Video Article

Generation of Aligned Functional Myocardial Tissue Through Microcontact Printing

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Abstract

Advanced heart failure represents a major unmet clinical challenge, arising from the loss of viable and/or fully functional cardiac muscle cells. Despite optimum drug therapy, heart failure represents a leading cause of mortality and morbidity in the developed world. A major challenge in drug development is the identification of cellular assays that accurately recapitulate normal and diseased human myocardial physiology *in vitro*. Likewise, the major challenges in regenerative cardiac biology revolve around the identification and isolation of patient-specific cardiac progenitors in clinically relevant quantities. These cells have to then be assembled into functional tissue that resembles the native heart tissue architecture. Microcontact printing allows for the creation of precise micropatterned protein shapes that resemble structural organization of the heart, thus providing geometric cues to control cell adhesion spatially. Herein we describe our approach for the isolation of highly purified myocardial cells from pluripotent stem cells differentiating *in vitro*, the generation of cell growth surfaces micropatterned with extracellular matrix proteins, and the assembly of the stem cell-derived cardiac muscle cells into anisotropic myocardial tissue.

Video Link

The video component of this article can be found at https://www.jove.com/video/50288/

Introduction

Despite recent advances in medical therapy, advanced heart failure remains a leading cause of mortality and morbidity in the developed world. The clinical syndrome arises from a loss of functional myocardial tissue and subsequently an inability of the failing heart to meet the metabolic demands of affected individuals. Since the heart has a limited regenerative capacity, autologous heart transplantation is the only current clinically accepted therapy directly aimed at replenishing lost functional heart tissue. Significant drawbacks of heart transplantation, including a limited number of donor hearts and the need for long-term immunosuppressive therapy, preclude the wide spread use of this therapy. As a result, medical therapy has been the mainstay of treatment for patients with heart disease. A major challenge in drug development is the identification of cellular assays that accurately recapitulate normal and diseased myocardial physiology *in vitro*.

The recent convergence of stem cell biology and tissue engineering technology raises new promising avenues for cardiac regeneration. Generating functional myocardial tissue from a renewable patient-specific source would be a major advance in the field. This would allow for the development of disease specific cellular assays for drug development and discovery and would lay the foundation for cardiac regenerative medicine. Human embryonic stem (ES) cells and, more significantly, human induced pluripotent stem (iPS) cells represent a potentially renewable patient-specific source of ventricular progenitor cells and mature ventricular myocytes. The combination of stem cell biology and tissue engineering strategies addresses this problem by generating functional heart tissue *in vitro* that can be used in the development of cellular assays for drug discovery or in cardiac regenerative approaches for the treatment of advanced heart failure.

A central challenge in cardiac regeneration has been the identification of an optimal cell type. A wide array of cells have been studied[1], however to date, although different multipotent cardiogenic precursors from various sources have been discovered, the identification of a cell source that meets critical requirements such as commitment to the myogenic cell fate, maintenance of the capacity to expand *in vivo* or *in vitro* and composition to a functional myocardial tissue has proven to be a central problem[2]. We have previously described a double transgenic murine system that enables the isolation of highly purified populations of committed ventricular progenitors (CVP) from ES cells differentiating *in vitro*. We generated a novel double transgenic mouse that expresses the red fluorescent protein dsRed under the control of an Isl1-dependent enhancer of the MEF2c gene and the enhanced green fluorescent protein under the control of the cardiac-specific Nkx2.5 enhancer. Based on this two-colored fluorescent reporter system, first and second heart field progenitors from developing ES cells can be isolated by means of Fluorescence Activated Cell Sorting (FACS) according to their expression of MEF2c and Nkx2.5 genes. CVP resemble cardiac myocytes

based on the expression patterns of myocardial markers and structural and functional qualities[3], what offers great promise for cardiac tissue regenerative purposes.

