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Original Article

Pulsatile tubular cardiac tissues fabricated by wrapping human iPS cells-derived cardiomyocyte sheets

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ABSTRACT

The purpose of this study was to fabricate pulsatile tubular cardiac tissue using cell sheet based-tissue engineering. First, we fabricated human induced pluripotent stem cell (hiPSc)-derived cardiomyocyte sheets and normal human dermal fibroblast (NHDF) sheets which are harvested from temperature responsive culture dishes only by lowering the temperature. Then tubular cardiac tissues are formed by wrapping one hiPSc-derived cardiomyocyte sheet and three NHDF sheets around an octagonal column, and both ends of the tubular tissue were covered with fibrin and collagen gel. The octagonal column with the tubular tissue was connected to an *in vitro* circulation system in a culture box. After four-day culture, the cardiac tissue survived and pulsated spontaneously in the circulation system. Furthermore, the analysis with a Millar catheter inserted into the cardiac tubes revealed significant inner pressure changes generated by their beating. In addition, the tubular cardiac tissue pulsated in response to the electrical stimulation. Although histological analyses demonstrated that cardiac troponin T-positive cells stratified the inner surface of the tubular tissues, gene expression analyses showed an immature state of these cardiomyocytes. Thus, cell sheet-based tissue engineering realized human pulsatile tubular cardiac tissue fabrication and we believe that these tubular cardiac tissues should contribute to future drug screening and regenerative therapy for heart diseases.

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1. Introduction

Recent remarkable advances in stem cell biology and tissue engineering have realized regenerative therapy and three-dimensional (3D) model fabrication. Especially, development of human induced pluripotent stem cells (hiPSc) has accelerated tissue model fabrication for basic research and drug screening. Application of iPSc to the cardiac research field has been actively pursued. For example, hiPSc-derived cardiomyocytes are used to examine arrhythmogenic cardiotoxicity of new drugs such as QT prolongation, one of the serious adverse events [1]. Recent research suggests that usage of hiPSc could help us to understand electrophysiological mechanisms of pathogenesis that cannot be accurately examined by animal experiments, and may shorten the time required for developing new drugs [2].

Clinically, in addition to electrophysiological analysis, functional assessments of wall motion and pulsatile inner pressure are routinely performed for checking heart condition. To measure the inner pressure of a heart, a pressure catheter is introduced in the heart ventricle. As mentioned above, current assessments of hiPSc-derived cardiomyocytes have been limited to electrophysiological analysis, because previous models using hiPSc-derived cardiomyocytes lack chamber-like structure (e.g. tubular structure, pouch structure). Therefore, some tissue engineering technologies are needed to realize functional heart tissues with a structure such that can generate pulsatile inner pressure.

On the other hand, as one of the tissue engineering technologies, we have developed “cell sheet-based tissue engineering“. Cell sheets are harvested from temperature-responsive culture dishes only by lowering the temperature. Cell sheet transplantation is more effective than isolated cell injection due to more cell survival in the target organ. Various types of cell sheet transplantation have

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been clinically applied for cornea, heart, esophagus, cartilage, gingiva, ear and lung diseases [3–9]. 3D tissues have been also fabricated *in vitro* by layering cell sheets to mimic *in vivo* heart and liver tissues [10,11]. Furthermore, functional cardiac tubular structure has been already realized by wrapping a neonatal rat cardiac cell sheet [12].

In the current study, we aimed to fabricate human tubular cardiac tissues by combining hiPSc-derived cardiomyocytes and cell sheet-based tissue engineering. The human cardiac tubular tissues were engineered by wrapping human cardiomyocyte and human fibroblast sheets and their surface electrical potential, inner pressure, histology and cardiac-specific gene expression were analyzed.

2. Methods

2.1. Preparation of a cardiomyocyte sheet and normal human dermal fibroblasts sheets

HiPSc (line 201B7) [13] purchased from RIKEN (Tsukuba, Japan) were cultured into a 250 mL stirred cultivation system (ABLE Co., Tokyo, Japan) (Fig. 1-A). In this study, we used hiPSc derived cardiomyocytes that expressed the puromycin-resistance gene under the control of the mouse α-myosin heavy chain promoter and the neomycin-resistance gene under the control of the rex-1 promoter [14]. Several days later, hiPSc formed embryoid bodies (EBs) in the

