Progressive Myofibril Reorganization of Human Cardiomyocytes on a Dynamic Nano-topographic Substrate

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Abstract: Cardiomyocyte (CM) alignment with striated myofibril organization is developed during early cardiac organogenesis. Previous work has successfully achieved *in vitro* CM alignment using a variety of biomaterial scaffolds and substrates with static topographic features. However, the cellular processes that occur during the response of CMs to dynamic surface topographic changes, which may provide a model of *in vivo* developmental progress of CM alignment within embryonic myocardium, remains poorly understood. To gain insights into these cellular processes involved in the response of CMs to dynamic topographic changes, we developed a dynamic topographic substrate that employs a shape memory polymer (SMP) coated with polyelectrolyte multilayers (PEM) to produce a flat-to-wrinkle surface transition when triggered by a change in incubation temperature. Using this system, we investigated cellular morphological alignment and intracellular myofibril reorganization in response to the dynamic wrinkle formation. Hence, we identified the progressive cellular processes of hiPSC-CMs in a time-dependent manner, which could provide a foundation for a mechanistic model of cardiac myofibril reorganization in response to extracellular microenvironment changes.

Keywords: Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs); Stimuli-responsive biomaterials; Nano-topography; Shape memory polymer (SMP); Cardiac mechanobiology

INTRODUCTION

Nearly a century of mechanobiology research has implicated that dynamic nanoscale structural cues from extracellular matrices (**ECM**) as critical regulators of cellular morphology, differentiation, and functions at each sequential phase of tissue development.[1–5] However, current understanding and conceptual models to study cell developmental mechanobiology are still largely based on the static experimental platforms, in which the mechanical or structural cues remain constant over time. To better study how these mechano-inductive cues regulate tissue development at the level of the cell, recent efforts in the field have pursued *in vitro* approaches that recapitulate time-dependent mechanical and structural cues of ECM remodeling during development.[6–8] With recent advances in smart biomaterials, it is now possible to design and fabricate a cytocompatible substrate with dynamic nanoscale topographic properties to investigate how a changing extracellular microenvironment influences the cellular development.[9–11] As a class of smart biomaterials, shape memory polymers (**SMPs**) can memorize the permanent shape through crosslinking, be programmed to a temporary shape by an immobilizing transition (e.g., vitrification or crystallization), and later undergo dramatic shape change to return to the permanent shape by external stimuli (e.g., temperature or light).[12,13] Inspired by dynamic, time-dependent microenvironments *in vivo*, dynamic cell culture platforms based on SMPs have enabled in-depth investigations of cell-matrix mechanobiology.

SMP-based dynamic cell culture platforms have demonstrated their capability of introducing dynamic changes in shape and surface topography to mimic the progressive changes of mechano-structural cellular environment in tissue development and remodeling. As the early demonstration of SMPs used for dynamic cell culture, a

polyurethane-based glassy SMP substrate embossed with parallel microgrooves was triggered to complete a textured-to-flat surface transition, which induced morphological changes and reorientation of mouse embryonic fibroblasts.[14] In reverse, we also achieved a flat-to-textured surface transition by creating a SMP of poly(*tert*-butyl acrylate-co-butyl acrylate) (***t*BA-co-BA**) coated with a thin gold film.[12] By compressive buckling of gold thin film during the shape change, dynamic nanoscale wrinkle formation affected the orientation of cell nuclei, position of subcellular organelles (Golgi apparatus) and cell polarized motility.[15] Similarly, a thermal-responsive PCL-based SMP was developed to transit from a topographic patterned surface to a flat surface, which enforced the morphological transformation of human mesenchymal stem cells from an aligned spindle-like shape to a rounded stellate shape.[16]

The nanoscale structural cues from cell microenvironments play a key role to regulate cellular properties of the cardiomyocytes (**CMs**) by promoting cell alignment, improving contractile functions, and facilitating electromechanical transductions across the cardiac myofibers.[17–20] For example, nanofibrous scaffolds produced by electrospinning technique have been used to successfully generate aligned CMs with enhanced calcium cycling and contractile synchronization.[21] Biomaterial substrates with different nanoscale structures (e.g. nano-grooves, nano-grids, or nano-wrinkles) have been found to promote CM alignment along the direction of nanoscale topographic cues.[22–27] A recent study showed that nano-topographic cues had more dominant effects on hiPSC-CM alignment than material chemistry by comparing two substrate materials (polyurethane *versus* polystyrene) with two depths (100 nm *versus* 350 nm).[28,29] However, most nano-topographic surfaces are still largely built upon static biomaterial substrates, and thus are not able to recapitulate the dynamic ECMs remodeling events throughout cardiac tissue development. Focusing on dynamic mechanical cues, a PDMS-based substrate with dynamic reversible stiffness was developed to interrogate cardiac mechanotransduction via YAP/TAZ signaling from a perspective of bulk mechanical properties.[10] For dynamic structural cues, SMP-based dynamic substrate has been reported to induce reorientation of neonatal rat ventricular cardiac tissues by an orthogonal change of topographic directionality.[30] However, this study focused on the use of SMP for tissue engineering and translational purposes, instead of CM mechanobiology in response to dynamic structural cues.

In this study, we developed a cytocompatible SMP substrate with programmable surface topography based on a *t*BA-co-BA copolymer and polyelectrolyte multilayer (**PEM**) thin film coating. Shape recovery of the SMP substrate, triggered by a temperature increase, induced the nanoscale wrinkle formation of the PEM thin film. As expected, we found that hiPSC-CMs exhibited an aligned morphology on the SMP-PEM substrates promoted by the parallel oriented nano-wrinkles. Using the dynamic substrates of flat-to-wrinkled transition, we profiled a series of consecutive biological processes of cell morphology reshaping and intracellular myofibril reorganization of hiPSC-CMs in response to the dynamic topographic changes. This study would provide new evidence to understand the mechanism of CM alignment based on an *in vitro* culture system, which, to some extent, mimics the dynamic changes of cardiac alignment in early fetal heart development. Hence, we envisage that the combination of SMP-based dynamic substrate and hiPSC technology could provide a great potential to establish new analytical tools and *in vitro* model systems for developmental cell mechanobiology.

