Enhancement of Cardiomyogenesis in Murine Stem Cells by Low-Intensity Ultrasound

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Objectives—Low-intensity ultrasound (LIUS) has been shown to enhance bone and cartilage regeneration from stem cells. The ease of its incorporation makes it an attractive mechanical stimulus for not only osteogenesis and chondrogenesis, but also cardiomyogenesis. However, to date, no study has investigated its effects on cardiomyogenesis from embryonic stem cells.

Methods—In this study, murine embryonic stem cells were differentiated via embryoid body formation and plating, and after 3 days they were subjected to daily 10 minutes of LIUS treatment with various conditions: (1) low-pulsed (21 mW/cm^2, 20% duty cycle), (2) low-continuous, (3) high-pulsed (147 mW/cm^2, 20% duty cycle), and (4) high-continuous LIUS.

Results—Low-pulsed and high-continuous LIUS had improved beating rates of contractile areas as well as increased late cardiac gene expressions, such as α- and β-myosin heavy chain and cardiac troponin T, showing its benefits on cardiomyocyte differentiation. Meanwhile, an early endodermal marker, α-fetoprotein, was significantly attenuated after LIUS treatments.

Conclusions—With these observations, it is demonstrated that LIUS simulation could enhance cardiomyogenesis from embryonic stem cells and increase its selectivity toward cardiomyocytes by reducing spontaneous differentiation.

Key Words—cardiomyogenesis; embryonic stem cells; low-intensity ultrasound (LIUS); spontaneous differentiation

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Abbreviations
AFP, alpha-fetoprotein; ANOVA, analysis of variance; cTNT, cardiac troponin T; EB, embryoid body; ESC, embryonic stem cells; HC, continuous LIUS at high output; HP, high output; LC, continuous LIUS at low output; LIUS, low-intensity ultrasound; LP, low output; mESC, murine embryonic stem cells; MHC, myosin heavy chain; MSC, mesenchymal stem cell; PBS, phosphate-buffered saline; PCR, polymerase chain reaction

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Therapeutic ultrasound has already found its wide applications in clinics and interests of research recently.[1] Ultrasound at high intensity of more than 100 W/cm^2 will generate a considerable amount of heat that causes detrimental effects on the cells and tissues.[2] Meanwhile, low-intensity ultrasound (LIUS) at a frequency of 1 to 2 MHz with an intensity of less than 2 W/cm^2 is becoming popular in rehabilitation, and has been approved by the Food and Drug Administration for use in orthopedics, such as promoting bone fracture healing, treating orthodontically induced root resorption, regrowing missing teeth, enhancing mandibular growth in children with hemifacial microsomia, promoting healing in various soft tissues such as cartilage and intervertebral disc, and improving muscle healing after laceration injury.[3] At such a low intensity, thermal effects generated by LIUS could be negligible. The repeated and rapid vibratory action of ultrasound will cause compression and rarefaction displacement of cells in the culture medium or in the tissue, which would stimulate and affect cellular fate through mechanotransduction.[4]

In tissue engineering and regenerative medicine, effects of LIUS on growth and differentiation of stem cells and progenitors have been studied recently. Low-intensity ultrasound was found to enhance proliferation[5] and viability[6] of human mesenchymal stem cells (MSCs) as an intervention for delayed union or nonunion. When transplanted MSCs in rat models were treated with LIUS, these rats had enhanced bone fracture healing shown by a high ratio of bone volume to tissue volume in microcomputed tomography, and a significantly faster remodeling in late-phase histomorphometry.[7] In addition, both in vivo and in vitro studies have demonstrated its effects on osteogenesis and chondrogenesis. Low-intensity ultrasound increased the expression of osteoblast genes in differentiating adipose-derived stem cells, myoblasts, and hematoma-derived progenitor cells toward osteogenic lineages.[8–10] Alkaline phosphatase activity, osteocalcin secretion, the expression of osteoblast-related genes, and the mineralization of hematoma-derived progenitor cells were significantly higher with LIUS treatment, but without a change in the proliferation of the hematoma-derived progenitor cells. mRNA expression levels of runt-related transcription factor 2, osteocalcin, alkaline phosphatase, and bone sialoprotein genes were higher with LIUS treatment than the control.[10] Conversion of differentiation pathway may be through activated phosphorylation of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase.[9] Similarly, LIUS could be a noninvasive, efficient, and cost-effective method to induce chondrogenic differentiation of MSCs to form hyaline cartilage-like tissue in cartilage tissue engineering.[11–14] Low-intensity ultrasound–treated MSCs gave rise to higher chondrogenic markers, sulphated glycosaminollycans, collagen, glycosaminoglycan content and mechanical strength, more intense and spreading extracellular matrix in histology, even without transforming growth factor-β, and less blood invasion into the constructs. Furthermore, many chemical cues were essential to control directed differentiation toward the intended lineage of either osteogenesis or chondrogenesis. In the investigation of the beneficial effects from LIUS on stem/progenitor cell differentiation, some of them were used to evaluate the outcome.[15,16]

The adult heart is composed primarily of postmitotic and terminally differentiated cells, which are established at birth and cannot be replaced by newly formed cells as a result of cardiomyocytes lost with age or cardiac diseases. Routine and mass production of human cardiomyocytes is important for both the pharmaceutical industry and clinical application. In vitro tests may replace heterologous cellular assays or even animal models in predicting the physiological activity of cardiac drugs and the potential cardiotoxic effects of nonrelated drugs and compounds before clinical trials. Well-characterized cardiomyocytes with proven safety and efficiency is a prerequisite in developing cell therapies in cardiology. At present, only two potential sources of human cardiomyocytes have been identified unequivocally: cardiac progenitor cells from the heart itself and human embryonic stem cells, derived from the inner cell mass of blastocyst stage embryos.[17,18] Although a subpopulation of myocardial cells have cardiac stem cell characteristics, their limited availability hinders therapeutic applications.[19] Embryonic stem cells (ESCs) represent a possibly unlimited source of functional cardiomyocytes, but their in vitro differentiation into cardiomyocytes involves a poorly defined, inefficient, and relatively nonselective process.[20] Stem cells derived from other tissues (ie, bone marrow) had the capability of repairing heart damage in animal models.[21] However, inefficient differentiation and possible fusion with somatic cells limit their use.[22]

