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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 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.

Keywords

Mechanical stretch; Pluripotent P19 embryonal stem cells; Cardiomyogenesis; Beating colony number and area; Beating rate

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Supporting information

Sample video files, procedure for obtaining contracting area (Fig. S1) and surface coverage by beating areas (Fig. S2) are given.

Disclosure

None.

1. Introduction

During the developmental process, cardiomyocytes (CMs) evolve in a 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 cue for myocardial regenerative medicine may thus suggest a strong biomimicking rationale for producing functional (or spontaneously beating) myocardial tissue construct (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 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 [8,9]). 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.

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 (Fig. 2B) showed overall comparable trends as with beating colony number. A persistent statistical result was detected that stretch alone had significantly larger beating areas than those of 5-Aza alone on all three days (#), again indicating a potentially more stimulatory effect from stretch. Contracting areas for stretched samples displayed large variabilities (and sometimes slight decrease with time in culture). This may be because for stretched cases a large number of beating colonies were continually formed comprising new and matured colonies with varying sizes. Also, some comparisons, especially with control having very few n number (e.g., $n = 6$ for the control on day 10), did not reach statistical significance in ANOVA with post-hoc test. Stretch+5-Aza did not have a significant additional increase in beating area from stretch alone. On the other hand, the surface coverage by beating areas (Fig. S2), a combination of the results of beating colony number (Fig. 2A) and area (Fig. 2B), suggests a synergistic effect of stretch and 5-Aza on day 14 and 18 (Ψ).

The beating rate (Fig. 2C), determined using the same video sets as with contracting area, also showed similar trends with stretch and 5-Aza but with less persistency relative to beating colony number (Fig. 2A) and surface coverage (Fig. S2). The beating rate showed in general relatively small standard deviations compared with contracting area, potentially illustrating a synchronized beating motion regardless of variations in the size of contracting areas. Difference in beating rate due to stretch and 5-Aza became less significant with time in culture, suggesting matured beating dynamics.

3.3. CM differentiation markers could be detected in stretched P19 ESCs

In addition to spontaneous beating, to further evaluate CM differentiation, GATA4 cardiac marker was observed by immunofluorescence. Fig. 3A shows that GATA4, known to localize in the nucleus for myocardial transcription [15,16], was evidently seen in stretch, 5-Aza, and stretch+5-Aza cases but not for the control, as is consistent with the beating data in Fig. 2. Again, it is intriguing that stretch alone could induce P19 ESCs to express key CM marker, GATA4. In assessment of the GATA4 nuclear localization, stretch, 5-Aza, and stretch+5-Aza showed significant increases than control (Fig. 3B). CM differentiation markers, GATA4, Troponin T, and Nkx 2.5, were further examined with western immunoblotting (Fig. 3C). GATA4 and Troponin T immunoblots evidence that P19 ESCs exposed to stretch alone clearly exhibit cardiomyogenic GATA4 and Troponin T bands with signals potentially stronger than those of controls. Note, since these western blots used whole cell lysates (that include beating colonies and non-beating parts as well), the actual difference may be seen in the blots as mitigated. Stretch+5-Aza induced the strongest immunoresponsive bands for GATA4 and Troponin T. Nkx 2.5 was also detected more for the stretch and 5-Aza co-treatment case.

4. Discussion

Since CM development and homeostasis are constantly exposed to mechanical loading milieu, mechanical stimuli may provide biomimetic cues for myocardial regenerative medicine. Determining mechanical and soluble exclusive/synergistic effects in triggering functional CM development will help design new and improved myocardial tissue engineering protocols. We aimed to reveal the role of stretch and 5-Aza in regulating pluripotent P19 ESC differentiation to CMs. Interestingly, stretch alone could trigger P19 EBs to form spontaneously beating colonies with significantly improved beating characteristics (more beating colony, larger contracting area) even compared with those from known soluble factor, 5-Aza. Stretch alone could also induce P19 ESCs to exhibit key CM differentiation markers comparable to 5-Aza treatment. Our data suggest that stretch-stimulated P19-derived CMs are functional in both beating dynamics and genomic development. In addition, stretch and 5-Aza provided in general synergistic effects in functional CM developments.