MATERIALS AND METHODS

hiPSCs culture

The human induced pluripotent stem cell (**hiPSC**) line WTC was obtained from Bruce R. Conklin lab at the Gladstone Institute of Cardiovascular Disease. The hiPSCs were grown on 6-well plates coated with Geltrex (Thermo Fisher Scientific, Ca# A1413302) in Essential 8 (E8) media (Thermo Fisher Scientific, Ca# A1517001) refreshed every 24 hours. The hiPSCs were passaged to a new plate every 3 days, at the seeding density of 2.5 x 10^4 cells/cm^2 in the E8 media supplemented with 10 μM ROCK inhibitor (Y-27632; BioVision, Ca# 1994) for the first 24 hours.

hiPSC-CMs differentiation and purification

The experimental procedures of hiPSC-CMs differentiation has been detailed in our previous publications.[31] In brief, differentiation was initiated at Day 0 by treating confluent hiPSCs with 6 μM GSK3 inhibitor (CHIR99021;

Stemgent, Ca# 04-0004) for 48 hours in RPMI 1640 (Thermo Fisher Scientific, Ca# A1517001) media with B27 supplement minus insulin (Thermo Fisher Scientific, Ca# A1895601) (**RPMI-B27-I**). Next, cells were treated with 5 μM Wnt inhibitor (IWP4; Stemgent, Ca#04-0036) in RPMI-B27-I media for another 48 hours. Last, cells were cultured in RPMI-B27-I for another 2 days, before switching to RPMI 1640 media with B27 complete supplement (Thermo Fisher Scientific, Ca# 17504044) (**RPMI-B27+C**) for continuing culture until Day 20. The differentiated hiPSC-CMs were dissociated on Day 20 for purification procedures. In brief, hiPSC-CMs were treated with STEMdiff™ cardiomyocyte dissociation kit (Stem Cell Technologies, Cat# 05025) per protocol provided by the vendor. The dissociated cells were collected and replated in Geltrex-coated 6-well plates with RPMI-B27+C media supplemented with 10 μM Y-27632. After 2-day recovery in RPMI-B27+C media, cells were incubated with purification media for 6 days, made of DMEM no glucose (Thermo Fisher Scientific, Ca# 11966-025), NEAA (Thermo Fisher Scientific, Ca# 11140050, GlutaMAX (Thermo Fisher Scientific, Ca# 35050061) and 4 mM lactate (Sigma Aldrich, Ca# L7022). After purification, cells were cultured in RPMI-B27+C media refreshed every two days.

Shape memory polymer (SMP) fabrication

The SMP, crosslinked poly(*t*BA-co-BA), was synthesized by UV polymerization of monomers, *tert*-butyl acrylate (*t*BA) and butyl acrylate (BA); photoinitiator, 2,2-dimethoxy-2-phenyl acetophenone (DMPA); and crosslinker, tetraethylene glycol dimethacrylate (TEGDMA). The monomer solution of 95 wt% *t*BA (AcRos Organics, Ca# 371130010) and 5 wt% BA (Sigma Aldrich, Ca# 234923) was prepared with 1 wt% DMPA (Sigma Aldrich, Ca# 196118) and 5 wt% TEGDMA (Sigma Aldrich, Ca# 86680) of total weight of *t*BA and BA.[12] The solution was mixed and injected into two Rain-X-coated glass-slides separated with a 1 mm thickness Teflon spacer, and then crosslinked in a UV light box (Black Ray, 365 nm, 2.0 mW/cm^2) for one hour. Next, the samples were immersed in a solution of 50% methanol and 50% water overnight to remove excess monomers. The crosslinked polymers were dried at room temperature in the chemical hood for 24 hours and then in the vacuum oven at -15 PSI at 40°C for another 24 hours. After drying, the polymers were cut into 25 mm by 5 mm rectangles. To program the strain that would later be recovered during shape-memory triggering, each piece of rectangular SMP was preheated to 70°C for 5 minutes, then stretched uniaxially to 140% of original length (40% strain) using an in-house designed manual polymer stretcher, and finally cooled down to room temperature for 10 minutes (**Figure 1a**).

Polyelectrolyte multilayer (PEM) coating

Each piece of SMP was coated with the PEM thin film, which would subsequently form the nano-wrinkles during SMP shape recovery. The SMP was placed onto the double-sided tape on a glass slide, which was then secured on a spin coater. Spin-coating of PEM film onto the SMP surface started with one layer of 3 wt% polyethylenimine (PEI) (Sigma, Ca# 408727) at 3000 rpm for 12 seconds. Next, 20 bilayers of poly(styrene 4-sulfonate) (PSS) as polyanion and poly(allylamine hydrochloride) (PAH) as polycation were consecutively spin-coated on the SMP surface with twice water rinse between each layer (**Figure 1b**). PSS (MW = 70 kDA and pkA ~ 2, Sigma Aldrich, Ca# 43457) and PAH (MW = 70kDa and pkA ~ 8.5, Sigma Aldrich, Ca# 283215) were made by dissolving the powders in 2M sodium chloride (NaCl, Sigma Aldrich, Ca# 57653) solution to make 0.1 M concentration stock solutions. The pH of both solutions was adjusted to 3.5 with 0.1 M Hydrogen Chloride (HCl, Sigma Aldrich, Ca# 320331) to create a fully ionized polymer. The spin-coating for each layer and water rinse was completed at 3000 rpm for 12 seconds.[32]

Cell seeding procedure

The unstrained (static flat), pre-recovered (static wrinkled) and dynamic (flat-to-wrinkled) SMPs with PEM coating (**SMP-PEM**) were coated by Geltrex for one day in the 48-well plate at room temperature. Purified hiPSC-CMs were dissociated with 0.025% trypsin for 10 minutes, centrifuged at 800 RPM for 5 minutes, re-suspended with RPMI-B27+C media, seeded to the SMP-PEM in the 48-well plates with a density of 30,000 cells per well, and incubated at 30°C with 5% CO_2 for two days, before the incubation temperature was raised to 37°C for SMP shape recovery (**Figure 1c**).

