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Enhanced cardiomyogenic induction of mouse pluripotent cells by cyclic mechanical stretch

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ABSTRACT

The cardiac milieu is mechanically active with spontaneous contraction beginning from early development and persistent through maturation and homeostasis, suggesting that mechanical loading may provide a biomimetic myocardial developmental signal. In this study, we tested the role of cyclic mechanical stretch loading in the cardiomyogenesis of pluripotent murine embryonic (P19) stem cells. A Flexcell tension system was utilized to apply equiaxial stretch (12% strain, 1.25 Hz frequency) to P19 cell-derived embryoid bodies (EBs). Interestingly, while control EBs without any further stimulation did not exhibit cardiomyogenesis, stretch stimulation alone could induce P19-derived EBs to become spontaneously beating cardiomyocytes (CMs). The beating colony number, average contracting area, and beating rate, as quantified by video capturing and framed image analysis, were even increased for stretch alone case relative to those from known biochemical induction with 5-Azacytidine (5-Aza). Key CM differentiation markers, GATA4 and Troponin T, could also be detected for the stretch alone sample at comparable levels as with 5-Aza treatment. Stretch and 5-Aza co-stimulation produced in general synergistic effects in CM developments. Combined data suggest that stretch loading may serve as a potent trigger to induce functional CM development in both beating dynamics and genomic development, which is still a challenge for myocardial regenerative medicine.

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1. Introduction

During the developmental process, cardiomyocytes (CMs) evolve in mechanically active environments generated by spontaneous contraction [1]. In the developed heart, the contraction and rhythmic beating also provide mechanical loading conditions for the cardiac tissue and CMs [2]. Exploring mechanical cues for myocardial regenerative medicine may thus suggest a strong biomimicking rationale for producing functional (or spontaneously beating) myocardial tissue constructs (see our reviews on the role of mechanical loading for tissue engineering in general [3] and myocardial regeneration and hypertrophy [4]).

Several multipotent/pluripotent cells have been tested for functional cardiomyogenesis, including mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), etc. [5–7]. Several efforts were made to identify soluble

factors to drive cardiomyogenesis, for example, a hypomethylating agent, 5-Azacytidine (5-Aza), was tested for MSCs [8] and P19 ESCs [9]. However, the demethylation effect by 5-Aza could be nonspecific, potentially involved in other cell functions such as modulating tumor regulatory genes [10]. While soluble factor-driven CM development is still challenging, a few recent studies attempted the use of mechanical stretch loading based on the rationale described above. Results with stretch loading, however, indicated varying outcomes. For example, adipose-derived MSCs exposed to stretch loading did not show CM gene markers nor spontaneous beating [11]. Bone marrow MSCs treated with stretch could display cardiac gene expressions (e.g., GATA4, Nkx 2.5) but did not form functionally beating CMs [12].

We exploit cyclic stretch as a biomimetic developmental cue to guide cardiomyogenesis and obtain functionally beating CMs by using a cell model, P19 embryonic stem cell-like cells. The P19 ESCs maintain an undifferentiated state without a feeder layer, indicating an advantage over other ESCs and some iPSC cultures. P19 cells demonstrated multipotency by differentiating into all three

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germ layers such as neurogenesis under retinoic acid and cardiomyogenesis under 5-Aza [9,13,14], but their mechanical control has not been tested. As a proof-of-concept study, we applied cyclic stretch to embryoid bodies (EBs) formed from P19 ESCs. Control EBs did not exhibit cardiomyogenesis, but interestingly stretch stimulation could trigger P19-derived EBs to form spontaneously beating CMs. Further, cardiac marker expressions and beating characteristics (beating colony number, contracting area, beating rate) under stretch alone were comparable or even superior to known soluble (5-Aza) induction.

2. Materials and methods

2.1. P19 embryonal stem cell culture and mechanical stretching

P19 murine embryonal stem cell-like carcinoma cells (ATCC CRL-1825) were maintained in regular culture dish using growth media (Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% penicillin-streptomycin). To form EBs, P19 cells were seeded on bacterial grade petri dish for 4 days using a cell suspension (1×10^6 cells/ml) made of growth media supplemented with 1% dimethyl sulfoxide. Formed EBs were gently collected by suctioning with 1 ml micropipette, transferred onto collagen-coated stretchable elastic membrane (Flexcell Bioflex 6-well plate), and allowed to adhere on the membrane for 24 h. About 200 EBs were allowed to attach on each 6-well stretch plate. P19 cell-derived EBs were then exposed for 24 h to stretch, 5-Aza (Sigma A2385, 10 μ M), or both stretch and 5-Aza. No stretch and no 5-Aza treatment was used as a control. For stretching, Flexcell FX-5000 system was used to equiaxially elongate the elastic membrane against a loading post. Sinusoidal stretching at 12% strain at 1.25 Hz frequency was chosen after preliminary screening (see Discussion section). The duration of stretch was set for 24 h as noted above to test comparative effects with 5-Aza (adopting the reported condition of 5-Aza treatment for 24 h to induce cardiomyogenesis [8]). After treatments (day 1), P19-derived EBs were maintained up to day 18 under simple growth media (changed every two days). The stretch plates were housed inside the incubator, so all four test conditions were conducted at 37 °C and 5% CO₂.

