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3D bioprinted functional and contractile cardiac tissue constructs

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Abstract

Bioengineering of a functional cardiac tissue composed of primary cardiomyocytes has great potential for myocardial regeneration and *in vitro* tissue modeling. However, its applications remain limited because the cardiac tissue is a highly organized structure with unique physiologic, biomechanical, and electrical properties. In this study, we undertook a proof-of-concept study to develop a contractile cardiac tissue with cellular organization, uniformity, and scalability by using three-dimensional (3D) bioprinting strategy. Primary cardiomyocytes were isolated from infant rat hearts and suspended in a fibrin-based bioink to determine the printing capability for cardiac tissue engineering. This cell-laden hydrogel was sequentially printed with a sacrificial hydrogel and a supporting polymeric frame through a 300- μ m nozzle by pressured air. Bioprinted cardiac tissue constructs had a spontaneous synchronous contraction in culture, implying *in vitro* cardiac tissue development and maturation. Progressive cardiac tissue development was confirmed by immunostaining for α -actinin and connexin 43, indicating that cardiac tissues were formed with uniformly aligned, dense, and electromechanically coupled cardiac cells. These constructs exhibited physiologic responses to known cardiac drugs regarding beating frequency and contraction forces. In addition, Notch signaling blockade significantly accelerated development and maturation of bioprinted cardiac tissues. Our results demonstrated the feasibility of bioprinting functional cardiac tissues that could be used for tissue engineering applications and pharmaceutical purposes.

Keywords

Cardiac tissue; Contractility; Heart failure; Bioprinting; Tissue engineering; *In vitro* tissue model; Body-on-a-chip

1. Introduction

Due to high mortality and morbidity, heart failure remains to be a leading cause of death in the United States [1]. Most heart failures exhibit cardiomyocyte loss, and the adult mammalian heart does not sufficiently restore cardiomyocytes after damage [2]. Currently, heart transplantation offers the best option at the end-stage of heart failure; however, replacing the damaged heart with a healthy one raises several limitations, including

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inadequate organ availability for transplantation, immune rejection, and surgical complications. Meanwhile, *in vitro* heart disease modeling, which is important for drug discovery and development, is particularly difficult due to the challenges of obtaining a functional cardiac tissue that accurately represents the structural and functional characteristics of native heart. Thus, strategies to improve heart regeneration and *in vitro* tissue modeling, especially through tissue engineering principle, have gained increasing attention [3,4].

Although bioengineered tissue constructs can be an effective tool for studying cellular function and tissue morphogenesis/function in a controllable microenvironment [5–7], the intrinsic limitations of tissue engineering technologies to recreate complexes and similarities from native tissues restrict their applications. Recent advances in three-dimensional (3D) bioprinting strategy show promise as a viable option for creating functional tissue constructs that are designed to regenerate or replace a damaged tissue and organ [8–14]. The bioprinting methods can place cell-laden hydrogel bioinks in a layer-by-layer fashion, replicating the 3D architecture of the tissues or organs of interest. Therefore, this strategy is especially suitable for creating arbitrary 3D tissue constructs, like human tissues and organs, by precise placement of multiple materials, including cells, biomaterials, and components of the extracellular matrix (ECM) [14].

In this study, we aimed to develop a functional cardiac tissue that can mimic the structural, physiological, and functional features of native myocardium. We hypothesized that this bioengineered cardiac tissue composed of dense and uniformly aligned cardiomyocytes could accelerate the cardiac tissue development with spontaneous and synchronous contraction. The bioprinted cardiac tissues were fabricated into centimeter-scale and examined by immunofluorescence, calcium imaging, and contractile force measurement. We also evaluated these bioprinted cardiac tissues by physiologic responses to known cardiac drugs and developmental responses to critical cues, including mechanical force and biochemical cues.

