Fabrication of 3D tissue constructs using standing post platform

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Abstract

3D tissue constructs offer a simplified model that mimics the in vivo tissues. Standing post platform has been established in this study to fabricate 3D tissues with a variety of shapes. Non-fouling PDMS molds cast from ABS templates were seeded by cell-laden collagen type I scaffolds. The scaffolds were aggregated to form the tissues within 24 hours. Human fibroblast, hiPSC-CM, and hiPSC-MSC tissue rings were successfully fabricated with this standing post platform. Ring contracture of fibroblasts and hiPSC-MSCs was observed and compared with different collagen density, different cell seeding density, and different cell culture concentration. In addition, the tissue constructs could also be cultured with serum-free media. By increasing the number of the standing posts, oval and triangle-shaped hiPSC-MSC tissue constructs have been also created. Macroscopically morphological changes were recorded and compared. With these results, the standing post platform was proven its consistency to fabricate tissue constructs, and ready to be used across different cell types suitable for quantitative analysis.
Fabrication of 3D tissue constructs using standing post platform

By

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B.S. Industrial Engineering, Kasetsart University, 2015

Thesis

Submitted in partial fulfillment of the requirements for the degree of Master of Sciences (M.S.) in Bioengineering

Syracuse University

August 2018
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1. Introduction

1.1. Overview of Tissue Engineering

The goal of tissue engineering is to create native-like tissues by using the combination of cells, materials, and biological cues\(^1\). The technological importance of tissue engineering is to study human-specific tissue development and diseases in living matters\(^2\text{-}^4\) and to understand the complexity of biological mechanism on the tissue level\(^5\text{-}^7\). Engineered *in vitro* models starts from 2D models, which have great reproducibility but lack of informational accuracy since native tissues are 3D\(^8\). On the other hand, although studies based on living animals produces physiological information\(^9\), it is difficult to quantitatively and consistently analyze the results to high variation of test subjects. Therefore, 3D tissue models offer us the opportunities to improve the experimental reproducibility, reduce the cost of animal testing, test with human cells, and provide the information that is absent in 2D models\(^10\text{-}^11\).

Conventionally, 3D tissue constructs are created by seeding biological cells into the biomaterial scaffolds fabricated by different methods. The advantages of tissue constructs are as follows: (1) The constructs can be formed by defined cell compositions, either from primary cells harvested from human and animals, or from immortal cells lines, such as fibroblasts (3T3\(^{12\text{-}14}\), endothelial cells (HUVEC\(^{15\text{-}17}\), smooth muscle cells (C2C12\(^{18\text{-}20}\), etc.; (2) The tissue constructs offer simplified models to investigate fundamental biological questions, while also can increase the model complexity via different techniques; (3) The tissue constructs can be used to emulate native developmental processes, such as wound healing\(^{21\text{-}23}\) and muscle development\(^{19,24,25}\); (4) The tissue constructs are readily accessible for studying tissue mechanics, structure and functions; and (5), The tissue constructs are reproducible and scalable in both size and quantity\(^{26}\).
However, tissue constructs also have disadvantages, which needs to be addressed in many research fields. The tissue constructs are fundamentally different from the native biological tissues. They are a simplified version of real organ/tissue because the constructs only contain specific types of cells and lack appropriate cellular interactions occurred in the native tissues. For instance, although, the majority of cells in hearts are cardiac muscle cells and fibroblasts that can be recreated in vitro, the incorporation of endothelial cells will need to be addressed to produce both vascularization and soluble biochemical products that can impact heart function and development. Moreover, dissimilarity in cell density and mechanical properties of the tissue constructs and targeted biological tissues can lead to the functional differences. Currently, one major challenge of tissue engineering is the lack of supportive infrastructures, such as vascular system, in the tissue constructs, which will result in insufficient nutrients and oxygen delivery available to the constructs 26.

1.2. Engineering Tissue Rings

Among all the tissue constructs, ring-shaped tissue constructs offer several advantages over conventional sheet-shaped tissue constructs: (1) The ring-shaped tissue constructs offer even force distribution leading to measuring precision since the designs circumvent force concentration in the center unlike other designs 27,28; (2) The ring-shaped tissue constructs are easier to be handled and sutured to the damaged native tissues with standard clinical procedures; (3) Specifically for cardiac tissue engineering, the ring-shaped constructs could potentially be used to mimic the structural feature of ventricular tissue sections for further analysis on contractile behaviors in a biomimetic manner. A network of ring-shaped constructs could be used to emulate human organs, such as vasculature and trachea, since a ring-shaped tissue could mimic the cross section of these organs for understanding their physiological and mechanical functions.
1.2.1. Muscle Tissue Rings

Coronary artery bypass grafting surgery (CABG), a surgery aiming to restore normal blood flow by replacing a problematic section of an artery, is performed hundreds of thousands of times yearly in United States\textsuperscript{29–31}. One in three patients is not suitable for autologous surgery because of the lack of available artery\textsuperscript{32}. Vascular tissue engineering is a way to help these patients by fabricating such an artery. Vascular tissue engineering also may provide a suitable model for studying heart diseases. Scaffold-free tissue ring fabrication has been presented for vascular tissue engineering\textsuperscript{32}. In summary, the protocol is as follow: (1) Polycarbonate template is created by traditional milling process; (2) Polydimethylsiloxane (PDMS) mold is fabricated by adding the polymer at a ratio of (10:1) (w/w) into the polycarbonate template. The PDMS mold will be ready after curing at 60°C for 4 hours; (3) Agarose mold is cast using the PDMS mold; (4) cells are seeded into the agarose mold; (5) Media is exchanged every other day beginning from 24 hours after the seeding; (6) A consistent ring should be formed within 7 days. The protocol is illustrated in Figure 1.

