Pneumatic Cell Stretching System for Cardiac Differentiation and Culture

Joose Kreutzer[1,3,*], Liisa Ikonen[2,3,*], Juha Hirvonen[1,3], Mari Pekkanen-Mattila[2,3], Katriina Aalto-Setälä[2,3,4], Pasi Kallio[1,3]

[1]*Department of Automation Science and Engineering, Tampere University of Technology, Korkeakoulunkatu 3, 33720, Tampere, Finland.*

Phone: +358 40 849 0018

Fax: +358 4219 198 1189

E-mail:joose.kreutzer@tut.fi

[2] *Institute of Biomedical Technology, University of Tampere, Biokatu 12, FI-33014, Finland*

[3] *BioMediTech, Tampere, Finland*

[4] *Heart Center, Tampere University Hospital, Biokatu 6, 33520 Tampere, Finland*

[*] Liisa Ikonen and Joose Kreutzer contributed equally to this work

Abstract This paper introduces a compact mechanical stimulation device suitable for applications to study cellular mechanobiology. The pneumatically controlled device provides equiaxial strain for cells on a coated polydimethylsiloxane (PDMS) membrane and enables real time observation of cells with an inverted microscope. This study presents the implementation and operation principles of the device and characterizes membrane stretching. Different coating materials are also analyzed on an unstretched membrane to optimize the cell attachment on PDMS. As a result, gelatin coating was selected for further experiments to demonstrate the function of the device and evaluate the effect of long-term cyclic equiaxial stretching on human pluripotent stem cells (hPSCs). Cardiac differentiation was induced with mouse visceral endoderm-like (END-2) cells, either on an unstretched membrane or with mechanical stretching. In conclusion, hPSCs grew well on the stretching platform and cardiac differentiation was induced. Thus, the platform provides a new possibility to study the effect of stretching on cellular properties including differentiation and stress induced cardiac diseases.

Keywords: *cardiomyocytes; human embryonic stem cells; human induced pluripotent stem cells; mechanical stimulation; PDMS; stretching*

1. Introduction

Cardiomyocytes (CMs) differentiated from human stem cells will have an important role in treating heart damage and screening pharmaceutical drug candidates [1]. CMs can be generated from human embryonic stem cells (hESCs) [2,3] and from human induced pluripotent stem cells (hiPSCs) [4]. Various methods for cardiac differentiation have been used, including embryoid body formation [5], co-culture with mouse visceral endoderm-like cells (END-2) [3], a combination of different growth factors [6], and factors modulating different signaling cascades [7]. However, there are several limitations in these protocols, for example, efficiency is still limited. Furthermore, mature CMs cannot be obtained with current methods, and pure CM populations cannot be achieved [8].

Mechanical stimulation affects cell fate, morphology, orientation, and differentiation, as has been shown in recent publications [9-22]. Several approaches of applying mechanical stimuli to cells, such as flow-induced shear forces, hydrostatic pressure, substrate topography and stiffness, cell indentation, and substrate stretching, have been reported [23-28]. This paper concentrates on active substrate stretching methods.

The magnitude and frequency of the applied stretching affect the cardiac cell fate, but reported results are not consistent. An increase in the expression of cardiac differentiation markers have been shown for example for murine and mouse ESC-derived CMs [11,18,19]. On the other hand, negative effects of stretching on the cardiac differentiation of murine, rat and mouse cells have also been reported [19,20,29]. Most of the studies have used animal cells, and the number of stretching experiments using human CMs is still very low [16]. Therefore, more studies on the topic are needed.

Several research groups have reported on custom-made stretching systems connecting a flexible membrane to an actuator. The approaches can be categorized into electric actuator and pneumatic systems. A majority of the developed systems have used an electric actuator,

such as a stepper motor, a DC motor, or a voice coil actuator to deform the cell cultivation membrane [14,20,21,30-34]. The electric motor systems are typically bulky and complex requiring wiring and parts that are easily rusted in humid environment inside the incubator, and thus, give rise to a toxic and contamination risks. Only few groups have reported the use of pneumatic systems in cell stretching devices, where either over pressure or partial vacuum pressure is used to stretch the cell culture substrate. In a direct approach, an over pressure buckles the membrane upwards [35]. In an indirect approach, an over pressure is applied to a loading post behind the membrane to facilitate planar stretching of the membrane [36]. In addition to over pressure, partial vacuum pressure has been used for providing membrane strain indirectly utilizing a loading post below the membrane, as in a commercially available cyclic tension device, Flexcell®. In the loading post approaches, lubricants are typically used between the membrane and the post to enhance the membrane sliding [36,Flexcell®]. For example in the Flexcell® device, lubricant disturbs the visualization of the cells and must be removed carefully not to stress the cells. In addition, large loading posts completely block visualization of the cells using inverted microscopy during the experiment. Indirect partial vacuum has also been used without the loading post by Huh et al. [37] similar to our system. However, in that study, cells are cultured inside a small channel and thus additional continuous perfusion is required.

To overcome the currently existing challenges, this paper introduces a novel vacuum-operated cell stretching device which includes a large medium container to maintain long-term nutrient supply without additional actuators and does not include corrosive parts or a loading post but provide purely planar membrane deformation to study differentiation of hPSCs into CMs (hPSC-CMs) under equiaxial strain. The paper describes working principle, implementation process, and experimental characterization of the stretching device made of polydimethylsiloxane (PDMS) elastomer. The paper demonstrates the applicability of the

developed stretching device for differentiating and culturing human pluripotent stem cells to CMs in long-term cell experiments. It also studies coating materials to optimize cell adhesion (Supplementary data 1) and identifies a functional stretching sequence (Supplementary data 2).