2. Materials and methods

2.1. Isolation of neonatal rat ventricular cardiomyocytes

All animal procedures were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Wake Forest University Health Sciences. Neonatal rat ventricular cardiomyocytes were isolated from 1- to 3-day-old Sprague-Dawley rats (Charles River Laboratories Inc., Wilmington, MA) using enzyme digestion with trypsin and collagenase as reported previously,[15,16] with minor modification. Neonatal rat hearts were harvested and immediately washed with ice-cold PBS. Hearts were pre-digested with 0.05% trypsin (Hyclone, GE Healthcare Bio-Sciences, Pittsburgh, PA) at 4 °C overnight. On the second day, hearts were washed with phosphate buffered saline (PBS, Hyclone) twice and then digested with pre-warmed 0.1% collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ) at 37 °C for 8 min. The supernatant was then transferred to 50 ml Corning® tube (Corning, NY) containing 10% fetal bovine serum (FBS, Hyclone) on ice. Fresh pre-warmed collagenase solution was added to hearts for additional digestion for 8 min. Digestion was repeated for a total of 5

cycles. Supernatant after digestion was pooled together, neutralized with 10% FBS, and then washed with PBS twice. The digested heart tissues were resuspended in 10 ml of Dulbecco's Modified Eagle's medium (DMEM, Hyclone) plus 10% FBS and then placed on ice for 5 min to allow larger tissue debris to precipitate. After cell counting, the supernatant was carefully transferred to another 50 ml Corning® tube and centrifuged at 300g for 5 min. The isolated cells were immediately mixed with a fibrin-based composite hydrogel for 3D bioprinting as described below.

2.2. Preparation of composite hydrogel as a bioink

The fibrin-based composite hydrogel was used as a bioink, which contained 20 mg/ml fibrinogen, 30 mg/ml gelatin, 20 µg/ml aprotinin, 10% glycerol, and 3 mg/ml hyaluronic acid (HA) in DMEM culture medium. Sacrificial hydrogel contained 30 mg/ml gelatin, 10% glycerol, and 3 mg/ml HA was used to support the cell-laden hydrogel in the printed constructs. The all components were thoroughly dissolved in DMEM using a rotor mixer at low speed (30 sec per rotation) for 30 min. The composite hydrogels were then sterilized by filtration through a 0.2 µm filter. All chemicals were obtained from Sigma-Aldrich Co. (St Louis, MO) unless stated otherwise.

2.3. Fabrication of cardiac tissue constructs using integrated tissue-organ printing (ITOP) system

Our integrated tissue-organ printing (ITOP) system consisted of an X, Y, Z-axis stage/controller and multiple dispensing modules. [14] A three-axis stage system (Aerotech, Inc., Pittsburgh, PA) having $200 \times 200 \times 100 \text{ mm}^3$ travel and controller was used to provide motions for the printing process. The dispensing modules had a precision pneumatic pressure controller (Musashi Engineering, Inc., Tokyo, Japan), a customized metal syringe, and micro-scale metal nozzle (TECDIA, Tokyo, Japan). Customized computer-aided design/manufacturing (CAD/CAM) process, including slicing, tool path generation, and motion program generation, was used to design the aligned cardiac tissue constructs for the printing process.

For the cardiac tissue constructs, we utilized three dispensing modules for cell-laden hydrogel, sacrificial hydrogel, and poly(ϵ -caprolactone) (PCL), separately. The isolated cardiomyocytes were mixed with the prepared composite hydrogel bioink at the concentration of 10×10^6 cells/ml before being placed on ice for 30 min. The cell-laden hydrogel was then placed in a bioprinter chamber (preset to 18°C) for 30 min to allow the hydrogel temperature to reach to 18 °C. All bioprinting processes were performed at 18 °C, which was maintained by the environmental control system of the bioprinter. PCL (MW = 4000–5000, Polysciences, Inc., Warrington, PA) was used to fabricate a frame structure, allowing the cell-laden hydrogel structure to be anchored. The cardiomyocyteladen hydrogel bioink was dispensed to form a string form or patch form of the printed cardiac constructs. Briefly, 3D printing of the cardiac tissue constructs was performed under the following conditions: the PCL frame was printed at 98 °C using a 300 µm metal nozzle with 750 kPa pneumatic pressure, and the cell-laden hydrogel and the sacrificial hydrogel were printed at 18 °C using a 200 µm Teflon nozzle with 100 kPa pneumatic pressure. Printing speed was 100 mm/min. The printed constructs were immediately immersed in DMEM medium

containing 50 U/ml thrombin and kept at room temperature for 20 min for gelation. Then, the constructs were cultured in Claycomb medium (Sigma-Aldrich Co.) supplemented with 10% FBS, 1% L-glutamine, 10 µg/ml aprotinin, and 1% antibiotic/antimycotic at 37 °C with 5% CO₂. Cell culture medium was replaced every 3 days.