This protocol can be used with various types of cells, such as rat smooth muscle cells (rSMCs) and human smooth muscle cells (hSMCs) by adjusting the protocol. The tissue rings with different cells resulted in different morphology, matrix composition, and mechanical properties. By performing tensile strength testing, they found Day-14 0.66 million rSMCs rings were stronger and thicker than Day-7 0.5 million rSMCs rings and Day-14 0.75 million hSMCs rings (97 ± 30 vs 113 ± 8 vs 160 ± 30 kPa for UTS (ultimate tensile strength) and 0.94 ± 12 vs 0.53 ± 0.02 vs 0.51 ± 0.05 mm for thickness). hMSCs rings were fabricated and broken during initial pre-cycling phases of uniaxial tensile testing due to the lack of mechanical strength. However, the protocol possesses several drawbacks: (1) the method depends largely on the operator; (2) the agarose mold is not reusable, so each cell seeding requires additional fabrication process of agarose molds from PDMS molds.
1.2.2. Mesenchymal Stem Cell Tissue Rings

Mesenchymal stem cells (MSCs) are immune-evasive multipotent stromal stem cells. MSCs can be acquired from sources such as bone marrow, cord cells, adipose tissue, molar cells, etc. MSCs can give rise to many lineages such as bone, fat, chondrocyte, muscle, neuron, islet cells, and liver cells. Because of harvest and differentiation potentials, MSCs offer a bright future, but studies need to be performed before using them clinically, especially studying on differentiation pathway and immune modulation.

MSCs are also located at perivascular locations surrounding blood vessels (on both arterial and venous vessels) in the bone marrow, thus 3D MSC tissue ring and tube might be
suitable to mimic native morphology and behavior *in vitro*. Additionally, hMSC-derived cartilaginous tissue rings were fabricated with the final goal of creating tissue tubes with multiple rings\(^3\). The fabrication protocol was improved by the agarose devices for increasing throughput of tissue rings from one single mold. The cells were seed with TGFβ-loaded microspheres to fabricate the hMSC rings as depicted in Figure 2. The microspheres helped to produce thicker and darker hMSC tissue rings than their counterpart as shown in Figure 3. In the mechanical testing, hMSC and hMSC + microspheres tissue tubes had equal and higher maximum load and tensile strength compared to the native rat trachea.

**Figure 2. Tissue ring and tube fabrication diagram\(^3\).** hMSCs + growth factor loaded microsphere were loaded into agarose wells. The rings were taken out on day 2 to manufacture tissue tubes by stacking tissue rings on silicon tubes. (A) hMSCs rings and (C) hMSCs + microsphere rings were in agarose well outlined by white dots with 2 mm post outlined by black dots. (D) hMSCs tubes and (D) hMSCs + microsphere tubes were produced by stacking 3 rings (white arrow) and 6 rings (black arrow) on silicon tubes.
1.2.3. Cardiac Tissue Rings

Cardiac tissue cells or cardiomyocytes are the muscle cells of the cardiac muscles. The cells are paramount to unison contraction mechanism to deliver blood to various organs and tissues. The inability of cardiomyocytes to proliferate after birth is an obstacle to cardiac tissue repairs, thus cardiomyocytes are required to be derived from stem cells for cell transplantation. To observe cardiac tissue functions in vitro, 3D engineered cardiac tissue is to fabricate cardiac tissue for a specific part of the heart. For example, decellularized heart tissue has been executed by decellularizing whole heart and filling them with cardiomyocytes. The decellularization leaves ECM intact including blood vessel and connective tissue and eliminates cells. This method presents alternative autologous heart transplantation. Another example is prefabricated matrices method referred to as a protocol which seeding cells into prefabricated porous solid matrices. The matrices can be manipulated to desired geometry such as cardiac sheet for repairing damaged heart and cardiac tissue ring to study biological questions.

Akiyama et al. have developed the cardiac tissue ring fabrication method and introduced the concept of seeding cardiomyocytes with magnetic particles. This method used polycarbonate pole, instead of conventional PDMS or agarose mold, as illustrated in Figure 3. The method begins with mixing suspended cardiomyocytes in medium containing MCL magnetic particles (Fe₃O₄; average particle size of 10nm). After that, the MCL labeled cardiomyocytes are mixed with collagen solution. The mixture is cast into the well with polycarbonate pole in the center. Then, the cardiomyocytes are accumulated onto culture bottom by placing a magnet beneath the well to attract the MCL labeled cardiomyocytes. The cardiomyocytes layer will be formed. Excess ECM are to be aspirated before incubating the remaining cardiomyocytes for an hour after which media are added. The cardiomyocyte layer will rapidly shrink into a cardiac tissue ring within 3-5 days. The results suggested that
80% cell viability at 200 MCL pg/cell. Contractile forces were observed by inducing electrical pulses on the cardiac tissue ring. The replicability of the method is excellent, but the tissue fabricated by this method has foreign magnetic particles embedded throughout the tissue preventing usages in drug discovery and tissue replacement.

![Diagram](image)

**Figure 3. Cardiomyocyte ring fabrication diagram using magnetic**, a mixture of MCL-labeled cardiomyocytes and ECM precursor were seeded into a well with polycarbonate post. After that immediately, cardiomyocytes were attracted to the well bottom by placing a magnet under the well forming cardiomyocytes layer. Excess ECM were to be aspirated before adding the medium. Rapid shrinkage of cardiomyocytes sheet was occurred resulting in cardiomyocyte tissue ring

1.3. Human Induced Pluripotent Stem Cell (hiPSC) Technology

Induced pluripotent stem cells (iPSCs) are pluripotent stem cells derived from animal and human somatic cells by reprogramming using four factors; Oct4, Sox2, Klf4, and c–Myc. iPSCs were introduced in 2006 and rejuvenated the stem cell field since previously the only pluripotent stem cells available to researchers were embryonic stem cells (ESCs) that are highly controversial in ethics. The iPSCs have differentiation potential to any cell types, which makes them a precious tool for stem cell research and regenerative medicine. Combining hiPSC technology and tissue engineering method, mimicking native tissue with
higher accuracy could be achieved. hiPSCs-derived tissue rings have been developed for engineering vascular and perivascular tissues in vitro.