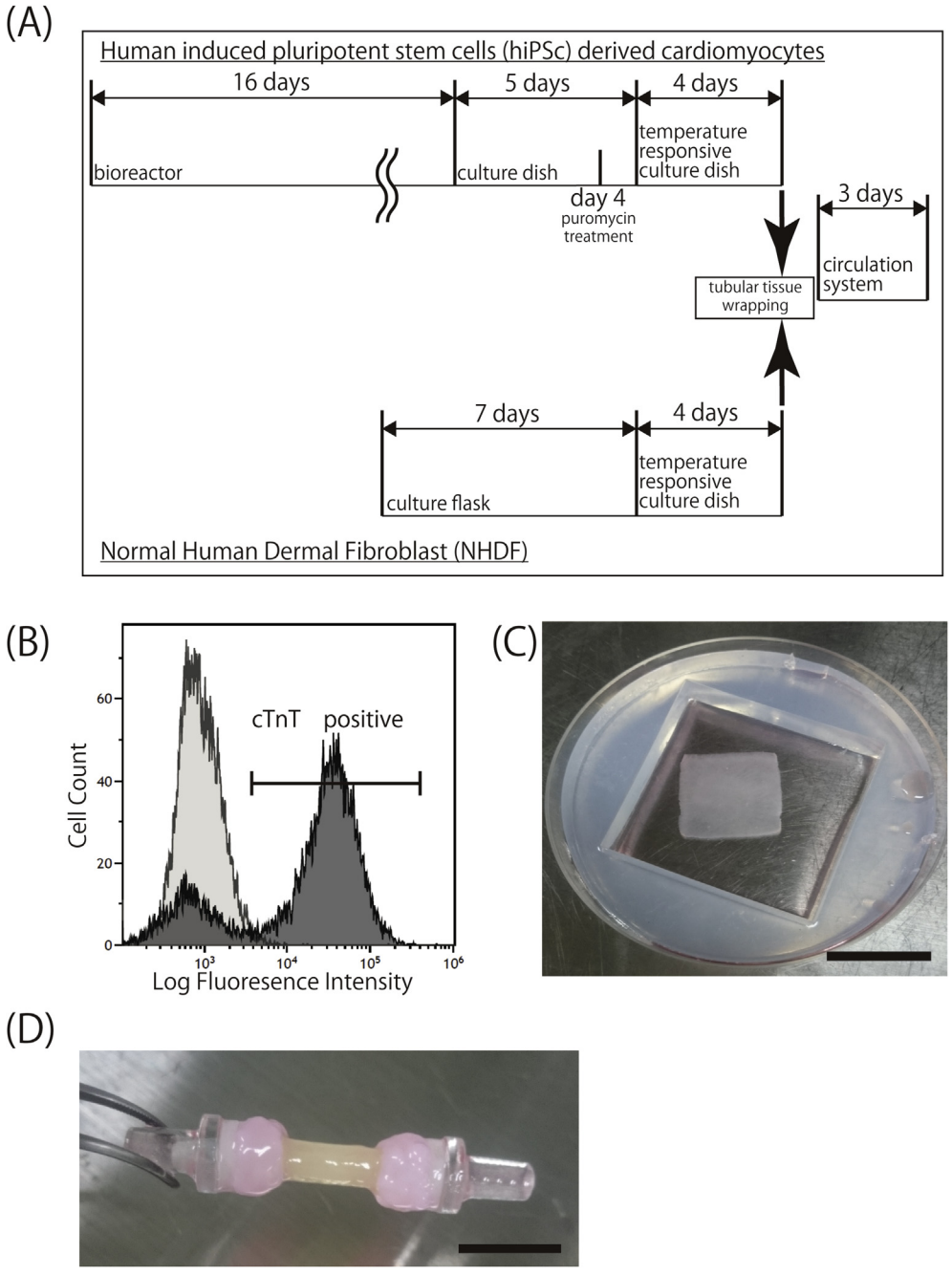


Fig. 1. Methods of tubular tissue formation. **(A)** Schematic illustration of a cell culture and fabrication for the tubular tissue. **(B)** Flow cytometric analyses on cardiac troponin-T expressing cells after the treatment with puromycin (79.9 ± 6.0%, n = 11). **(C)** The photograph shows a detached cardiomyocyte sheet. Bar = 20 mm. **(D)** The photograph shows the tubular tissue and an octagonal column. Bar = 10 mm.

cultivation system. To induce differentiation of EBs into cardiomyocytes, growth factors and a small molecule were added in accordance with the protocol suggested in a previous study on the cultivation system [15]. After 16 days, EBs of differentiated cardiomyocytes were collected from the cultivation system and isolated into single cells using Hanks' balanced salt solution (17460-15, NACALAI TESQUE Inc., Kyoto, Japan) containing 0.05% trypsin/EDTA (32777-44, NACALAI TESQUE Inc.). Next, the isolated cardiomyocytes were plated at 1.0×10^7 cells/dish onto a 100 mm diameter culture dish in Dulbecco's Modified Eagle's Medium (DMEM) (D6429, Sigma–Aldrich) supplemented with 10% FBS (Japan Bio Serum, Hiroshima, Japan) and 1% penicillin-streptomycin (15140122, Thermo Fisher Scientific, Rockford, IL, USA) at 37 °C in a humidified atmosphere with 5% CO_2 for four days. The medium used was replaced one day and three days after plating.

After four days of culture, puromycin (A11138-03, Thermo Fisher Scientific) was added at 1.5 mg/mL on the cardiomyocytes culture dish. One day after the addition of puromycin, cardiomyocytes were treated with Hanks containing 0.05% trypsin/EDTA and collected from the dish. After the treatment with puromycin, about 80% of cells were cardiac troponin-T positive cardiomyocytes (79.9% ± 6.0%, n = 11) (Fig. 1-B). Next, cardiomyocytes were plated on the 100 mm-diameter temperature-responsive culture dish (CS3015, UpCell, CellSeed, Tokyo, Japan) on a square silicon frame of 50 mm × 50 mm with 6×10^6 cells/dish. After four days, the cardiomyocyte sheet was detached from the temperature-responsive culture dish (Fig. 1-C). Spontaneous pulsation of a cardiomyocyte sheet was observed by an inverted microscope (ECLIPSE TE2000-U, NIKON, Tokyo, Japan). Simultaneously, we cultured Normal Human Dermal Fibroblasts (NHDF) (CC-2509, LONZA) in a culture flask (353143, CORNING, Tewksbury, MA, USA) to fabricate a NHDF sheet. The NHDF sheets were cultured following the manufacturer's instructions. After one week, we collected NHDF from the culture flask and plated them onto the 100 mm-diameter temperature-responsive culture dishes with 6×10^6 cells/dish. The NHDF sheets were detached from the dish four days later.

2.2. Fabrication of cardiomyocyte tube and construction of circulation system

Cell sheets were wrapped around a hollow tubular column. We designed the column using 3D CAD software (SoildWorks, Dassault Systèmes SolidWorks Corporation, Waltham, MA, USA) and created it with a rapid prototyping system (EDEN350, Objet Geometries, Billerica, MA, USA).