Chemical treatment

To analyze how cell mechanosensitivity affects with alignment of hiPSC-CMs, we selected four small molecules to inhibit the cell cytoskeletal tension, including Blebbistatin (Sigma-Aldrich, Ca# 203390), Y27632 (BioVision, Ca# 1994), FAK inhibitor 14 (Y15, Sigma-Aldrich, Ca# SML0837) and Cytochalasin D (Sigma-Aldrich, Ca# C8273). The small molecules were dissolved in dimethylsulfoxide (DMSO) and diluted in RPMI-B27+C media to yield the final concentrations of 10 μM and 20 μM. The hiPSC-CMs on the dynamic SMP-PEM substrate were treated by these chemicals. Specifically, after hiPSC-CMs were seeded on the dynamic substrate (flat) and cultured at 30°C for 2 days, cell culture media was replaced by RPMI-B27+C media with one chemical at different concentrations, and culture temperature was increased to 37°C to trigger the wrinkle formation. The treatment lasted for 36 hours, which covered the entire process of cell alignment and myofibril reorganization. At the end of the treatment, hiPSC-CMs were fixed and stained with α-actinin and F-actin for fluorescent imaging.

Atomic force microscopy (AFM)

To characterize the wrinkle morphology, strained SMP-PEM samples were placed on Teflon in an isothermal oven at 42°C for 30 minutes to trigger return to the original permanent shape. Then, the samples were placed in a vacuum oven to dry at room temperature at -15 PSI for 5 days and cut into 3.5 mm by 5 mm rectangles. Atomic force microscopy (AFM; Nano R-2 from Pacific Nanotechnology) with contact mode using a Si_3N_4 cantilever (spring constant: 5 N/m) was employed to characterize the wrinkle morphologies of these samples. A 50 μm square area was scanned for the wrinkled surface with a height of 300 nm and scan frequency of 4 Hz.

Scanning electron microscopy (SEM)

The hiPSC-CMs on SMP-PEM were fixed in 4% paraformaldehyde (PFA) overnight with 0.1M PBS at room temperature. After washing three times with PBS, samples were then dehydrated in a series of concentrations of ethanol (15%, 30%, 50%, 70%, 95% and 100%) at room temperature for 15 minutes at each concentration, plus two more 100% ethanol dehydration at last. After dehydration, samples were dried in the vacuum oven for one day. The polymer samples without cells did not require the dehydration procedures, and were only dried in the vacuum oven for one day. Prepared samples were placed on stubs, and sputter-coated a layer of gold thin film with a thickness of 10 nm. At last, scanning electron microscope (SEM, JSM-IT100LA, JEOL USA Inc.) was used to image the SMP-PEM surface with and without cells.

Immunocytochemistry

Cell samples for staining were fixed in 4% PFA solution for 20 minutes, permeabilized with 0.2% triton solution (Sigma Aldrich, Ca# T8787) for 5 minutes, and blocked with 2% bovine serum albumin solution (BSA; Sigma Aldrich, Ca# A8022) for 30 minutes. After removing the BSA solution, samples were incubated with primary antibodies (**Table S1**) for 2 hours, washed with PBS three times, and then incubated with secondary antibodies for 1.5 hours. Phalloidin staining for F-actin was performed with secondary antibodies incubation. After three washes of PBS, the samples were incubated with DAPI solution for 10 minutes for nuclei staining. For bright-field and epifluorescence microscopy, the images were taken with a Nikon Eclipse Ti microscope with Zyla 4.2 PLUS sCMOS camera. For confocal microscopy, the images were taken with a Zeiss LSM 710 confocal microscope at Blatt Image Center, Syracuse University.

Cell remodeling quantification

The cellular remodeling and myofibril reorganization were characterized based on fluorescent images of the individual hiPSC-CMs (**Figure 1d**). To analyze the cellular remodeling, an individual hiPSC-CM was surrounded by a rectangle. To determine the hiPSC-CM elongation, cell aspect ratio was calculated based on the ratio between length and width of the rectangle. To determine the hiPSC-CM alignment, cell orientation was measured based on the angle between the rectangle length and wrinkle direction. For the flat surface, cell orientation was measured based on the angle between the rectangle length and X-axis of the image. To analyze the nuclei morphological changes, the nucleus of an individual hiPSC-CM was surrounded by a rectangle, and nuclei aspect ratio and orientation were measured using the same methods as cell aspect ratio and orientation, respectively. The cell and nuclei orientation were analyzed and plotted as polar-histogram charts using MATLAB. The cell perimeter (P) and

cell area (A) were measured using ImageJ, and cell shape index (CSI) was calculated based on the equation of $CSI = \frac{4\pi \times A}{P^2}$.[33]

To analyze the myofibril reorganization, the myofibril components (Z-lines, thin filaments, thick filaments and focal adhesions) were stained and imaged using the confocal microscope. The measurement of length for each myofibril component was averaged from 4-6 random positions on one hiPSC-CMs and 3-5 hiPSC-CMs per image. The sarcomere length was measured between two adjacent Z-lines stained by sarcomere α-actinin. The length of thin filament was measured between two adjacent M-lines stained by Phalloidin (F-actin). The length of thick filament was measured as A-band stained by myosin heavy chain (β-MYH). The length of focal adhesion was measured based on the vinculin staining at the ends of thin filaments.