In 2016, Dash et al. have experimented on tissue-engineered vascular ring\textsuperscript{41}. The team used improved Dikina-modified Gwyther’s method\textsuperscript{36}, a scaffold-free tissue ring fabrication method. Briefly, hiPSC-derived vascular smooth muscle cells (hiPSC-VSMCs) were seeded into ring-shaped agarose well and formed tissue rings within a day after the seeding. Thinning and failure of the rings have been observed under the rings cultured with SmGM-2 within 4 to 7 days, however, the problem was solved when switching media to culture medium with 20% FBS, PDGF-BB, and TGF-β1 one day after seeding. A large amount of collagen type I was present in the rings. The average thickness was 0.84 – 0.87 mm with the inner diameter of 2 mm at day 14 – 17. The stress-strain plot was drawn in Figure 4(a). Contractility measurement was calculated by using force required to move a ring by a distance of 1.14 mm divided by its cross-sectional areas (Figure 4(b)). 1 mM carbachol and 50 mM KCl were added as an agonist to observe contractility reaction and the results were 67.35 ± 22.7 Pa and 44.44 ± 27.13 Pa respectively for different drugs (Figure 4(c)). Additionally, supravalvular aortic stenosis (SVAS) hiPSC-VSMCs have been generated by the same protocol. Contractility of control hiPSC-VSMCSs in response to carbachol was much higher than the disease hiPSC-VSMCs (42.01 ± 11.77 Pa and 5.51 ± 4.51 Pa respectively) (Figure 4(d)).
Figure 4. VSMC ring from modified Gwyther’s protocol\textsuperscript{41}. The top left image depicted the VSMC ring during the measurement (a). Stress & Strain were plotted (b). hiPSC-VSMCs contractility in response to agonists; carbochol and KCl (c). Healthy and diseased VSMC rings were compared, showing the healthy ones were stronger (d).

1.4. Goals

This study has 3 objectives: (1) Develop a protocol to generate tissue rings from several cell types (human fibroblast, hiPSC-derived MSC and hiPSC-derived cardiomyocytes); (2) Generate hiPSC-derived MSC tissue ring with chemical-defined culture media and achieve geometrical consistency and high reproducibility; (3) Create a variety of tissue shapes with a multi-posts system.
2. Methods

2.1. Overview

Figure 5 illustrated tissue ring fabrication protocol. Acrylonitrile butadiene styrene (ABS) molds were printed by a 3D printer. PDMS molds were created by using ABS molds to be a container for the cells suspension in collagen type I matrix with the expectation that cells would aggregate surrounding the post.

![Image of tissue ring fabrication protocol](image)

**Figure 5. Schematic of tissue ring fabrication.** (1) Printing ABS mold (scale bar = 3mm), (2) Casting PDMS mold (scale bar = 3mm) from ABS mold, (3) seeding cells with collagen into the PDMS mold (scale bar = 1mm), and (4) tissue rings aggregated within a day (scale bar = 1mm) were illustrated.

Different ABS molds could be designed to produce smaller tissue rings or multi-post system to produce 3D tissue structure such as triangle or ellipse. 2-post, 3-post, and 4-post molds were fabricated based on this concept. PDMS and curing agent ratio were used as 10:1 for tissue ring formation but increased to 5:1 to accommodate the multi-post system. The total amount of cell suspension mixture was adjusted linearly based on the volume of the PDMS molds. For instance, a 2-post PDMS mold was required a double amount of cells suspension mixture of a regular ring-shape PDMS mold.
2.2. PDMS Mold Fabrication

Figure 6. Schematic of a ABS mold (a) and its 3D representing image (b) and the corresponding reversed PDMS mold (c).

The ABS molds were designed using SolidWork, saved into STL files, and printed using MakerBot Replicator 2X 3D printer. The PDMS molds were designed to fit in the cell culture wells of the regular 24-well plate (21 mm in diameter). To fabricate PDMS molds with 3 mm trough and 2 mm center post designed in Figure 6, ABS molds were coated with silicone spray (stoner, G0093241) to help with PDMS detachment. PDMS and curing agent were mixed at a ratio of 10:1 (w/w) in weighing boat and poured into ABS molds. The molds containing PDMS were then transferred into a desiccator connecting to a vacuum pump and degassed for 3 hours to remove all bubbles that would negatively affect PDMS consistency. Next, the PDMS was cured in an oven at 60°C overnight.

To take the PDMS molds out of the ABS molds, the edge between the solidified PDMS and the ABS mold was traced with an X-ACTO knife gently. The PDMS molds were
removed by using a mini spoon depicted in Figure 7. Finally, the PDMS molds were rinsed in 70% Ethanol for 15 minutes for sterilization.

![Figure 7. Images of a mini spoon (a) used to remove PDMS mold, the ABS mold (b), and the PDMS mold (c).]

### 2.3. PDMS Mold Coating

The Pluronic solution was used to coat PDMS molds to have a non-fouling surface. 10% Pluronic acid was made by adding 1g of Pluronic F-127 (Pluronic® F-127, P2443) in 10 mL of distilled water and dissolving in the oven at 50°C overnight. Then, the 10% Pluronic acid was sterilized using Steriflip® Filter Unit and stored at room temperature. Before coating PDMS molds, the PDMS molds were washed with 70% Ethanol and PBS solution respectively. To coat the molds, 10% Pluronic acid was poured into the molds and incubated for 24 hours at room temperature.

### 2.4. Cell Culture

Human fibroblast cells were cultured in low glucose DMEM supplemented with 10% fetal bovine serum (v/v) (Thermofisher, 10437010), 1% Glutamax (Thermofisher, 35050061), and 1% Nonessential Amino Acids (Thermofisher, 11140076) and maintained at 37 °C in a 5% CO2 incubator. Passaging was performed approximately every week using Trypsin-EDTA (Thermofisher, 25200114) at 37 °C for 5 minutes to dissociate the cells and then transferred 10-15% cells available into each new culture flask with the same size.
hiPSC-MSCs were received from Tackla Winston. hiPSC-MSCs were maintained in serum-free StemPro MSC medium (Life technologies, A103320) at 37 °C in a 5% CO2 incubator. Passaging was performed approximately every week using Trypsin-EDTA (Thermofisher, 25200114) at 37 °C for 5 minutes to dissociate the cells and then transferred 10-15% cells available into each new culture flask with the same size. hiPSC-CMs were received from Chenyan Wang. hiPSC-CMs were maintained in RPMI media supplemented with B-27 complete.

2.5. Collagen preparation

Collagen scaffold was prepared by the combination of rat tail Collagen I (Thermofisher, A1048301), 1N NaOH (Sigma-Aldrich, 1091371000), distilled water, and 10X PBS (Thermofisher, AM9625) according to manufacturer’s instruction.

\[
\begin{align*}
\text{Volume of collagen needed (V1)} &= \frac{\text{Final conc. of collagen} \times \text{Total Volume (V1)}}{\text{Initial conc. of collagen}} \\
\text{Volume of 10X PBS needed (V2)} &= \frac{\text{Total Volume (V1)}}{10} \\
\text{Volume of 1N NaOH needed (V3)} &= (V1) \times 0.025 \\
\text{Volume of distilled water needed (V4)} &= (V1) - (V1 + V2 + V3)
\end{align*}
\]

**Figure 8. Collagen mixture formula.** Collagen, 10X PBS, 1N NaOH, and distilled water were used according to the calculation.