2.4. Gross observation and immunofluorescent analyses

Gross images of the printed cardiac tissue constructs were captured weekly until 4 weeks. Images and videos of printed cardiac tissue were taken at designated time points. The videos of spontaneous contraction of printed cardiac tissues were recorded at 50 magnification, using a Cannon G11 camera (Melville, NY) with a Zeiss microscope (Jena, Germany) under a high-quality video mode (640 480 resolution, 30 fps). The videos were processed by an OGG Video Converter 6 (FastSpring, Santa Barbara, CA) and converted into image sequences. ImageJ software (National Institute of Health, Bethesda, MD) with MTrackJ or MTrack2 plug-ins was used to process image sequences, identify motion of the bioengineered cardiac tissue, and graphically plot the results.

For immunofluorescent staining, the cardiac tissue constructs were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with protein blocker (Dako, Agilent Technologies, Santa Clara, CA), and incubated with primary antibodies diluted in antibody diluents (Dako) for 1.5 h at room temperature. Samples were washed in PBS and incubated with secondary antibodies for 30 min at room temperature, and mounted with Vector Vectashield Mounting medium (Vector Laboratories, Burlingame, CA). The primary antibodies were α -actinin (1:50, Abcam, Cambridge, MA) and connexin 43 (1:100, Abcam), and the secondary antibodies were TexRed-conjugated goat anti-rabbit IgG (1:200, Vector Laboratories) and FITC-conjugated goat anti-mouse IgG (1:200, Vector Laboratories). Imaging analyses, including cell coverage, perimeter, fiber alignment, and sarcomere length were performed by ImageJ software.

2.5. Calcium imaging analysis

In order to validate the progressive synchronous contraction along with tissue maturation in bioprinted cardiac tissues, calcium imaging analysis was performed at 1 and 3 weeks in culture. Bio-printed cardiac tissue constructs were incubated with 3 µM Fluo-8 AM (Abcam) for 15 min at 37 °C. The constructs were subsequently washed with DMEM medium supplemented with 10% FBS for 3 times and placed on a heating adaptor on a Zeiss inverted microscope system (Zeiss Axiovert 200). Calcium influx fluorescent videos were recorded by a Cannon G11 camera with the Zeiss microscope under high-quality video mode (640 480 resolution, 30 fps). Fluorescence intensity (F) was normalized based on basal tissue fluorescence after dye loading (F₀). ImageJ software was used for plotting and quantifying the beating of the bioprinted cardiac tissues.

Cardiac drug response on bioprinted cardiac tissues was examined by calcium imaging analysis. Known cardiac drugs, epinephrine (200 nM, Sigma-Aldrich Co.) and carbachol (10 µM, Sigma-Aldrich Co.) were exposed to bioprinted cardiac tissues at 3 weeks in culture, respectively. Calcium imaging was monitored to detect the synchronous contraction of bioprinted cardiac tissues, as described above.