The column was octagonal, with eight 0.5 mm × 1.0 mm slits on the sides. The inner diameter of the column was 2 mm, with a thickness of 0.75 mm. Next, three NHDF sheets detached from the temperature-responsive culture dish were layered and cut into 20 mm squares with a scalpel (No. 21, FEATHER, Osaka, Japan). The layered NHDF sheets were wrapped around the octagonal column. To prevent the NHDF sheets from peeling off, the NHDF sheets were incubated for an hour at 37 °C. After incubation, a monolayer hiPSc-derived cardiomyocyte sheet was detached from the temperature-responsive culture dish and wrapped around the outermost part of the octagonal column, and was incubated for 1 h at 37 °C. Next, to seal the gap between the edge of the wrapped cell sheets and the column (Fig. 1-D), fibrin gel (BOLHEAL, KAKETSUKEN, Kumamoto, Japan) and collagen gel (3% Atelocollagen, KOKEN, Tokyo, Japan) were applied on both ends of the octagonal column using a syringe and needle. The fibrin gel was dropped to coagulate between the sheet and the column, then the collagen gel was coated to fill the gap between the sheet and the column. To ensure the adhesion of the gel and the octagonal column, the octagonal column was incubated for an hour at 37 °C in DMEM with 10% FBS and 1% penicillin-streptomycin. In the next step, the cardiomyocyte tube was connected to the reactor tank, and the circulation system was constructed (Fig. 2-A). The circulation system including the reactor tank was established in a culture box with an observation window. The circulation system was created following the previous study [16]. The reactor tank with cardiomyocyte tube was connected to a syringe pump using a silicon tube, and the medium was circulated with 0.5 mL/min by steady flow. For circulation medium, the same medium used for the culturing cardiomyocyte sheet was used. In addition, to adjust the pH of the medium, the inside of the reactor tank was kept at 37 °C with a heater, and in 5% CO_2 with a gas mixing system (TOKAI HIT, Shizuoka, Japan). Furthermore, to evaluate the expansion of the cardiomyocyte tube, a small tip was connected to the rear end of the octagonal column after three days circulation. When the culture medium was circulated, the cardiomyocyte tube was swollen by inner pressure through the column slit (Fig. 2-B).

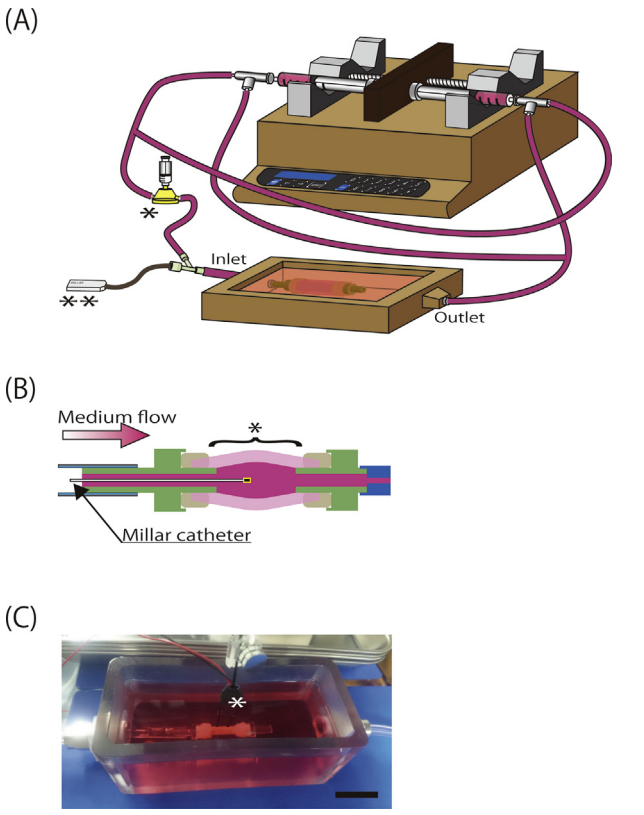


Fig. 2. Construction of the circulation system. **(A)** Overview of a bioreactor assembly and the circulation system. *: Bubble trap, **: catheter connector. **(B)** Schematic illusration of a cross-section of the tubular tissue. Millar catheter was inserted into the tubular tissue. *: Swollen tubular tissue. **(C)** The photograph shows a tube holding chamber and a microelectrode (*). Bar = 10 mm.

2.3. Observation of cardiomyocyte tube with microscope

A microscope (VB-7000, Keyence, Osaka, Japan) was used to observe the expansion of the cardiomyocyte tube and its spontaneous pulsation in the culture box. The lens was placed above the cardiomyocyte tube. To confirm that there was no leakage outside the cardiomyocyte tube, fluorescent bovine serum albumin

conjugates (A13110, Thermo Fisher Scientific) were added to the circulation medium.

2.4. Histological and immunohistochemical analyses

For histological analysis, the cardiomyocyte tube was fixed in 4% paraformaldehyde (3311-1, Wako Pure Chemicals, Osaka, Japan) three days after circulation and next day routinely processed into 6 mm thick paraffin-embedded sections. Hematoxylin and Eosin (HE) staining and Azan staining were carried out by conventional methods [17]. Sections were visualized with light microscopy (ECLIPSE E800, Nikon, Tokyo, Japan) and imaged using a software (NIS-Elements, Nikon). Immunohistochemical staining was carried out by dipping the myocardial tube in 4% paraformaldehyde for 5 min, and then stepwise immersing it in phosphate-buffered saline (PBS, 11482-15, NACALAI TESQUE Inc.) containing 10%, 20%, and 30% sucrose (196-00015, Wako, Osaka, Japan) for 1 h each. Next, the immersed tube was embedded in an optimum cutting temperature (O.C.T.) compound (4583, SAKURA FINETEK, Tokyo, Japan) and then routinely processed into 10 μm thick sections. Processed sections were incubated with either a 1/100 dilution of anti−troponin T mouse monoclonal antibody (MS-295-P1, Thermo Fisher Scientific) or a 1/100 dilution of anti-vimentin rabbit monoclonal antibody (ab92547, Abcam, Cambridge, UK) for 1 h at room temperature, and then with Alexa Fluor 488 conjugated secondary antibody (A11017, Thermo Fisher Scientific) and Alexa Fluor568 conjugated secondary antibody (A21069, Thermo Fisher Scientific) for 45 min at room temperature. Finally, sections were visualized with confocal laser microscopy (FV1200-IX83, OLYMPUS, Tokyo, Japan).