Since collagen rapidly gels at room temperature, other reagents needed to be mixed in advance before mixing with collagen. For instance, to produce 2 mg/ml collagen mixture for 100 µl, 66.67 µl of 3 mg/ml rat tail collagen I, 10 µl of 10X PVS, 1.67 µl of 1N NaOH, and 21.67 µl of distilled water were needed. NaOH, 10X PBS, and distilled water were loaded and mixed into a tube. When cell-suspension media was ready, the collagen was then added.
into the same tube. 200 µl pipette tips or larger were recommended, otherwise, the procedure should be done on ice to avoid collagen being clogged inside the tips.

2.6. Cell Seeding

Cells from culture flask were trypsinized for 5 minutes in the incubator at 37 °C with 5% CO2, the cell-suspension media was transported into a tube, and then cell culture media was added to the tube. Centrifuge was conducted to let cells form a pellet on the bottom. The supernatant was aspirated, and the cells were evenly distributed by adding new media. Cell counting was executed using hemocytometer. Cell-suspension media was adjusted to the desired concentration. For instance, to change the 2 ml cell suspension with 5 million (5 M) cells into the final cell concentration of 0.3 M cells per 50 µl, the media would be decreased to (5 M/ 0.3 M) x 50 µl = 833.33 µl. The media would be pelleted, subsequence supernatant would be aspirated, and 833.33 µl of media would be added. After adjusting, the cell suspension media was added into prepared collagen mixture with a ratio of 1:2. The cell-collagen mixture was loaded into PDMS wells and incubated at 37 °C with 5% CO2 for 40 minutes, and then media was filled to the top of the wells. The media were changed every 2 days.

2.7. Tissue Ring Extraction

Tissue rings could be extracted out of the PDMS wells when they became strong enough for the extraction. Fibroblast tissue rings were extracted on day 1, hiPSC-MSCs were extracted on day 3 for DMEM + 10% FBS and serum-free culture media, but on day 7 for DMEM + 5% FBS. hiPSC-CM rings were resilient enough on day 7, but they were strongly attached to the posts, resulting in difficulties for the extraction. To extract tissue rings, mini probe, shown in Figure 9, was used to push the ring up gently to the top of the posts.
Figure 9. The probe used for tissue ring extraction.

2.8. Tissue Shape Measurement

The microscopic images were captured using either 1 frame (tissue rings) or 4x4 frames (tissue constructs) to cover the entire tissues. The tissue rings were measured at least 3 times stochastically for thickness, inner diameter, and ring diameter, as shown in Figure 10, by Nikon Eclipse Ti microscope and NIS Elements software. The measurements were then averaged for each sample.

Figure 10. Tissue rings measurement. The tissue ring thickness and inner diameter were measured.

2-post 3D tissue constructs were measured for the gap between a post and the structure, and deflection of the structure (D) defined direction toward the structure as positive as depicted in Figure 11.
3D triangle tissue constructs were measured for the gap between a post and the structure (G), the angle between the center line and the direction of the gap (Angle), and deflection (D) of the structure as shown in Figure 12. Each value was also normalized by dividing the value by the distance between posts.

2.9. Statistics

Each batch was analyzed with a sample size of at least 3 sample per group. The data were represented as means ± standard deviation. Prism 6 software (version 6.01) was used to plot and perform one-way and two-way ANOVA with p-value < 0.05 considered significant.
3. Results

3.1. Tissue Ring

Previous studies had reported deriving tissue rings from scaffold-free method\textsuperscript{32,41}. However, the reports also stated the significance of cell aggregation. Dash et al. were switched to collagen-promoting supplemented media, otherwise hiPSC-VSMC were failed to hold its ring structure within 4-7 days. This highlighted the importance of scaffold, especially collagen, to form a solid tissue ring. Collagen had been selected to act as a scaffold, as it is abundant in ECM. To seed with collagen, a container was needed to enforce collagen to retain the desired shape. PDMS well with a center post would be appropriate to accomplish the task. PDMS was our material of choice of the well because of its inert, non-toxic, reusable and clear. 3D printed ABS mold negative was fabricated to be used as a primary mold to manufacture PDMS mold. Nevertheless, a combination of the collagen-cell mixture in a PDMS mold was not enough to produce a tissue ring, because collagen and cells attached to the mold surface subsequently unsuccessful to aggregate to the center and form a ring. Pluronic F127 was introduced to remedy the problem by coating the mold to induce non-fouling surface on the mold surfaces. Fibroblasts were the first cells to be experimented to polish seeding protocol due to their robust nature. hiPSC-MSCs and hiPSC-CMs were later seeded by using the established protocol. Fibroblast, hiPSC-MSC, and hiPSC-CMs tissue rings could be seen in comparison in Figure 13.
3.1.1. Fibroblast Tissue Rings

Experiments were completed using 0.5 M cells for each condition. Two collagen concentrations had been applied (0.5 and 2.0 mg/ml) to form the fibroblast tissue rings. To study the effect of the non-fouling surface on PDMS surface, cells were seeded into the wells with and without Pluronic acid coating. Each ring had been observed for a period of 14 days as shown in Figure 14.

![Figure 13](image13.jpg)

Figure 13. A fibroblast ring (a) comparing to hiPSC-MSC (b) and hiPSC-CM (c) rings (scale = 1 mm).

![Figure 14](image14.jpg)

Figure 14. A fibroblast ring from mixing method retaining its shape and size for 14 days (scale bar = 1 mm).

We found higher collagen concentration yielded no significant difference in ring diameter with p-value = 0.1281 at Day 14 (Figure 15(a)). The average ring diameters of 0.5
and 2.0 mg/ml were 2.382 ± 0.687 and 3.282 ± 0.398 mm. The results suggested seeding with 2.0 mg/ml collagen would offer thicker tissue ring.

