Microfabricated embryonic stem cell divider for large-scale propagation of human embryonic stem cells†

Minseok S. Kim, Janghwan Kim, Hyo-Won Han, Yee Sook Cho,* Yong-Mahn Han and Je-Kyun Park**

Received 17th July 2006, Accepted 7th December 2006
First published as an Advance Article on the web 4th January 2007
DOI: 10.1039/b617760n

We have developed a novel method for fabricating an embryonic stem cell divider (ESCD) constructed from a poly(dimethylsiloxane) (PDMS) replica with a square or hexagonal pattern, and have proposed a new dissociation method for human embryonic stem cells (ESCs). An aspect ratio of the device as high as 2 was perfectly replicated in the cutting line. Using the ESCD, human ESC colonies can be easily and efficiently dissociated into regular-sized ESC clumps without enzymatic treatment. The regularity of the ESC clumps dissociated by the ESCD was compared to that dissociated by a conventional mechanical method. Its quality and reliability were confirmed by maintaining undifferentiated ESCs up to the 15th passage. The ESCD will contribute to the advance quality control of in vitro ESC cultures and allow large-scale production of qualified ESCs with tremendous time- and work-saving.

Introduction

Human embryonic stem cells (ESCs), which have the capacity to differentiate into all types of cells both in vivo and in vitro, have tremendous impact on the fields of regenerative medicine, functional genomics and proteomics, as well as drug discovery.1 Such applications will require rigorous quality control of in vitro ESC culture. Significant challenges have been made providing clearly defined culture conditions, which maintain indefinite expansion without unintentional differentiation and are free from potential contamination with animal sources.2,3 However, a quality controlled and reproducible culture system, especially in terms of dissociation method, is still one of the biggest hurdles for the maintenance and large-scale propagation of ESCs.

Currently available enzymatic and non-enzymatic (chemical and mechanical) methods for ESC dissociation were mainly effective for detaching ESC colonies from the culture dish and producing ESC clumps without size-control. In enzymatic methods, human ESC colonies can be easily detached by incubation with enzymes such as collagenases or dispases. Detached ESC colonies are required to break into ESC clumps by gentle pipetting. Because of its high tendency for karyotypical abnormalities,4,5 this method is unbeneicial for long-term culture of ESCs. Although the mechanical method, which uses homemade-equipment or a cell scraper for cell passaging,6,7 has been considered the best way to maintain qualified ESCs with relatively uniformed clump size and karyotypical stability, it is impossible to apply the method for large-scale production of ESCs because of its huge time- and labor-consuming processes largely dependent on personal skills.

Microtechnology has supported innovative tools enabling micro-scale control so that a variety of tools have been applied for biological and medical applications.8,9 Recently, microwell-based human ESC culture has been used to control cluster size and cell differentiation.10,11 However, applications of microtechnology have rarely been studied for a large expansion of undifferentiated human ESCs and precise control of ESC clump size under intact culture conditions.

Here, we propose a simple cell dissociation method using an embryonic stem cell divider (ESCD) to support large-scale expansion with high efficiency and minimization of damage to human ESCs. A dissociation method for human ESCs and the fabrication of an ESCD will be described and the results of biological assays will be discussed.

Experimental

Configurations of an ESCD with square and hexagonal patterns are shown in Fig. S1†. The length of one side of square and hexagonal patterns was 200 μm and 124 μm, respectively. The height of both patterns was 40 μm and the cutting line to dissociate ESC colonies was 20 μm wide. The ESCDs were made to fit inside a 35 × 10 mm tissue culture dish with a 1 mm-margin to the wall of the dish to provide enough space for easy and free movement. Microfabrication processes for the ESCD are available as ESI.†

Human ESCs (CHA-hES4) used for the experiments were kindly provided from the Pochon Cha University, Korea. The cultivation methods of STO (mouse embryonic fibroblast cell line) and human ESCs are available as ESI.‡ The authors contributed equally to this work.

‡ Electronic supplementary information (ESI) available: Microfabrication, cell culture and ESC marker assays, Fig. S1, S2, S3, S4 and Table S1. See DOI: 10.1039/b617760n.
Review Board (IRB) at KRIBB, Korea. Attachment of ESCs onto ESCD can be avoided by soaking in 0.1% BSA/PBS solution and air bubbles trapped in ESCD can be easily removed by 70% ethanol washing. Prior to subculture, 2 ml of ESC culture medium per 35 mm dish was replaced with fresh and pre-warmed medium. To dissociate ESCs, ESCD was vertically pressed down onto the surface of culture dish. Complete dissociation was confirmed by observing it under a stereomicroscope (Nikon, Japan). Then, cell clumps were detached from the dish by using a rubber cell scrapper (Nalge Nunc, Rochester, NY) and transferred onto a STO feeder layer in a 35 mm dish. Small-sized ESC clumps produced from the boundary region of ESC colonies could be discarded by brief centrifugation or using a 100 μm cell strainer (BD Biosciences, NJ) if necessary. Fragments of ESC culture media were changed at day 2 after subculture and then daily.