2.5. Detection of the inner pressure and analysis of peak positive dP/dt and peak negative dP/dt

A Y connector (MAP101, MERIT MEDICAL, South Jordan, UT, USA) was connected to a silicon tube of the upper reactor tank. A pressure catheter (SPR-671-1.4Fr, Millar, Houston, TX, USA) was inserted into the Y connector to detect the inner pressure changes according to the spontaneous pulsation of the cardiomyocyte tube three days after circulation commencement. The tip of the inserted catheter via silicon tube was installed in the inner center of the cardiomyocyte tube. The inner pressure was collected and recorded with a pressure control unit (PCU-2000, Millar), and the output data was analyzed with a Power Lab (LP3508, ADInstruments, New South Wales, Australia) and Lab Chart 7 (ADInstruments). Peak positive dP/dt and peak negative dP/dt data were obtained based on the trend data of the inner pressure with a Lab Chart.

2.6. Measurement of cardiac electrical potential and electrical stimulation

To measure cardiac electrical potential, a microelectrode (EKL2-8020-C5, BIO RESEARCH CENTER, NAGOYA, Japan) was positioned on the surface of the cardiomyocyte tube (Fig. 2-C). First, a microelectrode was attached to a 3-axis manual micromanipulator (MM-3, BIO RESEARCH CENTER) so that the position of the microelectrode could be adjusted manually. Next, the position of the microelectrode was adjusted to confirm that its tip directly contacted the cardiomyocyte tube. The cable of the microelectrode was connected to a differential extracellular amplifier (EX-1, BIO RESEARCH CENTER), and then to the Power Lab to record the cardiac electrical potential. An additional microelectrode was attached to the cardiomyocyte tube to examine the contractility in response to electrical stimulation (10 msec, 100 mV) using an electronic stimulator (SEN-3401, NIHON KOHDEN, Tokyo, Japan). The pacing rate of electrical stimulation was set at a rate of 5 beat per minutes (bpm) faster than the pulsation rate of the cardiomyocyte tube. After observing for 5 min that the pulsation of the cardiomyocyte tube followed electrical stimulation, we increased the stimulation pacing rate by 5 bpm and observed for another 5 min until the pulsation could no longer follow electrical stimulation. We then reversed the process and started decreasing the pacing rate by 5 bpm and observed for 5 min until the pulsation could not follow electrical stimulation.

2.7. Real time reverse transcription quantitative polymerase chain reaction

Gene expression levels of the cardiomyocyte tube tissues after circulation and the static culture tissues were evaluated using real time reverse transcription quantitative polymerase chain reaction (RT-qPCR) and compared. The static culture tissues were prepared in the same method as the cardiomyocyte tubes were prepared. After layering the cardiomyocyte sheet and three NHDF sheets, the layered tissues were plated on the cell culture inserts (353090, CORNING). The cell culture inserts were placed in a 6-well culture plate (353502, CORNING), and then cell culture medium was added into the inserts and the plate. The cell culture medium was DMEM with 10% FBS and 1% penicillin-streptomycin. Both the inside and outside of the cardiomyocyte tube in the circulation system made contact with the culture medium, so we used the cell culture inserts to create a similar environment, in which the culture medium made contact with both the top and bottom surface of the static culture tissue. In addition, we covered the circumference area of the static culture tissue with collagen gel and fibrin gel as the cardiomyocyte tube was also covered with these gels. The period of the static culture was for three days, and the 6-well culture plate was incubated at 37 °C in a humidified atmosphere with 5% CO_2, in the same way as the cardiomyocyte tube circulation.

Total RNA of the cardiomyocyte tube and static culture tissue was isolated using an RNeasy Mini Kit (74104, QIAGEN, Venlo, the Netherlands). For RT-qPCR, a High Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems, Stockholm, Sweden) and TaqMan Fast Advanced Master Mix (4444557, Applied Biosystems) were used following the manufacturers' instructions. Experiments were performed on a StepOne and StepOnePlus RealTime PCR System (Applied Biosystems). For a housekeeping gene primer and cardiomyocyte related gene primers, TaqMan assays (Applied Biosystems) (Table 1) were used. For a housekeeping gene primer, ABL proto-oncogene 1, non-receptor tyrosine kinase with the smallest standard deviation using TaqMan Array Human Endogenous Controls Plate (4426700, Applied Biosystems) was selected.

Following the analysis method of Livak et al. [18], the value of one sample was the average of the triplicate value and the error bars of the result figures showed the standard deviation of 5 independent replicate experiments.

Table 1
Primers list for RT-qPCR

Gene name	Applied Biosystems TaqMan assay ID
ABL1	Hs00245445_m1
NPPA	Hs00383230_g1
NPPB	Hs01057466_g1
RYR2	Hs00181461_m1
TN NT	Hs00165960_m1
MYL2	Hs00166405_m1
MYL7	Hs01085598_g1
MYH6	Hs01 101425_m1
MYH7	Hs01 110632_m1

2.8. Statistical analysis

All date were expressed as mean ± standard deviation. Comparison of the two groups was done by Student's *t* -test. When the one-way ANOVA was significant, comparison of the three groups was done by Tukey–Kramer test. A *p*-value of less than 0.05 was considered as significant.

3. Results

3.1. Swollen cardiomyocyte tube in vitro demonstrated spontaneous pulsation after circulation

The structure of the octagonal column and the cell sheet size for fabricating cardiomyocyte tube was examined in advance (not shown in results). We used one to three sheets of hiPSc-derived cardiomyocyte and layered them without using the NHDF sheets. However, this caused a pinhole and as a result, the cardiomyocyte tube did not swell. The pinholes in the cardiomyocyte tube were verified with fluorescent conjugates leakage in circulation. Before the cardiomyocyte tube fabrication (Fig. 3-A), we used a light microscope and observed many pinholes present in the hiPSc-derived cardiomyocyte sheet. When the circulation medium leaked from the pin-holes, the cardiomyocyte tube did not swell, and the inner pressure from spontaneous pulsation of the cardiomyocyte tube was not be detected. Therefore, we fabricated tubular tissue using NHDF sheets to examine the number of cell sheets without leakage by putting fluorescent conjugates into the tubular tissue. By increasing the NHDF sheets one by one, we confirmed that the leakage did not occur when four sheets were wrapped around the octagonal column. Thus, in the current experiment, we used three NHDF sheets and one cardiomyocyte sheet around the octagonal column. Consequently, no pin-holes appeared after loading the inner pressure of the cardiomyocyte tube with three days of *in vitro* circulation and the tube was successfully swollen. In addition, we microscopically observed that the swollen hiPSc-derived cardiomyocyte tube repeated contraction and relaxation through spontaneous pulsation (Video. 1).