Shaking and mixing protocols were introduced to establish consistent rings. Pluronic acid was added to detach the collagen gel from the PDMS mold. The seeded wells with shaking protocol were shaken for 600 RPM x 3 minutes using Labnet Vortemp 56 EVC Shaking Incubator. Comparing to the shaking protocol, mixing protocols described in the method section were resulted in larger and consistent rings as shown by the mean and standard deviation, shown in Figure 15(b). The results didn’t conclude any significant differences between each group in diameter except shaking and pluronic + mixing with p-value = 0.0076. The ring diameters of control, shaking, Pluronic+ shaking, and Pluronic + mixing groups were 3.282 ± 0.398, 2.691 ± 0.367, 3.062 ± 0.371, and 3.518 ± 0.057 mm.

**Figure 15. Fibroblast rings diameter varied by collagen density and fabrication protocol.** The top left graph depicted ring diameter varying collagen concentration (a). The top right graph depicted the effect of Pluronic acid coating on the ring diameter (b). The bottom graphs showed the fibroblast rings seeded in Pluronic coated well using mixing
protocol and 2.0 mg/ml collagen over time and on the last day (c and d). The rings were measured on day 14.

Fibroblast tissue rings fabricated from mixing protocols were characterized in detail in Figure 15(c), 15(d) and Figure 16. The rings retained size and shape consistently throughout two-week culture after the first 2 days, therefore the protocol was finalized to be performed for seeding hiPSC-MSCs and hiPSC-CMs.

![Figure 16](image)

**Figure 16. Fibroblast rings thickness (a) and inner diameter (b).** The rings retained the openness over time.

### 3.1.2. Asymmetrical hiPSC-CM Ring

1 M hiPSC-CMs were seeded in each asymmetrical well to fabricate an asymmetrical cardiac ring imitating ventricular tissue as shown in Figure 17. Seeding with 0.3 and 0.6 M cells were also performed previously, but resulting in failure; cells were not able to aggregate into a ring. The hiPSC-CM rings were able to beat within 4 days without using any stimulant.
**Figure 17. A hiPSC-CM ring over 7 days.** The ring had uneven surface unlike other cell types (scale = 1 mm).

3.1.3. hiPSC-MSC Tissue Rings

hiPSC-MSC rings fabricated using mixing protocol with Pluronic coating were resulted in an unexpected occurrence that the rings became solid structure (closing inner diameter), 2-4 days after removing from the molds at Day 3, depicted in Figure 18-19. This phenomenon was not reported from Dikana et al MSC rings fabricated using the scaffold-free method, because their rings remained in the molds and were harvested right before the measurement. They didn’t characterize the ring growth and morphogenetic changes after removal from the devices.
Figure 18. A 0.6 M hiPSC-MSC cells ring turning into a spheroid within 7 days. The ring was removed from the PDMS mold on day 3 cultured with DMEM + 10% FBS (scale bar = 1mm).

Figure 19. A 0.3 M hiPSC-MSC cells ring turning into a spheroid within 4 days. The ring was removed from the PDMS mold on day 3 (scale bar = 1mm).

Histochemistry was performed immediately after the rings were extracted from the molds, showing positive MSC biomarkers of CD 90 and CD 73 and H&E staining, as illustrated in Figure 20.
Figure 20. Immunohistochemistry of H&E, CD73, and CD90. The top left (a) and the bottom left (c) pictures depicted expression of CD 90. The top right (b) was the result of H&E staining. The bottom right (d) exhibited a hiPSC-MSC ring expressing CD73.

hiPSC-MSC rings were seeded in two total amounts of cells (0.3 and 0.6 M cells), three different collagen concentrations (0.5, 1.0, and 2.0 mg/ml) and cultured with different serum concentration (5% FBS and 10% FBS) and serum-free media. The rings were harvested on day 3, except 5% FBS groups harvested on day 7, because of insufficient strength to be removed from the mold in earlier days.

Collagen concentration study had been depicted in Figure 21. In summary, collagen concentration affected thickness significantly (p-value = 0.0011) with significant difference between 0.5 and 1.0 mg/ml groups (p-value = 0.0008). Collagen concentration also influenced inner diameter significantly (p-value = 0.0032) with significant differences between 0.5 and 2.0 mg/ml groups (p-value = 0.0192), and 1.0 and 2.0 mg/ml groups (p-value = 0.0028). Lastly, collagen concentration impacted ring diameter (p-value = 0.0004) significant with significant differences among every 2 groups; 0.5 and 1.0 mg/ml (p-value = 0.00283), 0.5 and 2.0 mg/ml (p-value = 0.0003), and 1.0 and 2.0 mg/ml (p-value = 0.0304).
The data was also summarized in Table 2. Contrast to the Fibroblast rings which became thicker when seeding with higher collagen concentration, the hiPSC-MSC rings were thicker with lower collagen concentration.

![Figure 21. hiPSC-MSC rings seeded with different collagen density. The rings with 0.5, 1.0, and 2.0 mg/ml were resulted in different thickness (a), inner diameter (b), and ring diameter (c).](image)

The effect of cell number has been investigated in Figure 22. hiPSC-MSC rings were seeded with two different amounts (0.3 and 0.6 M cells). Both types of the hiPSC-MSC rings showed a similar trend in thickness, inner diameter, and ring diameter over time (Figure 22(a), 22(c), 22(e)). Thickness was drastically increased right after the rings were removed and gradually declined over time. Inner diameter was rapidly declined after the removal and reach to zero within 7 days for 0.6 M cells and 11 days for 0.3 M cells. Ring diameter was gradually shrinking at a different rate for each system over time corresponding to inner diameter shrinkage. However, the rings after the removal on day 3 depicted in a different manner. Although the two systems showed no significant difference in thickness (p-value = 0.5131), they had the difference on inner diameter with p-value = 0.0250 and ring diameter with p-value = 0.0077. The data was shown in Table 3.
Figure 22. hiPSC-MSC rings with 0.3 and 0.6 M cells. The thickness, the inner diameter, and the ring diameter of each cell number were shown over 11 days ((a), (c), (e)) and were plotted on day 3 on Figure ((b), (d), (f)).

hiPSC-MSC rings with 0.3 M and 0.6 M cells were cultured with two different FBS concentrations (5.0% and 10.0%) and serum-free media to inspect the effect of different culture media (Figure 23). Culture media had no significant effect on thickness for both 0.3 M (p-value = 0.2749) and 0.6 M cells (p-value = 0.4771) (Figure 23(a) and 23(b)). However, this did not apply to inner diameter as shown in Figure 23(c) and 23(d) (p-value = 0.0003 for 0.3 M cells and p-value = 0.0304 for 0.6 M cells). For the hiPSC-MSC rings with 0.3 M cells, the statistical difference on inner diameter between 10% and 5% FBS had p-value of 0.0007, between 5% FBS and serum-free media had p-value of 0.0010. The tissue rings seeded with 0.6 M cells only showed significant difference between 10% FBS and serum-free media groups with p-value of 0.0024. For ring diameter differences, 0.6 M cells were resulted in no significant difference (p-value = 0.2771) but 0.3 M cells had demonstrated significant
difference with p-value < 0.0001 with significant differences between 5.0% FBS to 10.0% FBS and serum-free media of p-value < 0.0001 for both comparisons (Figure 23(e) and 23(f)).