Quantitative sarcomere analysis

To analyze the sarcomere organization, an image-processing algorithm based on 2D fast Fourier transformation (**2D FFT**) was used for the fluorescent images of sarcomere α-actinin staining.[34] To ensure the robustness of this algorithm for sarcomere analysis at different conditions, we cropped individual hiPSC-CMs using a 300 pixels X 300 pixels frame (**Figure S1a**). Well-organized sarcomeres in a hiPSC-CM contain spatially repeating patterns of sarcomere α-actinin staining, which can be extracted as a periodic signal by 2D FFT (**Figure S1b**). The periodic high-frequency peak signals can be used to compute the "*sarcomere score*" for individual hiPSC-CMs (**Figure S1c**). We only compute the peak value at periodic term around 0.3 with largest peak value labeled with red triangle as the sarcomere score (**Figure S1d**).

Contractile function analysis

The beating videos of individual hiPSC-CMs were analyzed by Motion-Tracking algorithm.[35] The software is available at https://gladstone.org/46749d811. The positions of pixel macroblocks were tracked in each frame to generate motion vectors between adjacent frames to calculate the velocity. An average motion velocity heatmap was generated for each beating video. The algorithm can output the parameters of beat rate (beat per minute, BMP), time interval between contraction and relaxation (C-R interval), contraction velocity (μm per second), relaxation velocity (μm per second) and X/Y contraction ratio (X-direction contraction velocity divided by Y-direction contraction velocity).

Statistical analysis

Data was analyzed using the software GraphPad Prism 6. Data is presented as box plots showing the minimum, maximum, median, and 25th and 75th percentiles. One-way ANOVA with multiple t-test was used to compare the difference among different groups. The statistical significance was determined based on p-value < 0.05 (*), < 0.01 (**), < 0.001 (***) and < 0.0001 (****), respectively. For each condition, twenty hiPSC-CMs were selected for quantification. On dynamic surface, each hiPSC-CM was randomly selected six different positions for sarcomere length quantification, four random positions for actin and myosin length quantification. For vinculin length quantification, each hiPSC-CMs has been selected six to eight clear and bright long shape vinculin spots.

RESULTS

Nano-wrinkle formation on SMP-PEM surface

*t*BA-co-BA SMPs were UV crosslinked, stretched to 40% strain (140% of original length), coated with 20 bilayers of PEM, and cut into small pieces for cell seeding (**Figure 1**). In our previous study, we have shown that the SMPs under dry condition have a *Tg* at 42 °C, which decreases to ~ 37 °C after hydration in cell culture media.[12] Therefore, the SMPs can maintain the temporal shape (40% strain) under room temperature and recover to the original shape when the temperature increases above 42 °C (dry condition) or 37 °C (hydrated condition). Due to the shape recovery, the thin PEM film on the SMP surface buckles to form wrinkles. Wrinkle formation on the SMP-PEM surface is fully completed within 30 minutes at 42 °C under the dry condition. In the cell culture media (hydrated condition), wrinkle formation on the SMP-PEM surface is observed within 1 hours at 37 °C and fully completed within 4 hours. No wrinkle formation occurs on the surface at 30 °C under hydrated condition. To characterize the

wrinkle morphology of SMP-PEM substrates, we compared the flat surface before shape recovery (**Figure 2a, Figure S2a**) and wrinkled surface after shape recovery (**Figure 2b, Figure S2b**) under the dry condition using AFM. We found that shape-recovered SMP-PEM had apparent wrinkle formation on the surface with an average depth of ~ 200 nm and an average width of ~ 600 nm, and Geltrex coating had no effect on the wrinkle morphology (**Figure S2c**). We also confirmed the wrinkle formation using SEM for the shaped-recovered SMP-PEM substrate under the dry condition (**Figure 2c**) and under the hydrated condition with a hiPSC-CM growing on the top (**Figure 2d**).

Cellular alignment of hiPSC-CMs on the static nano-topographic surface

First, we tested whether nano-wrinkles on the SMP-PEM surface can effectively align individual hiPSC-CMs in culture. We seeded the hiPSC-CMs on the Geltrex-coated static SMP-PEM substrates with flat surface and wrinkled surface under 37 °C. The flat surface was created from unstrained SMP-PEM substrate, while the wrinkled surface was created from strained SMP-PEM substrate, which had been dry pre-recovered at 42°C for 30 min before media hydration and cell seeding. Based on fluorescent images and brightfield images (**Figure 2e, 2f**), we observed that hiPSC-CMs on the static wrinkled surface showed cell alignment parallel to the wrinkle direction, while the hiPSC-CMs on the static flat surface did not show any directionality preference. The static wrinkled surface promoted the hiPSC-CMs elongation with a significantly higher cell aspect ratio than the flat surface (**Figure 2g**). However, the nuclei aspect ratio did not show significant difference between flat and wrinkled surfaces (**Figure 2h**), indicated that nanoscale topography influenced more on the cell spreading along the wrinkle directions. Next, to determine cell orientation and nuclei orientation, we measured the angles between the wrinkle direction and the long axis of a cell or a nucleus, respectively (**Figure 2i**). On the static flat surface, hiPSC-CMs spread randomly into a large distribution of orientations, with only 5% of total cells in the range of -15° to 15° to the X-axis of each image. On the static wrinkled surface, hiPSC-CMs were well-aligned to the wrinkle direction, with about 75% of total cells in the range of -15° to 15° to the wrinkle direction. By measuring the nuclei orientation, we found ~ 75% of total nuclei on the wrinkled surface in the range of -15° to 15° to the wrinkle direction, but only 35% of total nuclei on the flat surface oriented into one direction.