2. Materials and methods

2.1. Implementation and operation of the stretching device

The stretching device has been implemented using PDMS (Sylgard 184, Dow Corning, USA). The device consists of four main parts: a thin (120 μm) PDMS membrane (1); an outer PDMS shell (2); an inner PDMS shell (3); and a rigid glass plate (4) (Fig. 1). By applying a partial vacuum pressure into the cavity between the shells, the elastic PDMS membrane deforms and buckles the inner shell symmetrically in the radial direction (Fig. 1(b)). Thus, the cells grown on the membrane are equiaxially stretched.

The wall thickness of the inner shell is small (1.5 mm) such that it can be easily deformed using the vacuum. The outer shell has thick walls (6.5 mm) to support the structure. The glass plate prevents the top parts of the shells from buckling. The height of the shells is 7 mm to provide sufficient volume for the cell culture medium.

The PDMS was prepared using a standard protocol. First, a silicone elastomer pre-polymer (base) and a cross-linker (curing agent) were thoroughly combined at a mixing ratio of 10:1 (weight ratio). Second, the mixture was placed in a vacuum for 20 minutes to remove air bubbles formed during the mixing and trapped in the uncured liquid silicone. Third, the mixture was casted to a mold. Finally, the mixture was cured in an oven (Binder GmbH, Tuttlingen, Germany) at 65°C for two hours.

The PDMS shells were fabricated by punching a 7-mm, bulk PDMS sheet using custom-made punching tools. The inner shell was punched using 12-mm and 15-mm diameter

punching tools and the outer shell was formed with 19-mm and 32-mm tools. The membrane was prepared by spinning liquid PDMS on polystyrene (PS) plates. The PDMS was poured on the plate and spun with 200 revolutions per minute (rpm) for 10 seconds, followed by five-second acceleration to 700 rpm, which was then applied for 30 seconds.

A 1-mm glass plate with a diameter of 32 mm was purchased from Aki-lasi Oy (Tampere, Finland). A 10-mm hole was drilled in the middle of the glass plate for seeding cells and supplying medium. A 2-mm hole was drilled at approximately 9 mm from the center for the vacuum connection. To guarantee a tight connection with silicone tubing (3 mm), a PDMS boss (6 mm) was bonded around the connection hole on top of the glass plate.

The parts were bonded together using oxygen plasma (Vision 320 Mk II, Advanced Vacuum Scandinavia AB, Sweden) with the following parameters: O_2 flow rate of 30 sccm, pressure of 30 mTorr, power of 30 W, and time of 15 s. Fig. 1(d) shows an implementation of the device.

2.2. Vacuum operation system

A partial vacuum pressure is applied to the stretching platform using a computer-controlled pressure-regulation system that was built in-house (performance: range from 2 bar over pressure to 392 mbar partial vacuum pressure, accuracy of 0.1%, repeatability of 0.1%, and rise time 20ms). The pressure-regulation system (Fig. 2) consists of a computer with a LabVIEW-based control software, an AD/DA measurement board (DAS16/16-AO, Measurement Computing Corporation, USA), a computer-controlled electro-pneumatic transducer (T-2000, Marsh Bellofram, USA), and an ejector pump (VAD-1/8 from Festo Oy, Finland).

2.3. Characterization of the stretching device

The stretching device has two main parameters to characterize: an in-plane strain and out-of-plane displacement of the membrane. The in-plane strain of the membrane is important, as the cells attached on the membrane are expected to experience the same deformation. Therefore, the strain is characterized in several locations on the membrane. The out-of-plane displacement affects the capability to image cells during stretching and needs therefore to be characterized.

The test setup to characterize the stretching device is illustrated in Fig. 2. A CCD camera (Sony XCD-U100), optics (12x motorized zoom with 3-mm motorized fine focusing, Navitar, Inc., Rochester, NY, USA), and led coaxial illumination (Navitar, Inc.) were used for imaging the membrane deformation.

The in-plane strain and the out-of-plane displacement were measured in five locations around the membrane: four symmetrical locations on the perimeter, and one in the middle (Fig. 1(c)). In the measurements, 16 different static partial vacuum pressures were applied between 0-392 mbar. After each pressure supply step, the motorized focusing system was used to focus on the membrane surface and to record an image.