![Graphs showing differences in thickness, inner diameter, and ring diameter](image)

Figure 23. hiPSC-MSC rings with 0.3 and 0.6 M cells with different serum concentration. The rings cultured with 10% FBS, 5%FBS, and Serum free were plotted showing the thickness, the inner diameter, and the ring diameter over 11 days ((a), (c), (e)) and upon removal ((b), (d), (f)) (day 3 for 10%FBS and serum-free media and day 7 for 5%FBS).

Two-way ANOVA was performed comparing the effect of a number of cells seeded and serum concentration to thickness, inner diameter, and ring diameter. The result showed no significant differences caused by a number of cells seeded, serum concentration, and their interaction affecting thickness. On the other hand, significant differences in inner diameter
caused by serum concentration (p-value = 0.0055) and the interaction between a number of cells seeded and serum concentration (p-value = 0.0071) were present. Finally, ring diameter was not influenced by a number of cells seeded or serum concentration but influenced by the interaction of the two factors with a p-value of 0.0179.

3.2. Multi-post hiPSC-MSC 3D tissue structure

Mechanical stimuli were known factor affecting cell behavior. Microscopically, the mechanical stimuli maintain and regulate chondrocyte phenotype. Macroscopically, the tissue structure could be remodeled. To understand the importance of mechanical stimuli, a model was needed. Our multi-post system could serve as a tool to pursue the question. The number of the post could be modified to provide a different kind of forces. In addition, hydrogel could be changed or modified to observe the interaction between the hydrogel and mechanical stimuli.

2-post system was used to represent the simple model, in which only bidirectional forces were presented to regulate the tissue morphology and structure. Tissue triangles represented a more complex model, in which forces were presented from several directions. The center post of the tissue triangles could be served as a stabilizer offering more evenly-distributed forces comparing to the triangles without the center post. The deflection and the gap of the structure had been chosen as parameters to be measured, because of their observability. These parameters could be compared under different tissue geometries and culture conditions to understand the role of mechanical forces on tissue remodeling.

Multi-post hiPSC-MSC 3D structures were fabricated using the same protocol as previous tissue rings by linearly increasing required cell suspension volume (Figure 24). Despite fabricating them with identical protocol, the gap between tissue and posts in 2-post
system was narrowing, while the gap in triangle posts was widening. In following experiments, three multi-post systems (2, 3, and 4 posts) had been performed.

**Figure 24. 2-post hiPSC-MSC oval shape and 4-post hiPSC-MSC triangle:** Day-4 hiPSC-MSC 2-post structure was shown in the figure a (the distance between the post was 4 mm) and day-5 hiPSC-MSC 4-post triangle was shown in figure b (the triangle was equilateral with 8 mm on each side and the center post point of origin was at the center of the triangle) (scale bar = 1 mm).

### 3.2.1. 2-post hiPSC-MSC structure

2-post hiPSC-MSC structures with 5 and 6 mm post distances had shown no significant differences. Deflection was gradually increasing, while the gap was decreasing in the same manner over time (Figure 25 and 26).

**Figure 25. A 2-post hiPSC-MSC tissue shape images over 13 days** (scale bar = 1mm).
Figure 26. 2-post hiPSC-MSC tissue shapes with different post distance gap and deflection measurements comparison over 13 days; Gap (a) and deflection (b) of 5 and 6 mm post distance 2-post hiPSC-MSC tissue shapes portrayed no significant differences over time.

3.2.2. hiPSC-MSC triangle

hiPSC-MSC triangles seeded in 6 mm, 8 mm with and without center post were experimented to study the effect of the center post and the post distance on the tissue remodeling (Figure 27 and Table 4).

Figure 27. A triangle hiPSC-MSC tissue structure over 13 days. (scale bar = 1mm).
The gap was influenced by both the center post and the post distance with the significance of p-value = 0.0041 (Figure 28(a), 28(b)). The normalized gap between 6 and 8 mm without the center post, which could indicate the importance of the post distance, was found significantly different with the p-value of 0.0069 (0.234 ± 0.032 and 0.313 ± 0.021 mm). The normalized gap between with and without the center post (same 8 mm post distance), which could indicate the importance of the center post, was found significantly different with the p-value of 0.0054 (0.222 ± 0.031 and 0.313 ± 0.021 mm).

Deflection was only impacted by the post distance since the existence of the center post did not affect the deflection as supported by Figure 28(c) and 28(d), which indicated no significant difference between 8mm hiPSC-MSC structure with and without the center post. However, the post distance influenced the deflection, since 6 mm without the center post was the difference to 8 mm with and without the center post indicated by the p-value of 0.0143 and 0.0180 (0.064 ± 0.021, 0.108 ± 0.024, and 0.110 ± 0.010 mm respectively).

Angle was not determined differently by either the presence of the center post or the distance between the posts as shown in Figure 28(e) and 28(f).

Comparing the tissue remodeling characteristics between 2-post structures and triangle structures, the gap displayed in opposite manner, which showed that the gap of the triangles was increasing over time, while the gap of 2-post structures was decreasing over time. Yet, the deflection was increasing over time in both systems. Clearly, the mechanism behind the disparity could be further studied by expanding the experiment.
Figure 28. hiPSC-MSC triangles with different post system angle, gap, and deflection measurements comparison over 13 days. The hiPSC-MSC triangles with 6 mm without center post, 8 mm with and without center post were measured for normalized gap, normalized deflection, and angle over the duration of 13 days ((a), (c), (e)) and on day 13 ((b), (d), (f)).