Embryonic stem cells are capable of unlimited proliferation in culture in the undifferentiated state, and under the proper conditions can differentiate into spontaneously beating cardiac myocytes. The spontaneous beating rate may vary at different stages of the cell and is absent in the mature atrial or ventricular myocytes. Meanwhile, ESC-derived cardiomyocytes express cardiac genes in a developmentally controlled manner. As in early myocardial development, mRNAs-encoding Nkx2.5 transcription factors appear in embryoid bodies (EBs) before mRNAs-encoding atrial natriuretic factor, myosin light chain–2v, α-myosin heavy chain (α-MHC), β-myosin heavy chain (β-MHC), Na^{+}-Ca^{2+} exchanger, and phospholamban. Sarcomeric proteins of ESC-derived cardiomyocytes are also established developmentally in the following order: titin (Z disk), α-actinin, myomesin, titin (M band), MHC, α-actin, cardiac troponin T (cTNT), and M protein. Cardiomyocytes with characteristics of fetal/neonatal type express slow skeletal muscle troponin I isoforms and a greater ratio of β-MHC to α-MHC, whereas those with more rapidly contract preferentially express cardiac troponin I and α-MHC. Thus, the measurement

of beating rate and cardiac-associated gene products is for assessing the differentiation time during cardiomyogenesis.[20] Embryonic stem cells intend to spontaneously differentiate into multiple lineages, but with only a small fraction into the cardiomyogenic lineage, which could reduce the clinical efficacy of cardiomyogenesis. Another major consideration would be the potential of ESCs to form teratomas, which could impair recovery of heart function after myocardial infarction.[23] Therefore, the controlled differentiation to endodermal, mesodermal, and ectodermal lineages is of importance.

In a recent study, LIUS was applied to osteoblasts at different stages of maturation, and chemokine and cytokine expression in the osteoblasts were analyzed.[24] Intriguingly, a mechanoreceptor in cardiomyocytes was detected in osteoblast, and the level increased significantly during cell maturation. Therefore, it is suggested that constant mechanical stress generated by LIUS may have a great potential in enhancing cardiomyocytes differentiation and maturation. However, until now this hypothesis has not been evaluated. In this study, effects of LIUS on cardiomyocyte differentiation of murine embryonic stem cells (mESCs) were investigated. The cells were subjected to a daily treatment of LIUS for 10 minutes at two different intensities (21 and 147 mW/cm^2) and two different wave generation modes (continuous wave and pulsed wave). The beating rate of differentiated mESCs, expression of cardiac proteins, cardiac gene expression, and spontaneous differentiation of mESCs toward endodermal and ectodermal lineages were measured and compared with the control. It is found that LIUS stimulation can significantly enhance cardiomyogenesis but reduce endodermal differentiation, which indicates a possibly high cardiomyocytes purity and yield. The effects are dependent on both the number of treatment days and LIUS parameters.

Materials and Methods

Expansion and Cardiac Differentiation of mESC

Murine embryonic stem cells (E14TG2a, ATCC, Singapore) were expanded on 0.1% porcine-gelatine (Sigma-Aldrich, Singapore) coated flasks under ESC maintenance medium. The maintenance medium consists of high-glucose Dulbecco-modified Eagle's medium (Gibco cell culture, Thermo Fisher Scientific, Singapore), 10% gold-standard fetal bovine serum (PAA Laboratories GmbH, Haidmannweg, Germany), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich), 2 mM L-glutamine (Gibco cell culture, Thermo Fisher Scientific), 1 × penicillin-streptomycin (PAA Laboratories GmbH), and 1000 U/mL leukemic inhibitory factor (Chemicon, Merck Millipore, Singapore). The medium (5 mL) was changed daily using a pipettor (SP 10000-AUTO, Aciquip, Newton, United Kingdom) with an accuracy of ±10 µL and cells were passaged every 2 to 3 days. Murine ESCs were primed toward the mesodermal lineage by culturing on gelatine-coated flasks in HepG2-CM for 3 days. Then the formation of EBs was initiated. Murine ESCs were gently detached from the gelatine-coated flasks using 0.25% Trypsin-EDTA (Gibco cell culture, Thermo Fisher Scientific) and small clumps of mESCs were replated onto bacteriological-grade Petri dishes containing EB medium, which consists of high-glucose Iscove's modified Dulbecco's medium (Gibco cell culture, Thermo Fisher Scientific), 20% gold-standard fetal bovine serum, 2 mM L-glutamine, 1 × penicillin-streptomycin, and 450 µM 1-thioglycerol (Sigma-Aldrich). The EBs grew for 5 days with medium change every 2 days. To induce cardiomyocyte differentiation, suspended EBs were plated onto gelatine-coated 6-well plates (polystyrene material, a diameter of 34.8 mm, bottom thickness of 1.27 mm, Corning Costar, Sigma-Aldrich), and 0.25 µM Cardiogenol C (Sigma-Aldrich) was added into the EB medium as the cardiomyogenic-inducing agent. This differentiation medium was used for the rest of the culture until Day 21 with medium change every 2 days.

Low-Intensity Ultrasound Treatment Conditions

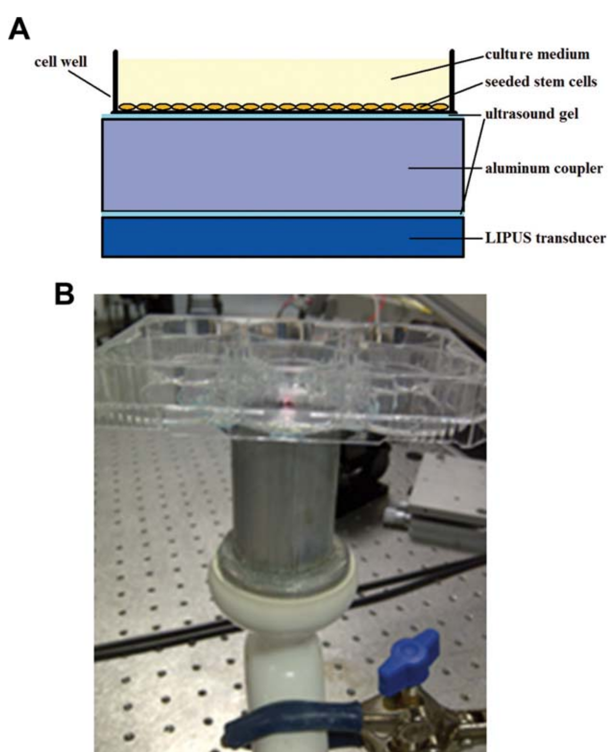
On Day 11, plated EBs were subjected to daily treatments of LIUS for 10 minutes till Day 21. The LIUS transducer (Model X Ultrasound, Rich-Mar, Chattanooga, TN) has a working frequency of 1 MHz and an active area of 10 cm^2. To avoid the great pressure variation in the near field due to acoustic interference, a cell well was placed at the transition between the near field to the far field:

$$z_R = \frac{a^2}{\lambda} \quad (1)$$

where a is the radius of LIUS piston transducer, and λ is the wavelength. A piece of an aluminum cylinder with a height of 50.8 mm was used as a stand-off and acoustic coupler between LIUS transducer and cell-seeded plates, and ultrasound coupling gel was filled uniformly among

the transducer, aluminum coupler, and well plate for acoustic transmission, as illustrated in Figure 1. In this study, various treatment conditions were tested: (1) LIUS at pulse repetition frequency of 1 Hz, duty cycle of 20%, and low output (LP); (2) continuous LIUS at low output (LC); (3) LIUS at pulse repetition frequency of 1 Hz, duty cycle of 20%, and high output (HP); and (4) continuous LIUS at high output (HC), as listed in Table 1. Spatial averaged acoustic intensity of 30 mW/cm^2 is a condition commonly used in studies of chondrogenesis and osteogenesis.[25] I_{SPTA}, spatial peak temporal average intensity, is limited by the Food and Drug Administration for cardiac radiology at 430 mW/cm^2. The cell culture and differentiation process timelines for 2D cultures are briefly summarized in Figure 2. To avoid the effect of ultrasound exposure to cells in the neighboring wells, mESCs were seeded in only one well in the 6-well plate. At the end of 21-day culture, cells from 2D cultures were extracted and analyzed. Sham group (attached to LIUS transducer with no activation for 10 minutes) was used as the control.

Figure 1. (**A**) Schematic diagram and (**B**) photograph of LIUS exposure to seeded cells in 6-well plates.



Quantitative Polymerase Chain Reaction

The total RNA was isolated from extracted cells using the RNeasy mini kit (Qiagen, Germantown, MD) and quantified with a ultraviolet spectrophotometer (Evolution 500, Thermo Fisher Scientific, Grand Island, NY). The first strand of complementary DNA was synthesized from 1 μg of RNA with oligo-dT primers, M-MLV reverse transcriptase, dNTP mix, RNasin, and nuclease-free water (all reagents from Promega, Madison, WI) according to the manufacturer's instructions. Quantitative polymerase chain reaction (PCR) was carried out using the SensiFAST SYBR Fluorescein Kit (Bioline, Singapore). The PCR amplification and measurement were conducted in an iQ5 PCR machine (Bio-rad, Singapore) for 2 minutes of denaturation at 95°C, and 40 cycles of denaturation at 95°C for 10 seconds, and annealing/extension at 60°C for 30 seconds. All gene primers used in this study are listed in Table 2.

Qualitative Evaluation of Beating Colonies and Cell Viability

Beating colonies of the cultured mESCs were observed microscopically using an inverted optical microscope (Axiovert 200M, Carl Zeiss, Singapore) at 10 × magnifications. Beating rates of 10 or more contractile areas using each LIUS exposure condition were counted for

Table 1. Treatment Ultrasound Parameters Used in the Experiment

Parameter	LP	LC	HP	HC
Acoustic intensity setting (mW/cm^2)	30		300	
Measured peak acoustic pressure (kPa)	25.1 ± 1.8		66.5 ± 1.5	
Measured spatial-peak temporal-peak acoustic intensity (mW/cm^2)	21.0 ± 3.0		147.4 ± 6.7	
Duty cycle (%)	20	100	20	100
Pulse repetition frequency (Hz)	1			
Exposure time (minutes)	10			

Note: The acoustic impedance of culture medium in determining the acoustic intensity from the measured acoustic pressure was assumed to be 1.5 MRayl.

30 seconds and recorded. Cell viabilities of mESCs in 2D and 3D cultures after 21 days of culture were evaluated using calcein-AM (Sigma-Aldrich), a dye staining the esterase activity of live cells, and propidium iodide (Sigma-Aldrich), a dye binding to nuclei of dead cells. Cells or hydrogel beads were washed with phosphate-buffered saline (PBS) twice and incubated with 4-μM propidium iodide and 2-μM calcein-AM for 30 minutes at room temperature in the dark. The samples were then washed with PBS twice. Samples were visualized with an inverted fluorescence microscope (Axiovert 200M, Carl Zeiss) and captured with the installed camera (AxioCam MRC, Carl Zeiss).

Immunocytochemistry

On Day 21, differentiated cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS for 1 hour at room temperature and then treated with 0.1% Triton-X (Ultrapure grade, USB Corp, Cleveland, OH) for 45 minutes at room temperature. Cell samples were stained with the primary antibody of sacromeric α-actinin overnight at 4°C. After overnight incubation, the samples were washed three times before incubating with its fluorescein isothiocyanate–conjugated secondary antibody for 1 hour at room temperature. After extensive washing, a second primary antibody for cardiac Troponin I and Tetramethylrhodamine-conjugated secondary antibody was used. All antibodies were purchased from Santa Cruz Biotechnologies (Dallas, TX) and diluted 100× before use. After immunostaining, samples were incubated with DAPI (Thermo Fisher Scientific) for 5 to 10 minutes to stain for the nucleus. After briefly washing off excess DAPI, the samples were finally viewed and captured with the inverted fluorescence microscope.

Statistical Analysis

The sample size in each group was at least six. The statistical differences among experimental groups in this study were carried out using the Tukey-Kramer multiple-comparisons posttest one-way analysis of variance (ANOVA) test at 95% level of significance and professional statistical software (IBM Corp, Chicago, IL). The degree of freedom was 4. Error bars on the charts represent the standard deviation in each experimental group.