To demonstrate the organization of sarcomere and myofibril structures on the nano-topographic surface, we performed immunostaining and confocal microscopy for the hiPSC-CMs on the static wrinkled surface. Well-organized Z-lines (also called Z-discs) were stained by sarcomere α-actinin, and myofibril filaments were stained by F-actin and myosin heavy chain (β-MYH). Z-lines were located in the middle of thin filaments, indicating that actin thin filaments tightly connected to the Z-disc structures (**Figure 2j**). The thick filaments stained by β-MYH were located in the center of each sarcomere, partially overlapped with actin thin filaments, but did not directly connect with Z-discs, showed as distinct bands (**Figure 2k**). Troponin complex is a group of proteins on the thin filaments, sensitive to intracellular calcium concentration for initiation of cardiac contractions, and Troponin T staining in our images revealed substantial overlap with actin thin filament (**Figure 2l**) but not with Z-lines. Finally, sarcomeres and myofibrils are anchored to the cell membrane through focal adhesions. Our vinculin staining at the membrane periphery of hiPSC-CMs showed clear association with actin thin filament (**Figure 2m**). Especially, repeating patterns of focal adhesion proteins of paxillin, vinculin, and zyxin spatially associated with the middle line of each actin thin filament, indicating the formation of costameres (**Figure S3**). All these images demonstrated the spatial relationship of different components of sarcomeres and myofibrils of hiPSC-CMs. These results suggested that the SMP-PEM substrate supported normal cell growth and spreading, and, more importantly, nano-wrinkles promoted the alignment of intracellular cardiac myofibrils and parallel organization of sarcomere structures.

Cellular alignment of hiPSC-CMs on the dynamic nano-topographic surface

To elucidate how hiPSC-CMs would respond to the dynamic topographic changes on the SMP-PEM, we first compared the hiPSC-CM alignment between static and dynamic surfaces. Three surfaces were set up as a static flat surface, a static wrinkled surface, and a dynamic flat-to-wrinkled surface (**Figure S4a**). All three groups of SMP-PEM substrates were coated with Geltrex at room temperature, seeded with hiPSC-CMs, and incubated at 30°C for 2 days for cell spreading. Next, all the samples were moved to 37°C incubation for another 36 hours with media

refreshment. During this period of time, the dynamic surface formed nano-wrinkles underneath the hiPSC-CMs, while static surfaces had no topographic change. Based on actin and α-actinin staining, we could identify clear sarcomere structure from the hiPSC-CMs on all three surfaces, while hiPSC-CMs had a preferential orientation to the wrinkle directions on the surfaces with wrinkle formation (**Figure S4b**). The hiPSC-CMs exhibited elongated morphology with higher cell aspect ratio on the surfaces with wrinkle formation than the ones on flat surface. Static wrinkled surface induced higher cell elongation than the dynamic surface (**Figure S4c**). Similar to previous experiments with static surfaces (**Figure 2h**), we did not observe significant difference on the nuclei aspect ratio amongst different surfaces (**Figure S4d**). For myofibril analysis, we measured the sarcomere length between two adjacent Z-lines, and determined the sarcomere organization using the "*sarcomere index*" calculated based on a 2D FFT algorithm (**Figure S1**). Interestingly, we found no significant difference on both sarcomere length and sarcomere index amongst three different surfaces (**Figure S4e, S4f**). These results indicated that hiPSC-CM alignment on the dynamic surfaces at 36 hours post shape-recovery triggering could reach to a stable myofibril reorganization, comparable to the hiPSC-CMs on the static surfaces.

To study how different surfaces would impact on the cell shape of individual hiPSC-CMs, we measured the cell perimeter, cell area, and cell shape index (CSI). We found no significant difference on cell perimeter (**Figure S5a**), but the cell area and CSI significantly decreased for the hiPSC-CMs on the wrinkled surface (**Figure S5b, S5c**). The decrease of CSI indicated that hiPSC-CMs exhibited an anisotropic cell shape (**Figure S5c**), which was consistent with the result of cell aspect ratio, showing an elongated morphology (**Figure S4c**). To study the contractile functions of single hiPSC-CMs (**Figure S6a**), we found significant difference on beat rate of the hiPSC-CMs between static flat and static wrinkled surfaces (**Figure S6b**), but not on the C-R interval (**Figure S6c**), contractile motion velocity (**Figure S6d, S6e**). We found a slight increase of X/Y contraction ratio, indicating better anisotropic contraction on the wrinkled surfaces (**Figure S6f**).

Next, with a focus on the dynamic surface, we recorded the progressive remodeling of hiPSC-CMs in response to the topographic changes within 24 hours. After 2 days incubation at 30°C, the dynamic SMP-PEM substrate with hiPSC-CMs was moved to a 37°C incubator to trigger the shape recovery and wrinkle formation, which is the first time point (hour 0). Within the next 24 hours, samples were collected every 4 hours for analyzing cellular alignment and myofibril reorganization of the hiPSC-CMs. For cell orientation, hiPSC-CMs had no preferential directionality within hour 0 – 12, while hiPSC-CMs slowly reoriented to the wrinkle direction as a preferential cellular alignment starting at hour 16 (**Figure 3a**). However, no preferential directionality was observed for the nuclei orientation during the entire 24-hour experiments (**Figure 3b**). In addition, we measured the cell and nuclei aspect ratios to analyze the cell morphological elongation within 24 hours. From the results, we found that cell aspect ratio slightly increased from hour 16 (**Figure 3c**), but nuclei aspect ratio remained the same during the entire 24-hour experiments (**Figure 3d**). The difference in changes of shape and directionality between cells and nuclei suggested that cell bodies might have a higher plasticity than the nuclei, which makes them more responsive to the dynamic topographic changes. Consistent with the result of cell aspect ratio, we also observed a trend of slight decrease in CSI, indicating an anisotropic cell shape, through cell perimeter and cell area showed no significant difference during the entire 24-hour experiments (**Figure S5d-f**). For the temporal changes of contractile functions, we found that hiPSC-CMs gradually increased the beat rates, and showed significant differences on the late timepoints of hour 16 – 24 (**Figure S6g**), which might have resulted from the temperature increase that triggered the shape recovery. However, we found no significant changes on C-R interval, contraction and relaxation velocity, and X/Y contraction ratio for the hiPSC-CMs on the dynamic surfaces (**Figure S6h-k**), which indicated that the slight changes on cell anisotropic morphology (**Figure 3c and Figure S5f**) had not yet translated into the changes on anisotropic contractile functions.