Supplementary video related to this article can be found at https://doi.org/10.1016/j.reth.2019.09.001

3.2. Detection of inner pressure and electrical potential of the cardiomyocyte tube, and analysis of pulsation rate and peak dP/dt

The inner pressure of the swollen cardiomyocyte tube was collected via inserting a pressure catheter after three days of circulation (0.13 ± 0.11 mmHg, n = 10). At the same time, the electrical potential was detected in the cardiomyocyte tube, and the pulsation rate was calculated from the peak interval of the electrical potential (99 ± 46 bpm, n = 10). The maximum pulsation rate was 180 bpm, and the minimum pulsation rate was 34 bpm. The cycle of the inner pressure change was consistent with the cycle of the electrical excitation (Fig. 3-B). Peak positive and negative dP/dt data were 1.47 ± 0.67 mmHg/sec (peak positive dP/dt, n = 8) and 1.25 ± 0.54 mmHg/sec (peak negative dP/dt, n = 8), respectively (Fig. 3-C).

Table 2
Analysis of contractile response to electrical stimulation

	spontaneous pulsatile	Followed interval to electrical stimulation	
		Min	Max
Exp. 1	72	60	100
Exp. 2	58	60	80
Exp. 3	60	60	110
Exp. 4	54	50	120 [bpm]
(Ave)	(61)	(57.5)	(102.5)

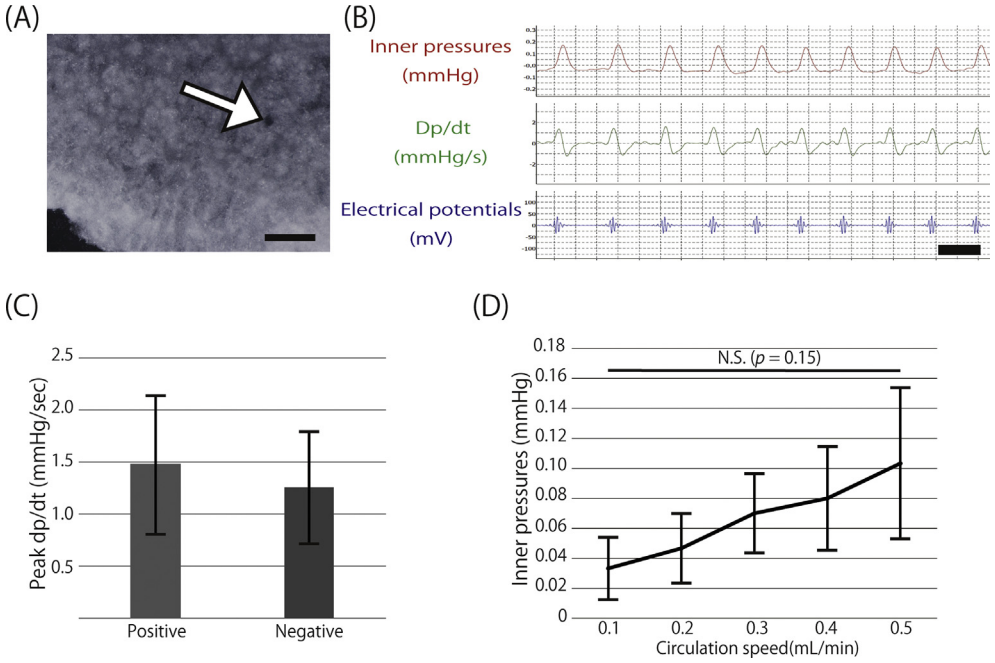


Fig. 3. Functional analyses of tubular tissue. **(A)** The photograph shows a detached hiPSc-derived cardiomyocyte sheet. Black arrows indicate a pinhole in a cardiomyocyte sheet. Bar = 5 mm. **(B)** Inner pressures, dP/dt, and electrical potentials from the beating tubular tissue. Bar = 1 s. **(C)** A graph shows peak positive dP/dt and peak negative dP/dt (n = 8). **(D)** A graph shows the relationship between inner pressures changes from tubular tissue and circulation speed (n = 3).

3.3. Pulsation of cardiomyocyte tube followed external electrical stimulation

In addition to the pressure catheter and a microelectrode for detecting the electrical potential, another microelectrode for electrical stimulation was positioned on the surface of the cardiomyocyte tube. Electrical stimulation protocol was used for evaluating following capability, and the electrical potential of the cardiomyocyte tube followed the electrical stimulation as described in Table 2. Moreover, the waveform of the inner pressure followed the waveform of the pulsation during stimulation pacing (Video. 2).

Supplementary video related to this article can be found at https://doi.org/10.1016/j.reth.2019.09.001

3.4. Physiological functions of pulsatile cardiomyocyte tube

Cardiac stroke volume increases in response to myofiber stretch because of increased pre-load. In cardiovascular physiology, this is known as the Frank-Starling Law [19]. For imitating physiological pre-load, the inner diameter of the column was 2.0 mm and was connected to the tip whose inner diameter was narrowed to 0.5 mm. The more the circulation speed increased, the more the inner pressure increased. It expanded cardiomyocyte tubes leading to the increase of pre-load. In the current experiment, the circulation speed was increased from 0.1 to 0.5 mL/min in increments of 0.1 mL and the electrical stimulation was conducted at 80 bpm for a stable beating. Then pulsatile inner pressure changes were detected by the pressure catheter. The smallest pulsatile inner pressure changes were observed at 0.1 mL/min flow speed, and the pulsatile inner pressure changes increased according to the circulation speed (Fig. 3-C). The increased inner pressure changes suggested that the cardiomyocyte tubes might respond to pre-load, conforming to the Frank-Starling Law, although the inner pressure changes were not significantly related to the circulation speed ($p = 0.15$).