4. Conclusion and Discussion

A protocol for tissue ring fabrication that can be applied to several cell types had been created. The protocol can be improved by minimizing tissue ring size to serve as an economically sound test sample for drug discovery research. One way to achieve this is to reduce the size of the mold. This, in turn, could bring problem concerning cell seeding, since the high viscosity of collagen gel would make it challenging to use smaller pipette tips lesser than 200 µl. Another aspect of minimizing tissue ring was the consistency. Ring cultured with a third size of normal tissue ring with 2mm diameter had been performed resulting in
geometrically inconsistent rings (data not shown). Lastly, the mold could be put together in a productive manner by creating a block featuring numerous wells within the block.

Contracture in hiPSC-MSC and fibroblast rings after the removal was also observed in fibroblasts with free-floating collagen model\textsuperscript{43,44}. Briefly, the cell-laden collagen scaffold was seeded and detached from the well to observe its contracture over time, which was exhibited inclusively when using collagen as a scaffold\textsuperscript{44}. The free-floating fibroblast-collagen was contracting into a ball, but our fibroblast rings were contracting for 3 days after the removal and retained their morphology. Given that fibroblast could acquire myofibroblast phenotype under mechanical tension\textsuperscript{45}, the situation with the fibroblast rings might be because fibroblasts differentiated into myofibroblasts that induced by the external force from the center post. If that was the case, the model could be used to study the importance of α-SM actin in myofibroblast to fibroblast differentiation which was not answered yet\textsuperscript{45}.

Tissue contracture was also correlated with various cytokines. Extracellular matrix metalloproteinase inducer (EMMPRIN) expression increased the contractile properties in fibroblasts and α-SM actin production, which was a marker of myofibroblasts\textsuperscript{45}. Transforming growth factor β (TGF-β) also induced contracture in fibroblast\textsuperscript{45}, increased EMMPRIN output\textsuperscript{46}, and enhanced rings strength\textsuperscript{36}. Finally, IL (interleukin) 4 and 13 prompted MSC-laden collagen type I gel contracture\textsuperscript{47}. In future, supplementing these cytokines in culture media could be used to investigate the contracture across different cell types.

The majority of tissue structures were cultured by basal medium supplemented with an animal-derived serum that is not chemical-defined culture condition. This adds unknown factors for fabrication and analysis since animal serum has batch-to-batch variation leading to inconsistency results\textsuperscript{48} and unplanned interactions with the test subjects\textsuperscript{49,50}. Furthermore,
tissues cultured with animal-derived serum could be exposed to the risk of (virus, bacterial, and fungal) contamination\textsuperscript{51,52}. This risk would be eliminated if serum-free media is used. The serum also contains a large amount of high molecular weight protein, which is an uncommon environment for somatic cells\textsuperscript{53}. Thus, chemical-defined culture media has been chosen to fabricate hiPSC-MSC rings resulting in geometrical consistency and high reproducibility.

Tissues with different shapes have been fabricated from the multi-post system, which could be an important tool to observe and study matrix remodeling and cell contractility. Our previously described structures fabricated from 2-post oval and 4-post triangle gaps remodeling in contrast manner; the 2-post shapes remodeled to the direction of the post, but the 4-post shapes remodeled in opposite direction to the posts. Yet, the deflection of these two shapes progressed in the same inward direction.

Similar matrix remodeling study has been done with cardiac cells within collagen/fibrin 3D matrices in PDMS well platform containing 2 cantilevers on each side to fabricate dog-bone tissue shapes\textsuperscript{54}. Dog-bone tissue shapes width changes had been observed over time and were explained by current stress comparing to maximum contractile stress (stall stress) of the cells vs strain rate relation which is like that of a sarcomere. The contractile stress was occurred at the junction between each adherent cell and its substrate and measured with traction microscopy\textsuperscript{55}. Shortly, in any portion of tissue shape, if the portion receives the stress more than the stall stress, that portion will extend in the same axis of the stress and in turn narrowing that portion, and vice versa. The stress could be calculated from the static force from cantilevers and cross-sectional area of the tissue shape. The static force was derived from stiffness and deflection of the cantilevers, while cross-sectional areas were measured visually. Increasing cantilever stiffness would increase tissue tension and stress\textsuperscript{54,56}. Matrix stiffness also affects the amount of stress\textsuperscript{56}; the denser the matrix, the lesser
the stress. Surprisingly, an attempt to temporary stiffen the cantilever by attaching magnetic bead on the posts and using a magnetic field to counter the bending were resulted in increasing cell contractility of valvular interstitial cells\textsuperscript{57}. The strain rate could be quantified by tracking the deformation on each point of the tissue shape visually. The maximal principle stress direction affecting cell tension was another remodeling direction indicator as the nuclei aligned to the direction\textsuperscript{58}.

Expanding from dog-bone shape to other shapes, Schell et al discovered that Matrix geometry influencing maximal principle stress directions, and in turn cell tension as observed by the nucleus directions which aligned to the directions\textsuperscript{58}. Agarose hydrogel molds with different geometry, number, and position of agarose posts were seeded with chondrocytes and fibroblasts and used to predict the maximal principle stress directions via finite element method by treating the tissue as neo-Hookean material and the posts as a rigid body. The predicted stress directions were aligned to the nucleus directions. For instance, hexagonal equibiaxial shapes had the equal nucleus directions in every direction in the center as predicted by the calculation as do the dog-bone shapes in the rod section, which were predicted to have mostly horizontal stress directions. Both cell types had the same nucleus directions according to the mold design but the fibroblast tissue shapes, especially dog-bone shapes, were more deflected and elongated than the chondrocytes tissue shapes since fibroblasts are more contractile.

The matrix remodeling also affects cell contractility\textsuperscript{59}. The matrix with and without cells under magnetic force was compared and shown that the matrix with cells returned its former stress and strain values before the force was applied, while the matrix without cells did not have the same recovery ability. Polacheck and Chen explored further and found that the deflection of the posts/cantilevers were from the combination of cells generated forces within the tissue\textsuperscript{60}. With these concepts, we could explore the relation behind the matrix
remodeling and cell contractility using different tissue shapes by modifying post position and quantity, mechanical forces by varying matrix stiffness, cell types, post stiffness, and likely size.

To further characterize the tissue morphology, the tissue height and volume will need to be measured and calculated. We have encountered some difficulties to measure the height directly through actin staining and upright confocal microscope, since the standing posts will interfere lowering down the objective for imaging the bottom of the tissues. A potential approach to measure the height can be completed by cutting the mold boundary and visualized sideway, which will sacrifice the PDMS molds. The tissue volume could be estimated based on the height and area measurements.

