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Development of vascularized iPSC derived 3D-cardiomyocyte tissues by filtration Layer-by-Layer technique and their application for pharmaceutical assays

Yuto Amano [a], Akihiro Nishiguchi [a], Michiya Matsusaki [a], Hiroko Iseoka [b], Shigeru Miyagawa [b], Yoshiki Sawa [b], Manabu Seo [c], Takashi Yamaguchi [c], Mitsuru Akashi [a,d,*]

[a] Department of Applied Chemistry, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan
[b] Department of Cardiovascular Surgery, Graduate School of Medicine E1, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
[c] Advanced Technology Research & Development Center, Ricoh Institute of Technology, RICOH Company Ltd., 16-1 Shinei-cho, Tsuzuki-ku, Yokohama, Kanagawa 224-0035, Japan
[d] Building Block Science Joint Research Chair, Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan

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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.

Statement of Significance

In vitro fabrication of vascularized three-dimensional (3D) human cardiomyocyte (CM) tissues derived from human induced pluripotent stem cells (iPSCs) has attracted much attention owing to their requirement of much amount of nutrition and oxygen, but not yet published. In this manuscript, we report construction of vascularized 3D-iPSC–CM tissues by a newly-discovered filtration-Layer-by-Layer (LbL) technique. The filtration-LbL fabricates nanometer-sized fibronectin and gelatin (FN–G) films onto iPSC–CM surfaces. The FN–G nanofilms induce cell–cell interactions via integrin molecules on cell surfaces, leading to construction of 3D-tissues. The constructed vascularized 3D-iPSC–CM tissues would be a promising tool for tissue regeneration and drug development. We believe that this manuscript has a strong impact and offers important suggestions to researchers concerned with biomaterials and tissue engineering.

* Corresponding author at: 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan.
E-mail address: akashi@chem.eng.osaka-u.ac.jp (M. Akashi).

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1. 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 do not 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*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*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.

2. Materials and methods

2.1. 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% glutarardehyde 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 μm, 6 well cell culture insert with 0.4, 3, and 8 μm 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).

2.2. Isolation of neonatal rat cardiomyocytes

The rat-cardiomyocytes (rCMs) were prepared from 0 to 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 mm^3 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.

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

The preparation of iPSC–CMs has previously been reported [28]. Briefly, the human iPSCs (253G1) purchased from RIKEN (Tsukuba,

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