCl solution were added into 3 wells of 6 well cell culture plates respectively. $1, 5, 10$, or 50×10^5 isolated rCMs and iPSC-CMs were suspended in 500 μ l of 0.2 mg / ml FN and G / Tris-HCl solution and taken into a 6 well culture insert of 0.4, 3, or 8 μ m pore membrane. The insert was set at the well containing FN and G / Tris-HCl solution and alternately incubated for 1 min using a shaking incubator (SI-300, As One, Japan) through washing steps. In order to collect the coated cells during each step, the insert was moved to an empty well and shaken horizontally at 1.1 g to filtrate the suspension.

1.6. Construction of CM tissues using a cell accumulation technique

After 9 steps of coating, nanofilms of FN-G were coated on each of the cell surfaces. These cells were suspended in 0.4 ml of medium with 10% FBS and seeded into the 24 well cell culture inserts with a semipermeable membrane which was set in a 6 well cell culture plate, following addition of 6 ml of medium into the cell culture plate. After 2 hours of incubation, a further 6 ml of medium were added into each well to connect the inner and outer media of the inserts and incubated in 5% CO₂ at 37 °C. After 1 day of incubation, CM tissues were constructed. Medium199 and

DMEM were employed as media for rCM or iPSC-CM tissues culture respectively. In the case of iPSC-CM tissues containing NHCs, NHCs and iPSC-CMs were coated with FN-G nanofilms by filtration-LbL separately and co-cultured by mixing them just before seeding. In the same manner, 1×10^5 NHCMECs, coated NHCs, and coated iPSC-CMs were co-cultured to introduce blood capillary networks into the iPSC-CM tissues. Normally, these tissues were cultured for 4 days and fixed with 25% glutaraldehyde for Hematoxylin-Eosin (HE) and azan staining and with 4% paraformaldehyde for immunostaining. The NHCs (passages: 4–8) were cultured in fibroblast growth medium (FGM-3). The NHCMECs (passages: 3–7) were cultured in endothelial basal medium (EGM-2MV).

1.7. Immunofluorescent staining and quantification of tubular structures

The cardiomyocytes were immunostained with an anti-TnT antibody. Briefly, the tissues were underwent the treatments of permeabilization with 0.2% Triton-X for 15 min and blocking with 1% BSA / PBS for 1 hour. The tissues were incubated with the primary antibodies (1:100) overnight. After washing step, the secondary antibodies (1:200) were added to the tissues. The endothelial cells were immunostained with an anti-CD31 antibody. The tissues were incubated with the primary antibodies (1:50) for 1 hour after blocking step. After washing step, the secondary antibodies (1:200) were added to the tissues. The NHCs were immunostained with an Alexa Fluor 488 conjugated anti-Vimentin antibody. The tissues were incubated with the antibody (100:1) for 1 hour after blocking step. The obtained tissues were observed by confocal laser scanning microscopy (CLSM, FLUOVIEW FV10i, Olympus, Japan) and confocal disk scan microscopy (DSU-IX81-SET, Olympus, Japan). For measurements of occupied area percentage, a Metamorph software version 6.2r6 (Molecular Devices, USA) and WimTube (Wimasis, Germany) were used.

1.8. ELISA measurements of angiogenic factors

VEGF-A secreted from iPSC-CM tissues composed of 1×10^6 iPSC-CMs containing 0%, 25%, 50%, and 75% NHCs (NHCF0, NHCF25, NHCF50, and NHCF75) and 1×10^5 NHCMECs were measured by ELISA assay. The 12.4 ml of supernatant tissues in the 6 well plates were collected and added into a microplate of each ELISA assay kit.

1.9. Drug assessment of doxorubicin

1, 50, 1000 nM doxorubicin (Dox) / DMEM was administered to the vascularized NHCF50 iPSC-CM tissues cultured for 1 day by medium change. After 3 days of incubation from administering, beats per minute (BPM) was counted and the tissues were immunostained with anti-CD31 antibody. The obtained tissues were observed and analyzed with occupied area percentage as in paragraph 1.7. In order to compare the 2D cell culture model and the 3D tissue model, 2×10^4 iPSC-CMs, NHCs and 4×10^3 NHCMECs were seeded in a 24 well insert as 2D.

1.10. Statistical analysis

All data were expressed as means $\pm$ SD unless otherwise specified. The values represent the mean $\pm$ SD from three independent experiments. Statistical comparisons between groups were analyzed using a Student's t-test. A p value < 0.05 was considered to be statistically significant.