Cell reshaping and reorientation are tightly related to the intracellular cytoskeletal reorganization in response to the extracellular environment changes. The remodeling of focal adhesions and cytoskeletal structures regulated by the wrinkle formation might direct the cellular alignment of hiPSC-CMs on the dynamic PEM-SEM substrate. Especially, hiPSC-CMs have unique cytoskeletal structures of well-organized repeating patterns of contractile myofibril and sarcomere units (**Figure 3e**), which makes it important to study the processes of myofibril

reorganization during the cellular alignment. In this study, we focused on four main components of myofibril structures, including thin filaments stained by F-actin, thick filaments stained by β-MYH, Z-discs stained by sarcomere α-actinin, and focal adhesions stained by vinculin (**Figure 3f**). To analyze myofibril responsiveness to the wrinkle formation, we measured the length for each component every 4 hours after shape-recovery triggering and compared to the original length at hour 0. We found that sarcomere index showed a noticeable drop at hour 4 – 8, indicating a temporal correlation between sarcomere disorganization and wrinkle formation (**Figure 3g**). Thin filament length significantly increased within hour 8 – 24 (**Figure 3h**), while thick filament length remained the same during the entire 24-hour experiments (**Figure 3i**). An increase of sarcomere length of hiPSC-CMs was found within hour 16 – 24 compared to hour 0, but a significant decrease of length was also observed from hour 20 to hour 24 (**Figure 3j**). Next, we measured the length of vinculin staining to estimate the maturity and stability of focal adhesions, and found that wrinkle formation had a remarkable influence on the focal adhesion dynamics. A decrease of vinculin length at early time points (hour 4 and hour 8) indicated the disassembly of focal adhesions due to surface topographic changes, while recovery of vinculin length to the original level at hour 12 indicated the reassembly of mature focal adhesions for stable cell attachment to the substrate (**Figure 3k**).

Disruption of myofibril remodeling on dynamic topographic surface

To test how cell mechanosensitivity and cytoskeletal tension would affect the hiPSC-CM remodeling induced by dynamic topographic changes, we used four small molecules to inhibit myosin kinase (blebbistatin), RhoA/ROCK kinase (Y27632), actin polymerization (cytochalasin D) and focal adhesion kinase (FAK inhibitor 14, Y15) at two different concentrations (10 μM and 20 μM), together with DMSO controls to each chemical (**Figure 4a**). The chemicals were applied individually at hour 0, when the incubation temperature was raised for shape recovery and wrinkle formation, and terminated at hour 36, when the hiPSC-CMs on the dynamic surface reach a stable myofibril reorganization. We found that these chemicals significantly affected the response of hiPSC-CMs to the nano-topographic changes for cellular alignment, shown as lower cell aspect ratio (**Figure 4b**). However, the nuclei aspect ratio was not affected by the chemical treatment (**Figure 4c**). From the fluorescent images, we observed that blebbistatin and Y27632 treatment increased the cell area by reducing cell contractility, while cytochalasin D dramatically decreased the cell area by inhibiting actin polymerization (**Figure 4d**).

We found that inhibition of cell contractility by blebbistatin and Y27632 increased the sarcomere length, which might have resulted from the impediment of neo-myofibril formations but extension of existing myofibrils within a spreading hiPSC-CMs. In contrast, inhibition of actin filament stability by cytochalasin D and Y15 decreased the sarcomere length, which might be induced by the disassembly of myofibrils within the hiPSC-CMs (**Figure 4e**). Though different chemicals showed different effects on sarcomere length, all the treatments resulted in a decrease of sarcomere index, indicating a high level of sarcomere disarray (**Figure 4f**). Overall, interference on cell mechanosensitivity via small molecule inhibition not only disrupted the cellular response to the wrinkle formation, but also induced severe sarcomere disorganization due to the loss of cytoskeletal tension and mechanical integrity.

DISCUSSION

Cardiac tissues are generally characterized as a well-organized cellular architecture with highly aligned CMs, with anisotropic elongated cell shape and parallel organization of intracellular myofibrils and sarcomeres.[36–38] However, such coherent structure of CM alignment is not developed until gestational age 14 week for humans,[39] or embryonic day 16 for mice.[40] While more evidence has indicated that ECM structural cues play a pivotal role in the evolution of emergent myocardial cytoarchitecture, the progression of CM alignment in response to dynamic ECM cues during tissue development remains poorly understood and characterized. In this study, we successfully established a dynamic SMP-PEM substrate system, which enabled the changes of surface topography by temperature-controlled flat-to-wrinkle transition. This dynamic nano-topographic system not only preferentially facilitated the alignment and elongation of hiPSC-CMs, but also enabled profiling the progressive cell reshaping and myofibril reorganization in a time-dependent manner (**Figure 5a**). This allowed us to gain a better understanding of cellular processes of hiPSC-CMs in response to the dynamic structural cues from cell microenvironment.

By anchoring to the transmembrane integrin, cell focal adhesions are directly affected by external mechano-structural stimuli and then regulate downstream cytoskeletal dynamics.[41,42] It has been well documented that focal adhesions play a role in the CMs as the mechano-structural sensor to extracellular changes and transduce the mechanical signals through Z-discs to stabilize the contractile myofibrils.[43,44] In our study, disassembly and reassembly of long-stable focal adhesions was the first biological event occurred during hour 4 - 8, when hiPSC-CMs responded to nano-wrinkle formation on the dynamic substrate (**Figure 3k**). Focal adhesion kinase (**FAK**) is the essential signaling machinery of focal adhesion dynamics.[45–48] It has been reported that FAK inhibition could enhance the stability of cell focal adhesions to the substrate, and thus promote the assembly of neo-myofibrils.[49,50] In our results, Y15-treated hiPSC-CMs obviously recruited large focal adhesion sites on the edge of cells, indicating that FAK inhibition stabilized the costameres consisting of focal adhesions and interconnected Z-discs. However, some sarcomeres located at the center of cell bodies of Y-15-treated hiPSC-CMs lacked clear actin thin filament associated with the Z-discs. Instead, actin thin filaments are more likely appearing at the cell periphery regions. This phenomenon is consistent with a previous report that retrograde flow of myofibrils from cell periphery to nucleus region was impaired by the FAK inhibition.[51] It seems that FAK inhibition by Y15 could stabilize the focal adhesions but impede retrograde flow of actin filament, which eventually led to the loss of sarcomere organization.

