Electronic Supplementary Information (ESI)

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

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Microfabrication processes for the ESCD

Photoresist (PR) AZ-1512 (Microchem, Newton, MA) was spincoated at 1500 rpm for 5 s and 2000 rpm for 6 s to make a 1.5 μ m thick deep reactive ion etching (DRIE) masking layer. After PR patterning, dry etching for the cutting line was performed by inductively coupled plasma (ICP) DRIE (Unaxis VL-DSE, FL) equipment. The remnant PR was stripped out and the fabricated mold was immersed into sulfuric acid at 95 °C for 3 h. Then, the silicon mold was diced by 22.5 mm × 22.5 mm and the pieces were sonicated in ethanol for 15 min. After drying the pieces, they were sufficiently sprayed with a mold release agent (Nambang CNA Co., Korea) and the mixture of poly(dimethylsiloxane) (PDMS) prepolymer and curing agent (Sylgard 184; Dow Corning, MI) was poured onto the diced molds where the ratio of mixture was 5:1. The polymer was cured at 70 °C for 3 h on a hot plate to complete cross-linking.

Culture of STO cells

The STO cell line (Mouse embryonic fibroblast cell line) was purchased from American Type Culture Collection (ATCC; Manassas, VA). STO cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine (Sigma, St. Louis, Mo), 0.1 mM β-mercaptoethanol (Sigma), and 1% nonessential amino acid (GIBCO-BRL). Cells were treated with 10 µg/ml of mitomycin C (Sigma) for 1.5 h, washed with phosphate-buffered saline (PBS), and then replated into 0.1% gelatin-coated 35-mm culture dishes.

Culture of human ESCs

Human ESCs (CHA-hES4 derived at the Pochon Cha University, Korea) were plated at an initial

density of 50 colonies per 35-mm dish. Undifferentiated ESCs were maintained by co-culture with mitomycin C (Sigma)-treated STO feeder cells in DMEM/F12 medium (Invitrogen, Carlsbad, CA) containing 20% knockout serum replacement (Invitrogen), 1% non-essential amino acids (Invitrogen), 0.1 mM mercaptoethanol (Sigma), 100 U/ml penicillin-streptomycin (Invitrogen), and 4 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen) at 37°C, 5% CO₂ in air. Before we developed ESCD, ESCs were mechanically passaged to fresh feeders every 5 days. For mechanical transfer, home-made tool was made of Pasteur pipettes and sterilized. After carefully isolating ESC colonies from surrounding STO feeder layers, ESC colonies were disaggregated into small ESC clumps by scrapping with home-made tool (mechanical method).^{6,7}

ESC counts

Number of ESCs was adopted from the nuclei counts obtained by 4, 6-diamidino-2-phenylindole (DAPI) staining. Briefly, after fixed with 4% paraformaldehyde/PBS for 30 min, the cells were incubated in 0.5 μ g/ml DAPI solution for 15 min in the dark. Stained nuclei were visualized with an inverted fluorescent microscope (Olympus, Japan).

Alkaline phosphatase assay

Staining for alkaline phosphatase (AP) was performed at room temperature using AP detection kit containing Naphthol/Fast Red Violet solution (Chemicon, Tmecula, CA) as recommended by the manufacturer. During reaction, culture dishes were protected from drying and direct light. The cells were rinsed with deionized water, and air-dried. Images were observed under an inverted microscope (Olympus, Japan).

Immunocytochemistry analysis of ESC markers

Human ESCs were washed with Ca²⁺- and Mg²⁺-free PBS, and then fixed in 4% paraformaldehyde at 4°C for 30 min. The cells were then blocked by the respective normal serum for each primary antibody, and incubated with different primary antibodies against the human pluripotent ESC markers, Oct4, SSEA-1, -4, Tra-1-60, or Tra-1-81 (Chemicon, Temecula, CA). After washing with a washing buffer (10 mM Tris, 100 mM NaCl, 0.05% Tween 20, and 0.3% BSA), the cells were incubated with Alexa 488- or Alexa 568-conjugated secondary antibodies (Molecular probes; Eugene, OR) for 30 min. The cells were washed again, and then observed under an inverted fluorescence microscope (Olympus, Japan). SSEA-1 was used as a negative control.



Fig. S1 Schematic diagrams of an ESCD. (A) PDMS divider with a square pattern. (B) PDMS divider with a hexagonal pattern. (C) Cross-sectional view of square pattern (see Fig. S1A, a-a'). A side length of a square pattern is 200 μ m. (D) Cross-sectional view of hexagonal pattern (see Fig. S1B, b-b'). A side length of a hexagonal pattern is 124 μ m. Both patterns have the same height and width of cutting line (40 and 20 μ m, respectively).



Fig. S2 Phase-contrast images of an ESCD and an ESC colony. (A and D) The surface images of ESCD. Images of human ESC colony after pressing with ESCD with a square (B and C) or hexagonal pattern (E and F). Images were taken from the brightfield at $100 \times$ (A, B, D and E), or $200 \times$ magnification (C and F).



Fig. S3 Human ESC marker expression after 10 passages. After passaging with the ESCD, the undifferentiated state of human ESCs was characterized by examining ESC marker expressions. Using specific primary antibodies, expressions of Oct-4, SSEA-1,-4, Tra-1-60, and Tra-1-81 were analyzed by immunohistochemistry as mentioned in the Experimental. The alkaline phosphatase (AP) expression was measured using an AP detection kit. The SSEA-1 expression was examined as a negative control. Magnifications, $40 \times$ (AP), $200 \times$ (Oct-4, SSEA-1,-4, Tra-1-60, and Tra-1-81).



Fig. S4 Human ESC marker expression after passaging with a mechanical method. After passaging with the mechanical method, the undifferentiated state of human ESCs was characterized by examining ESC marker expressions. Immunohistochemistry analysis was performed using specific primary antibodies against Oct-4, SSEA-1,-4, Tra-1-60, or Tra-1-81 as mentioned in the Experimental. The alkaline phosphatase (AP) expression was measured using an AP detection kit. The SSEA-1 expression was examined as a negative control. All images were acquired at 40× magnification.

 Table S1 Characterization of the fabricated silicon mold and PDMS replica with square and hexagonal patterns.

	Silicon mold		PDMS replica	
	Square	Hexagon	Square	Hexagon
Side length	198.6 ± 2.5	122.9 ± 2.9	187.4 ± 0.5	120.6 ± 4.2
Cutting line	20.1 ± 0.4	20.1 ± 0.6	19.9 ± 0.7	20.0 ± 1.3
Height	40.8 ± 0.3	39.3 ± 0.8	39.9 ± 0.4	37.0 ± 2.3