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Controlled-size embryoid body formation in concave microwell arrays

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ABSTRACT

Embryonic stem (ES) cells hold great potential as a renewable cell source for regenerative medicine and cell-based therapy. Despite the potential of ES cells, conventional stem cell culture methods do not enable the control of the microenvironment. A number of microscale engineering approaches have been recently developed to control the extracellular microenvironment and to direct embryonic stem cell fate. Here, we used engineered concave microwell arrays to regulate the size and shape of embryoid bodies (EBs)—cell aggregate intermediates derived from ES cells. Murine ES cells were aggregated within concave microwells, and their aggregate sizes were controlled by varying the microwell widths (200, 500, and 1000 μm). Differentiation of murine ES cells into three germ layers was assessed by analyzing gene expression. We found that ES cell-derived cardiogenesis and neurogenesis were strongly regulated by the EB size, showing that larger concave microwell arrays induced more neuronal and cardiomyocyte differentiation than did smaller microwell arrays. Therefore, this engineered concave microwell array could be a potentially useful tool for controlling ES cell behavior.

1. Introduction

Embryonic stem (ES) cell is a powerful cell type for studying regenerative tissues and cell-based therapies, because it can self-renew and differentiate into a variety of specific lineages [1], including cardiomyocytes [2–4] and neurons [5–9]. ES cells possess greater proliferative and differentiation potential than do adult stem cells, and can recapitulate early embryonic development. Because of these features, ES cells are of great interest to researchers in the fields of regenerative medicine and tissue replacement. One critical issue in realizing the potential of ES cells is obtaining large uniform populations of clinically relevant cell types. When allowed to grow under certain conditions, ES cells generate embryoid bodies (EBs) that form the three primary germ layers: ectoderm, mesoderm, and endoderm [10–14]. Cell-lineage specification during embryonic development is largely controlled by temporally and spatially regulated signals mediated by these three germ layers [11,15,16]. If effectively harnessed, these promising characteristics make ES cells a potentially renewable source of cells for regenerative medicine and chronic disease treatment.

Despite the great clinical promise of ES cells, a number of technical challenges associated with culture of the ES cells stand in the way of realizing their therapeutic potential. The most significant challenge is the inability to control the microenvironment —a prerequisite for achieving homogeneous lineage-specific differentiation from heterogeneous EBs. Unfortunately, traditional hanging drop and suspension culture methods do not lend themselves to resolution of such problems. These limitations of traditional culture techniques have led to the development of various microscale technologies that possess the potential to regulate the microenvironment of ES cells [17,18].

ES cell differentiation is regulated by microenvironmental stimuli, such as cell–cell, cell-extracellular matrix, and cell-soluble factor interactions. Thus, controlling cell–microenvironment interactions is of paramount importance in directing ES cell differentiation. Non-adhesive polyethylene glycol (PEG) microwell arrays have been previously used to control the homogeneity of EB size and shape [19,20]. Recent studies on PEG microwell-mediated control of EB size have also investigated the effects on ES cell fate determination, specifically addressing cardiogenesis and vasculogenesis via

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WNT signaling pathways [21]. These studies showed that WNT11 was highly expressed in larger microwells (450 μm in diameter) and ES cells cultured therein exhibited cardiogenesis. In contrast, higher expression of WNT5a in smaller microwells (150 μm in diameter) was associated with endothelial cell differentiation. In addition to PEG, polyurethane microwells containing self-assembled monolayers have been used to culture human ES cells and have been shown to allow ES cell pluripotency to be maintained [22]. In such microwells, a triethylene glycol-terminated alkanethiol self-assembled monolayer prevents cell and protein attachment. Moreover, a polydimethylsiloxane (PDMS)-based hollow sphere method has been developed for culturing EBs [23]. The resulting hollow sphere structure contains 500 μL medium, allowing for long-term (10–15 days) culture of EBs *in vitro* without medium depletion.

A surface-patterning technique (i.e. microcontact printing) has also been developed to regulate EB size-dependent ES cell differentiation and to explore the underlying stem cell biology [24–27]. For example, microcontact-printed substrates (200–800 μm in diameter) have been shown to provide control over colony size-dependent human ES cell differentiation [24]. The small interfering RNA (siRNA) and inhibition assays have shown that Smad1 activation was involved in this effect. Furthermore, microfabricated adhesive stencils have been used to form murine ES cell aggregates within micropatterned substrates (100–500 μm in diameter) [25]. These studies demonstrated that mesoderm and endoderm differentiation were highly induced in larger cell aggregates, whereas ectoderm differentiation was enhanced in smaller aggregates. However, previous microscale engineering approaches still have some limitations, such as cylindrical microstructures of PEG microwells are not similar to contour of EBs and microcontact-printed substrates can only control the initial size of EBs.

We have previously developed concave and convex-based thin PDMS membrane arrays (~10 μm thickness) for culturing the cells [28]. Cell attachment and proliferation were affected by concave and convex surface topography. However, our previous approach analyzed the adherence and growth of single cells, not the behavior of cell aggregates. Here, we used the concave PDMS microwell array, which was similar to the contour of EBs, to culture EBs in a controlled homogeneous manner. EBs were retrieved from concave microwells after culturing for 4 days *in vitro*.