3.5. The characterization of cardiomyocyte tubular section

Sections of the cardiomyocyte tube were created after three days of circulation and were sliced at the mid portion of the tubular tissue along the short axis. The cleavage surface of the tissue was stained with HE staining. The tubular tissue was found to be circular, and the survival of the cells in the tissue was observed (Fig. 4-A, B). Also, following AZAN staining of the cleavage surface of the tissue, the cytoplasm was found to be stained red in both the outermost and innermost part of the cardiomyocyte tube, which indicated high cell density and rich cytoplasm (Fig. 4-C). These results showed that the cardiomyocyte tubes maintained their structure under the circulation flow. Next, after immunohistochemical stain, we found that the inside of the cardiomyocyte tube was stained with vimentin and the outside was stained with cardiac Troponin T (Fig. 4-D). This result showed that the 3-layer NHDF sheets and the cardiomyocyte sheet wrapped around the octagonal column maintained the stratified tissues.

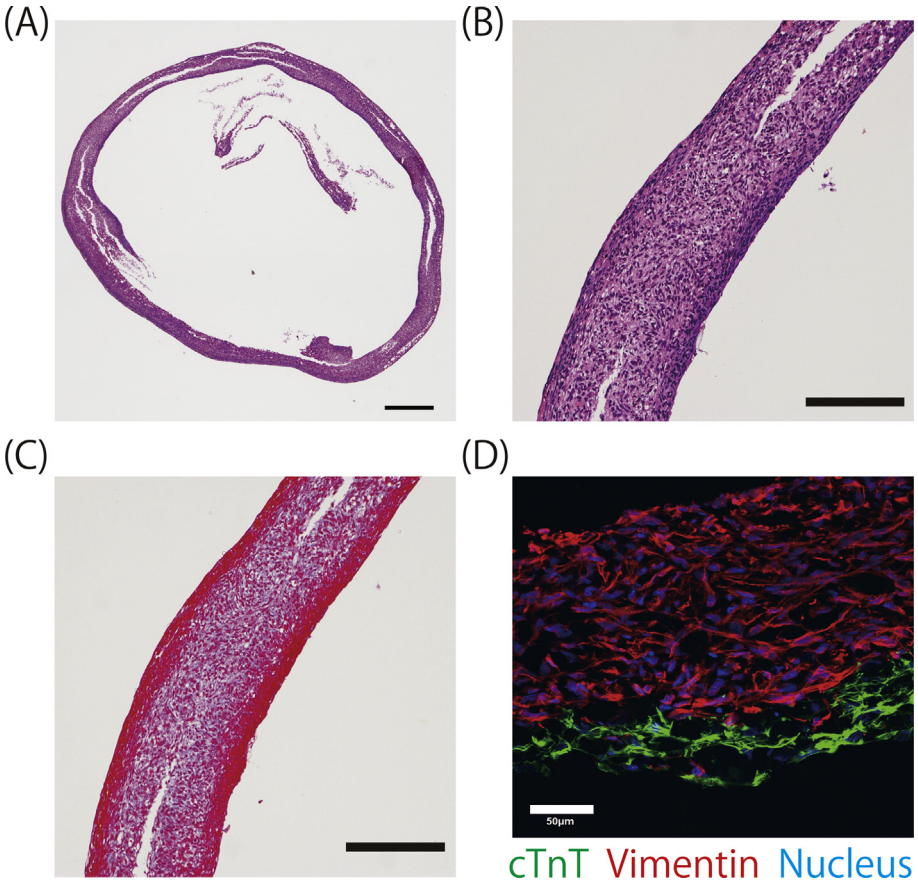


Fig. 4. Histological and immunohistochemical analyses of the tubular tissue, 3 days after circulation in the bioreactor. (A), (B) Cross-sectional view of the HE stained tubular tissue. Bar (A) = 500 μm, (B) = 200 μm. **(C)** Cross-sectional view of the AZAN stained tubular tissue. Bar = 200 μm. **(D)** Immunohistochemical staining of the tubular tissue. A hiPSc-derived cardiomyocyte sheet was stained with cardiac troponin-T (cTnT, green). NHDF sheets were stained with Vimentin (red), and nuclei were stained with DAPI. Bar = 50 μm.

3.6. Comparison of RT-qPCR in the cardiomyocyte tube and the static culture tissue

Gene expression levels in the cardiomyocyte tube were elucidated by RT-qPCR (Fig. 5). Expression levels of the cardiomyocyte tubes were significantly increased in comparison with the levels of the static cultured tissues in NPPA (Natriuretic Peptide A), MYL 2 (Myosin Light Chain 2) and MYL 7 (Myosin Light Chain 7). On the other hand, expression levels of the cardiomyocyte tube were equivalent to the static cultured tissue in NPPB (Natriuretic Peptide B), RYR2 (Ryanodine Receptor 2), TNNT (Troponin T), MYH6 (Myosin Heavy Chain 6), MYH7 (Myosin Heavy Chain 7).