2.6. Contractile force measurement

For direct measurement of contractile force from the printed cardiac tissues, a micro-spring device integrated with the construct was printed under the same printing conditions as described above. After 4 weeks in culture, printed micro-springs with the cardiac tissue constructs were tested using a DMT 750TOBS tissue organ bath system (DMT-USA, Ann Arbor, MI) to generate standard curves of force versus the motion of the scale on the micro-spring stem, shown in the following equation.

$$F_i = k D_i$$

Where k refers to the spring deformation stiffness, F represents the force generated by the micro-spring, i refers to the particular image in the image sequence, and D represents the distance of motion scale on micro-spring traveled from baseline in a given image in the sequence. Videos of printed micro-spring movement were recorded at 200× magnification, 30 frames per second at 640 × 480 resolution. Original color videos (.MOV file) were processed by Microsoft Movie Maker (Microsoft, Redmond, WA) to generate gray videos. Then, the videos were processed by an OGG Video Converter 6 and converted into image sequences. ImageJ software was used to crop the motion scales from the empty background on stacked images. Movement of motion scales was traced by ImageJ software with MTrackJ or MTrack2 plug-ins and plotted to force schemes.

Force measurement responding to epinephrine was performed with bioprinted cardiac tissues at 3 weeks in culture. The constructs were subsequently washed with DMEM supplemented with 10% FBS for 3 times and epinephrine with different concentrations (a range of 2 nM–200 μM) was added to the culture medium. The constructs were incubated at 37 °C for 15 min and then placed on a heating adaptor on Zeiss inverted microscope system for video capturing. The contractile force was measured as described above.

2.7. Notch signaling interruption on bioprinted cardiac tissues

Notch inhibitor, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (5 μM DAPT, Abcam) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Co.) and added into the culture medium for bioprinted cardiac tissues. The culture medium with DAPT was changed every two days. Immunofluorescence and calcium image analysis were performed at 1 week in culture of bioprinted cardiac tissues as described above.

2.8. Statistical analysis

Data were analyzed with Student's t -test or one-way ANOVA using GraphPad Prism software version 3.0a (GraphPad Software, Inc., La Jolla, CA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Bioprinted cardiac tissue constructs

Bioprinted cardiac tissues were fabricated by processing three components; cell-laden hydrogel, sacrificial hydrogel, and PCL polymer, using 3D bioprinting method. The constructs composed of cardiomyocytes were printed layer-by-layer as designed with customized CAD/CAM software (Fig. 1A) and our custom-made ITOP system (Fig. 1B,C). The PCL frame structure and hydrogels were sequentially dispensed to fabricate the designated printing patterns (Fig. 1D). The cell-laden hydrogel was designed to be printed crossing over the PCL frame to maintain its pattern alignment along with the printed pattern direction. Bioprinted cardiac tissue constructs were fabricated into string form and patch form depending on the numbers of printing beams in the constructs (Fig. 1E,F). The numbers of beams were 16 for the string form and 76 for the patch form. The entire construct was $1.8 \times 1.6 \text{ cm}^2$ with 0.6 mm thick. Total printing times per a construct was 7 min for the string form and 12 min for the patch form.

3.2. Gross observation and immunofluorescent analyses

On gross observation during the culture after bioprinting, bio-printed cardiac tissue constructs gradually shrunk on the two sides that were not attached to the PCL frame (Fig. 2A). After shrinking in the first 4 weeks after bioprinting, the dimension of the cardiac tissue constructs stayed stable for up to 9 weeks of culture, maintaining 55% of the initial tissue size. To assess whether our 3D printed cardiac tissue constructs are functional, we examined their contractile function. At 3 days in culture, bioprinted cardiac tissues exhibited weak, localized, and spontaneous contraction. The spontaneous and synchronous beating of bioprinted cardiac tissues started at 1 week in culture and was maximized at 3–4 weeks in culture (Fig. 2A and Supplementary Movie 1(a)).

The most profound histologic feature of bioprinted cardiac tissue was the formation of dense and uniformly aligned heart muscle bundles. At the earliest stages, a group of round shaped cells were dominant in the construct at 4 days in culture. During the culture, a few cells showed elongation, and the elongated α -actinin positive cells increased within the construct at 1 week. Eventually, the bioprinted cardiac constructs were developed aligned cardiomyocytes with clear sarcomere structure, which filled up the entire space of the construct at 3 weeks in culture (Fig. 2B). Connexin 43 was also expressed at 3 weeks of bioprinted constructs. We found a rapid increase in α -actinin positive cell area, cardiomyocyte perimeter, and the alignment of muscle fibers (Fig. 2C). Cardiomyocyte sarcomere length did not significantly change during tissue development, implying this phenotype may be determined by cell genetic background instead of the microenvironment where tissues developed. Overall, bioprinted cardiac tissues developed from separated individual cardiomyocytes into connected, aligned and densely stained cardiac tissue, filling the entire constructs in both string and patch forms. This *de novo* cardiac tissue formation suggested that 3D bioprinting strategy could mimic the structural feature of the cardiac tissue.