Although there have been many advances in the field of tissue engineering, mimicking native cellular architecture remains a key challenge. Conventional methods to address this challenge include seeding biological or synthetic scaffolds with cells *in vitro*. Due to a number of disadvantages of scaffolds, including rapid degradation, limited physical and mechanical stability and low cell density[1,4,5] , we have attempted to engineer scaffold-less tissue. Although cardiac cells can modify their local microenvironment by the secretion of extracellular matrix proteins, they have a more limited capacity to organize themselves to rod shaped cardiac myocytes in the absence of extracellular cues. Thus, a template that provides cells appropriate spatial and biological cues to compose functional myocardial tissue is required. Microcontact printing addresses this challenge by providing a simple and inexpensive technique to precisely control cell shape, organization, and function[6-8], all of which are crucial for the generation of aligned functional myocardial tissue. It comprises the use of microtextured Polydimethylsiloxane (PDMS) stamps with feature sizes ranging down to 2 μm[9] that enable the deposition of extracellular matrix proteins onto PDMS substrates in precise patterns and thus to affect cell adhesion spatially.

Herein, we propose to combine tissue bioengineering technology with stem cell biology to generate anisotropic functional myocardial tissue. Accordingly, we here demonstrate our recently published approach for the following: (1) the generation of micropatterned protein surfaces on PDMS substrates by microcontact printing for the generation of a template for aligned myocardial tissue, (2) the isolation of highly purified cardiac progenitors from ES cells differentiating *in vitro*, and (3) the combination of both techniques to generate aligned functional myocardial tissue.

Protocol

The protocol to generate aligned functional myocardial tissue can be divided into three major parts. The fabrication of the micropatterned master using soft lithography techniques is not considered part of the following protocol but can be made based on established method[6].

1. Microcontact Printing of Fibronectin onto PDMS Substrates

1. Mix Sylgard 184 (Dow Corning) PDMS elastomer at a 10:1 base to curing agent ratio and degas the assembly using a desiccator to eliminate any air bubbles.
2. Cure PDMS against a previously fabricated master with 20 μm wide and 2 μm tall ridges, separated by 20 μm spacing for either 2 days at room temperature or 4 hr at 65 °C to obtain microtextured PDMS stamps.

** Curing in a desiccator is highly recommended to eliminate any air bubbles.*

3. Spin coat PDMS onto glass cover slips (*e.g.* 22x22 mm) at 5,000 rpm for 2 min using a Headway Spin Coater to produce thin and even PDMS substrates of 15 μm thickness.
4. Clean stamps with 70% Ethanol to remove dust and dirt particles. Let them air dry.

** Stamps can be used several times, however, renewing PDMS stamps on a regular basis is recommended due to a surface abrasion over time.*

5. Place stamps in a SPI Plasma-Prep II Plasma Cleaner and process for 1 min at 275 mTorr to render PDMS surfaces hydrophilic.

** Oxygen plasma treatment should be watched. Longer treatment times can result in cracking of the elastomer.*

6. Sterilize stamps with 70% Ethanol for 1 min. Dry quickly with compressed air.

** Further steps should be performed in a biological safety cabinet to maintain sterility.*

7. Cover stamp surfaces with Fibronectin solution (50 μg/ml in ddH_2O). Let it adsorb at least 10 min.
8. Shake off Fibronectin solution from stamps and dry quickly with compressed air.
9. Establish conformal contact between stamps and PDMS substrates for 2 min and press firmly.
10. Rinse micropatterned substrates with ddH_2O. Store them in ddH_2O for a maximum of 2 weeks at 4 °C unless initially used. Store PDMS stamps in ddH_2O.

2. Maintenance of Double Transgenic Embryonic Stem Cell Lines

First, prepare the following media for culturing mouse ES cells:

Mouse Embryonic Fibroblast (MEF)-Medium: Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS) ES cell- or Differentiation-grade, 1% Penicillin-Streptomycin (P/S)

Embryonic Stem Cell (ESC)-Medium: DMEM, 15% FBS ES cell-grade, 1% Non-Essential Amino Acids (NEAA), 0.5% P/S, 0.2% Leukemia Inhibitory Factor (LIF), 0.0007% 2-Mercaptoethanol (2-ME)

1. Coat 6-well plates with sterile 0.1% Gelatin in ddH_2O and let it adsorb 15 min at 37 °C.
2. Add MEFs from a thawed aliquot to 50 ml PBS in a conical tube and centrifuge them at 1,000 rpm for 5 min. Resuspend MEFs in MEF-medium.
3. After adsorption, aspirate excess Gelatin and add MEFs into the wells. Allow MEFs 1 day to attach.