2.3.1. In-plane strain of the membrane

The in-plane strain of the membrane was determined using pattern recognition with manual landmark labeling. In each five locations (See Figure 1(c)), three manually selected landmark points were recognized and tracked. An image-processing toolbox of Matlab (Mathworks, Natick, MA, USA) was used to analyze the images. The pixel coordinates (x, y) of the centroids of the landmarks were recorded and the distance between them was calculated for each pressure (Fig. 3), The engineering strain was calculated from the changes in the distances. Eq. 1 describes the notations used in the calculation of the distance, L_{p12}, between Landmarks 1 and 2 in pressure p.

$$L_{p12} = \sqrt{(x_{p1} - x_{p2})^2 + (y_{p1} - y_{p2})^2} \quad (1)$$

where (x_{p1}, y_{p1}) and (x_{p2}, y_{p2}) are the pixel coordinates of Landmarks 1 and 2, respectively, in pressure p. The engineering strain between two landmarks is given for Landmarks 1 and 2 in Eq. 2.

$$\varepsilon_{p12} = \frac{\Delta L}{L} = \frac{L_{p12} - L_{012}}{L_{012}} \quad (2)$$

where ε_{p12} is the strain between Landmarks 1 and 2, L_{p12} is the distance between Landmark 1 and Landmark 2 in pressure p, and L_{012} is the distance between the landmarks in zero pressure (p=0). The in-plane strain (ε_p) in pressure p was calculated by taking the average of the three strain components, as shown in Eq. 3. Standard deviation was calculated from the three strain components. The same analysis was done for each location, thus resulting in five different ε_p curves.

$$\varepsilon_p = \frac{\varepsilon_{p12} + \varepsilon_{p23} + \varepsilon_{p13}}{3} \quad (3)$$

An average strain (ε_{tot} = average from all 15 strain components) was used for presenting the data with one number. Similarly, x- and y-axis strain components (ε_{xtot} and ε_{ytot}, respectively) were calculated to analyze the equiaxial behavior of the strain.

2.3.2 Out-of-plane displacement of the membrane

The out-of-plane displacement was estimated by performing the focusing in steps of 22.5µm and observing the landmarks on the membrane from a computer screen. When the membrane was in focus, the total displacement of the motorized focusing system was recorded.

2.4 Human pluripotent stem cells

The H7 (embryonic stem cell line, WiCell, USA) hESC line and a hiPSC line [38] were used. They behave similarly and thus are later called hPSCs. To maintain the undifferentiated state, the hPSCs were cultured on top of mouse embryonic fibroblast (MEF) cells, as described by Thomson et al. [39], with some modifications. The hPSCs were cultured in KSR-medium [Knockout DMEM (Invitrogen), 20% serum replacement (SR, Invitrogen), 2 mM GlutaMax (Invitrogen), 1% non-essential amino acids (NEAA, Lonza), 50 U/ml P/S, 0.1 mM 2-mercaptoethanol (Invitrogen) and 8 ng/ml basic fibroblast growth factor (bFGF, R&D Systems, USA)] and enzymatically passaged with type IV collagenase (1 mg/ml, Invitrogen) once a week. The hPSCs were differentiated into CMs using the protocol described by Pekkanen-Mattila et al. [40].

At the end of the stretching experiments, the cells were collected and characterized using quantitative real-time polymerase chain reaction (qPCR). Total RNA was isolated from the collected cells using a NucleoSpin® RNA II kit (Macherey-Nagel GmbH & Co, Germany). The concentration and quality of the isolated RNA was monitored with a spectroscope (NanoDrop ND-1000, Technologies Inc., USA). The RNA was then transcribed to cDNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). A qPCR was performed according to the standard protocols on Abi Prism 7300 instrument (Applied Biosystems). Table 1 lists the primers (biomers.net GmbH, Germany) used.

The RNA samples were collected from three experiments and these three biological replicates were analyzed as triplicates. The relative gene expression levels of stretched and unstretched control samples were determined using the $2^{-\Delta\Delta C\tau}$ method [41]. The housekeeping gene, peptidylpropyl isomerase G (PPIG), was used for normalization of the data and the unstretched control was used as the calibrator. The statistical significance of the difference between gene expression levels of stretched and unstretched control cells was calculated by using the Mann-Whitney U Test.

3. Results

3.1. Characterization of the stretching device

3.1.1 In-plane strain of the membrane

Fig. 4(a) shows the in-plane strains (ε_p) for each five locations on the membrane. It demonstrates that the strain in the different locations on the PDMS membrane is uniform and possesses a nearly linear response to the pressure. With the maximum applied pressure, an average in-plane strain ε_{tot} = 9.5% was achieved. The variation in the strain in the different locations was very small with the largest standard deviation of 0.3% (p = 288 mbar). The corresponding x- and y-axis strains were ε_{xtot} = 9.6% ± 0.2% and ε_{ytot} = 9.4% ± 0.4%. These results demonstrate that the membrane is equiaxially and uniformly stretched.

3.1.2 Out-of-plane displacement of the membrane

The measurement result is depicted in Fig. 4(b) and it shows that the out-of-plane displacement is nearly constant on the membrane edge. The membrane moves out of plane 315 µm (± 22.5 µm), while the partial vacuum pressure varies from 0 to 392 mbar. As the membrane is loose without input pressure, a small initial partial vacuum pressure is needed to tighten it. Therefore, the out-of-plane displacement in the middle location (5) differs from the displacement in the edge locations (1-4) with no pressure.

3.2. Cell stretching experiments

The hPSCs were differentiated on END-2 cells on gelatin coated PDMS membranes. Each experiment contained four parallel stretching devices, and each experiment was repeated three times. Parallel to stretching, hPSCs were cultured on unstretched, coated, PDMS multi-well plates that served as the control (Supplementary data 1). The cells were allowed to attach for four days before stretching was applied.

Various stretching sequences were investigated. Stretching too early or too fast reduced cell attachment and survival (Supplementary data 2). A sinusoidal signal with a gradually increasing strain amplitude and frequency was applied (Table 2). The stretching started with 1% strain and 0.2 Hz frequency, and increased daily by 1% and 0.2 Hz until the values of 5% and 1 Hz were achieved. This reduces the rapid changes at the beginning of cell differentiation and thus enhances the cell attachment.