Results

2. 1. Establishment of Filtration-LbL

To determine the experimental conditions for cell coating by filtration-LbL, the pore size of the filter membrane and the initial cell number in cell coating were optimized (Supplementary Fig. 1). We selected the filter membrane with a 3 μ m pore size for the separation because 0.4 μ m pores were permeable to neither cells nor solutions and 8 μ m of pores passed both (The diameter of CMs is approximate 10 μ m). When we used 5×10^7 rCMs as an initial cell number in a 6 well of

insert (33 mm²) with 3 µm pore size membrane, it was difficult to remove the solution due to jamming of cells. Therefore, we decided to use the 3 µm pore size and less than 1×10^7 cells in filtration-LbL. To compare the effects on cellular functions between the filtration-LbL and the centrifugation-LbL, yield, viability, and damage in cell membrane of rCMs were tested (Fig. 2). As shown in Fig. 2a, the yield in the filtration-LbL was much higher than that in the centrifugation-LbL after 9 step coating (over 70~75%) even if the initial number of rCM was small. **In the case of centrifugation-LbL, cells could not be collected at the condition of smallest initial cell number. The reason of this phenomenon was unclear, but it might be due to the lower aggregation property at small cell number and total 18 times of centrifugation.** Although both methods maintained high viability after the coating (Fig. 2b), the leakage of lactate dehydrogenase (LDH), which is the intracellular enzyme, in the filtration-LbL after 9 steps was one-third lower than that in the centrifugation-LbL, indicating the filtration-LbL did not damage cell membranes (Fig. 2c). After filtration-LbL, 1×10^6 rCM cells coated with FN-G nanofilms were seeded into a 24 well culture insert and cultured with MED199 containing 10% FBS. After 4 days of incubation, the structural evaluation of 3D-rCM tissues was performed by HE staining and immunostaining with TnT and Cx43. Within 24 hours from cell seeding, the obtained CM tissue displayed partially-synchronous beating and maintained beating over 10 days (Supplementary Movie 1). The formation of an approximately 5 layered (5L)-structure was confirmed from the histological image (Fig. 2d). Moreover, the tissue was positive for TnT which is a differentiation marker of **myocytes** (Fig. 2e) and Cx43 which is a gap junction marker (Fig. 2f), indicating that beating of rCM tissue was regulated through the expression of ion channels and skeletal proteins similar to a living CM tissue.

2. 2. *The construction of 3D-iPSC-CM tissues using filtration-LbL*

We employed the filtration-LbL to fabricate the 3D-iPSC-CM tissues. In order to confirm the formation of FN-G nanofilms onto cell membranes, we employed flow cytometry measurement with anti-FN antibody conjugated with Alexa Fluor 488. Fig. 3a showed that the number of **coated**

iPSC-CMs displaying over 10^1 values of green fluorescence intensity, increased during each step of filtration-LbL as well as the previous centrifugation-LbL. This indicated that the adsorption amount of FN increased with increasing step number and FN-G nanofilms were successfully coated onto individual surfaces of CMs using the filtration-LbL. The yield and viability of iPSC-CMs in filtration-LbL were compared to those in centrifugation-LbL. The yield and viability after filtration-LbL showed the same tendency as the results of rCMs and FN-G coated iPSC-CMs were obtained with high efficiency and viability as compared to centrifugation-LbL (Fig. 3b,c). HE staining and immunostaining of TnT and Cx43 of the tissues composed of 5×10^5 or 1×10^6 iPSC-CMs highlighted successful construction of the 3D-iPSC-CM tissue with the expression of TnT and Cx43 as well as rCM tissues (Fig. 3d-f). The obtained 3D-iPSC-CM tissues showed beating after 1 day from seeding (Supplementary Movie 2) and maintained for over 90% of either cell number or viability after 1 week.

2. 3. The effect of the introduction of NHCFs on tissue organization.