3.3. Synchronous contraction of bioprinted cardiac tissues

Tissue development correlated to the contractile of bioprinted cardiac tissues was confirmed at 1 and 3 weeks in culture by α -actinin immunostaining (Fig. 3A). At 1 week in culture, the cellular orientation occurred, but limited tissue development was represented in comparison with 3-week cultured cardiac constructs. Synchronous contraction of these cardiac tissue constructs was confirmed by the global alignment and connection of cardiomyocytes on calcium imaging, with a calcium influx spreading across the entire tissue (Fig. 3B and Supplementary Movie 1(b)). Indeed, such synchronous beating occurred gradually after bioprinting and the tissue development was directly associated with its contractility. Bioprinted cardiac tissues at 1 week in culture only had limited aligned cardiac cells with irregular beating on calcium imaging, while developing into dense and uniformly aligned cardiomyocytes at 3 weeks in culture, bioprinted cardiac tissues exhibited strong and synchronous contractility. Our results indicated a gradual progression of tissue development of *de novo* bio-printed cardiac tissue with uniformly aligned and dense cardiac tissues with increased ability for synchronous beating capability in later stages.

To investigate the feasibility using bioprinted cardiac tissues as an *in vitro* 3D tissue model, the constructs were examined drug response with known cardiac drugs, including epinephrine and carbachol. We performed drug response assays using calcium imaging analysis to monitor and quantify spontaneous beating of bioprinted cardiac tissues. Bioprinted cardiac tissues showed a typical response to cardiac drugs (Fig. 4A,B and Supplementary Movie 1(c)). The classical androgen agonist epinephrine increased the beating frequency from 80 to 110 BPM, while the androgen antagonist carbachol (10 μ M) decreased the beating frequency to 40 BPM. These effects were reversible when the drugs were removed from bioprinted cardiac tissues, indicating that bioprinted cardiac tissues could physiologically respond to cardiac drugs.

3.4. Contractile force of bioprinted cardiac tissues

We fabricated the micro-spring device that integrated with bio-printed cardiac tissue to measure the contractile force (Fig. 5A). When bioprinted cardiac tissue contracted, it pulled the micro-spring to move back and forth. These motions were transformed into the force generated by bioprinted cardiac tissues. To convert the movement of micro-springs to the contractile force of bio-printed cardiac tissues, we loaded them into the organ bath system to assess the force of the micro-spring. The micro-spring showed a consistency of bending stiffness ($k = 55 \mu\text{N}/\mu\text{m}$) within a broad linear range of up to 450 μm (Fig. 5B), which covered the range of motion by the cardiac tissue contraction. To evaluate the dosage effect on the contractile force from bioprinted cardiac tissue, epinephrine with a dose ranging from 20 nM to 200 μM was added. At the range from 20 nM to 2 μM of epinephrine, the contractile forces (2.80 mN) of bioprinted cardiac tissues were stayed. When epinephrine concentrations reached 20 μM or 200 μM , the contraction force of bioprinted cardiac tissues increased to 7.26 mN and 6.80 mN, respectively (Fig. 5C,D and Supplementary Movie 2).

3.5. Notch signaling blockade on cardiac tissue development

We investigated the effects of Notch signaling inhibition on bio-printed cardiac tissues which contained neonatal cardiomyocytes. Notch signaling on bioprinted cardiac tissues was

interrupted by adding Notch inhibitor (5 μ M DAPT). At 7 days in culture, the tissue development of bioprinted tissues treated with DAPT was enhanced, resulting in burst peak fluorescent signals on calcium imaging and synchronous contraction, while the control group (non-treated cardiac tissue) showed isolated activation of calcium influx and lack of synchronous contraction (Fig. 6A,B). Histologically, DAPT-treated cardiac tissues showed a higher percentage of α -actinin expression, cardiac muscle fiber occupancy, and cellular alignment compared with non-treated cardiac tissues (Fig. 6C,D). These results indicated that Notch signaling inhibition could accelerate earlier formation of synchronous contraction in bioprinted cardiac tissues with highly aligned muscle fibers.