Attempts to differentiate cell models into myocardial lineage have mostly depended on soluble signals. Bone marrow MSCs exposed to neonatal ventricular myocyte-conditioned media could display CM markers [5]. Inhibiting p38 α mitogen-activated protein kinase via SB203580 imidazole analogues was recently attempted to induce ESC cardiomyogenesis

[6]. Another recent study using iPSCs tested the exposure to glycogen synthase kinase-3 β inhibitor followed by insulin-transferrin-selenium-X to obtain beating CMs [7]. The treatment with a cytosine analog, 5-Aza, has been attempted for MSCs [8] and P19 ESCs [9] to achieve cardiomyogenesis, while ignoring potential side effects of 5-Aza such as nonspecific DNA demethylation. Overall, soluble factor-driven CM differentiation is still challenging with no unified protocols established for varying precursor cells.

The heart and its component cells are exposed to a series of mechanical stimuli beginning from organogenesis and throughout maturation and adulthood [17]. Mechanical loading may thus act as a biomimicking signal for proper myocardial development. Exploiting this concept, a few recent studies begun to test the effects of mechanical stretch in inducing cardiomyogenesis, but reported varying outcomes. Adipose MSCs exposed to stretch (12% cyclic strain at 1 Hz for 96 h) failed to express cardiac markers [11]. For bone marrow MSCs treated with stretch (5–20% strain, 1 Hz, 24–72 h), CM markers (GATA4, Nkx 2.5, MEF2c) could be detected but beating cells were not produced [12]. When ESCs were stretched (10% strain, 1 Hz, 24 h) on day 9 of differentiation, stretching had actually a negative effect on cardiomyogenesis [18]. In the soluble factor-driven iPSC development into CMs, stretch co-treatment was briefly tested [7], in which added stretch (30% strain, 1 Hz, 5000 cycles/day from day 8 to 15) enhanced the formation of beating CMs. In their study, however, the stretch alone case was not tested. Combined, while the potential biomimetic role of stretch loading to trigger functional CM development is becoming recognized, stretch data with varying precursor cells are inconsistent. Additionally, these studies have made very little effort to rigorously characterize the beating motion with using parameters of beating colony number, contracting area, beating rate, etc.

Here we explored the CM differentiation ability of P19 ESCs to test the stretch effect. This cell model is known to undergo differentiation into all three germ layer derivatives via forming three-dimensional aggregates, EBs [13]. Though the cellular mechanism behind P19 aggregation and multipotent differentiation is not fully understood, it is fair to consider that the establishment of EBs and the paracrine signaling under given microenvironments might direct the fate decision [19]. For the successful differentiation of P19 cells into different lineages, the EB formation is not enough by itself since it subsequently requires other external cues (as we demonstrated with no beating colony formation for control P19 EBs). So far, studies have tried to generate different embryonic tissues (cardiac or neural) from the P19 aggregates by soluble factors of 5-Aza or retinoic acid [9,20]. For successfully generating beating CM clusters, it may be imperative to recapitulate the mechanically active in vivo cardiac environment as relative to toxic chemical induction such as with 5-Aza. Hence, we tested the stretch cue in directing P19 EB development toward myocardial lineage.

We evidenced that stretch stimulation alone could trigger the formation of beating CMs with pluripotent P19 cells. Using P19 ESC model, we could also compare mechanical (stretch) and soluble (5-Aza) induction of spontaneously beating CMs (through adjusting the duration of treatment for both stimuli the same at 24 h). We observed that stretch alone could produce beating colonies (Fig. 1) relative to almost no contracting area formation for the control case. Some of the quantified beating properties were even superior for stretch alone than 5-

Aza (Figs. 2,S2). The beating cells produced by stretch stimulation alone could also express key CM markers (Fig. 3).