Results

Acoustic pressure exposed to seeded cells plate was measured by a needle hydrophone (HNP-0400, Onda Corp, Sunnyvale, CA) placed close to the bottom of 6-well plates (distance of ~0.1 mm) filled with 5-mL deionized water and connected with a 3D translational stage (PT3, Thorlabs, Newton, NJ). The LIUS pressure waveform was recorded by a digital oscilloscope (WaveSurfer 44MXs-B, Teledyne LeCroy, Chestnut Ridge, NY), and the hydrophone was scanned across the well

Figure 2. Timeline of cardiac differentiation and LIUS treatments for 2D conventional cultures.

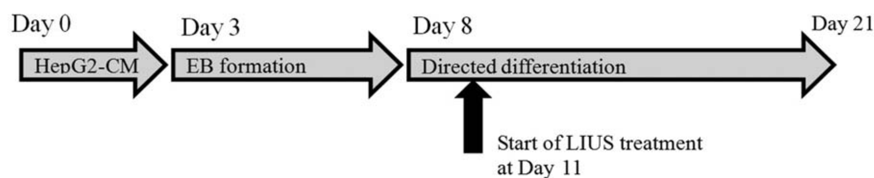
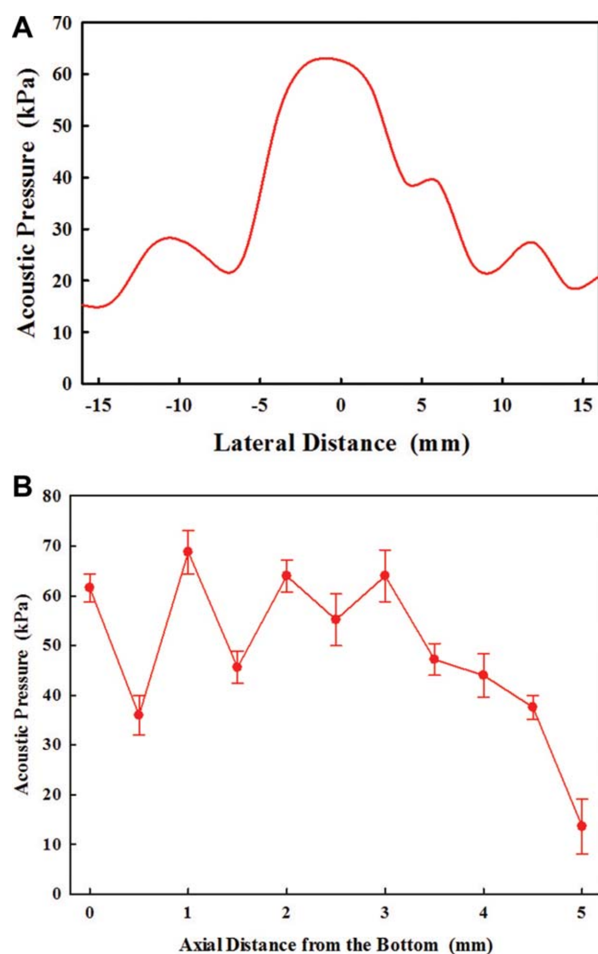


Table 2. Sequences of Forward and Reverse Primers Used for Quantitative PCR

Gene	Forward Primer	Reverse Primer
Glyceraldehyde 3-phosphate dehydrogenase	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA
Brachrury T	AAGGAACCACCGGTCATCG	CGTGTGCGTCAGTGGTGTGTAATG
Nkx2.5	AGCAACTTCGTGAACTTTG	CCGGTCCTAGTGTGGA
cTNT	CTCCATCGGGGATCTTGGGT	CAGAGGAGGCCAACGTAGAAG
β-MHC	ACCCCTACGATTATGCG	GGTGACGTACTCGTTGCC
α-MHC	ACCGTGGACTACAACAT	CTTTCGCTCGTTGGGA
AFP	GAAGCAAGCCCTGTGAACTC	CCGAGAAATCTGCAGTGACA
Nestin	AGGACCAGGTGCTTGAGAGA	TCCTCTGCGTCTTCAAACCT

to find the maximum pressure. The maximum acoustic pressures at the setting of 30 and 300 mW/cm^2 were determined as 25.1 ± 1.8 kPa and 66.5 ± 1.5 kPa, respectively, using the sensitivity-calibration data provided by the hydrophone manufacturer. The discrepancy between the measured acoustic intensities and the settings may be the result of the energy loss at the interfaces (transducer/aluminum and aluminum/well plate). The hydrophone was further moved axially in a step size of 0.5 mm. The lateral pressure distribution across the well close to the bottom and axial pressure distribution from the bottom are shown in Figure 3. The great variation may be caused by the surface agitation at the water interface.

Figure 3. (**A**) Lateral distribution close to the bottom and (**B**) axial distribution from the bottom of acoustic pressure in the cell well filled with 5-mL deionized water measured by a hydrophone at the acoustic intensity setting of 300 mW/cm^2.



Beating Rate of Differentiated ESCs

On Day 18 and 21, beating colonies found in differentiated mESCs were quantitatively evaluated by measuring the beating rate. Figure 4 shows the beating rate of cells corresponding to the trends observed in the upregulation of cardiac genes. On Day 18, the beating rate of cells under HC-LIUS treatment was measured as 41.5 ± 12.6 beats per minute, which was almost twice as fast as the non-LIUS treated controls (19.3 ± 4.7). Meanwhile, there were no significant differences among the other experimental groups and the control ($P > .05$) at this moment. In comparison, on Day 21, the LP- and LC-LIUS treatments also produced a higher beating rate than that of the controls (36.2 ± 9.7 and 42.3 ± 8.2 versus 20.5 ± 6.8, respectively). The beating rate of HC-LIUS had a slight increase to 46.8 ± 8.0, but that of HP-LIUS had almost no change from 23.0 ± 5.9 to 22.8 ± 3.4. These results briefly show that LIUS could generally enhance ESC-differentiated cardiomyocyte contractile function, and the enhanced differentiation effects at different stages depend on the LIUS parameters.