4. Discussion

Bioengineered cardiac tissues have been developed by either seeding cells on polymeric substrates or condensing cells in a plastic mold [15,17–20]; however, their utilizations have been limited due to lack of cellular organization, uniformity, and scalability. To overcome these limitations, we have investigated the feasibility using 3D bioprinting strategy to develop the functional cardiac tissues with spontaneous synchronous contraction and spatial organization similar to native myocardium. In this study, we were able to print a variety of shapes of cardiac tissues with distinct geometries, demonstrating the capability of 3D bioprinting in geometry control. Compared with the other engineering approaches such as solid substrate seeding [21–23] or hydrogel molding [20,24], 3D bioprinting strategy has inherent advantages in fabricating a highly organized cardiac structure. Although several studies have reported cardiac tissue engineering and bioprinting in the past several years [25–28], the cardiac constructs reported in past were small scale with low-level tissue maturation and disoriented muscle fibers, resulting in weak contractility. Therefore, we utilized 3D bioprinting principle to fabricate uniformly organized, centimeter-scaled cardiac tissues. We designed a simple aligned cardiac structure composed of cardiomyocyteladen hydrogel and supporting PCL polymer frame that allowed bioprinted cardiac tissues to develop into dense and universally aligned cardiac muscle bundles with synchronous contraction. We also demonstrated that bioprinting approach could be used as a tool to directly fabricate force-sensors together with tissue. Here, simultaneous printing of the micro-spring device and cardiac tissue construct enabled integrated, real-time force measurements of cardiac contractility. In addition, bioprinted cardiac tissue physiologically responded to known cardiac drugs and displayed improved tissue formation by inhibition of Notch signaling.

In vitro tissue models based on 2D cell culture could not provide a microenvironment that resembles 3D features of native cardiac tissue. Recently, a variety of 3D tissue culture methods has been utilized to provide a proper microenvironment for cardiac tissue development [20–24]. Moreover, 3D bioprinting methods have shown the ability to fabricate a complex, composite structure of any shape and size [14]. Since tissue engineering approaches aim to fabricate a tissue construct highly resembling native tissues structurally and functionally, 3D bioprinting strategy can be a powerful tool to generate completely aligned, dense cardiac muscle tissues. In this study, the multiple dispensing capacities of our 3D ITOP system made possible the multilayered design of the cell-based constructs for bioengineered cardiac tissues.

Regarding the design concept, anchoring points to attach cellularized constructs are critical for successful bioengineering of organized tissue structures. These anchors allow the intrinsic force to induce the compaction phenomenon within the tissue construct, and in turn, the force facilitates the development of muscle fibers and guides their alignment [14,15,24,29]. Here, we applied this principle by using the PCL frame to anchor bioprinted cardiac construct. When tissues were not anchored to the PCL frame, bio-printed cardiac cells failed to develop. It was shown that bioengineered cardiac tissue did not develop into functional and beating function unless it was attached to the PCL frame (Supplemental Fig. 1). We interpreted this result to mean that the intrinsic force generated by PCL frame after bioprinting was essential for cardiac tissue development and maturation. Although the compaction phenomenon is required for cardiac tissue development, we believe that the aligned architecture generated by 3D printing strategy can synergistically affect the cellular orientation and maturation of the bioprinted cardiomyocytes along with the PCL frame.