** An aliquot of 10 million MEFs suffices for 3 6-well plates.*

4. When MEFs have attached, add ES cells from a thawed aliquot to 50 ml PBS in a conical tube and centrifuge them at 1,000 rpm for 5 min. Resuspend ES cells in ESC-medium and add them to the wells containing MEFs. Culture ES cells in ESC-medium until confluence is reached.

** Once ES cells have reached confluence, passaging is required to avoid overgrowth and to maintain ES cell colonies.*

5. Prepare ES cells for passaging. Aspirate ESC-medium and wash once with sterile PBS.
6. Aspirate PBS and add 500 µl of Trypsin. Process 3.5 min at 37 °C.
7. Pipette the Trypsin solution up and down several times to break down ES cell colonies and neutralize it with 500 µl of ESC-medium.
8. Passage ES cells according to your desired ratio, *e.g.* transfer 33 µl to another well with previously prepared MEFs to passage 1/30. Maintain ES cells in ESC-Medium.

** A passage ratio of 1/30 allows ES cells to reach confluence within 3 days. Passage ES cells according to your in vitro differentiation schedule.*

3. *In vitro* Differentiation of Double Transgenic Embryonic Stem Cell Lines and Isolation and Seeding of Cardiac Progenitors

First, prepare the following media required for the *in vitro* differentiation of ES cells:

Adaptation-Medium: Iscove's Modified Dulbecco's Medium (IMDM), 15% FBS ES cell-grade, 1% NEAA, 0.5% P/S, 0.2% LIF, 0.0007% 2-ME

Differentiation-Medium: IMDM, 15% FBS Differentiation-grade. 1% NEAA, 0.5% P/S, 0.35% of 0.1M Ascorbic Acid, 0.0007% 2-ME

1. Start *in vitro* differentiation when ES cells have reached confluence. Coat 10 cm culture dishes with sterile 0.1% Gelatin in ddH_2O and let it adsorb 15 min at 37 °C.

** Be aware of the duration of the in vitro differentiation process. It takes 8 days until cardiac progenitors can be isolated. Plan your experiments accordingly.*

2. Harvest ES cells as before. After adsorption, aspirate excess Gelatin and add approximately 1/3-1/2 of the ES cell suspension to prepared culture dishes containing Adaptation-medium. Culture cells for 2 days.

** Choose the amount of ES cells for adaptation according to your experiments.*

3. Harvest adapted ES cells into a 50 ml conical tube containing PBS using 1. 5 ml of Trypsin. Centrifuge dissociated ES cells at 1,000 rpm for 5 min and resuspend them in Differentiation-medium.
4. Make embryoid bodies (EBs) of approximately 1,000 cells per 10 µl drop in 15 cm culture dishes using a multi-channel pipette. Invert the plates and culture EBs as hanging droplets for 2.5 days at 37 °C.
5. After 2.5 days, pool EBs of 6-8 15 cm culture dishes into one using Differentiation-medium and culture them for a further 3.5 days at 37 °C.
6. After 3.5 days, collect EBs in a conical 50 ml tube and let them sink to the bottom for approximately 5 min. Suck up the supernatant and wash EBs with sterile PBS. Let EBs sink again to the bottom for approximately 5 min.
7. Dissociate EBs by processing them with 2. 5 ml of Trypsin for 2 min in a water bath at 37 °C shaking the tube softly. Then, add 2. 5 ml of sterile PBS to the Trypsin solution and pipette up and down with a 10 ml serological pipette approximately 8-10 times to break down cell clusters. Neutralize Trypsin with 10 ml of FACS buffer containing 7.5% FBS ES cell- or Differentiation-grade and 0.01% DAPI in PBS.
8. Centrifuge dissociated EBs at 1,000 rpm for 5 min and resuspend them in approximately 1 ml of FACS buffer. Pipette up and down with a 1 ml pipette approximately 8-10 times to break down cell clusters.