4. Discussion

In the present study, we fabricated human functional tubular cardiac tissues by using hiPSc-derived cardiomyocytes and cell sheet technology. The laminated tubular tissues pulsated

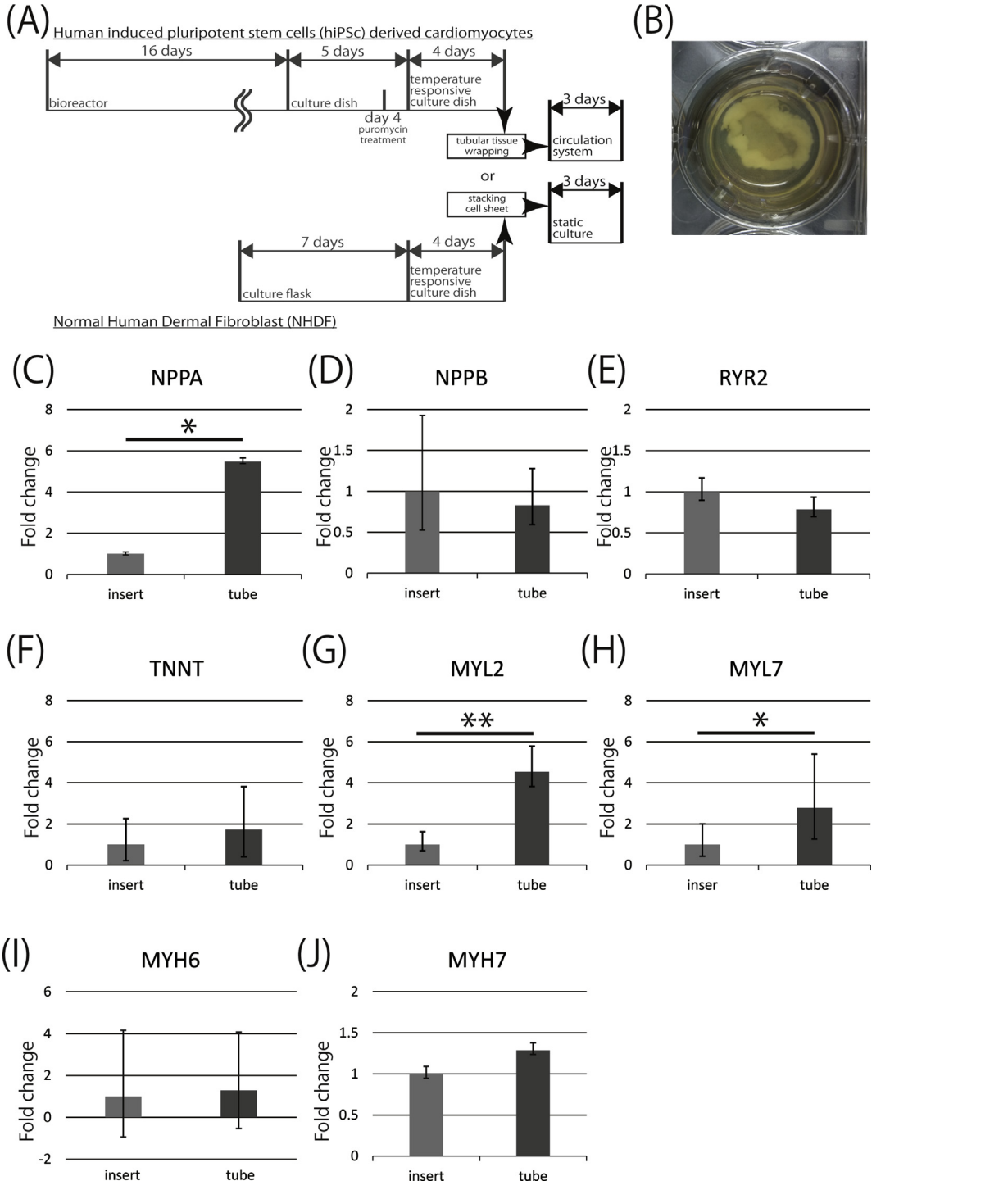


Fig. 5. RT-qPCR analyses of the tubular tissue and the static cultured tissue. **(A)** Schematic illustration of a fabrication for the tubular tissue and static cultured tissue. **(B–J)** Gene expression levels in the tubular tissue and the static cultured tissue. Expression levels of NPPA, MYL2, MYL7 were significantly increased in the tubular tissue. Relative expression of RNAs was calculated using the two delta CT method *: $p < 0.05$, **: $p < 0.01$.

spontaneously and evoked significant inner pressure in accordance with surface electrical potential. They followed external electrical stimulation and showed partial maturation in gene expression.

In our previous study, a pulsatile myocardial tube was engineered by wrapping a neonatal rat cardiac cell sheet around a fibrin tube [12]. On the other hand, in this study, we used hiPSc-derived cardiomyocyte sheet and a scaffold-free wrapping technique to realize human heart tissue model having a chamber-like structure. In place of fibrin gel, the octagonal column with slits was used as a wrapping core. The column initially supported fragile cell sheets and then the slits enabled wrapped cell sheets to release from the column by applying inner pressure.

Although the scaffold-free swollen tubular cell sheet was successfully fabricated by using the column, some pin-holes of the cell sheet caused circulation medium leakage resulting in shrinkage of the tubular tissue. These pin-holes occurred depending on cell types, seeding cell density and extracellular matrix among cells. Tubular cardiac tissues using only cardiomyocyte sheets frequently shrank due to the pin-holes; on the other hand, the tubular tissues consisting of a cardiomyocyte sheet and NHDF sheets did not shrink. This seems to be because of less intercellular adhesion in cardiomyocyte sheets than in NHDF sheets. Fibroblasts synthesize extracellular matrix including collagen and forms durable tissue with tight intercellular adhesion. Actually, dermal fibroblast sheets have been already clinically applied to close air-leaks in lung diseases [9]. Therefore, fibroblast sheets may be useful as supportive parts in tissue engineering.