2.2. Characterizing spontaneous contraction of beating colonies

Spontaneously beating cell colonies were observed as early as about day 10, and the beating was characterized on day 10, 14, and 18. The beating colony number was manually counted throughout the 6-well plate. Videos were captured to analyze the beating motion. The area of contraction was quantified by analyzing framed images with NIH ImageJ software. The images were converted to 8-bit grayscale images, and the beating areas were outlined thus generating 8-bit inverting LUT images with a binary mask. The scale of a known distance (in μ m) was used to calculate the beating area from the masks created (Fig. S1 for detailed steps). The substrate coverage by beating areas were obtained by multiplying the beating colony number with average contracting area followed by division with substrate area. The beating rate was also counted from the same videos. Note, the n numbers for beating area and rate quantifications for each test condition were different (see Fig. 2 caption), as depending on stretch or 5-Aza treatment different numbers of beating colonies were formed.

Supplementary video related to this article can be found at <http://dx.doi.org/10.1016/j.bbrc.2017.05.092>.

2.3. Immunofluorescence and western blot

GATA4, one of the key cardiomyogenesis markers, was observed on day 18 by immunofluorescence. Cells were washed with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and probed with mouse monoclonal antibody for GATA4 (Santa Cruz sc-25310) at 1:200. Cells were washed with PBS and exposed to fluorescein isothiocyanate (FITC)-tagged secondary antibody (Abcam ab6785) at 1:1000. Nuclei were double-stained with DAPI (Santa Cruz sc-3598). A Leica DMI 4000B fluorescent microscope was used. The percentage of cells showing GATA4 nuclear localization was counted by comparing GATA4 and DAPI single/composite images. Protein expressions relevant to cardiomyogenesis were further assessed by western blotting. Briefly, on day 18 proteins were lysed from the entire substrate with protein lysis buffer and examined to detect GATA4 (sc-25310), Troponin T (sc-8121), and Nkx 2.5 (sc-8697) with corresponding antibodies (noted in parenthesis, Santa Cruz). After exposing to secondary antibody, chemiluminescence was performed to detect immunoresponsive bands with GAPDH as a loading control.

2.4. Statistics

Statistical significance was tested using one-way analysis of variance (ANOVA) with a Tukey-Kramer post-hoc test. All data are presented in the figures as means $\pm$ standard deviations. Notations in the figures, *, #, and ψ , denote statistical significance compared with control, 5-Aza, and stretch, respectively.

3. Results

3.1. Mechanical stretch triggers P19 ESCs to form spontaneously beating colonies

We first confirmed the 5-Aza induction of P19 cell differentiation into spontaneously beating CMs, as was reported [9]. See example videos in supplementary data for all four test groups (day 10). For the control sample, beating was hardly seen throughout the substrate (note, the video shown for the control was taken from such a scarce spot). For other conditions (stretch, 5-Aza, stretch+5-Aza), large number of beating colonies could be easily found (see quantified beating colony number, Fig. 2A). We for the first time evidenced that cyclic stretching of P19-derived EBs could trigger the formation of spontaneously beating colonies. An example of the beating colony for the stretch case is shown in Fig. 1 (dotted line).

3.2. Stretch stimulation increases beating colony number, contracting area, and beating rate

The control (no stretch, no 5-Aza) displayed almost negligible beating colony formation up to day 18 (Fig. 2A), indicating that EB formation with P19 ESCs is not sufficient to produce beating CMs. For stretch and 5-Aza conditions, the beating colony number showed increasing trends with time in culture up to day 18. Interestingly, stretch stimulation alone resulted in significantly more number of spontaneously beating colonies than control (**) and 5-Aza (##) for all three days of measurements, suggesting that stretch cue may play a role to trigger beating CM formation and stretch effect may be even stronger than the known soluble factor induction with 5-Aza. Stretch+5-Aza appeared to produce more beating colonies relative to stretch alone but not reaching statistical significance.

Based on framed image analysis from videos, we could quantify various characteristics of CM beating. The area of contraction

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