** Add FACS buffer according to the estimated amount of dissociated EBs. Too highly concentrated cells may clog during FACS and too low concentrated cells may prolong FACS time unnecessarily.*

9. Transfer cells to a sterile round-bottom tube with a 35 µm cell strainer to obtain a single cell suspension.
10. Sort differentiated ES cells using a FACSAria Flow Cytometer and obtain purified populations of CVP.

We developed a multi-step gating strategy to isolate highly purified colored cells. In brief, we first gate on Forward Scatter Amplitude (FSC-A) and Side Scatter Amplitude (SSC-A) (Figure 1A**). This allows us to separate whole cells from cellular debris. We then gate on FSC-A and Forward Scatter-Width (FSC-W) (**Figure 1B**). This allows us to isolate cell singlets from doublets and other cellular aggregates. We then gate on SSC-A and Side Scatter-Width (SSC-W) (**Figure 1C**). This increases the purity of cell singlets. We then gate on DAPI staining to isolate viable cells (DAPI-negative) from non-viable cells (**Figure 1D**). We then gate on Phycoeryhthrin Amplitude (PE-A) and PE-Cyanine dye 7 Amplitude (PE-Cy7-A) to obtain true red-positive (R^+) and true red-negative (R^-) cells and to exclude autofluorescent red-negative cells (**Figure 1E**). R^+ and R^- cells are then gated separately on Fluorescein isothiocyanate Amplitude (FITC-A) and PE-A to sort true green-positive (G^+) and true green-negative (G^-) cells within these populations (**Figure 1F+1G**). This results in 4 populations of cells as follows: R^+G^+, R^+G^-, R^-G^+ and R^-G^- (**Figure 1H**).*

11. Passivate micropatterned substrates with 1% Pluronic F-127 in ddH_2O for 10 min. Then, wash them 3x with sterile PBS.

** Pluronic F-127 is a surfactant that blocks cell adhesion. It supports confinement of cell adhesion to micropatterned area.*

12. Attach PDMS gaskets around the patterned region and press firmly. Then, seed CVP onto Fibronectin micropatterned substrates.

Representative Results

FACS-purification of *in vitro* differentiated ES cells revealed four distinct populations of progenitors (**Figure 1**). The presence of micropatterned Fibronectin was confirmed by immunofluorescence microscopy that displayed a complete transfer of continuous Fibronectin lines (**Figure 2**). Plating of FACS-isolated progenitors onto Fibronectin micropatterns resulted in alignment of the R^+G^+ and part of the R^-G^+ populations. Although Pluronic F-127 as a supportive blocker of cell adhesion was used, the R^+G^- and R^-G^- populations did not respect the anisotropic architecture of Fibronectin and grew into the space between adjacent protein lines (**Figure 3**).