Although both rCM- and iPSC-CM tissues constructed above possessed multilayered structures and CM functions like beating, there were still gaps in the tissues and the beatings were heterogeneous. To improve the homogeneity of structures, cell density, thinness and the synchronization of beating, NHCFs that are considered to support the organization of CMs were introduced into the iPSC-CM tissues because NHCFs are expected to secrete ECM such as collagen [29]. The total seeding cell number per one sample was set to 5 or 10×10^5 cells and the ratio of NHCFs was adjusted to 0%, 25%, 50%, 75%, and 100% (NHCF0, NHCF25, NHCF50, NHCF75, and NHCF100) for total cell number. As a result, thickness and cell density of the obtained 3D-iPSC-CM tissues increased with increases in the NHCF ratio (Fig. 4a,b). Azan staining displayed that the amount of collagen fiber (blue) increased and the amount of muscle fiber (red) decreased with increases in the NHCF ratio, which suggested that the introduction ratio of NHCF and iPSC-CM was correlated with the obtained structures (Fig. 4a,c). To evaluate the population

and localization of iPSC-CMs and NHCFs in each tissue, vimentin which is an intermediate filament protein specific to mesenchymal cells was immunostained to observe NHCFs (Fig. 5). The NHCF0 tissues showed strong red color (TnT), but small amount of green (vimentin) positive cells were found even NHCFs were not added because we employed iPSC-CM with 50 – 60% differentiation in this experiment. The NHCF25 tissue possessed the cluster structures of TnT positive cells, while iPSC-CMs in NHCF75 tissue were organized in a state of single cells (Fig. 5a). Quantitative analysis of the CLSM images revealed that the change in cell population was dependent on NHCF ratio (Fig. 5e), which was in agreement with Fig. 4c.

Interestingly, the NHCF25 and NHCF50 tissues showed synchronous beating, while NHCF0 and NHCF75 tissues did not (Supplementary Movie 2-5). The NHCF100 tissue without CMs did not beat (data not shown). To perform the quantitative image analysis of beating, motion vector analyses based on a block matching method were utilized as a non-invasive method. In this method, images are divided into small blocks and cross correlations between consecutive two images are calculated about every block. The displacement vector of each block is derived from the peak position of the cross correlation signal. A similar analysis method was employed to investigate CM beating recently [30,31]. The surface motions in each 21 μm square block were calculated from phase contrast image sequences (Fig. 6). The arrows indicate the direction of the movement and the colors show the speed of movement (the maximum detection speed is about 130 $\mu\text{m} / \text{s}$ in NHCF25 and 13 $\mu\text{m} / \text{s}$ in the others). Almost all points of the surface in NHCF25 and NHCF50 tissue moved quickly and synchronously in the same direction. In contrast, some points on the surfaces of NHCF0 and NHCF75 tissue moved in various directions heterogeneously. To investigate these differences quantitatively, average velocity and time intervals of beating were analyzed. The maximum speed of beating was the fastest in NHCF25 tissue (Fig. 6c). The time interval of beating was shortened and the time interval of contraction and relaxation was delayed by increasing the ratio of NHCFs (Fig. 6d,e). The delay of the time interval of contraction at over 50% NHCF was considered to be involved in the increase of stiffness by the introduction of NHCFs which was

predicted from the increase of collagen fibers (Fig. 4a,c). These results indicated that the introduction of appropriate NHCFs promoted the tissue organization where ion channels were connected through the whole tissues.

2. 4. Vascularization of the 3D-iPSC-CM tissues

We tried to fabricate the 3D-iPSC-CM tissues containing blood capillary networks. To introduce the blood capillaries, iPSC-CMs and NHCFs coated with FN-G nanofilms were co-cultured with 1.0×10^5 NHCMECs (10% as compared to iPSC-CMs + NHCFs). The total iPSC-CMs and NHCFs number was 1×10^6 cells and NHCFs were adjusted to NHCF0, NHCF25, NHCF50, and NHCF75. After 4 days of incubation, the NHCMECs in the tissues were immunostained with anti-CD31 antibody to investigate the formation of blood capillary networks (Fig. 7, Supplementary Fig. 2). NHCMECs in the tissues containing NHCFs formed blood capillary networks with lumen structures (Fig. 7a,b), while the tissue without NHCF did not display any vascularization (Supplementary Fig. 2a). Notably, the density of blood capillaries increased clearly with increases in the NHCF ratio and the secreted amount of VEGF-A (a major angiogenesis factor) also increased with increasing NHCF ratio (Fig. 7c,d). Since it is known that angiogenic factor is one of the most essential factors for *in vitro* vascularization [26], it is considered that the introduction of NHCFs into 3D-iPSC-CM tissues enhanced not only the tissue organization but also the ability to form the capillary networks. In order to investigate the effect of blood capillaries on cell viability, vascularized or non-vascularized iPSC-CM tissues (NHCF50) were cultured in DMEM without FBS for 5 days (Supplementary Fig. 5). The vascularized 3D-tissues clearly showed higher viability (ca. 95%) than that of non-vascularized one (ca. 88%), suggesting that the lumen structures of the blood capillary acted as a tunnel for supplying nutrition and oxygen to the cells inside 3D-tissues.