To measure the mechanical properties of the tissue rings, the rings would be mounted on tensile testing machine such as Instron EPS 1000 in the horizontal position and attached on load cell of 1N ±1mN and custom wire grips. The rings would be extended to tare load and recorded for the gauge length. After entering cross-sectional area of the rings calculated from tissue thickness, stress-strain curve would be ready to be plotted. Appropriate amount of pre-cycling would be performed, and the rings would be pull to failure. Ultimate tensile strength and fracture strength would be recorded, and elastic modulus would be calculated from the stress-strain curve.
### 5. Appendix

#### Table 1. Measurement of fibroblast rings cultured with 2.0 mg/ml collagen density using mixing protocol in Pluronic-coated wells.

<table>
<thead>
<tr>
<th>Day</th>
<th>Ring Diameter (mm)</th>
<th>Thickness (mm)</th>
<th>Inner Diameter (mm)</th>
<th>Diameter (mm)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.90 ± 0.42</td>
<td>1.04 ± 0.22</td>
<td>1.82 ± 0.11</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.61 ± 0.12</td>
<td>1.08 ± 0.12</td>
<td>1.46 ± 0.17</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3.58 ± 0.20</td>
<td>1.12 ± 0.12</td>
<td>1.35 ± 0.11</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>3.51 ± 0.12</td>
<td>1.13 ± 0.18</td>
<td>1.26 ± 0.26</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3.49 ± 0.13</td>
<td>1.10 ± 0.14</td>
<td>1.28 ± 0.19</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>3.52 ± 0.06</td>
<td>1.11 ± 0.10</td>
<td>1.30 ± 0.18</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

#### Table 2. Measurement of hiPSC-MSC rings with different collagen density.

<table>
<thead>
<tr>
<th>Collagen Density (mg/ml)</th>
<th>Thickness (mm)</th>
<th>Inner Diameter (mm)</th>
<th>Ring Diameter (mm)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.92 ± 0.08</td>
<td>2.37 ± 0.35</td>
<td>3.34 ± 0.42</td>
<td>5</td>
</tr>
<tr>
<td>1.0</td>
<td>0.50 ± 0.19</td>
<td>2.65 ± 0.44</td>
<td>3.08 ± 0.29</td>
<td>5</td>
</tr>
<tr>
<td>2.0</td>
<td>0.73 ± 0.08</td>
<td>1.59 ± 0.21</td>
<td>2.26 ± 0.31</td>
<td>4</td>
</tr>
</tbody>
</table>

#### Table 3. Measurement of hiPSC-MSC rings with different cell seeding number and serum concentration.

<table>
<thead>
<tr>
<th>Cell Number</th>
<th>300,000</th>
<th>600,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thickness (mm)</td>
<td>Inner Diameter (mm)</td>
<td>Ring Diameter (mm)</td>
</tr>
<tr>
<td>10% FBS</td>
<td>0.58 ± 0.03</td>
<td>1.34 ± 0.17</td>
</tr>
<tr>
<td>5% FBS</td>
<td>0.64 ± 0.08</td>
<td>1.81 ± 0.8</td>
</tr>
<tr>
<td>Serum-free</td>
<td>0.58 ± 0.07</td>
<td>1.3± 0.17</td>
</tr>
<tr>
<td>Cell Number</td>
<td>600,000</td>
<td></td>
</tr>
<tr>
<td>Thickness (mm)</td>
<td>Inner Diameter (mm)</td>
<td>Ring Diameter (mm)</td>
</tr>
<tr>
<td>10% FBS</td>
<td>0.68 ± 0.13</td>
<td>1.73 ± 0.26</td>
</tr>
<tr>
<td>5% FBS</td>
<td>0.59 ± 0.07</td>
<td>1.42 ± 0.55</td>
</tr>
<tr>
<td>Serum-free</td>
<td>0.77 ± 0.32</td>
<td>1.06 ± 0.11</td>
</tr>
</tbody>
</table>
Table 4. Measurement of hiPSC-MSC triangles.
(nG = normalized gap, nD = normalized deflection).

<table>
<thead>
<tr>
<th></th>
<th>6 mm without cp</th>
<th></th>
<th>8 mm</th>
<th></th>
<th>8 mm without cp</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day</td>
<td>nG</td>
<td>Nd</td>
<td>nG</td>
<td>nD</td>
<td>nG</td>
<td>nD</td>
</tr>
<tr>
<td>1</td>
<td>0.16 ± 0.01</td>
<td>0.00 ± 0.02</td>
<td>0.14 ± 0.02</td>
<td>0.04 ± 0.01</td>
<td>0.15 ± 0.03</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>3</td>
<td>0.16 ± 0.01</td>
<td>0.00 ± 0.01</td>
<td>0.13 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>0.15 ± 0.04</td>
<td>0.02 ± 0.00</td>
</tr>
<tr>
<td>5</td>
<td>0.16 ± 0.01</td>
<td>0.00 ± 0.01</td>
<td>0.14 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>0.16 ± 0.02</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>7</td>
<td>0.19 ± 0.02</td>
<td>0.01 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.18 ± 0.02</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>9</td>
<td>0.21 ± 0.04</td>
<td>0.04 ± 0.01</td>
<td>0.20 ± 0.03</td>
<td>0.07 ± 0.02</td>
<td>0.26 ± 0.05</td>
<td>0.08 ± 0.00</td>
</tr>
<tr>
<td>11</td>
<td>0.22 ± 0.04</td>
<td>0.05 ± 0.02</td>
<td>0.21 ± 0.03</td>
<td>0.09 ± 0.02</td>
<td>0.30 ± 0.02</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>13</td>
<td>0.23 ± 0.03</td>
<td>0.06 ± 0.02</td>
<td>0.22 ± 0.03</td>
<td>0.11 ± 0.02</td>
<td>0.31 ± 0.02</td>
<td>0.11 ± 0.01</td>
</tr>
</tbody>
</table>
6. References


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Publication

Tackla S. Winston§, Kantaphon Suddhapas§, Chenyan Wang, Rafael Ramos, Pranav Soman, Zhen Ma. Serum-Free Manufacturing Mesenchymal Stem Cell Tissue Rings Using Human Induced Pluripotent Stem Cells. Stem Cells International (in progress)

§These two authors equally contribute to this work.