Cardiac Proteins by Differentiated Cells

We also evaluated whether LIUS would cause any deleterious effects on the development of structural and cardiac proteins on the cell surface. Surface expressions of structural proteins and cardiac markers in mESC-differentiated cells were evaluated via immunostaining for sacromeric α-actinin and cardiac troponin I on Day 21 in the control, LP-LIUS and HC-LIUS samples, respectively (Figure 5). Sacromeric α-actinin is a binding protein that attaches actin filament to dense striations in cardiomyocytes, and is represented by the green fluorescence to show a typical periodicity in the Z line of cardiac myofibrils. Cardiac troponin, a regulatory protein involved in calcium signaling for the contractile motion of cardiomyocytes, is represented by the red fluorescence to illustrate the cardiomyocyte injury. The intense and extensive nuclei-stained images, and uniform cardiac troponin I–stained image of all samples, demonstrated the viability of mESCs. No significant myocardial cellular damage, and more intense α-actinin-stained images of LIUS-treated samples in comparison to that of control, showed the enhanced cardiac myofibrils, which is in good agreement with the increased beating rate.

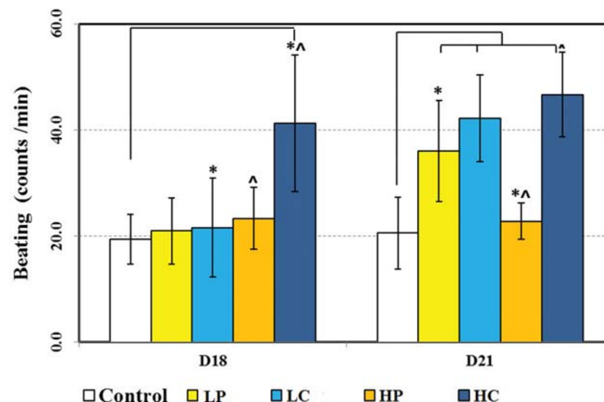
Cardiac Gene Expressions

After 3, 7, and 10 days of LIUS treatment (Day 14, 18, and 21 of culture, respectively), cardiac gene expressions were compared among treatments with each experimental group being normalized to the controls on Day 14 (Figure 6). There are time-dependent relationships of cardiac gene expressions in the controlled stem cells, Brachrury T and Nkx2.5 decreasing slightly to approximately 0.5, β-MHC increasing slightly to approximately 2.3, and cTNT and α-MHC increasing greatly to approximately 5.2 and 7.0, respectively. After only 3 days of LIUS treatment (Day 14), the upregulation of Brachrury T, a marker for mesodermal commitment of ESCs, was found to be higher in all LIUS experimental groups as compared with the control (Figure 6A), which suggests that LIUS could induce mesodermal lineage commitment and would have further beneficial effects for cardiomyogenesis. However, treated groups at the intensity of 30 mW/cm^2, either pulse wave or continuous wave, had similar Brachrury T expressions as the control on Day 18 and 21, whereas those treated at the intensity of 300 mW/cm^2 could still have high levels. Nkx2.5 transcripts are detected at early headfold stages in myocardiogenic progenitor cells, and its expression precedes the onset of myogenic differentiation, and continues in cardiomyocytes of embryonic, fetal, and adult hearts.[26] All LIUS experimental groups had a similar Nkx2.5 expression as the control, although the average values of 30 mW/cm^2 are statistically low on Day 14 ($P < .05$). Furthermore, markers for late cardiomyocyte differentiation, the two isoforms of the cardiac myosin heavy chains (α-MHC and β-MHC) and cTNT, were also evaluated. Specifically, α-MHC is a part of cardiac myosin that will move along the cardiac actin filament to generate contractile action. On Day 18, cells treated with HC-LIUS showed significant upregulation of three late cardiomyocyte genes compared with the controls. Although cells treated by LP-LIUS showed even lower cardiac gene expressions than the control on Day 18, their expressions were the highest among the LIUS experimental groups on Day 21 and significantly different from the control in cTNT and α-MHC. Overall, it shows that the LP-LIUS exposure could also enhance cardiomyogenesis of mESCs, but to a lesser degree than HC-LIUS, and the observations in cardiac gene expressions coincide with the comparisons of beating rates in Figure 4.

Spontaneous ESC Differentiation Toward Endodermal and Ectodermal Lineages

The spontaneous differentiation toward endodermal and ectodermal lineages was evaluated using the gene expression of early endodermal marker alphafetoprotein (AFP) and early ectodermal marker Nestin, respectively (Figure 7). It is found that the levels of AFP were much lower in all LIUS-treated cells compared with the control (Figure 7A). Cells that did not undergo LIUS treatment (the control) increased the levels of AFP by 16.8 ± 6.0 and 31.1 ± 4.0 fold on Days 18 and 21, respectively. In contrast, the upregulation level of AFP in the LIUS-treated cells

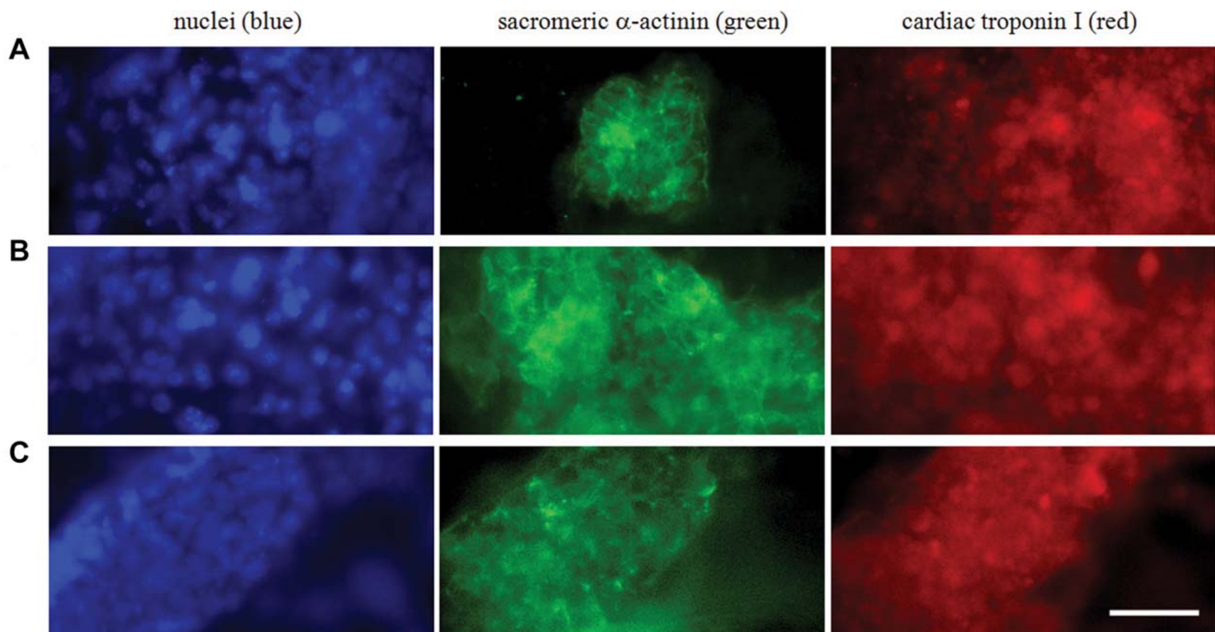
Figure 4. Beating counts in 1 minute for different LIUS-treated samples and controls on Days 18 and 21. Scale bar represents a significant difference ($P < .05$) of the indicated sample from its respective control. * represents a significant difference between the indicated sample and its intensity counterpart (ie, low and high LIUS); ^ represents a significant difference between the indicated sample and its pulsing counterpart (ie, LP- and LC-LIUS).