Although the *in vitro* evaluation of cardiomyocytes and cardiac tissues had been limited to electrophysiological analyses, recent advances in tissue engineering have opened the door to functional analyses of beating motion, contraction force and inner pressure. Eschenhagen and Zimmermann's group have created functional ring-shaped cardiac tissues by mixing cardiac cells with collagen gel and realized the measurement of their contraction force [20]. We have also developed contractile force measurement system using hiPSc-derived cardiac cell sheets on flat fibrin gel [21]. However, it is difficult in both constructs to measures inner pressure due to lack of chamber-like structure. Regarding chamber-like structure, it has been reported that functional pouched cardiac tissues were fabricated by casting collagen gel and cardiomyocytes [22]. In that report, the main object was to assist cardiac function by covering the pouch-like cardiac tissue over a rat heart; therefore evaluation of inner pressure was not clearly demonstrated. On the other hand, we have demonstrated significant inner pressure changes as a heart tissue model in both previous and present studies using a cell sheet wrapping technique. Cardiomyocyte sheets which are composed of electrically communicated cardiomyocytes can beat simultaneously. This synchronized beating is maintained even after wrapping the cell sheets. It should contribute to the induction of inner pressure changes.

Recently many researchers including us have developed human cardiac tissue models to assess the drug cardiac toxicity and genomic pathological phenotypes [21,23–26]. However, almost all of them are ring-shape [23], bundle [24,25] and squared [21] tissues and contractile force is mainly measured. On the other hand, this study presented the tubular cardiac tissues that are capable of measuring inner pressure changes. In the clinical setting, intraventricle pressure changes are usually measured using catheter to evaluate heart function and hemodynamics. Inner pressure and inner pressure changes are affected by the pre-load and after-load, and functional changes and drug responses according to changes of pre-load and after-load will lead to understand the characteristics of human cardiac tissue model and drug development.

In this study, for examining the Frank-Starling Law, the tubular cardiac tissues were expanded by narrowing the outlet tip and increasing the flow speed. Thus, some physiological conditions have been reproduced; however, more developments will be needed to imitate native ventricular function. One of the inevitable issues is to introduce valve functions in both the outlet and inlet of tubular cardiac tissues. If the functional valves are equipped, pressure-volume loop studies may be conducted. The effort to introduce valves into the tubular constructs is now on going. Furthermore, since recent study has reported the chamber specific differentiation of cardiomyocyte from human pluripotent stem cells and fabrication of heteropolar atrioventricular tissues [25], two chamber cardiac tissue will be fabricated in the future.

The results of RT-qPCR showed that the expression of some genes including NPPA, MYL2 and MYL7 significantly increased in the cardiomyocyte tube rather than in the static culture. NPPA gene expression is an early and specific marker for differentiating cardiomyocyte [27]. MYL2 and MYL7 are specific markers for ventricular and atrial cardiomyocytes, respectively. Cardiac tissues in the present study were mainly composed of cardiomyocytes and we previously reported that almost all of cardiomyocytes were MLC2v positive ventricular cardiomyocytes [21]. Electrical stimulation has been reported to promote cardiomyocyte maturation [24] and recent report has suggested that ventricular cardiomyocytes after the chamber specific differentiation from human iPS cells show the upregulation of not only ventricular genes but also atrial genes after the electrical stimulation [25]. Therefore, upregulation of MYL2 and MYL7 expression in tubular cardiac tissues might be due to the certain extent levels of maturation. Mechanical stimulation, electrical stimulation, hypertrophic signal activation with growth factor and metabolism modification have been reported to introduce the maturation of hiPSc-derived cardiomyocytes [27]. These methodologies are also applicable to our tubular cardiac tissue model. Furthermore, our previous reports on *in vivo* cardiac cell sheets wrapped around large blood vessels demonstrated significant maturation in histology and gene expression [14,17]. Mechanical stretch due to large vessel pulsation and appropriate supply of growth factors and nutrients from blood may contribute to cardiac tube maturation. As with these *in vivo* phenomena, pulsatile media flow through the tubular cardiac tissue and perfusion media modification with molecules accelerating tissue maturation may enable immature tubular cardiac tissue to mature enough for an adult cardiac tissue model.

In addition to tissue maturation, more cardiomyocyte sheets layering may improve the function of tubular myocardial tissues. However, layering number is limited due to nutrient and oxygen deficiency in thick tissues [28]. To solve this obstacle, perfusable blood vessels must be introduced. It is more difficult to introduce functional blood vessels *in vitro* rather than *in vivo*. Cell sheets co-cultured with endothelial cells formed endothelial networks; however, the networks lack continuous luminal structure. *In vivo*, these pre-vascular structures change into true blood vessels connecting to host blood vessels and contribute to vascular formation in cell sheets. On the other hand, *in vitro*, these pre-vascular structures never form true perfusable blood vessels in a static culture condition. Therefore, we have developed the original methodology layering co-cultured cell sheets step-by-step on the vascular bed which include a perfusable micro luminal structure and is perfused with culture media in the bioreactor system [29]. *Ex vivo* femoral tissues including a connectable artery and vein, and collagen gel including micro channels are used as vascular beds. Co-cultured endothelial cells form perfusable blood vessels *in vitro* as same as *in vivo*, and thicker tissues are gradually fabricated by the multi-step procedure. Combination of the cell sheet wrapping technique and

vascularization technique may realize in the future more functional tubular cardiac constructs.

5. Conclusion

This study demonstrated the original functional heart tissue model for detecting inner pressure using hiPSc-derived cardiomyocytes and cell sheet-based tissue engineering. Scaffold-free tubular cardiac tissues were successfully fabricated by wrapping cardiomyocyte sheets around the octagonal column with slits. NHDF sheets were used for supporting the tubular structure. Engineered human cardiac tubes evoked spontaneous beating, electrical potential and inner pressure changes. Additionally myocardial tube followed electrical stimulation. We believe that the fabricated pulsatile cardiac tube will contribute to future drug screening and regenerative therapy for heart diseases.

Conflict of interest

Shinpei Tsuruyma is a shareholder of SHIBUYA CORPORATION. Tatsuya Shimizu was a member of the scientific advisory board and a shareholder of CellSeed, Inc. Tatsuya Shimizu and Katsuhisa Matsuura are inventors of the bioreactor systems. Tokyo Women's Medical University, Tatsuya Shimizu and Katsuhisa Matsuura received a license fee from ABLE Corporation. Tokyo Women's Medical University receives research funds from CellSeed, Inc.

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