After 21 days, the cells were monitored by observing beating areas in the phase contrast images in both the stretched and the unstretched static control cultures. The images indicate successful differentiation of hPSCs into CMs. This was verified by qPCR analysis of the various CM-associated genes (Fig. 5). Although qPCR analysis did not show statistically significant differences between expression levels, the results demonstrate that the device is suitable for mechanical stimulation of hPSCs and that the cells differentiate into CMs. Moreover, these results are consistent with results by Saha et al. [16].

4. Discussion and conclusions

The aim of this study was to introduce a compact, pneumatically actuated, stretching device for CM differentiation and to study the effect of stretching on differentiation efficiency. The device does not require any separate operational actuators, but only a connection with vacuum tubing. The pressure operation system can be located in a far distance from the stretching device outside an incubator, and thus, only components which can be easily sterilized are placed inside the incubator to avoid contamination. Furthermore, the device includes a large medium reservoir, which allows for long term static culture without additional perfusion. The device does not require any supporting posts or lubricants behind the membrane. Because of that, it facilitates the use of an inverted microscope for observing the cells during the entire

experiment. This is essential for the morphological inspection of the culture. Multiple parallel stretching devices can be operated synchronously at the same time. In this current study, four parallel devices were used simultaneously. The proposed stretching device is simplified to perform only mechanical stretching for cells and can be used to study a relatively large cell population, similar to a commercial Flexcell® device. However, our device enables real time cell observation with an inverted microscope, as in the study of Huh et al. [37], but without the continuous perfusion requirement and also without lubricants that usually blur the visibility.

The stretching characterization demonstrates a uniform in-plane strain (ε_{tot} = 9.5% ± 0.3%) around the membrane, as shown in Fig. 4(a). The equiaxial nature of the strain was verified by measuring the *x*- and *y*-axis strain components ε_{xtot} and ε_{ytot} as 9.6% ± 0.2% and 9.4% ± 0.4%, respectively. As the variations are small and all the measured strain values ε_{tot}, ε_{xtot}, and ε_{ytot} are close to each other, we can assume that the strain is equiaxial and uniform. The reasons for the small variations in the strain along the membrane could be the following. First, punching is manually performed through thick and soft PDMS material. Thus, the shells are not in an ideal shape. Another reason is a manual concentric alignment of the shells. It is challenging to align the shells truly symmetrically without computer-aided visualization or automation. Finally, the image based analysis contributes to the error. The analysis was done automatically tracking the landmarks and finding the coordinates of their centroids. Quantization of images and limited contrast can affect the landmark recognition, leading to errors of a pixel or two for centroids, and thus cause small deviations in the calculated strain.

In the proposed platform, the membrane moves approximately 315 µm out of plane with maximum stretching compared to zero strain. This membrane displacement does not have any consequences when using static stretching because a microscope can be manually focused on the cells. When observing the cells during cyclic stretching, the out-of-plane

displacement does not disturb low-magnification observation of the entire cell population. However, the out-of-plane displacement disturbs the observation in high-magnification and will therefore need to be reduced and minimized in the future.

In this study, the stretching did not have any major impact on cell differentiation. Stretching hPSCs equiaxially with 1 Hz and 5% strain provided beating cell areas indicating successful differentiation of hPSCs into CMs. This was further validated with qPCR analysis of CM-associated genes (MYH6 expressed primarily in mature CMs, MYH7 expressed primarily in fetal CMs, or genes expressed in functional CMs, Cx43and Trop I) that showed similar cardiac gene expression levels as compared to controls. These results are in accordance with the findings by Saha et al. [16]. They reported that the cyclic 5% average membrane strain and 10 cycles/min (= 0.17 Hz) frequency had no significant effect on the differentiation gene-expression factor (SSEA-4) of differentiated hESCs, as compared to controls. However, Radisic et al. showed that the expression level of MYH6 rose compared to the expression level of MYH7 when the cells were maturing [42]. Our results do not support this finding but there was a large deviation between the samples, which might be due to the fact that the qPCR samples had to be taken earlier than planned because the cells began to detach at the end of the experiments. It is possible that if the cells were differentiated few days longer, there could have been significant differences on gene-expression levels.

In conclusion, the hPSCs differentiated well into CMs and started to beat when stretched with gradually increasing amplitude and frequency. Furthermore, the cells stayed alive on the membrane for several weeks including ten days continuous stretching. This proves that the platform can be used in applications to study cardiac mechanobiology. However, optimal stretching parameters are still to be found.

Acknowledgement The study was part of the projects: STEMFUNC funded by the Academy of Finland (Grant numbers 123762 and 126888) and Human Spare Parts funded by the

Finnish Funding Agency for Technology and Innovation (TEKES). Also Finnish Cultural Foundation, Finnish Foundation for Cardiovascular Research, Competitive Research Funding of the Pirkanmaa Hospital District, Paavo Nurmi Foundation, Foundation of Aarne and Aili Turunen, and CHEMSEM Graduate School provided financial support. We sincerely thank Ms Henna Venäläinen for technical assistance. We would also like to acknowledge the personnel of the animal facilities of University of Tampere.