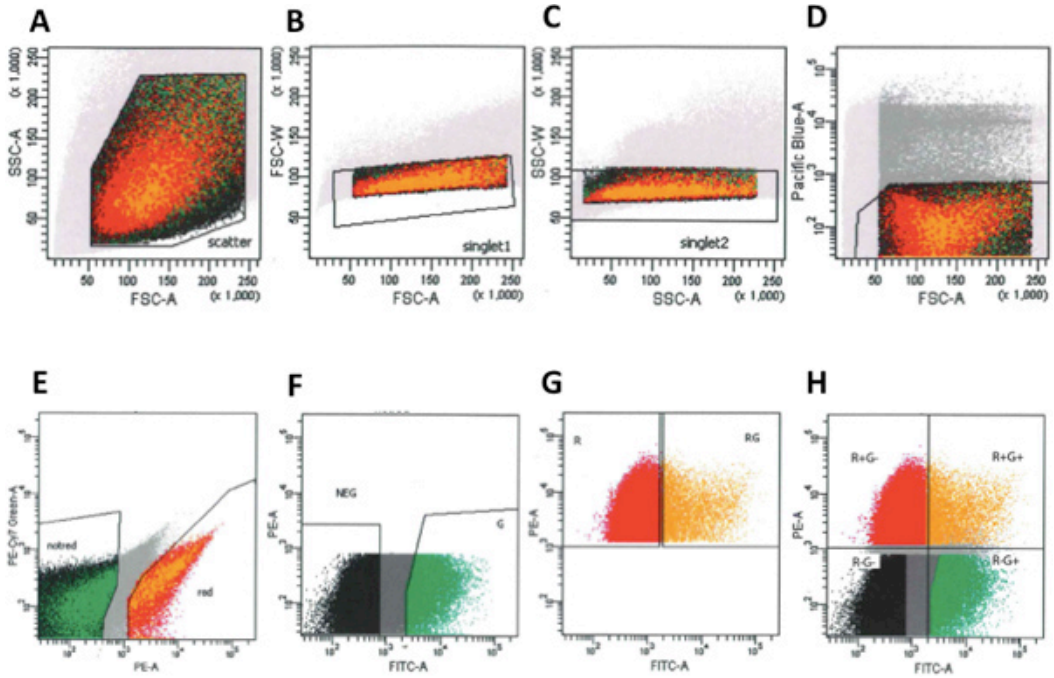

Figure 1. Representative flow cytometry chart of day 6 *in vitro* differentiated ES cells. (A) Gating on Forward Scatter Amplitude (FSC-A) and Side Scatter Amplitude (SSC-A). **(B)** Gating on FSC-A and Forward Scatter-Width (FSC-W). **(C)** Gating on SSC-A and Side Scatter-Width (SSC-W). **(D)** Gating on DAPI staining. **(E)** Gating for Phycoeryhthrin (PE) and PE-Cyanine dye 7 (PE-Cy7). **(F and G)** Gating for Fluorescein isothiocyanate Amplitude (FITC-A) and PE-A. **(H)** Flow cytometry plot revealing four distinct populations of progenitors: R^+G^+, R^+G^-, R^-G^+ and R^-G^-. Click here to view larger figure.

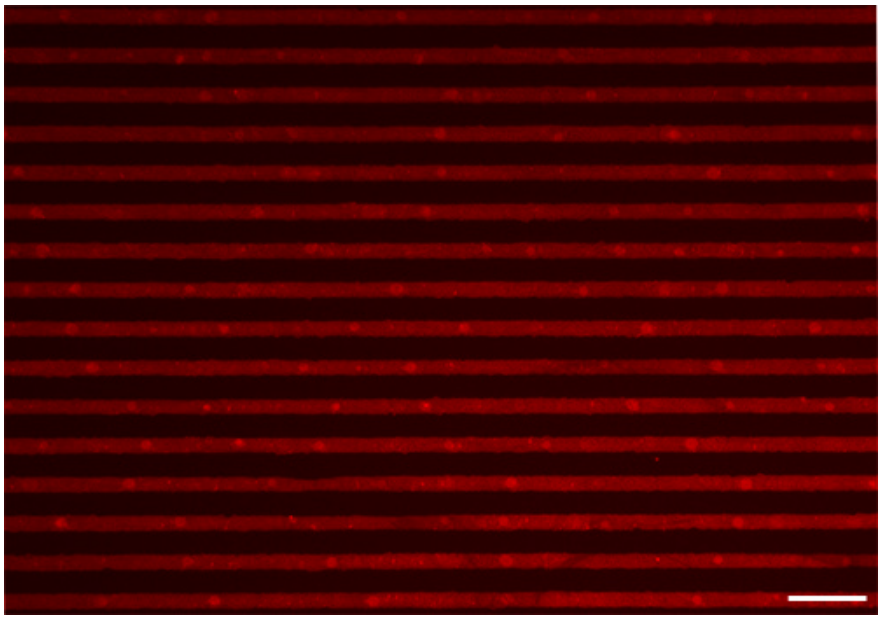

Figure 2. Representative immunofluorescence microscopy of micropatterned Fibronectin substrates. Scale bar, 80 µm.