There is growing evidence that focal adhesions could coordinate the force transmission between CMs and ECMs, and thus direct the myofibril growth '*myofibrillogenesis*' of the CMs.[41,52,53] At hour 8, we observed an increase of actin filament length, following the reassembly of focal adhesions, which indicated the close relationship between costamere assembly and myofibril extension. At hour 16, sarcomere length between two Z-lines significantly increased, which was coincident with hiPSC-CM alignment to the wrinkle directions (**Figure 3a & 3j**). There is a clear sequential order of wrinkle formation (hour 4), focal adhesion dynamics (hour 4 – 8), actin filament elongation (hour 8), sarcomere extension (hour 16), and cell alignment (hour 16 – 24) (**Figure 5b**). Surprisingly, we did not observe length change on myosin thick filaments during the entire myofibril reorganization processes (**Figure 3i**). It is possible that cytoskeletal tension that triggered the extension of actin filament and sarcomere was conserved by the titin structure, thus not transduced to the myosin filaments. Titin, known as the molecule spring in the sarcomeres, can release most tension from Z-discs by unfolding it elastic region at myofibril I-bands.[54–56] In addition, we observed a decrease of sarcomere length starting at hour 20 (**Figure 3j**), which was further confirmed at hour 36, showing no significant difference in sarcomere length between static and dynamic substrate (**Supplemental Figure 3e**). These results indicated that sarcomeres only temporally extended during myofibril reorganization in response to the wrinkle formation, and would return to the initial length after external microenvironment became steady again. Overall, focal adhesion dynamics and myofibril reorganization were the key time-specific biological events that directed the alignment and reorientation of hiPSC-CMs, because of the changes of nanoscale surface topography on the dynamic substrate.

For myofibril development during cardiomyocyte alignment and maturation, a three-step myofibrillogenesis model has been well established to explain the progression of pre-myofibrils (actin filament assembly associated with Z-bodies and non-muscle myosin II), nascent myofibrils (Z-line alignment with incorporation of muscle myosin and titin), and eventually mature myofibrils (completion of myofibril organization with addition of myomesin and C-protein).[57–60] Especially at the early stage of pre-myofibril assembly, actin filament polymerization is the first step to provide stable scaffolds to recruit α-actinin to form Z-bodies, which is consistent with our observation of actin filament growth prior to Z-disc extension.[61] Previous studies have shown that cardiac myofibrillogenesis occurs on the timescale of hours. Chick embryonic CMs showing clear formation of neo-myofibrils requires 2.5 hours, while full formation of mature myofibrils requires more than 4 hours.[62] Our present study focused on cell morphological alignment, which requires a longer timescale than monitoring molecule dynamics inside the cells, but more subtle changes on myofibril reorganization might be identified with time interval less than 4 hours. Time-lapse recording of live hiPSC-CMs on the dynamic substrate could provide us more accurate and time-sensitive cellular behaviors, which will be the key investigations of this study in the next phase.

During early heart formation, the changing mechanical properties of the developing myocardium also play an important role in regulating myofibril organization and contractile functions.[63,64] Inspired by the developmental

mechanobiology, it has been well documented that hiPSC-CMs respond to the cellular microenvironments provided by both passive biomaterial properties (e.g. stiffness, micropatterning, and topography) and active mechanical stimulation (e.g. stretching, compression, and shear stress). For example, optimal substrate stiffness was required to promote the myofibril assembly, sarcomere organization and contractile force generation. For passive mechanical stretching, neonatal rat CMs showed peak myofibril remodeling events at 3 – 4 hours after being applied 10% mechanical strain.[65] Similarly, another study based on neonatal rat CMs under 10% mechanical strain also reported the observation of sarcomere growth on the timescale of 40 – 80 minutes.[66] Both these studies demonstrated that new sarcomeres can be added to the end of myofibrils, or inserted in the middle of exist myofibrils. In addition, active cyclic mechanical stimulation has been widely used to mimic the mechanical loading during the cardiac cycles,[67–69] resulting in a fast-changing mechanical input, which is fundamentally different from the developmental timescale of structural ECM cues. Normally, these systems are not designed for the study of the dynamic cellular processes during tissue development, since tissue morphogenesis events occur on a timescale longer than mechanical loading during contraction.

It has been reported that cell contractility of CMs is required not only for assembling new myofibrils but also for maintaining existing myofibrils.[70] To test how cytoskeletal tension would affect hiPSC-CM sarcomere structures in our myofibril reorganization model, we treated the cells with different chemicals, including blebbistatin (myosin kinase inhibitor), Y27632 (ROCK kinase inhibitor) and cytochalasin D (actin polymerization inhibitor). Overall, the decrease of cytoskeletal tension impaired the integrity of sarcomere structures, and biochemical inhibition could abolish the hiPSC-CM mechanosensitivity to the nano-topographic changes for effective cell alignment (**Figure 4**). However, inhibition of cell contractility (blebbistatin and Y27632) and inhibition of actin polymerization (cytochalasin D) had distinct effects on cell area and sarcomere length. We believed that the increase of sarcomere length of blebbistatin/Y27632-treated hiPSC-CMs was related to the spreading of cell area, since insufficiency of neo-myofibril formation would induce the extension of existing myofibrils to compensate the expansion of cell periphery area. In contrast, cytochalasin D treatment would significantly reduce the cell area by disrupting actin filament polymerization, which resulted in the disassembly of sarcomere structures with a decrease of sarcomere length.