References

1. Freund C, Mummery CL: **Prospects for pluripotent stem cell-derived cardiomyocytes in cardiac cell therapy and as disease models.** *J Cell Biochem* 2009, **107**(4)**:**592-599.
2. Boheler KR, Czyz J, Tweedie D, Yang H, Anisimov SV, Wobus AM: **Differentiation of Pluripotent Embryonic Stem Cells Into Cardiomyocytes.** *Circ Res* 2002, **91**(3)**:**189-201.
3. Mummery C, Ward-van Oostwaard Dorien, Doevendans P, Spijker R, van den Brink S, Hassink R, van der Heyden M, Opthof T, Pera M, de la Riviere AB, Passier R, Tertoolen L: **Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells.** *Circ* 2003, **107**(21)**:**2733-2740.
4. Zhang J, Wilson GF, Soerens AG, Koonce CH, Yu J, Palecek SP, Thomson JA, Kamp TJ: **Functional cardiomyocytes derived from human induced pluripotent stem cells.** *Circ Res* 2009, **104**(4)**:**e30-41.
5. Kehat I, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, Gepstein A, Livne E, Binah O, Itskovitz-Eldor J, Gepstein L: **Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes.** *J Clin Invest* 2001, **108**(3)**:**407-414.

6. Kattman SJ, Witty AD, Gagliardi M, Dubois NC, Niapour M, Hotta A, Ellis J, Keller G: **Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines.** *Cell stem cell* 2011, **8**(2)**:**228-240.
7. Lian X, Hsiao C, Wilson G, Zhu K, Hazeltine LB, Azarin SM, Raval KK: **Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling.** *Proc Natl Acad Sci USA* 2012, **109**(27)**:**1848-57.
8. Wong SSY, Bernstein HS: **Cardiac regeneration using human embryonic stem cells: producing cells for future therapy.** *Regen Med* 2010, **5**(5)**:**763-775.
9. Biehl JK, Yamanaka S, Desai TA, Boheler KR, Russell B: **Proliferation of mouse embryonic stem cell progeny and the spontaneous contractile activity of cardiomyocytes are affected by microtopography.** *Dev Dyn* 2009, **238**(8)**:**1964-1973.
10. Boerboom RA, Rubbens MP, Driessen NJB, Bouten CVC, Baaijens FPT: **Effect of strain magnitude on the tissue properties of engineered cardiovascular constructs.** *Ann Biomed Eng* 2008, **36**(2)**:**244-253.
11. Gwak S, Bhang SH, Kim I, Kim S, Cho S, Jeon O, Yoo KJ, Putnam AJ, Kim B: **The effect of cyclic strain on embryonic stem cell-derived cardiomyocytes.** *Biomaterials* 2008, **29**(7)**:**844-856.
12. Lee WC, Maul TM, Vorp DA, Rubin JP, Marra KG: **Effects of uniaxial cyclic strain on adipose-derived stem cell morphology, proliferation, and differentiation.** *Biomech Mod Mechanobiol* 2007, **6**(4)**:**265-273.

13. Maul TM, Chew DW, Nieponice A, Vorp DA: **Mechanical stimuli differentially control stem cell behavior: morphology, proliferation, and differentiation.** *Biomech Mod Mechanobiol* 2011, **10**(6)**:**939-953.
14. McMahon LA, Reid AJ, Campbell VA, Prendergast PJ: **Regulatory effects of mechanical strain on the chondrogenic differentiation of MSCs in a collagen-GAG scaffold: experimental and computational analysis.** *Ann Biomed Eng* 2008, **36**(2)**:**185-194.
15. Park JS, Chu JSF, Cheng C, Chen F, Chen D, Li S: **Differential effects of equiaxial and uniaxial strain on mesenchymal stem cells.** *Biotechnol Bioeng* 2004, **88**(3)**:**359-368.
16. Saha S, Ji L, de Pablo JJ, Palecek SP: **Inhibition of human embryonic stem cell differentiation by mechanical strain.** *J Cell Physiol* 2006, **206**(1)**:**126-137.
17. Salameh A, Wustmann A, Karl S, Blanke K, Apel D, Rojas-Gomez D, Franke H, Mohr FW, Janousek J, Dhein S: **Cyclic mechanical stretch induces cardiomyocyte orientation and polarization of the gap junction protein connexin43.** *Circ Res* 2010, **106**(10)**:**1592-1602.
18. Schmelter M, Ateghang B, Helmig S, Wartenberg M, Sauer H: **Embryonic stem cells utilize reactive oxygen species as transducers of mechanical strain-induced cardiovascular differentiation.** *FASEB* 2006, **20**(8)**:**1182-1184.
19. Shimko VF, Claycomb WC: **Effect of mechanical loading on three-dimensional cultures of embryonic stem cell-derived cardiomyocytes.** *Tissue Eng Part A* 2008, **14**(1)**:**49-58.
20. Wan C, Chung S, Kamm RD: **Differentiation of embryonic stem cells into cardiomyocytes in a compliant microfluidic system.** *Ann Biomed Eng* 2011, **39**(6)**:**1840-1847.

21. Wang JH-, Yang G, Li Z: **Controlling Cell Responses to Cyclic Mechanical Stretching.** *Ann Biomed Eng* 2005, **33**(3)**:**337-342.

22. Giridharan GA, Nguyen M, Estrada R, Parichehreh V, Hamid T, Ismahil MA, Prabhu SD, Sethu P: **Microfluidic cardiac cell culture model (μCCCM)**. *Analytical chemistry* 2010, **82**(18):7581–7587.