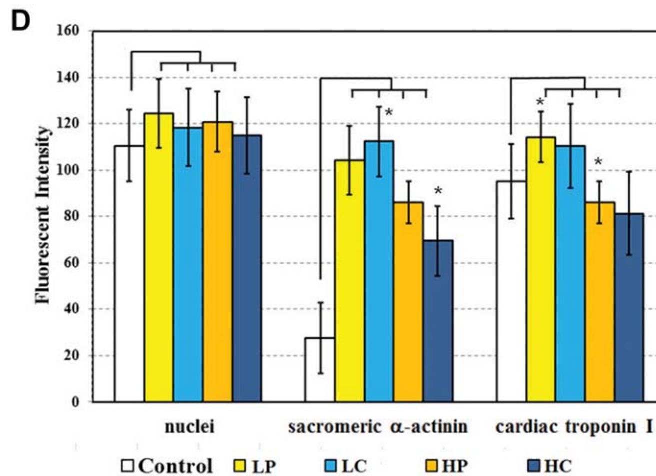


was only 1.2 to 6.0 and 7.8 to 20.4 fold on Days 18 and 21, respectively. There was no significant difference in the expression of Nestin between the controls and LIUS experimental groups throughout the whole culture, except the significantly lower level of LC on Day 21 (Figure 7B). Altogether, undesired differentiation toward endodermal lineages could be reduced, and eventually, high cardiomyocyte purity and yield could be achieved after LIUS treatment.

All statistical analysis results in this study using ANOVA are listed in Table 3. It is found that LIUS exposure to murine stem cells has certain effects on cardiomyogenesis except fluoresce in nulcei, β-MHC on Day 21, Nkx2.5 on Day 18, and Nestin on Day 14.

Figure 5. Representative fluorescence micrographs of nuclei (blue), sacromeric α-actinin (green), and cardiac troponin I (red) of differentiated cells in control (**A**), LP-LIUS sample (**B**) and HC-LIUS sample (**C**). Scale bar represents 50 μm. **D**, Comparison of the fluorescent intensities of nuclei, sacromeric α-actinin, and cardiac troponin I in differentiated cells. Scale bar represents a significant difference ($P<.05$) of the indicated sample from its respective control. * represents a significant difference between the indicated sample and its intensity counterpart.

