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Technical note

Pneumatic cell stretching system for cardiac differentiation and culture

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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.

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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

Abbreviations: MYH6, alpha myosin heavy chain 6; MYH7, alpha myosin heavy chain 7; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; Trop I, cardiac troponin I; CM, cardiomyocyte; Cx43, connexin 43; FBS, fetal bovine serum; PPIG, peptidylpropyl isomerase G (housekeeping gene); hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell; hPSC, human pluripotent stem cell; MEF, mouse embryonic fibroblast; END-2, mouse visceral endoderm-like cell; MCS, multichannel systems; NRC, neonatal rat cardiomyocytes; PFA, paraformaldehyde; PDMS, polydimethylsiloxane; PDL, poly-D-lysine; PS, polystyrene; qPCR, quantitative real-time polymerase chain reaction; RIE, reaction ion etching.

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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).

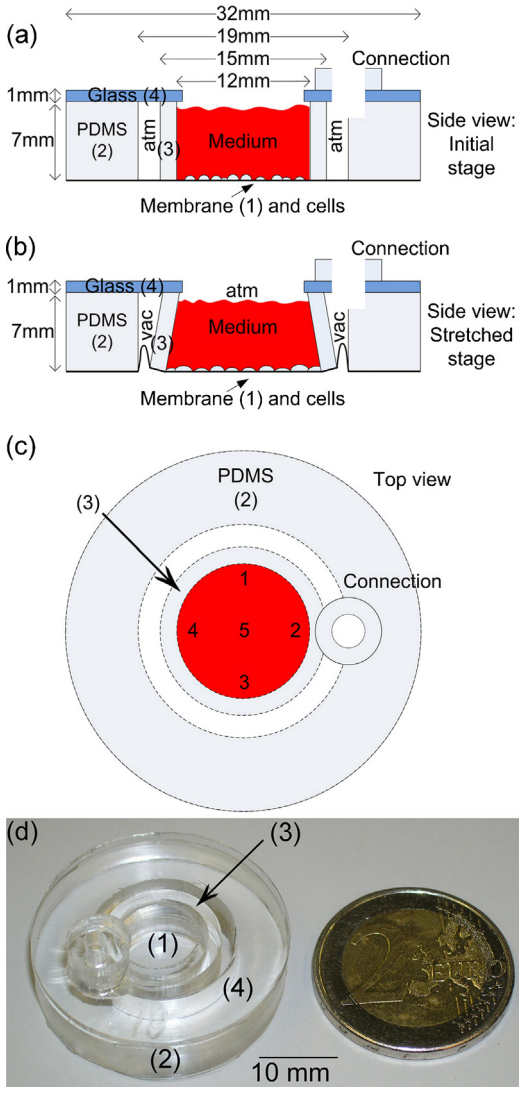

Fig. 1. Structure of the mechanical stimulation platform consisting of: (1) a thin PDMS membrane; (2) an outer PDMS shell; (3) an inner PDMS shell; and (4) a rigid glass plate. (a) Side view in an initial state with zero pressure. (b) Side view in a stretched state with applied partial vacuum pressure.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

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 min 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 2 h.

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 s, followed by five-second acceleration to 700 rpm, which was then applied for 30 s.

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

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