Biomaterials 103 (2016) 1–11

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Effect of biophysical cues on reprogramming to cardiomyocytes

Junren Sia [a, b, *], Pengzhi Yu [c], Deepak Srivastava [c], Song Li [a, b]

[a] Department of Bioengineering, University of California, Berkeley, USA
[b] UC Berkeley & UCSF Joint Graduate Program in Bioengineering, Berkeley, San Francisco, USA
[c] Gladstone Institute of Cardiovascular Disease, University of California, San Francisco, USA

ARTICLE INFO

Article history:
Received 25 March 2016
Received in revised form
6 June 2016
Accepted 15 June 2016
Available online 23 June 2016

Keywords:
Direct reprogramming
Microtopography
Induced cardiomyocyte

ABSTRACT

Reprogramming of fibroblasts to cardiomyocytes offers exciting potential in cell therapy and regenerative medicine, but has low efficiency. We hypothesize that physical cues may positively affect the reprogramming process, and studied the effects of periodic mechanical stretch, substrate stiffness and microgrooved substrate on reprogramming yield. Subjecting reprogramming fibroblasts to periodic mechanical stretch and different substrate stiffness did not improve reprogramming yield. On the other hand, culturing the cells on microgrooved substrate enhanced the expression of cardiomyocyte genes by day 2 and improved the yield of partially reprogrammed cells by half fold at day 10. By combining microgrooved substrate with an existing optimized culture protocol, yield of reprogrammed cardiomyocytes with striated cardiac troponin T staining and spontaneous contractile activity was increased by one fold. We identified the regulation of Mkl1 activity as a new mechanism by which microgroove can affect reprogramming. Biochemical approach could only partially recapitulate the effect of microgroove. Microgroove demonstrated an additional effect of enhancing organization of sarcomeric structure, which could not be recapitulated by biochemical approach. This study provides insights into new mechanisms by which topographical cues can affect cellular reprogramming.

1. Introduction

Fibroblasts were first demonstrated to be capable of being reprogrammed into cardiomyocytes by the forced expression of three transcription factors: Gata4, Mef2c and Tbx5 [1]. However, the efficiency of this process is low and only about 0.01%–0.1% of the starting population become beating cardiomyocytes. Since this possibility was demonstrated about 5 years ago, much effort has been undertaken to improve the efficiency of this process, as reviewed in Ref. [2]. These approaches can be broadly classified into addition of miRNAs [3–5], small molecules [6], proteins [7] and modification of transcription factors [8–11]. More recently, it has also been shown that the addition of fibroblast growth factor (FGF) 2, FGF10 and vascular endothelial growth factor (VEGF) to serum free culture media significantly increased the yield of beating cardiomyocytes [12], demonstrating that culture conditions could also affect reprogramming efficiency.

Cells sense and respond to physical cues in their environment. It has been shown that physical cues can affect reprogramming outcome for other target cell types. For instance, softer hydrogel can improve efficiency of reprogramming to pluripotent stem cells by promoting mesenchymal to epithelial transition [13]. Microtopography can enhance reprogramming to both pluripotent stem cells [14] and neurons [15]. Such physical cues have been demonstrated to have positive effects on the development of cardiomyocytes. Mechanical stretching improves the maturation of cardiomyocytes that are differentiated from mesenchymal stem cells [16]. Softer substrates favors the maturation of neonatal cardiomyocytes in terms of sarcomere assembly, calcium transients and gene expression [17,18]. Microgrooves have been found to enhance the differentiation and maturation of cardiomyocytes [19–21] from stem cells. The effects of physical cues on cell reprogramming and on cardiomyocyte development have been separately established, but their effects on direct reprogramming from fibroblasts to cardiomyocytes have not been studied.

Using αMHC-GFP reporter mouse [1], we screened the effect of cyclical mechanical stretch, substrate stiffness and microgroove on reprogramming yield of cardiomyocyte from fibroblasts, and found an enhancement for microgroove. Long term culture on microgroove combined with an optimized culture protocol [12] further

* Corresponding author. Department of Bioengineering, University of California, Berkeley, USA.
E-mail address: siajunren@gmail.com (J. Sia).

http://dx.doi.org/10.1016/j.biomaterials.2016.06.034
0142-9612/

increased the yield of beating cells compared to flat surface. We also identified the regulation of Mkl1 activity as a new mechanism by which microgroove can affect reprogramming. Biochemical approach could only partially recapitulate the effect of microgroove. Microgroove demonstrated an additional effect of enhancing organization of sarcomeric structure which could not be recapitulated by biochemical approach.

2. Materials and methods

2.1. Cell culture and reprogramming

To obtain tail tip fibroblasts, neonates of αMHC-GFP transgenic mouse [1] were sacrificed by decapitation and the tail tips excised. Tail tips were digested with 2 mg/ml collagenase for 30 min and plated for explant culture. After a week, cells were frozen down into aliquots. To obtain Thy1+/GFP- cardiac fibroblasts, hearts from neonates were digested with 2 mg/ml collagenase and cultured for 3 days in growth media. Cells were then passed through a 40 μm cell strainer and incubated with APC conjugated anti-Thy1 antibody (eBioscience, 17-0909-41) before sorting with BD Bioscience Influx Sorter.

To make Gata4, Mef2c, Tbx5 and Mkl1 retroviruses, Platinum E cells were transfected with pMX plasmids encoding these genes using Fugene 6 overnight. Media was replaced the next day, collected after 24 h and passed through a 0.45 μm syringe filter. This virus containing media was used within the same day to transduce seeded fibroblasts.