2. 5. Drug assessment of doxorubicin

Finally, to confirm whether the tissue models can be employed for the pharmaceutical tissue models, a safety evaluation of doxorubicin (Dox) was performed using monolayer-cultured CMs (2D-CM model) as an existing model and the vascularized NHCF50 iPSC-CM tissues (3D-CM model). Dox, which induces apoptosis by the inhibition of DNA and RNA synthesis, is widely employed as an anti-cancer drug and it is known to cause serious side effects in the heart [32]. Each 2D- and 3D-CM model was exposed to 1, 50, 1000 nM Dox during 3 days of incubation and the effects on beating and capillary formation of the CM tissues were examined. As shown in Fig. 8a, the BPM of the 3D-model exposed to 1 and 50 nM Dox was the almost same (around 40), while BPM of the 2D-model drastically decreased with increases of Dox concentration (BPM: 27 and 12 in 1 and 50 nM Dox). Both 2D- and 3D-CM models exposed to 1000 nM Dox showed no beating due to the toxicity (Fig 3a,b). We found that 3D-CM tissues enhanced drug resistance to the anti-cancer drug, which is in agreement with the HT29 (human colon adenocarcinoma) spheroid model reported previously [33]. Moreover, the vascularization in the 3D-CM model was also affected by the exposure to Dox in relation to Dox concentration, and total cell death in blood capillaries was found at 1000 nM Dox (Fig. 8c,d). These results suggested importance of toxicity evaluation to blood capillaries in 3D-CM tissue in drug development.

Discussion

We have reported the cell-accumulation technique to construct 3D-tissues with only cells using ECM-nanofilms on cell surfaces [22]. This technique is able to control a single layer level without complicated procedures and also introduce blood capillary networks into tissues. Compared with many other studies of 3D-tissues, such as those using hydrogel or nanowire scaffold, this technique would be useful for creating the dense tissues like CM tissues containing blood capillaries. However, this technique requires 18 times of centrifugation and its damage to cell membranes causes the leakage of cytosol molecules. It has been reported that the viability of HepG2 decreases to about 6% after 18 times of centrifugation without ECM proteins [34]. Therefore, modification of

the coating method with ECM-nanofilms in a cell-accumulation technique was required. Actually, as shown in Fig. 2c, it became clear that rCMs were damaged and leaked LDH by the centrifugation-LbL while novel filtration-LbL did not damage the rCMs. Although rCM and iPSC-CM tissues were fabricated by this intact method (Fig. 2d-f, 3c-e), we found that the introduction of NHCFs was also an important factor in improving tissue structures. We considered that this improvement was attributable to the difference in cell population from *in vivo* tissue and shortage of connective tissue like collagen. It has been reported that the heart in human body is composed of about 60-70% NHCFs, 30% CMs, and 10% other cells such as endothelial cells and fat cells [35]. NHCFs are known to secrete ECMs such as collagen type I and III and support the structures and functions of myocardial tissues [35-37]. As shown in Fig. 4, high dense tissues could be obtained by the introduction of NHCFs and homogeneous beating was confirmed in NHCF25 and NHCF50 tissues. This result suggested that the NHCFs could support not only tissue structures but also tissue functions of the 3D-iPSC-CM tissues. On the other hand, the beating of NHCF75 tissue was not homogeneous (Supplementary Movie 5). In the living body, overgrowth of NHCFs such as after CMs death due to cardiac infarction causes cardiac fibrosis by secretion of large amounts of type I and III collagen [38]. The NHCF75 tissue might imitate that state and the introduction ratio of NHCFs could determine the quality of the CM tissue model. Therefore, these results suggest that our 3D-iPSC-CM model may be applicable to heart disease models including myocardial fibrosis models.