Identifying stretch conditions that can maximize the formation of beating CMs from precursor cells will provide an important consideration in developing advanced myocardial tissue engineering protocols. Comparison of stretch regimens to induce functional CM development may not be fully feasible at this stage with limited number of reports using different precursors [7,11,12,18]. In our preliminary screening, stretch amplitudes of 5% and 10% at 1 Hz for 24 h provided less strong beating colony formation and relatively slower beating. While 12% strain and 1.25 Hz frequency adopted improved the production of beating colonies, more study is needed for further optimization of strain, frequency, daily repetition, etc. It is notable that while we focus on the stretch induction of functional CM differentiation from precursors, there have been studies on the stretch control of “already-committed” or “matured” CMs [21–23] (see our review [4]). These studies have dealt with physiological stretch conditions which already-differentiated CMs will see in vivo (about 10% strain and 1 Hz frequency, values similar to our study and others described above for differentiation purpose [7,11,12,18]). While these ranges are generally accepted as physiologically relevant, our prescreening result suggests a need for fine tuning to better trigger beating CM development.

Effectively differentiating functional CMs, in both beating dynamics and gene profile, is still a challenge. Our study demonstrates that mechanical stretch cue may have a significant implication in triggering precursor cell fate decision into functional CMs. Strategies for myocardial tissue engineering may thus be improved based on this outcome (e.g., developing cardiac patch comprising precursor cells and stretchable scaffold and exposed to stretching at identified regimens). A further study on the molecular mechanosensors that govern the stretch control of beating CM formation would reveal the molecular mechanotransduction mechanism and signaling pathway.

In conclusion, we showed that mechanical stretch loading could upregulate the pluripotent P19 cell differentiation into spontaneously beating CMs. We determined the efficiency of CM induction by stretch and 5-Aza via characterizing the beating patterns of the cell colonies. Stretch alone resulted in more beating colonies with larger contraction area and faster beating rate even when compared with 5-Aza soluble induction. The co-treatment of stretch and 5-Aza induced in general synergistic effects in beating colony formation. Expression of key CM differentiation markers such as GATA4 and Troponin T supported functional CM development by stretch. Our data suggest that stretch cue may be explored with potential benefits for cardiac repair and regenerative medicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Embryoid body formation with P19 ESCs is not sufficient for inducing cardiomyogenesis
- Stretch stimulation alone triggers spontaneously beating colony formation from P19 ESCs
- Beating colonies from stretch show beating characteristics even greater than 5-Aza induction
- Key CM differentiation markers (GATA4, Troponin T) are detected for stretch alone sample

Fig. 1. Cyclic mechanical stretch induces P19 embryonic stem cells to form spontaneously beating colonies

An example of beating cell colony for stretched sample on day 10. Sequential frames from the video are shown to represent the contraction motion. Inkscape software was used to draw dotted lines for the beating area.

Fig. 2. Stretch stimulation enhances beating characteristics (beating colony number, contracting area, beating rate)

(A) Beating cell colony number counted for 6-well stretch plate (9.62 cm²/well). (B) Area of contraction was computed from the framed images and NIH ImageJ software. In calculating the area from the binary mask (Fig. S1), calibrations of 2.08 pixel/μm and 1.04 pixel/μm were used for 20X and 10X magnifications, respectively. (C) Beating rate was obtained as beats/min. *: $p < 0.05$, **: $p < 0.01$ compared with control. #: $p < 0.05$, ##: $p < 0.01$ compared with 5-Aza alone. Ψ: $p < 0.05$, ΨΨ: $p < 0.01$ compared with stretch alone. Contracting area and beating rate were assessed using the same videos having n beating

colonies. The analyzed n number was 6, 45, 21, and 35 on day 10; 11, 32, 20, and 40 on day 14; and 15, 31, 24, and 40 on day 18 in the order of control, stretch, 5-Aza, and stretch+5-Aza.

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Fig. 3. CM specific markers could be detected for stretch alone sample

(A) GATA4 (green) and DAPI (blue) immunofluorescent co-staining on day 18. (B) GATA4 nuclear localization counted by comparing GATA4 and DAPI single and composite images. See Fig. 2 legend for notations of statistics ($n = 3$). (C) CM differentiation markers (GATA4, Troponin T, Nkx 2.5) were assessed on day 18 by western blotting of whole cell lysates with GAPDH loading control. GATA4 and Troponin T could be detected in stretch alone sample.