For reprogramming experiments, fibroblasts were seeded onto tissue culture plates and then transduced with virus overnight. Transduced fibroblasts were then reseeded onto the various substrates, which have been prepared as described below, at 10000 cells/cm^2. Media was changed to DMEM/M199, 10% FBS, 1% NEAA, 1% Pen/Strep (reprogramming media) and replaced every 3 days.

For optimized culture to obtain beating cells [12], transduced fibroblasts were cultured in reprogramming media for the first 2 weeks. For the next 2 weeks, media was switched to StemPro-34 SF medium (GIBCO) with 1X GlutaMAX (GIBCO), 1% Pen/Strep, 50 μg/ml ascorbic acid (Sigma Aldrich), 5 ng/ml recombinant human VEGF (R&D Systems), 10 ng/ml recombinant human bFGF (R&D Systems), and 50 ng/ml recombinant human FGF10 (R&D Systems).

2.2. Stretching device fabrication

Refer to Fig. S1.

2.3. Polyacrylamide gels fabrication

Protocol for fabricating hydrogel substrate was adapted from previously published work [22]. 22 mm by 22 mm coverslips were soaked in 2 N NaOH for 20 min, rinsed in DI water and dried by N_2. Coverslips were then treated for 5 min in a 1% (v/v) methacryloxypropyltrimethoxysilane (Gelest), 94% (v/v) methanol (MeOH), 5% (v/v) water solution with glacial acetic acid added to a final concentration of 1 mM. Thereafter, coverslips were rinsed thoroughly in methanol before being oven baked at 110 °C for 30 min. Polyacrylamide gel solutions were prepared with varying concentrations of acrylamide and Bis acrylamide to vary rigidity. *N*-acryloyl-6-aminocaproic acid (ACA, TCI) monomer was added to a final concentration of 100 mM. Rigidities for the various formulations were confirmed by rheometry (Table S1). Ammonium persulfate (Biorad) and TEMED (Sigma Aldrich) were added to the gel solutions to a final concentration of 0.1% and mixed briefly. 25 μl of the polymerizing solutions were quickly placed on a Gel Slick™ (Lonza) treated glass slide before the silanized coverslips were placed on top. The gels were allowed to polymerize for 2 h before being peeled off from the glass slide. The gels were sterilized in 70% ethanol for 15 min and rehydrated in MES buffer (0.1 M2-(*N*-morpholino) ethanesulfonic acid, pH 6.2). Then, the carboxyl groups of the ACA were activated with 0.2 M EDC and 0.5 M NHS (Thermofisher Scientific) in MES buffer for 30 min at room temperature. Gels were then washed quickly with PBS before being reacted with 0.25 mg/ml Matrigel diluted in PBS overnight at 4 °C. Cells were seeded the next day.

2.4. Microgroove membrane fabrication

SU 8 mold was fabricated with standard photolithographic technique, as previously described [19]. Briefly, SU 8 photoresist (Microchem) was spin-coated onto a silicon wafer and polymerized by exposure to UV through a patterned chrome mask. The unpolymerized photoresist was subsequently washed away in developer solution. PDMS was spin coated over the SU 8 mold and cured. Cured membrane was washed with 70% ethanol, oven dried overnight and then oxygen plasma treated. Immediately afterwards, membrane was coated with 0.25 mg/ml Matrigel for 30 min before cell seeding.

2.5. Real time quantitative polymerase chain reaction

At the indicated time, culture was lysed with Trizol (ThermoFisher Scientific) and RNA was extracted following the manufacturer's instruction. RNA concentration was quantified with Nanodrop 1000 (Thermo Scientific) and equal amount was loaded for cDNA synthesis using Maxima First Strand cDNA Synthesis Kit (ThermoFisher Scientific). cDNA was then loaded into 96 well PCR plate with primers and Maxima SYBR Green qPCR Master Mix (ThermoFisher Scientific). Primer information is listed in Supplementary Table 2. Thermal cycling and data acquisition was performed on iQ5 system (Biorad). GAPDH was used as housekeeping gene. Data was analyzed with ΔΔCt method.

2.6. Immunostaining, imaging, flow cytometry and quantification

For immunostaining, cells were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton-X 100, both for 15 min. Cells were then incubated with Phalloidin (ThermoFisher Scientific), anti-cardiac troponin T (MS-295-P, Thermo Scientific) or anti-Mkl1 (sc-21558, Santa Cruz) antibodies overnight at 4 °C. Secondary antibody (Alexafluor 546, Thermofisher Scientific) were added for at least 1 h before the nuclei were stained with Hoechst. Imaging was performed on a Zeiss AxioObserver epi-fluorescent microscope.

To quantify reprogramming efficiency at day 10, samples were trypsinized and analyzed for number of GFP + cells using Guava. This number was then normalized by the number of cells seeded to give reprogramming yield. For quantification of cTnT + cells by flow cytometry, samples were trypsinized and fixed with 4% PFA for 15 min. Samples were then permeabilized and blocked concurrently with 0.4% saponin and 5% donkey serum diluted in PBS. Anti-GFP (GFP-1010, Aves Labs) and anti-cardiac troponin T (MS-295-P, Thermo Scientific) antibodies were added followed by secondary antibodies (Alexafluor 546, Thermofisher Scientific & F-1005, Aves Lab). In between every steps, cells were spun down and washed with PBS once.

For quantification of cTnT + cells by imaging, plates containing stained adherent cells were loaded onto ImageXpress Micro High-Content Analysis System (IXM, Molecular Devices) for automated whole well imaging at 10× magnification. The images were then