Results and discussion

We constructed an ESCD that can easily dissociate human ESCs. In order to produce high quality ESC clumps, we tried various shapes and sizes of ESCD. Fig. 1 shows a well-detached square and hexagonal PDMS replica from a silicon mold. While the replica with the square pattern had a clear cutting line (Fig. 1A–C), that of the hexagonal pattern sometimes had more or less irregular shapes at the center of some sides owing to the directional angle of detachment (Fig. 1D–F).

Table S1† shows the characterization results for the silicon mold and PDMS replica \((n = 8)\). The hexagonal PDMS replica showed more or less larger variations in cutting line and height which were caused by detachment from the mold as mentioned above. However, the replica showed a satisfactory dimension with a high aspect ratio.

Fig. 2 shows the dissociated images of the ESC colony after pressing the ESCDs with square and hexagonal patterns. Under our experimental conditions, the single ESC colony cultured for 5 days could be divided into 25–30 pieces (each piece equals 40 000 μm\(^2\) in area) of cell clumps. Clear dissociation of ESC colonies was immediately confirmed by observing under a phase-contrast microscope (Fig. 2A and B), and DAPI staining (Fig. 2C and D) after pressing. It was confirmed that the cell number of the individual ESC clump was similar to counting of the DAPI-stained nuclei. The pressing time values (2–10 s) and vertical pressure to dissociate ESC colonies were varied depending on personal skill. The customized cell number of the ESC clump can be easily set by the size of unit pattern. Although the ESCD with an hexagonal pattern showed minor height variation of cutting lines as much as 5 μm, the hexagonal clumps of ESCs could also be uniformly made owing to their elastic characteristic (see Fig. S2†). Recently, although an automated mechanical passaging (AMP) method has been developed by modifying the McIlwain chopper, it is restricted only to square cutting forms of ESC clumps.

ESCD-derived cell clumps were easily detached by a rubber cell scrapper, transferred to a new culture dish for maintenance or further experiments (Fig. 3A). Fig. 3B shows that much more variable-sized ESC clumps and remnant fragments were produced when using a conventional (mechanical) method compared to the ESCD method. To confirm the quality of the ESCD during maintenance of the ESCs, fifty cell clumps were randomly selected from a suspension, transferred to a new 35 mm dish containing inactivated STO feeder cells. The attachment rate was measured by manually counting well-attached ESCs under an inverted microscope 2 or 3 days after subculture. The ESCD method increased the attachment rate up to over 95% and maintained a stable proliferation rate. In a conventional method, the attachment rate would not exceed 70% (data not shown). The regularity of re-cultivated ESCs in colony size was...
calculated by measuring the area of the individual ESC colony stained with alkaline phosphatase (AP) in a 35 mm culture dish. Using an ESCD, it was inevitable that irregular-sized ESC clumps at the boundary region of individual ESC colonies were produced and they could simply be discarded by brief spin-down or using a commercially available cell strainer. Even without such further processes, the ESCD method provided a higher number of similar sized ESC colonies and less ESC fragments than a conventional mechanical method (Fig. 3C).

During maintenance of the ESCs using the ESCD, the undifferentiated state of the ESCs was confirmed by monitoring expressions of pluripotent ESC markers including AP, transcription factors Oct-4, and cell surface markers SSEA-4, Tra-1-60, and Tra-1-81. As shown in Fig S3, all ESC specific markers were highly expressed in the ESCD-derived ESCs. The expression of SSEA-1, negative control was not detected (see Fig. S4). Cells were maintained using this tool for up to the 15th passage. During serial passaging, ESCs were maintained at a similar cell number, proliferation and attachment rate, and the undifferentiated state without significant variation.

Conventional mechanical passaging of human ESCs is known to be the best way to retain normal karyotype preserving genetic integrity, even after the 100th passage. However, this method consumes a large amount of time and requires highly skillful operation. By saving substantial time for culture and enhancing the attachment rate, the ESCD provides an extraordinary advance on large-scale propagation of undifferentiated human ESCs without enzyme treatment.

Our tool makes it possible to control the cell number of cell clumps, which will contribute to many areas such as development of ESC expansion and differentiation protocol, and quantitative analysis.

Acknowledgements

The authors thank Wonhye Lee for microfabrication and Hyungmin Chung for kindly providing the CHA-hES4 cell line. This work was supported by the Stem Cell Research Center of the 21st Century Frontier Research (SC2090), KRFC/KRIBB Research Program, KOSEF Stem Cell Research Program (M106410200206N410200210), the CHUNG Moon Soul Center for BioInformation and BioElectronics, and the Ministry of Commerce, Industry and Energy (MOCIE).

References


Fig. 3  Phase-contrast images of suspended ESC clumps produced by using an ESCD (A) and a conventional mechanical method (B). (C) Quantitational analysis of re-cultivated ESC colonies. The graph shows size distribution of individual AP stained-ESC colonies. The efficiency of the ESCD method was compared with a mechanical method (con). Data represent the mean ± SEM (** P < 0.001). To calculate the p-value, the values were compared using the T-test (paired two samples for means) using Microsoft Excel.