Biomechanical interactions exist in the ECM and neighboring cells regulate developmental processes of the myocardial tissue [30]. Rhythmic mechanical stretching was also reported to increase cardiomyocyte proliferation and maturation in bioengineered tissues [31]. Thus, the tissue development of bioprinted cardiac tissues implied not only the changes in cells and ECM components, but also in biomechanical properties of bioprinted cardiac tissues. Contractility is one of the most important indicators for determining the cardiac tissue development. Up to now, the contractile measurement of bioengineered cardiac tissues was mostly done by measuring the curvature of supporting substrate or micro-cantilever [17,18], or using customized micro-organ chambers [20,32]; however, these attempts may not be suitable for centimeter-scale tissues or high-throughput screening. In this present study, we were able to simultaneously print the micro-spring device, a force biosensor, integrated with bioprinted cardiac tissue, which could measure the real-time contractibility of bioprinted cardiac tissue. By using the printed micro-spring, we observed that the intrinsic force that generated within bioprinted cardiac tissue was up to 1.5 mN, indicating that bioprinted tissue has being constantly “stretched” by a significant force. In the future studies, we will utilize the capability of 3D bioprinting in fabricating multiple controlling and sensing devices such as micro-channels [33–35] and micro-electrical circuits [36,37] into a printed tissue construct. This can make possible to obtain real-time monitoring of multiple physiologic readings during the tissue development or pharmacologic testing.

Although thin slices of adult human hearts have been reported as an *in vitro* model for pharmacologic testing [38], apparent shortcomings, including the shortage of organ donors and low viability of heart tissues *in vitro*, limit its applications. Adult human cardiomyocytes are also limited due to their short live span *in vitro* with a poor proliferation capability [39]. Recently, the human induced pluripotent stem cell (iPSC)-derived cardiac cells have been used as an alternative cell source. Such an *in vitro* tissue model containing human-derived cardiac cells has great potential in exploring heart pathology and drug discovery, such as long QT syndrome (LQTS) [40] and familial dilated cardiomyopathy [41].

Our current work shows that bioprinted cardiac tissue is not structural and functional like native tissue immediately after printing; instead, the tissues are gradually matured after 3 – 4

weeks in culture. Also, the tissue formation during the culture could be modulated by biochemical and physical cues. Notch signaling plays an important role in heart development and regeneration [42–45]. Previous *in vivo* studies in Notch knock-out transgenic mice demonstrated that Notch inhibition diminished proliferation in neonatal cardiomyocytes and promoted maturation [46]. It is unclear whether *in vitro* Notch signaling regulates the formation of bioengineered cardiac tissues derived from neonatal heart cells. In this study, we found that Notch signaling and force cues could alter the tissue development and maturation. Thus, our future studies should be focused on the tissue culture stage after bioprinting to find an optimal culture condition to accelerate the tissue development with various biochemical and biomechanical signals.

5. Conclusion

We demonstrated the feasibility of 3D bioprinting strategy to bioengineer the functional cardiac tissue that possesses a highly organized structure with unique physiological and biomechanical properties similar to native cardiac tissue. Moreover, 3D bioprinting is the powerful tool for spatial patterning and micro-sensing and controlling. This bioprinting strategy has great potential to precisely generate functional cardiac tissues for use in pharmaceutical and regenerative medicine applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.actbio.2018.02.007>.

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Statement of Significance

Cardiovascular disease remains a leading cause of death in the United States and a major health-care burden. Myocardial infarction (MI) is a main cause of death in cardiovascular diseases. MI occurs as a consequence of sudden blocking of blood vessels supplying the heart. When occlusions in the coronary arteries occur, an immediate decrease in nutrient and oxygen supply to the cardiac muscle, resulting in permanent cardiac cell death. Eventually, scar tissue formed in the damaged cardiac muscle that cannot conduct electrical or mechanical stimuli thus leading to a reduction in the pumping efficiency of the heart. The therapeutic options available for end-stage heart failure is to undergo heart transplantation or the use of mechanical ventricular assist devices (VADs). However, many patients die while being on a waiting list, due to the organ shortage and limitation of VADs, such as surgical complications, infection, thrombogenesis, and failure of the electrical motor and hemolysis.