In order to provide sufficient nutrition, blood capillary networks are indispensable for the construction of 3D-thick tissues *in vitro*. Although cell sheets containing iPSC-CMs and iPSC-endothelial cells have been recently reported, the tissue could obtain capillary networks only after transplantation [20]. *In vitro* vascularization of heart tissue is the central challenge in the application of 3D-CM tissues for quick connection to the host blood capillary after transplantation and for evaluation of angiogenesis in heart, however 3D-iPSC-CM tissue containing blood capillary networks *in vitro* has never been reported to our knowledge. Recently, we reported that the

angiogenic factors secreted from surrounding fibroblasts are important for the formation of tubular blood capillary networks [26]. We introduced cardiac fibroblasts with NHCMECs into the 3D-iPSC-CM tissues to promote vascularization. As shown in Fig. 7, blood capillary networks could be introduced into 3D-iPSC-CM tissues *in vitro* by co-culture with NHCFs addition to iPSC-CMs and NHCMECs (Supplementary Fig. 2), and the amount of secreted VEGF-A increased in parallel with the increase in the number of NHCFs. These data indicate that the NHCFs are important not only for obtaining stable structures but also for formation of blood capillaries in 3D-iPSC-CM tissues. It is possible to control the density of blood capillary networks only by adjusting the seeding NHCMEC number (Supplementary Fig. 3) so that the tissue models could be applied to vascularization and ischemia models.

In the drug assessment experiment, we investigated the tissue response to Dox because of the cardiotoxicity of this drug [39,40]. There were drastic differences in the responses to Dox between in the 2D- and 3D-CM models. **We evaluated the encapsulated amounts of Dox in 3D and 2D cells by flow cytometry, and the amount in 3D and 2D were almost the same (data not shown).** The cell viability after treatment with Dox was measured by trypan-blue staining method (Supplementary Fig. 6). The viability in both 2D-monolayer and 3D-tissues was over 90%, indicating that Dox affected the functions of cardiac cells, not viability. Since the efficacy of anti-cancer drugs such as Dox depends on the ability of cell proliferation, it is considered that 3D-microenvironments around CMs that control cell proliferation significantly affect drug responses. It has been reported that drug resistance of 3D cultured tissue correlates with that of *in vivo* tissue [41]. These results indicate that the tissue models mimicking native 3D-tissue structures might obtain tissue responses comparable with living organs. The vascularization models could not be obtained in 2D-cultured models, hence this would be promising heart angiogenesis tissue models for pharmaceutical assays.

To further model the functions of heart tissue, it is necessary to adjust the amount and population of ECMs and imitate tissue structures at the level of ECM proteins. Actually, the

expression level of each integrin receptor and ECM component depend on the kind of cells. We need to arrange the ECMs suitable to heart tissue like laminin and type I collagen which are major components in CM tissue. Our technique could fabricate various kinds of tissues including diseased models like atherosclerosis and cancer only by arranging the cell types (data not shown) so that it could be applied to not only safety evaluation using normal CM tissue models but also drug discovery using iPSC-derived disease specific CM models including long QT syndrome and cardiomyopathy.

Conclusions

In conclusion, we produced high yield and low damage nanofilms coating onto single cell surfaces using 'filtration-LbL' for rat neonate and iPSC derived CMs. Furthermore, we also constructed 3D-iPSC-CM tissues using this method. Though the tissues composed with only iPSC-CMs had some cracks or gaps within the tissue, introducing the NHCFs into the tissues made them denser. Moreover, we successfully introduced blood capillary networks into the 3D-iPSC-CM tissues by co-culture with iPSC-CMs, NHCFs, and NHCMECs. The more NHCFs were co-cultured, the more VEGF-A was secreted in whole tissue resulting in the developed formation of blood capillary networks. In addition to these results, introducing the NHCFs has an effect on the beating. NHCF25 and NHCF50 tissue showed more synchronous beating than the NHCF0 and NHCF75 tissues. 1000 nM doxorubicin affected the beating and formation of blood capillaries in the toxicity assays of vascularized NHCF50 tissues. These results suggested that not only iPSC-CMs but also NHCFs are crucial for vascularized 3D-iPSC-CM tissues. We found for the first time that these 3D-iPSC-CM tissues containing blood capillary networks would be important for drug toxicity assay because strong toxicity of model drug, Dox, to the blood capillary was observed dependent on Dox concentration. Moreover, the vascularized 3D-iPSC-CM tissues would have a potential for drug efficacy assessments in drug discovery and transplantation in regenerative medicine.

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Figure Captions:

Fig. 1. Schematic illustration s of (a) centrifugation-LbL and filtration-LbL for nanofilm coating with Fibronectin (FN) and Gelatin (G) on cell surfaces and (b) schematic illustration of construction of vascularized 3D iPSC-CM tissues by cell accumulation technique.