23. Brown TD: **Techniques for mechanical stimulation of cells in vitro: a review.** *J Biomech* 2000, **33**(1)**:**3-14.

24. Desai TA: **Micro- and nanoscale structures for tissue engineering constructs.** *Med Eng Phys* 2000, **22**(9)**:**595-606.

25. Desmaële D, Boukallel M, Régnier S: **Actuation means for the mechanical stimulation of living cells via microelectromechanical systems: A critical review.** *J Biomech* 2011, **44**(8)**:**1433-1446.

26. Kim D, Wong PK, Park J, Levchenko A, Sun Y: **Microengineered platforms for cell mechanobiology.** *Annu Rev Biomed Eng* 2009, **11:**203-233.

27. Lee Da, Knight MM, Campbell JJ, Bader DL: **Stem cell mechanobiology.** *J Cell Biochem* 2011, **112**(1)**:**1-9.

28. Moraes C, Sun Y, Simmons CA: **(Micro)managing the mechanical microenvironment.** *Integr Bio* 2011, **3**(10)**:**959-971.

29. Rana OR, Zobel C, Saygili E, Brixius K, Gramley F, Schimpf T, Mischke K, Frechen D, Knackstedt C, Schwinger RHG, Schauerte P, Saygili E: **A simple device to apply equibiaxial strain to cells cultured on flexible membranes.** *Am J Physiol Heart Circ Physiol* 2008, **294**(1)**:**532-40.

30. Ahmed WW, Kural MH, Saif TA: **A novel platform for in situ investigation of cells and tissues under mechanical strain.** *Acta biomater* 2010, **6**(8)**:**2979-2990.

31. Huang L, Mathieu PS, Helmke BP: **A stretching device for high-resolution live-cell imaging.** *Ann Biomed Eng* 2010, **38**(5)**:**1728-1740.

32. Kurpinski K, Chu J, Hashi C, Li S: **Anisotropic mechanosensing by mesenchymal stem cells.** *Proc Natl Acad Sci USA* 2006, **103**(44)**:**16095-16100.

33. Pfister BJ, Weihs TP, Betenbaugh M, Bao G: **An In Vitro Uniaxial Stretch Model for Axonal Injury.** *Ann Biomed Eng* 2003, **31**(5)**:**589-598.

34. Seefried L, Mueller-Deubert S, Schwarz T, Lind T, Mentrup B, Kober M, Docheva D, Liedert A, Kassem M, Ignatius A, Schieker M, Claes L, Wilke W, Jakob F, Ebert R: **A small scale cell culture system to analyze mechanobiology using reporter gene constructs and polyurethane dishes.** *Eur Cells Mater* 2010, **20:**344-355.

35. Shimizu K, Shunori A, Morimoto K, Hashida M, Konishi S: **Development of a biochip with serially connected pneumatic balloons for cell-stretching culture.** *Sens Act B: Chem* 2011, **156**(1)**:**486-493.

36. Moraes C, Chen J-H, Sun Y, Simmons CA**: Microfabricated arrays for high-throughput screening of cellular response to cyclic substrate deformation.** *Lab Chip* 2010, **10:**227–34.

37. Huh D, Matthews BD, Mammoto A, Montoya-Zavala M, Hsin HY, Ingber DE: **Reconstituting organ-level lung functions on a chip.** *Science* 2010, **328**(5986):1662-1668

38. Lahti AL, Kujala VJ, Chapman H, Koivisto A, Pekkanen-Mattila M, Kerkelä E, Hyttinen J, Kontula K, Swan H, Conklin BR, Yamanaka S, Silvennoinen O, Aalto-Setälä K: **Model for long QT syndrome type 2 using human iPS cells demonstrates arrhythmogenic characteristics in cell culture.** *Dis Mod Mech* 2012, **5:**220-230.

39. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM: **Embryonic Stem Cell Lines Derived from Human Blastocysts.** *Science* 1998, **282**(5391)**:**1145-1147.

40. Pekkanen-Mattila M, Kerkelä E, Tanskanen JM, Pietilä M, Pelto-Huikko M, Hyttinen J, Skottman H, Suuronen R, Aalto-Setälä K: **Substantial variation in the cardiac differentiation of human embryonic stem cell lines derived and propagated under the same conditions - a comparison of multiple cell lines.** *Ann Med* 2009, **41**(5)**:**360-370.

41. Livak KJ, Schmittgen TD: **Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.** *Methods* 2001, **25**(4)**:**402-408.

42. Radisic M, Park H, Shing H, Consi T, Schoen FJ, Langer R, Freed LE, Vunjak-Novakovic G: **Functional assembly of engineered myocardium by electrical stimulation of cardiac myocytes cultured on scaffolds.** *Proc Natl Acad Sci USA* 2004, **101**(52)**:**18129-18134.