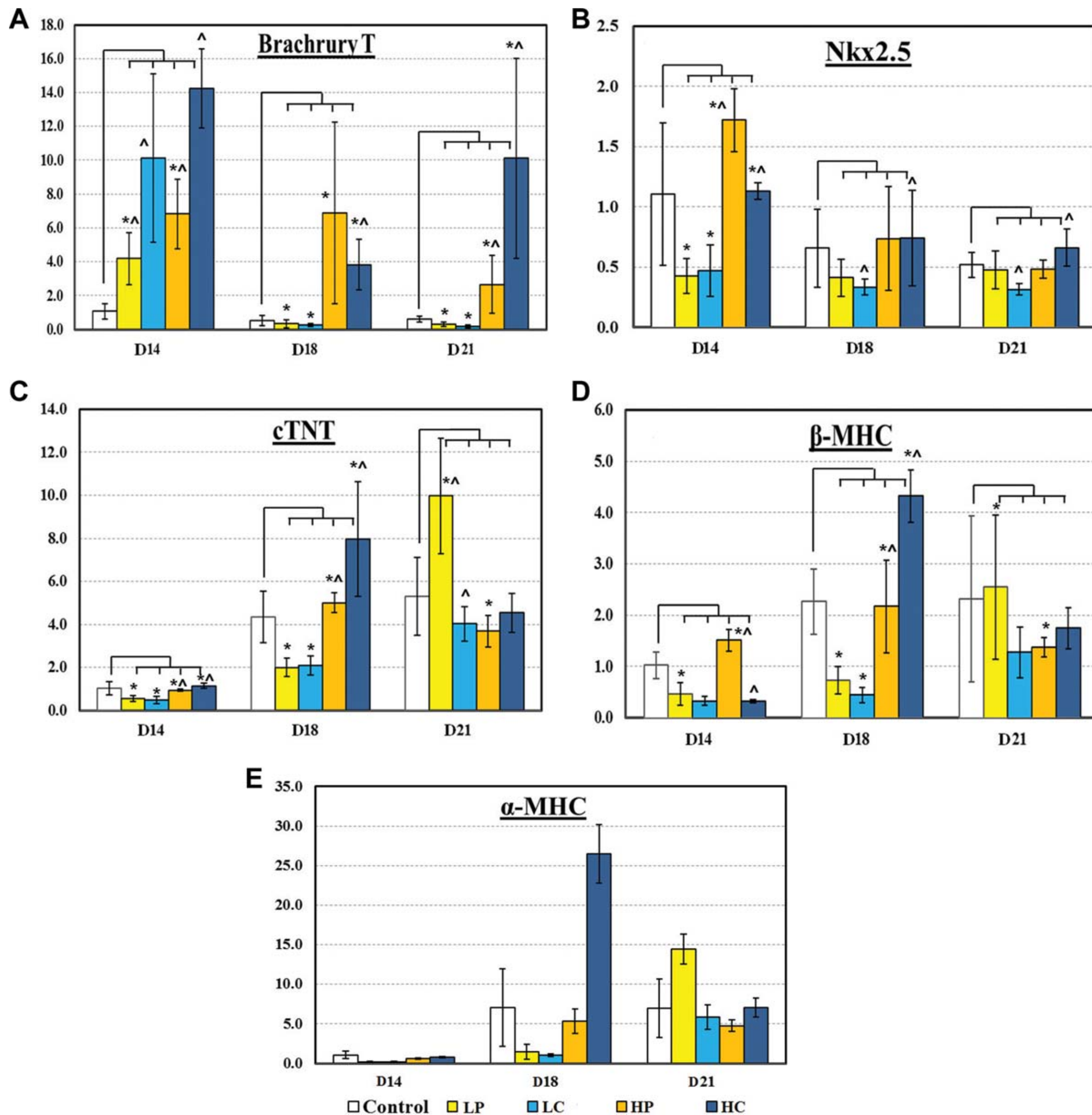




Discussion

In this study, LIUS was used as a novel mechanical stimulus to enhance mESC differentiation toward cardiomyocytes. Mechanical stimulation has been proven to improve cardiomyocyte differentiation from neonatal cardiomyocytes, MSCs and ESCs.[27–29] Contractile mechanical stretching provided a much stronger stimulus than the passive mechanical load for protein synthesis, secretion of growth promoting factors, upregulation of markers of cardiomyocyte maturity (ie, connexin-43 and myosin heavy chain). A possible reason for the beneficial effects of LIUS on cardiomyogenesis from ESCs was that high-frequency acoustic wave could induce force and motion in the medium to create a form of mechanical stimulation on the ESCs, and then

Figure 6. Fold changes in expression of cardiac markers during the LIUS treatment period: (**A**) Brachrury T, (**B**) Nkx2.5, (**C**) cTNT, (**D**) β-MHC, (**E**) α-MHC. Scale bar represents a significant difference ($P<.05$) of the indicated sample from its respective control. * represents a significant difference between the indicated sample and its intensity counterpart; ^ represents a significant difference between the indicated sample and its pulsing counterpart.

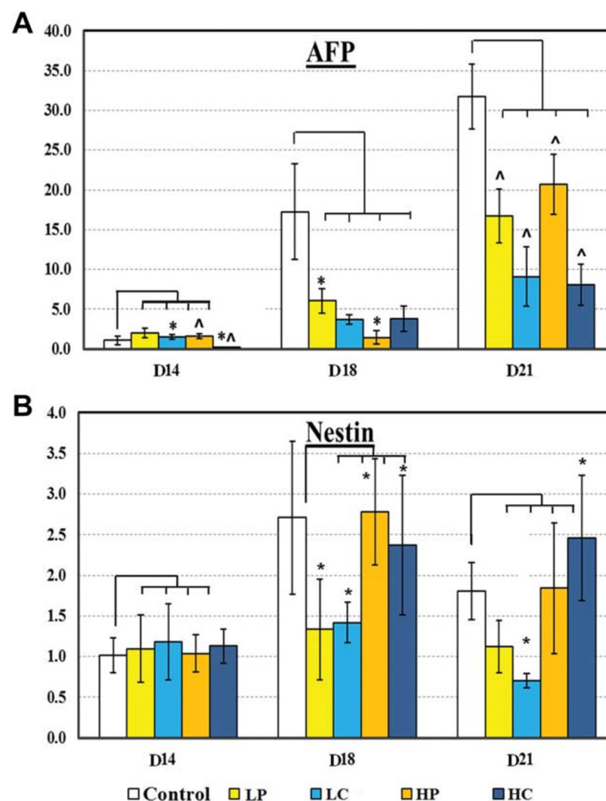


subsequently elicit biophysical effects. Cyclical stretching would result in a parallel orientation of cardiomyocytes and their intracellular myofibrils, whereas myofibrils that orientated perpendicular to the cyclical stretch direction emerge at prolonged stimulation.[30] However, the pathway by which mechanical stimuli is transduced into a biochemical signal that initiates cardiomyogenic differentiation has not yet been elucidated. In comparison to most mechanical stimulus designs using a revolution platform,[31–34] LIUS transducer can be easily and flexibly installed into different bioreactors and culture vessels. In addition, LIUS has great potential for future in vivo application because of its noninvasiveness, large penetration into soft tissue, nonionization, low cost, and portability for use even in rural health-care facilities.

One of the distinct differences between the LIUS-treated samples and the control was reduced spontaneous differentiation toward endodermal germ layer, regardless of the LIUS conditions used. Differentiation of pluripotent stem cells on stiffer substrates favored mesodermal commitment over endodermal commitment.[35,36] A stiffer substrate would impose higher counteracting forces on the cultured cells, and subsequently affect the cellular fate.[36] This deduction substantiated the observation from in vivo developing *Drosophila* gastrula, where localized tensional forces would increase to promote proliferation and cell migration into primitive streak formation.[37] Hence, the mechanical stress produced by LIUS might produce forces through particle motion that impedes spontaneous endodermal differentiation of ESCs, thus increasing the specificity toward cardiomyocyte differentiation. Such increase will result in an easier separation of cardiomyocytes from undesired differentiated products (such as those from endodermal lineage) and eventually higher cardiomyocyte purity and yield.

Low-intensity ultrasound has produced a temporal increase in Brachrury T expression as the mesodermal

Figure 7. Fold changes in the expression of (**A**) endodermal marker, AFP, and (**B**) ectodermal marker, Nestin, during LIUS treatment period. Scale bar represents a significant difference ($P<.05$) of the indicated sample from its respective control. * represents a significant difference between the indicated sample and its intensity counterpart; ^ represents a significant difference between the indicated sample and its pulsing counterpart.



commitment of differentiating ESCs, late cardiac gene expressions (ie, α-MHC, β-MHC, and cTNT) and high beating rate as enhanced cardiomyogenesis. The LP- and HC-LIUS appear significantly different from the other experimental groups on Days 18 and 21, respectively. Therefore, enhanced cardiomyocyte differentiation may be time dependent, which means that LP-LIUS requires a longer treatment period than HC-LIUS to produce the similar bioeffects. The reason may be that high acoustic pressure through the medium generated by HC-LIUS could have high mechanical stress and a strong mechanotransduction effect on the ESCs. Although the concept of using LIUS for enhanced cardiomyogenesis was proven here, the parameters of LIUS (ie, intensity, duty cycle, daily treatment time) will be investigated for their influences and parameter optimization. It is noted that optimal LIUS conditions will vary for different applications in tissue engineering and regenerative medicine. Different cardiac gene expressions depend on the differentiation stage of ESCs.