Fig. 2. Comparison between filtration-LbL (closed circles) and centrifugation-LbL (open circles) in (a) yield, (b) viability, and (c) LDH leakage of neonatal rCMs. Living cell number was measured by trypan-blue staining. LDH leakage in the supernatant at each LbL step was estimated using a LDH assay kit. (d) HE staining images of 3D-rCM tissues with 1.0×10^6 cells after FN-G coating by Filtration-LbL. The tissues were cultured for 4 days. (e, f) CLSM images of the 3D-rCM tissues with immunostaining using a troponin T and connexine43 antibodies. Nuclei were stained with DAPI. *denotes yield and viability after 5 steps of LbL because cells could not be collected after 9 steps.

Fig. 3. (a) Coated cell count by flow cytometry analyses of iPSC-CMs after 0, 1, 5, and 9 steps of centrifugation- and filtration-LbL, respectively. The cells were stained with anti-FN antibody. The maximum fluorescence intensity of non-coated iPSC-CMs was adjusted to 10^1 as a control, and cell number of the coated cells with over 10^1 fluorescence intensity was counted. (b, c) Comparison between filtration-LbL (closed circles) and centrifugation-LbL (open circles) of iPSC-CMs in (b) yield and (c) viability of iPSC-CMs. Living cell number was measured by trypan-blue staining. (d) HE staining images of 3D-iPSC-CM tissues with 5.0×10^5 cells after FN-G coating by filtration-LbL. The tissues were cultured for 4 days. (e, f) CLSM images of the 3D-iPSC-CM tissues with immunostaining using a troponin T and connexine43 antibodies. Nuclei were stained with DAPI. *denotes yield and viability after 5 steps of LbL because cells could not be collected after 9 steps. † indicates $p < 0.05$ compared to 0 step of each LbL.

Fig. 4. (a) HE and azan staining images of 3D-iPSC-CM tissues with different NHCF ratios, 0, 25, 50, 75, 100%, respectively. The tissues were fabricated with 5×10^5 cells and cultured for 4 days. (b) The thickness and (c) color area of the 3D-iPSC-CM tissues with different NHCF ratios. The values were estimated from histological images. Red and blue areas in (c) indicate muscle and collagen fibers, respectively.

Fig. 5. (a) CLSM images of 3D-iPSC-CM tissues with different NHCF ratios by immunological staining with troponin T and vimentin antibodies. The tissues were fabricated with 1×10^6 cells and cultured for 4 days. (b) The quantitative analyses of the area of each region in CLSM images of immunological staining.

Fig. 6. (a) The motion analyses of surface movement of 3D-iPSC-CM tissues with different NHCF ratios. **The arrows indicate the direction of the movement of separated $21 \mu\text{m}$ square block and the colors show the velocity of the movement.** (b) Average velocity, (c) **maximum contraction and relaxation velocity**, (d) time interval of beating, and (e) time interval contraction and relaxation in relation to NHCF ratios **from the motion analysis (a).**

Fig. 7. (a, b) CLSM images of blood capillary-like networks in 3D-iPSC-CM tissues composed of 5×10^5 iPSC-CMs, 5×10^5 NHCFs (NHCF50), and 1×10^5 NHCMECs. NHCMECs in the tissues were immunostained with anti-CD31 antibody and nuclei were stained with DAPI. (b) is top and x-z reconstructed cross section images. The arrow indicates lumen structure. (c) Area of CD31 positive capillary networks in the images of the 3D-tissues with different NHCF ratios. (d) Secreted amount of VEGF-A from the 3D-tissues with different NHCF ratios. † and †† indicate $p < 0.05$ and < 0.01 , respectively.

Fig. 8. The drug responses of the vascularized 3D-iPSC-CM tissues to the cancer drug, doxorubicin. (a) Beating number of 3D-iPSC-CM tissues (NHCF50) and 2D-iPSC-CMs (NHCF50) after 3 days of incubation in DMEM containing 1, 50, or 1000 nM doxorubicin. *indicates 0. (b) CLSM images of the 3D-iPSC-CM tissues and 2D-iPSC-CMs after incubation with doxorubicin by immunostaining with troponin T antibody. (c) CLSM images of blood capillary-like networks in 3D-iPSC-CM tissues after treatment with doxorubicin at different concentration. The networks were immunostained with anti CD31 antibody. (d) Capillary area in images of (c). †† indicates $p < 0.01$ compared to 2D in (a).

Figure. 1

Figure. 2

Figure. 3

Figure. 4

Figure. 5

Figure. 6

a CD31

b CD31 DAPI

Figure. 7

Figure. 8

Filtration-LbL technique