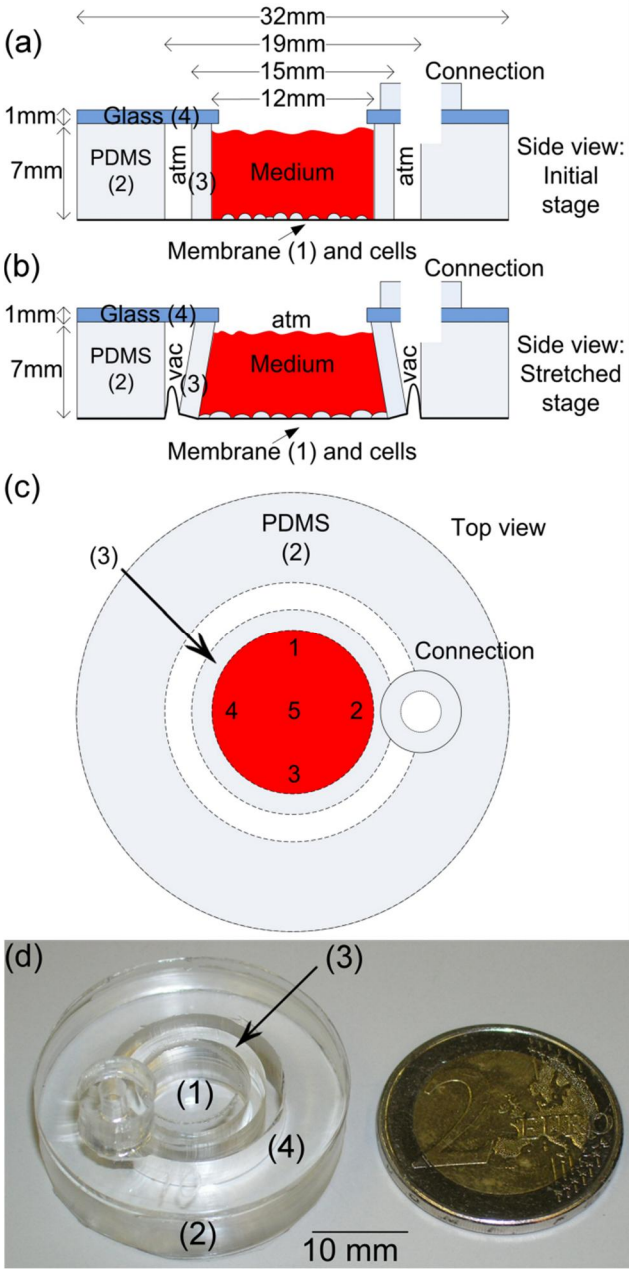

Fig. 1 Structure of the mechanical stimulation platform consisting of: (1) a thin PDMS membrane; (2) an outer PDMS shell; (3) an inner PDMS shell; and (4) a rigid glass plate. (**a**) Side view in an initial state with zero pressure. (**b**) Side view in a stretched state with applied partial vacuum pressure. (**c**) Top view of the structure illustrating also five locations where the membrane strain was measured. (**d**) Actual mechanical stimulation platform

