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# Optimizing human embryonic stem cells differentiation efficiency by screening size-tunable homogenous embryoid bodies

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ABSTRACT

Human embryonic stem cells (hESCs) are generally induced to differentiate by forming spherical structures termed embryoid bodies (EBs) in the presence of soluble growth factors. hEBs are generated by suspending small clumps of hESC colonies; however, the resulting hEBs are heterogeneous because this method lacks the ability to control the number of cells in individual EBs. This heterogeneity affects factors that influence differentiation such as cell–cell contact and the diffusion of soluble factors, and consequently, the differentiation capacity of each EB varies. Here, we fabricated size-tunable concave microwells to control the physical environment, thereby regulating the size of EBs formed from single hESCs. Defined numbers of single hESCs were forced to aggregate and generate uniformly sized EBs with high fidelity, and the size of the EBs was controlled using concave microwells of different diameters. Differentiation patterns in H9- and CHA15-hESCs were affected by EB size in both the absence and presence of growth factors. By screening EB size in the presence of various BMP4 concentrations, a two-fold increase in endothelial cell differentiation was achieved. Because each hESC line has unique characteristics, the findings of this study demonstrate that concave microwells could be used to screen different EB sizes and growth factor concentrations to optimize differentiation for each hESC line.



## 1. Introduction

The potential applications of human embryonic stem cells (hESCs) to the fields of regenerative medicine, tissue engineering and developmental biology have long been recognized given the pluripotent nature of these cells [1,2]. Pluripotency can be characterized in vitro through the formation of embryoid bodies (EBs), which are multicellular aggregates that spontaneously differentiate into cells representing the three germ layers and thereby recapitulate events that occur during early development [3]. Because EBs play a pivotal role in differentiation, hESCs are often induced to differentiate through EB formation, especially in the presence of soluble growth factors, to generate specific cell types [4,5]. Traditionally, EBs have been formed by suspending small clumps of hESC colonies in the absence of self-renewing factors because single hESCs are vulnerable after enzymatic dissociation [6]. Despite the simplicity and scalability of this method, the resulting EBs contain different numbers of cells, and each EB varies in size and shape [7]. This heterogeneity could be the primary cause of inconsistent differentiation efficiency because microenvironmental stimuli such as cell–cell contact and cell-soluble factor interactions are dependent on the size of the EB [8,9].

To date, several attempts have been made to develop simple and robust methods to control and regulate EB size and shape in a reproducible manner. EB formation by the hanging drop method has reportedly yielded homogeneously sized EBs; however, this technique is highly laborious, and technical challenges limit scalability [10]. Commercially available 96-well round-bottom plates have also been used to generate uniform-sized EBs, but seeding cells into individual wells is tedious and error prone [11–14]. The seeded cells are also forced to aggregate by centrifugation, but the effects of centrifugal force on the viability of EBs remain unknown.

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Use of a bioreactor for large-scale generation of EBs is less labor intensive, but the ability to form uniform EBs is insufficient [15,16]. Three-dimensional cross-linked alginate inverse opal scaffolds of uniform pore size have been used to generate uniformly sized EBs, the size of which was controllable by changing the pore size [17]. Although such scaffolds are advantageous in terms of scalability for uniform EB generation, recovery of the resulting EBs is difficult because the process requires disintegration of the scaffolds. In recent years, a few microfluidic platforms, including arrayed-well and channel platforms, have been developed to generate EBs of uniform size and shape [18]. Although these systems facilitated the production of uniform EBs, controlling EB size over a wide range is difficult, and the process is complicated. Previously, we reported a simple and reproducible technique for generating uniform-sized murine EBs (mEBs) using concave microwells [19,20]; however, the ability of this system to force single hESCs to aggregate into viable and functional EBs remains untested. In contrast to murine ESCs, human ESCs cannot be maintained as single cells with the exception of several cell lines [21]. Thus, homogeneous EB formation and EB size regulation from single hESCs are critical, and it is important to define the optimal differentiation conditions for each cell line.

In this study, we fabricated concave microwells of different sizes for the formation of size-tunable EBs, which were induced to differentiate in the presence of various growth factor concentrations in order to screen for optimal differentiation conditions in terms of EB size and growth factor concentration.

## 2. Materials and methods

### 2.1. Concave microwell fabrication

The concave microwell array structure was fabricated by exploiting the surface tension of the PDMS prepolymer. A PDMS microwell sheet (10 × 10 × 3 mm) containing approximately 100 arrayed cylindrical well structures was prepared using a standard soft lithography process [22,23]. We poured a PDMS prepolymer consisting of a 10:1 mixture of silicon elastomer (Sylgard 184) and curing agent onto the PDMS sheet and allowed it to completely fill all cylindrical microwells (Fig. 1A; a). After the sheet was completely filled with the PDMS prepolymer, we raked out the PDMS prepolymer using a slide glass by applying slight pressure to the soft PDMS microwell sheet (Fig. 1A; b). The softness of the PDMS sheet resulted in the removal of approximately half of the PDMS prepolymer by wiping, with the remaining half filling the cylindrical microwells. The surface tension of the PDMS prepolymer caused a meniscus to self-organize in the cylindrical microwells (Fig. 1A; c). The PDMS prepolymer in each well was polymerized by thermal curing on a hot plate (80 °C for 1.5 h), thereby forming the final concave structure (Fig. 1A; d). This fabrication procedure produced concave wells simultaneously (Fig. 1A; e) without the use of any specialized tools or complicated procedures. Fig. 1A; f shows scanning electron microscopy (SEM) images demonstrating the hemispherical structure of the microwells. The PDMS sheet containing concave wells was replicated using SU-8 (MicroChem, MA, USA) by pouring SU-8 (MicroChem, MA, USA) onto the concave array and exposing it to UV, thus creating a convex master mold. The PDMS concave microwell arrays were finally fabricated using the SU-8 replica (Fig. S2).