2. Materials and methods

2.1. Fabrication of concave microwell arrays

The fabrication process of concave microwell arrays is illustrated in Fig. 1 The thin PDMS membrane (~10 μm thickness) was spin-coated on the substrate, which contained a microscale through-hole. The membrane was deflected to form convex microstructures by applying vacuum pressure through the through-holes. A SU-8 50 prepolymer solution was uniformly applied at a thickness of 1 mm to the deformed PDMS membrane, and subsequently photo-crosslinked by exposure to UV light (365 nm wavelength). Afterward, we separated the solid SU-8 on which the convex microstructure was engraved and used this SU-8 plate as a master mold for the fabrication of concave microwell arrays as we have previously described [28]. In this study, we used PDMS concave microwell arrays with three different geometries: 200 μm width/150 μm thickness, 500 μm width/200 μm thickness, and 1000 μm width/300 μm thickness. In a parallel study, PDMS cylindrical microwells with three

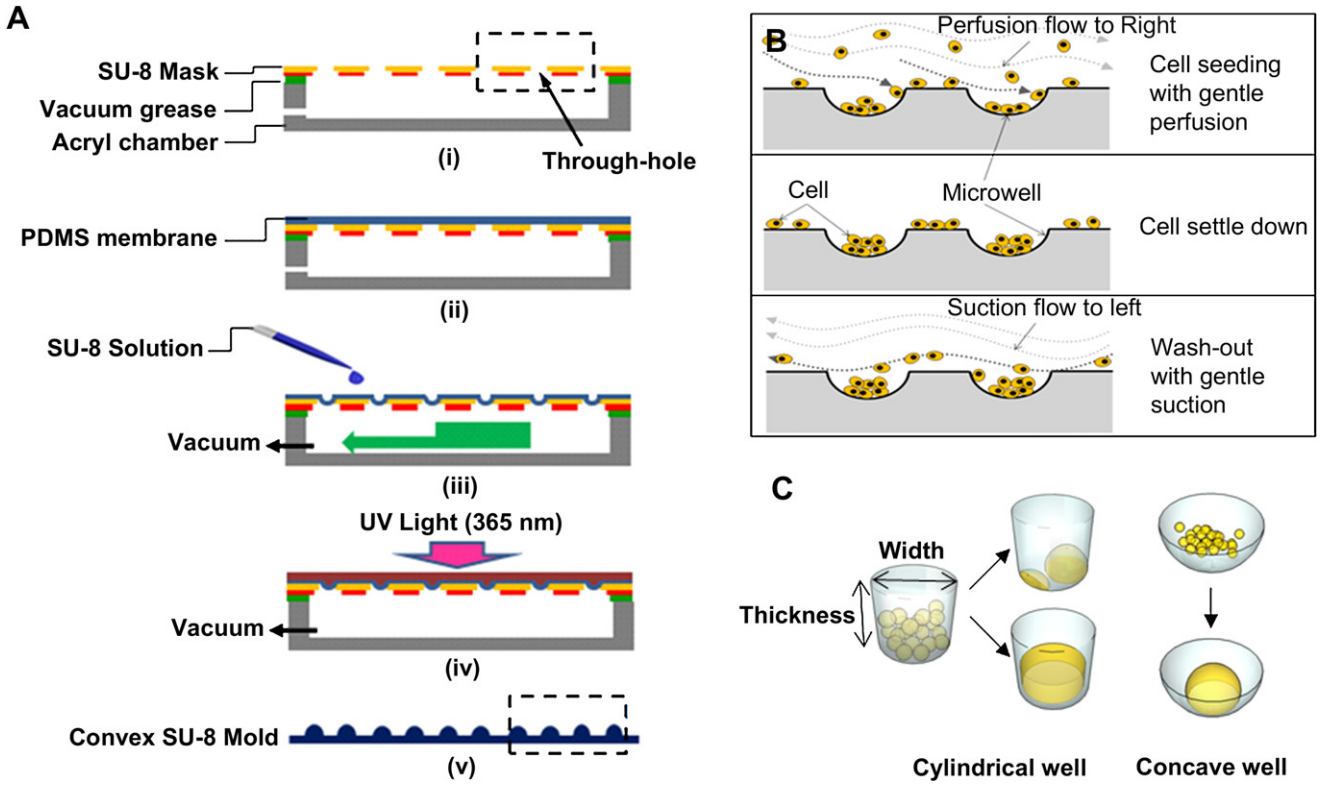

Fig. 1. (A) Schematic depiction of the process for fabricating concave microwell arrays. (i) The system consists of an acrylic chamber, vacuum grease, and a SU-8 mask surface that contains multiple through-holes. (ii) PDMS (~10 μm thick) is coated on the SU-8 mask surface. (iii) A SU-8 solution is layered on the PDMS membrane and negative pressure is subsequently applied through the acrylic chamber to deflect the PDMS membrane. (iv–v) The SU-8 solution is exposed to UV light (365 nm wavelength) during vacuum aspiration to create convex-shaped microstructures. The thickness of the convex-shape microstructures was controlled by the applied negative pressure. (B) Schematic process of cell seeding and docking within concave microwell structures. After gently aspirating cells that were not docked, the remaining cells docked within concave microwells were cultured as described in Materials and Methods. (C) Schematic drawing of cylindrical and concave-shaped microwell structures. The concave-shaped microwell structure is similar to the curved surface of cell aggregates.