Ultimately, 3D bioprinting strategy aims to create clinically applicable tissue constructs that can be immediately implanted in the body. To date, the focus on replicating complex and heterogeneous tissue constructs continues to increase as 3D bioprinting technologies advance. In this study, we demonstrated the feasibility of 3D bioprinting strategy to bioengineer the functional cardiac tissue that possesses a highly organized structure with unique physiological and biomechanical properties similar to native cardiac tissue. This bioprinting strategy has great potential to precisely generate functional cardiac tissues for use in pharmaceutical and regenerative medicine applications.

Fig. 1. Bioprinting of cardiac tissue constructs. (A) Basic motion program and (B,C) our customized ITOP system containing three components for bioprinting cardiac tissues. (D) Timelapse image sequence of cardiac tissue printing. Bioprinted cardiac tissue constructs: (E) string form and (F) patch form.

Fig. 2.

(A) Gross appearance and cardiac beating measurement with time: (left) progress tissue development of the patch formed cardiac tissue was observed and (right) plotting of spontaneous beating based on bright field video with time. (B) Immunofluorescent images of bioprinted cardiac tissues: α -actinin at 4 days and 1 week in culture and α -actinin/connexin 43 at 3 weeks after bioprinting. Bioprinted cardiomyocytes expressed α -actinin (red), connexin (green) and cell nuclei (blue). (C) Quantification of cardiac tissue development at 4 days, 1, and 3 weeks in culture ($n = 3$). * $P < 0.05$ compared with control. ** $P < 0.05$ compared with others. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Calcium imaging analysis of bioprinted cardiac tissues at 1 and 3 weeks in culture. (A) Immunofluorescence for α -actinin of bioprinted cardiac tissues at 1 and 3 weeks in culture, representing different levels on cellular organization and tissue development: α -actinin (red) and cell nuclei (blue). (B) Calcium imaging analysis for synchronization of bioprinted cardiac tissues at 1 and 3 weeks in culture (Supplementary Movie 1(b)). At 3 weeks, bioprinted cardiac tissue showed regular synchronous contractile behavior. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Cardiac drug responses. (A) Bioprinted cardiac tissue responded to epinephrine (200 nM) and carbachol (CCH, 10 μ M) as confirmed by changes in beating frequency and amplitude (Supplementary Movie 1(c)). (B) Quantification of beating frequency (beats per minute, BPM) of bioprinted cardiac tissues, responding to cardiac drugs ($n = 3$). *, ** $P < 0.05$ compared with control (baseline).

Fig. 5. Contractile force measurement of bioprinted cardiac tissues by micro-spring integration. (A) Printed micro-spring device used to measure contractile force generated by bioprinted cardiac tissues: (a) Illustration of micro-spring motion during diastolic and systolic phases of cardiac contraction cycle in bioprinted cardiac tissue. (b) Gross images of micro-spring for force measurement. (B) Motion (μm) derived from the contraction of bioprinted cardiac tissue was converted to force (mN). Dashed line shows 95% confidence range of linear aggregation. (C) Representative plots of the force generated by bioprinted cardiac tissues in response to different doses of epinephrine (Supplementary Movie 2). (D) Quantification of peak force generated by bioprinted cardiac tissues in response to different doses of epinephrine ($n = 3$). *P < 0.05 compared with control.

Fig. 6.

Notch signaling blockade on bioprinted cardiac tissues. (A) Calcium images on synchronous contraction of bioprinted cardiac tissues with and without DAPT treatment at 1 week in culture, Notch signaling blockade (DAPT) resulted in early formation of synchronous contraction, while no synchronous contraction in control (non-treated). Scale bar = 100 μm. (B) Plotting of beating frequency of bioprinted cardiac tissues from Notch signaling blockade and control groups at 1 week in culture. (C) Immunofluorescent analyses of bioprinted cardiac tissues with and without DAPT treatment at 1 week in culture: α-actinin (red) and cell nuclei (blue). Scale bar = 100 μm. (D) Quantification of cardiac tissue development by measuring frequency of α-actinin positive cells, cardiomyocyte area, cardiac muscle cell alignment, and cardiomyocytes perimeter in Notch blockade and control groups (n = 3). **P < 0.05 compared with control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)