### 2.2. hESC culture

Undifferentiated hESC lines (H9- and CHA15-hESCs) [1,8] were grown on mitotically inactivated mouse embryonic fibroblasts in DMEM/F12 (50:50%; Gibco BRL, Gaithersburg, MD) supplemented with 20% (v/v) serum replacement (Gibco) and basic hES medium components, including 1 mM L-glutamine (Gibco), 1% nonessential amino acids (Gibco), 100 mM beta-mercaptoethanol (Gibco) and 4 ng/ml bFGF (Invitrogen, Grand Island, NY). The medium was changed every day, and hESC colonies were transferred to new feeder cells every 7 days using dissecting pipettes.

### 2.3. EB formation using the suspension method

To generate EBs, hESCs were detached from feeder cells using 1 mg/ml dispase (Gibco), and they were cultured in a DMEM/F12 suspension supplemented with 10% SR (Gibco), 1% nonessential amino acids (Gibco), 1 mM L-glutamine, 1% penicillin-streptomycin and 0.1 mM mercaptoethanol in low-attachment dishes (Corning, Kennebunk, ME). The EB medium was changed every 2 days for 10 days.

### 2.4. Hanging drop method for EB formation

Undifferentiated hESCs were dissociated with TryLE (Gibco) and passed through a 40 m cell strainer (BD Biosciences, Bedford, MA) to produce a single-cell suspension. Single hESCs in batches of 500, 1500 and 3000 were counted and applied to the inner side of a 100-mm Petri dish (BD Bioscience) in 20 μl drops of EB medium containing 10 μM Y-27632 (Tocris, Ellisville, MO). The bottom of the dish was filled with 1 ml of PBS to prevent the drops from evaporating, and the dish was incubated at 37 °C for 72 h in a $CO_2$ incubator.

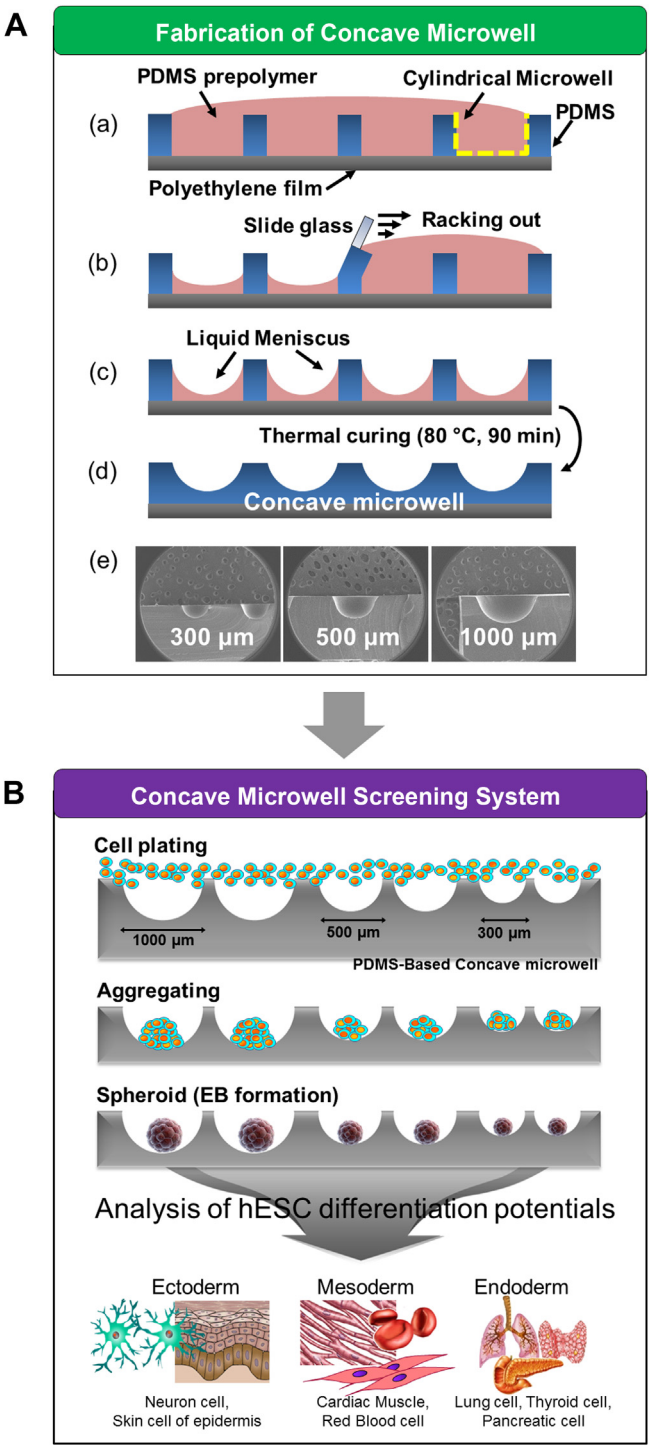


**Fig. 1.** (A) Procedure for concave microwell fabrication, and SEM images of the microwells. (B) Strategy for developing a potential screening system for hESC differentiation using size-tunable concave microwells.

### 2.5. EB formation using concave microwells

To initiate aggregation in concave microwells, single hESCs were seeded at 2.0 × $10^5$/ml in DMEM-F12 supplemented with 10% SR and 10 μM Y-27632. After