Table 3. Summary of ANOVA Statistical Results Between Testing Groups

		F-value	*P*
Beating in Figure 4	D18	6.90	<.001
	D21	15.48	<0.001
Fluorescence in Figure 5	Nuclei	0.71	.583
	Sacromeric α-actinin	37.33	<.001
	Cardiac troponin I	7.08	<.001
Brachrury T in Figure 6A	D14	24.36	<.001
	D18	9.27	<.001
	D21	15.31	<.001
Nkx2.5 in Figure 6B	D14	16.25	<.001
	D18	3.04	.036
	D21	9.35	<.001
cTNT in Figure 6C	D14	9.34	<.001
	D18	20.86	<.001
	D21	16.23	<.001
β-MHC in Figure 6D	D14	97.97	<.001
	D18	49.53	<.001
	D21	1.756	.169
α-MHC in Figure 6E	D14	10.34	<.001
	D18	9.52	<.001
	D21	21.58	<.001
AFP in Figure 7A	D14	14.25	<.001
	D18	30.95	<.001
	D21	45.18	<.001
Nestin in Figure 7B	D14	0.829	.214
	D18	5.44	.003
	D21	9.823	<.001

Cellular effects of nonthermal LIUS, such as increased diffusion rates and membrane permeability, are usually associated with acoustic streaming and stable cavitation.[38,39] However, cavitation by LIUS has not been confirmed, and is unlikely given the low mechanical index (0.095 at the acoustic intensity of 300 mW/cm^2 and frequency of 1 MHz). Therefore, ultrasound contrast agents (microbubbles) were sometimes added to the culture medium to provide cavitation nuclei for ultrasound exposure, but not in this study. Enhanced protein synthesis[40,41] and collagen synthesis[42] were observed in human fibroblasts in vitro after 500 mW/cm^2 ultrasound stimulation. Ultrasound could also stimulate ESCs to synthesize extracellular matrix through calcium channels. Calcium signaling was required for ultrasound-stimulated aggrecan synthesis by rat chondrocytes.[43] Integrins and stretch-activated cation channels have been known to be candidates for converting mechanical signals to chemical signals on the cell surface.[44] Molecules such as mitogen-activated protein kinase and NF-κB, and the calcium concentration may mediate their signal transduction pathways. In comparison, bubbles collapse quickly and release large amounts of energy at inertial cavitation, resulting in significant and transient shearing and microstreaming fields,[38] which could explain the effect of very low energy to cellular reactions. Exogenous free radicals and reactive oxygen species produced by inertial cavitation may also be used to direct the cardiomyogenic differentiation of stem cells in vitro, as reactive oxygen species are important intracellular messengers during cell growth and differentiation.[45] Incubation of ESCs with free radical scavengers and antioxidants had an inhibitory effect on cardiomyogenic differentiation.[23] However, higher ultrasound intensities may inhibit the synthesis of collagen and noncollagen proteins, which results in the observed cartilage cell proliferation.[46] Several other theories exist concerning the mechanism by which the ultrasound stimulates biochemical events at the cellular level.

In our setup, no acoustic absorbing material, such as rubber sheet, was tilted at the surface of the medium to absorb and deflect the incoming ultrasound burst. Thus, the standing wave could be established in the dish by the interference of incident and reflected waves. It is found that the presence of standing wave in the dish resulted in a much higher efficiency of sonoporation to cells than the absent case.[47] The standing wave-induced bioeffects vary with the ultrasound frequency and the

height of the medium. Therefore, it is not easy to compare the experimental conditions and results among different setups. It is difficult to presume the true acoustic power applied to the sonicated samples as well as the axial distribution in the cell well. Measurement close to the interface of medium and air has large variation, which may be the result of the surface agitation effect. However, the production of standing wave in the human body is difficult for real clinical use, except for structures around the bone. In a future study, to investigate the effect of ultrasound on the stem cells and simulate the in vivo conditions, the standing wave should be minimized.

There are several limitations in the current investigation. First, only four LIUS settings were tested here. Optimal parameters, such as driving frequency, acoustic intensity, duty cycle, and exposure time, should be found by more studies to make a quantitative conclusion. Ultrasound transducer may also be improved to ensure more uniform pressure distribution in the dish. Second, the role of LIUS-induced stable and inertial cavitation on the cardiomyogenesis from ESCs is in a great need. Varied concentrations of microbubbles will be added to the culture medium, and the acoustic emission signals from cavitation can be picked up using the approach of passive cavitation detection, from which a quantitative dose of stable and inertial cavitation can be calculated. According to the mechanism understanding and observed correlation, the controlled differentiation for high production of cardiomyocytes may be possible by a closed-loop feedback control. Third, growth factors, such as the transforming growth factor-β family, the Wnt family, and the fibroblast growth factors family may be used to regulate cardiac development. However, the precise pathway and interaction remain unclear. Fourth, only monolayer differentiation was tested here. The formation of EBs, in which ESCs are clustered into multicellular 3D aggregates that will differentiate to comprise all three germ layers, and co-culture of undifferentiated ESCs with a supportive stromal layer (ie, endoderm-like cell line, END-2) could increase cardiac differentiation.[48] Finally, animal models of cardiac injury are required to determine the efficacy and safety of the LIUS-enhanced cardiomyogenesis. Undifferentiated stem cells impose the risk of tumorigenicity after implantation.

In conclusion, LIUS has been applied in osteogenesis and chondrogenesis in the regenerative medical field for its mechanotransduction and mechanical cues. The beneficial effects of LIUS on cardiomyogenesis from ESCs were first evaluated in this study, and cardiac gene expressions and beating rate were improved with different LIUS treatment. In addition to enhanced cardiomyogenesis, endodermal differentiation was also significantly reduced upon LIUS stimulation. Altogether, LIUS has a high possibility of achieving higher cardiomyocyte purity and yield. With these benefits and the ease in incorporating LIUS stimulation into different culture platforms and in vivo study, LIUS may be a useful and effective tool for producing cardiomyocytes for clinical therapy in the future. More investigations are required for translating the qualitative conclusions made here to quantitative ones.

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