CONCLUSIONS

In this study, we have established and employed an *in vitro* SMP-based platform with a dynamic nano-topographic surface to study how changes in the structure of extracellular environment influence cell development of hiPSC-CMs. Based on this dynamic substrate system, we established a mechanistic basis of time-specific myofibril reorganization of hiPSC-CMs in response to the changes of surface nano-topographic properties, which could potentially provide better understanding of CM dynamic mechanobiology. To advance the dynamic cell culture systems, we are developing more advanced SMP-based substrates with multifaceted configurations and multi-time programmability, in order to achieve complex wrinkle patterns, bi-directional transition, and triggering by other stimuli. In future, genome-engineered hiPSC lines with molecular reporters of myofibril and sarcomere proteins will make it possible to track cellular processes of live hiPSC-CMs in response to the dynamic changes of nano-topographic cues. This could provide us more accurate and time-sensitive cellular behaviors, which will be the key investigations of this study in the next phase. Moreover, additional characterization on other structural components (e.g., titin, desmin, paxillin, and talin) in a time-dependent manner will provide more evidence on our CM myofibril reorganization model.

SUPPORTING INFORMATION

Table for the antibodies used in this study and figures for additional surface characterization and complementary results on cell shape, sarcomere alignment, focal adhesions and contractile functions for hiPSC-CMs.

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DATA AVAILABILITY STATEMENT

The raw and processed data required to reproduce these findings will be made available via contact with corresponding author Z.M. (zma112@syr.edu).

AUTHOR CONTRIBUTIONS

S.S., H.S. and Z.M. conceived and designed the experiments. S.S. and H.S. performed the polymer sample fabrication, biological experiments and data analysis. S.M. performed the polymer sample fabrication and atomic force microscopy. S.S. performed scanning electron microscopy. A.S., P.T.M. and J.H.H. developed shape memory polymer system. S.S., H.S. and Z.M. wrote the manuscript with discussions and improvements from all authors. Z.M. supervised the project development and funded the study.

CONFLICT OF INTEREST

All the authors claim no conflict of interests for the publication of this article.

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FIGURE CAPTIONS

Figure 1. Schematics of experimental procedures for SMP-PEM fabrication and dynamic cell culture. Poly(*t*BA-co-BA) SMPs were (a) fabricated with 40% strain and (b) 20 bilayers of PEM coating. (c) The hiPSC-CMs were seeded onto static wrinkled SMP-PEM substrate and dynamic flat-to-wrinkled SMP-PEM substrate for (d) cell alignment analysis based on shape and orientation of cell bodies and nuclei.

Figure 2. hiPSC-CM alignment on static SMP-PEM substrate. AFM images of (a) a static flat surface and (b) a static wrinkled surface (scale bar: 5 μm). SEM images of (c) a wrinkled surface and (d) a hiPSC-CM on the wrinkled surface (scale bar: 10 μm). The hiPSC-CMs were (e) randomly orientated on a flat surface, (f) but highly aligned on a wrinkled surface, confirmed by the fluorescent images (scale bar: 50 μm) with inserts of brightfield images. The hiPSC-CMs showed (g) higher cell aspect ratio on the wrinkled surface than the ones on flat surface, (h) but no difference on nuclei aspect ratio. (i) The hiPSC-CMs showed better alignment to the wrinkle direction based on the measurement of cell orientation and nuclei orientation on flat and wrinkled surfaces (axis for the polar graphs: percentile of cells). Confocal images of hiPSC-CMs showed well-organized myofibril structures aligned with wrinkle direction, including (j) α-actinin, (k) myosin heavy chain, (l) troponin T and (m) vinculin together with F-actin (scale bar: 10 μm). Arrows indicated wrinkle direction. ***$p < 0.001$.

Figure 3. hiPSC-CM alignment on dynamic SMP-PEM substrate. The hiPSC-CMs in response to dynamic wrinkle formation showed (a) cell body reorientation starting at hour 16 post shape-recovery triggering (b) but no preferential directionality of nuclei orientation. The hiPSC-CMs also showed (c) an increase of cell aspect ratio due to wrinkle formation (d) but no change of nuclei aspect ratio. (e) A schematic illustration of cardiac myofibril and sarcomere structure. (f) Fluorescent images and length measurement (arrows) of four key myofibril components, including actin thin filaments, myosin thick filaments, Z-discs and focal adhesions (scale bar: 10 μm). (g) Sarcomere index showed a mild sarcomere disarray at hour 4 and hour 8 post shape-recovery triggering. (h) Thin filament length significantly increased starting from hour 8, (i) while thick filament length maintained the same throughout 24 hours post shape-recovery triggering. (j) Sarcomere length showed an increase from hour 16, following a decrease at hour 24. (k) Disassembly and reassembly of focal adhesions were observed at hour 4 – 12, which was coincident with sarcomere disarray and actin filament extension. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

Figure 4. Inhibition on cytoskeletal tension disrupted myofibril remodeling. (a) Fluorescent images of hiPSC-CMs on the dynamic substrate treated with small molecule inhibitors on myosin kinase (blebbistatin), RhoA kinase (Y27632), actin polymerization (cytochalasin D) and focal adhesion kinase (FAK inhibitor 14) (scale bar: 20 μm). Reducing cytoskeletal tension by these four chemicals inhibited cell responses to the wrinkle formation with (b) lower cell elongation, (c) but no effect on the nuclei morphology. Treatment with blebbistatin and Y27632 resulted in an increase of (d) cell size and (e) sarcomere length, but (e) treatment with cytochalasin D and FAK-14 resulted in a decrease of sarcomere length. (f) Treatment with these mechanosensitivity inhibitors induced severe sarcomere disarray for individual hiPSC-CMs. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

Figure 5. The schematics of myofibril reorganization model. (a) The wrinkle formation that resulted from SMP shape recovery disrupts organized myofibrils through the disassembly of focal adhesions. As stable focal adhesions reassemble, myofibrils reorganize to direct cell alignment to the wrinkle orientation. (b) A timeline to show progressive myofibril reorganization of hiPSC-CMs in response to the changes of nano-topographic surface properties.