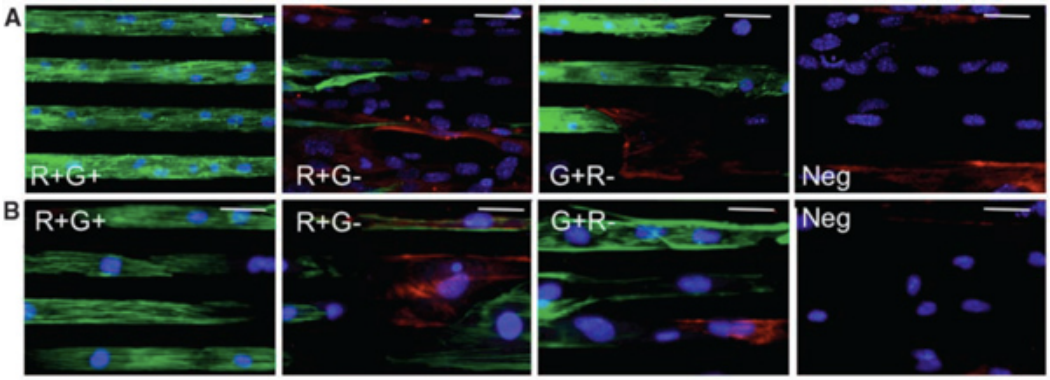

Figure 3. Representative immunofluorescence microscopy of embryonic- (A) and ES cell-derived (B) FACS-isolated progenitors after an additional 5 days of culture on Fibronectin micropatterns. Nuclei, blue; smMHC, red; sarcomeric α-actinin, green. Scale bar, 40 μm. Reproduced from Domian *et al.* (2009).

Discussion

In this protocol, we presented a method to isolate purified populations of cardiogenic progenitors and to seed them on micropatterned Fibronectin substrates what allows them align and take a cardiac myocyte-like rod shape. Normal cellular organization is critical for normal tissue function[8,10], in particular for myocardial tissue. Cardiac myocytes have been shown to develop improved mechanical[11,12] and electrophysiological properties[11,13] when forming anisotropic cell arrays. Since cardiac progenitors cultured on micropatterned Fibronectin substrates differentiate into rod-shaped myocardial cells *in vitro*,this represents apivotal step within cardiac tissue bioengineering.

Microcontact printing is a simple and inexpensive tool that can be used to generate 2-dimensional templates resembling myocardial tissue *in vitro*. However, efficient and successful patterning of extracellular matrix proteins relies critically on the protein of choice. Whereas a large variety of different proteins have been successfully stamped onto PDMS substrates, certain proteins, such as Collagen Type I, require surface modification of PDMS substrates prior to microcontact printing[14]. In addition, the amount of pressure applied to the stamp as well as the duration of stamping can also greatly impact the efficiency and fidelity of the microcontact printing.

Another critical step in this protocol is the usage of a plasma cleaner to render the surfaces of PDMS stamps hydrophilic. It is well-known that microcontact printing does not occur when both PDMS stamps and substrates do not undergo oxygen plasma treatment[15], and several groups found that tuning the wettability of PDMS substrates is required in order to make a transfer of protein possible[3,6,15] . However, we found that increasing the hydrophilicity of the PDMS stamp instead of the PDMS substrate results in more uniform protein micropatterns with higher quality, such as less disruptive or incomplete protein lines.

For the presented protocol, micropattern width of 20 μm was chosen, according to the reported cell width of murine neonatal cardiac myocytes[16]. However, the fabrication of the micropatterns should be carried out in a species- and heart developmental stage-dependent[16-20] manner in order to prevent opposite effects on cell viability due to limited space.

The discovery and purification of ES cell-derived CVP, particularly the R^+G^+ population, enable reliable studies within cardiac tissue bioengineering. Notably, we have observed our ES cell-derived cardiac progenitors to be capable of forming contracting myocardial tissue with maintaining their cellular alignment. Given that constructing three-dimensional functional tissue is a key goal in the field of tissue bioengineering, our generated two-dimensional anisotropic myocardial tissue could be effectively used to layer multiple cell sheets, as other groups previously described for other cell types[21,22] . However, to date, one limitation of the transgenic reporter system is the outcome of the *in vitro* differentiation of ES cells. FACS available CVP, in particular the strongly cardiogenic R^+G^+ population, currently account for an approximate average of 0.5% of all differentiated cells. Thus, improving the *in vitro* differentiation of ES cells into CVP will be a major step towards generating sufficient quantities of CVP to build larger anisotropic cardiac tissue consisting of longitudinally aligned myocardial fibers.

Disclosures

The authors declare that they have no competing financial interests.

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