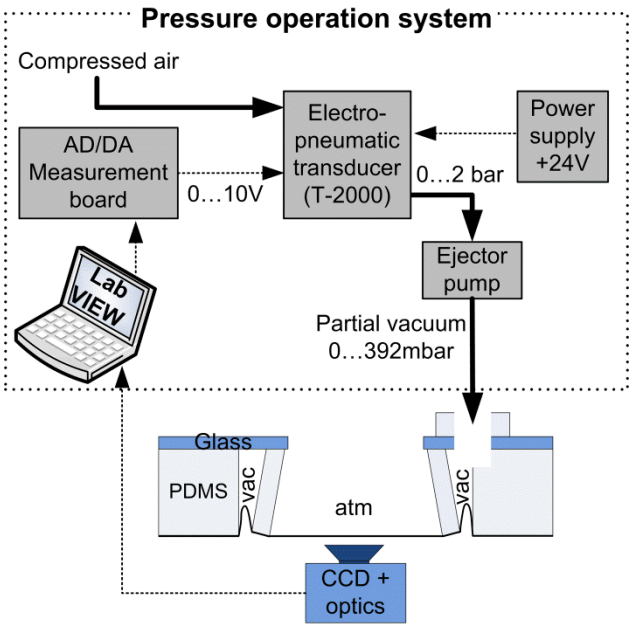

Fig. 2 Vacuum operation system connected to mechanical stimulation platform

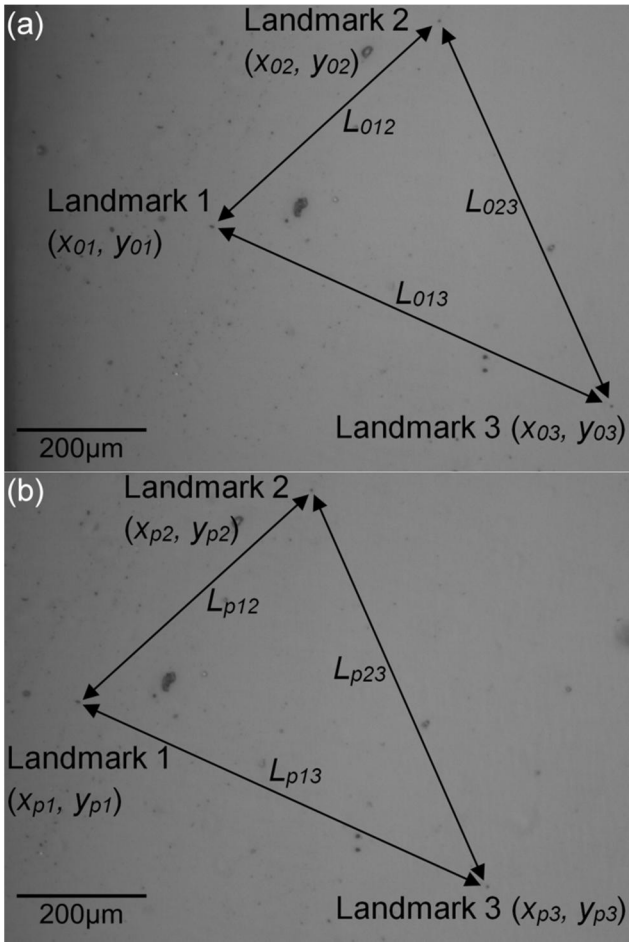

Fig. 3 Example of the in-plane strain measurement in the middle of the membrane (Location five). The locations and distances of three landmarks are illustrated on the membrane (**a**) before (zero pressure) and (**b**) during the static strain (pressure applied). Similarly, in-plane strain measurements are done for each location around the membrane

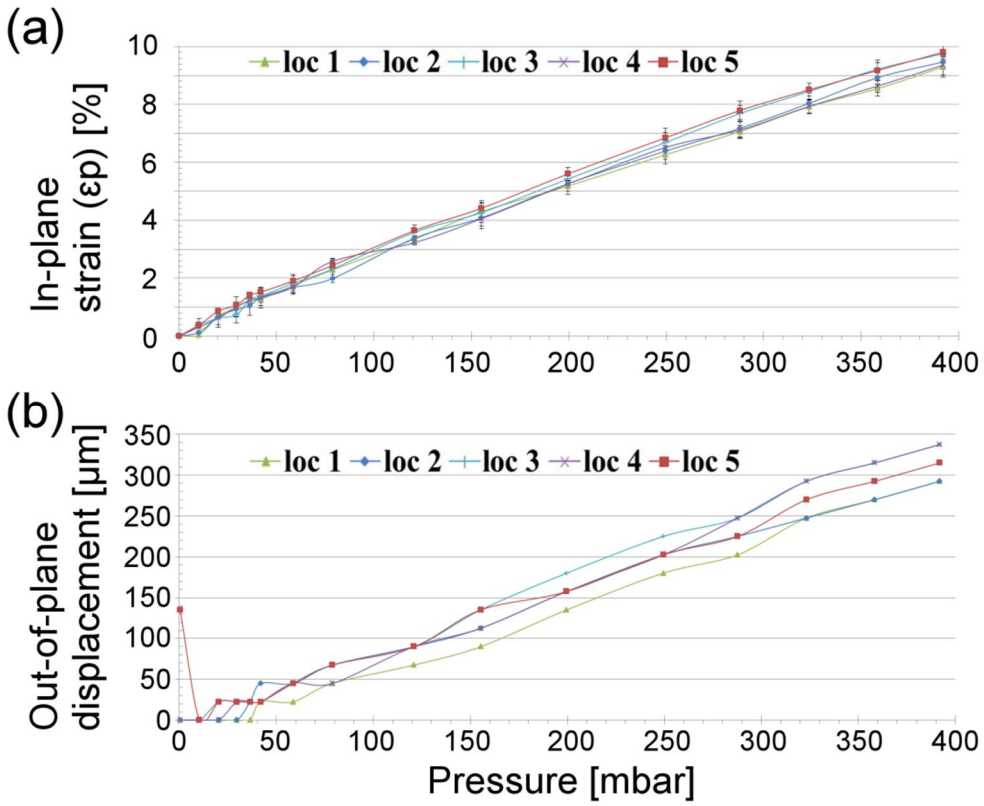

Fig. 4 (**a**) Average in-plane strain of the membrane calculated from three landmarks in five locations over various pressures and (**b**) out-of-plane displacement in the five locations

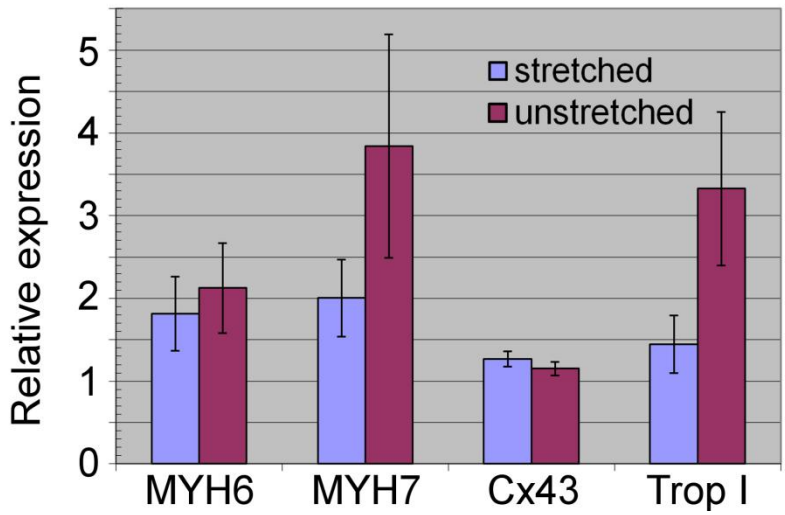

Fig. 5 The expression levels of cardiac genes were studied by qPCR with no significant differences between the gene expressions of the stretched and unstretched control cells. Twelve samples were analyzed in each group. The error bars represent SEMs