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Supporting Material

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SUPPORTING MATERIAL

I. SUPPLEMENTARY MATERIALS AND METHODS

Culture of hESCs and assessment of undifferentiated markers

hESCs were cultured in Matrigel coated plates (BD) in mouse embryonic fibroblast-conditioned medium (MEF-CM) supplemented with 12 ng/mL human recombinant basic fibroblast growth factor (bFGF) (BD). hESC colonies were dissociated with collagenase IV (Invitrogen) for 5~10 minutes and passaged every 5 to 7 days. Expression of Oct4, Nanog, Tra-1-60, Tra-1-81, stage-specific embryonic antigen-3 (SSEA-3) and SSEA-4, assessed by either immunocytochemical staining or flow cytometry, was considered as indicators of undifferentiated hESCs as previously described (1-4).

Time-lapse microscopy, quantitative cell tracking and motility analysis

Cells were seeded onto Matrigel (R&D) coated plates and cultured in MEF-CM supplemented with bFGF (12 ng/mL). The plate was housed in a LiveCell+ system (Pathology Devices, Inc. MD, USA) and monitored using a 10x objective (Leica HC PL FLUOTAR 10× NA 0.3). The individual cell trajectories (migration tracks) were recorded by tracking the centroid position of each cell on unprocessed movies using the Track Point function of Volocity software. Cell velocity = total length of cell trajectory/time of recording (μm/h). The D/T directionality ratio = the linear distance from the start point to the end point [D] /the total length of cell trajectory [T] (5) (Fig. S1 *B*). The travel radius represents the maximum linear distance from the starting point to the point farthest away from the starting point (6). Time-lapse movies were exported to AVI format at 3~30 frames per second.

The doubling time of hESCs has been found to be approximately 36 h (24 h - 48 h) (7). Cell division (proliferation) occurred in < 3% of cells for the 20 h duration of the experiments.

This often occurred during cell tracking, which can be identified by time-lapse microscopy and excluded from the analyses.

Derivation of E-cadherin Tet-ON expression sub-clones

The human E-cadherin coding sequences were amplified by PCR from pEYFP-N1-hE-cadherin (a gift from Dr. Yap, University of Queensland, Australia) using primers: 5'-GGCCGCGGCCGCTGTGAGCTTGCGGAAGTCAGTTCAGA-3' (forward), 5'-GGCCACGCGTCTAGTCGTCCTCGCCGCCTCCGTACATGT-3' (reverse). The resulting PCR product was inserted into pLVX-Tight-Puro vector after NotI and MluI digestion (Clontech) and verified by sequencing. Production of Lentivirus from Lenti-X^{TM} 293T packaging cells (Clontech) was performed according to the manufacture's instructions. Fresh lentiviral supernatant was collected and concentrated by centrifugation. hESCs cultured in 24-well Matrigel-coated plates at 50-60% confluence were transduced in the presence of 12 μg/mL polybrene (hexadimethrine bromide, Sigma) with pLVX-E-cadherin or TetON (as control) supernatant, or with pLVX-E-cadherin+TetON and cultured in MEF-CM supplemented with bFGF (12 ng/mL), G418 (50 μg/mL), and puromycin (1.0 μg/mL) for 10 days. Stable transduced hESC colonies were selected based on the response to doxycycline, expanded and maintained in the above culture medium in the presence of G418 (25 μg/mL) and puromycin (0.5 μg/mL). To up-regulate E-cadherin expression, hESC cells were treated with 1 μg/mL of doxycycline for 24 hours. Gene expression of E-cadherin was examined by Q-PCR, and protein expression levels on plasma membrane by quantitative image analysis following immunocytochemistry.

Immunocytochemical and alkaline phosphatase (AP) staining

Immunofluorescence staining was conducted as described previously (1; 2; 4). In brief, hESCs cultured on Matrigel coated chamber slides (Nunc) or culture plates (BD) were fixed, permeabilized, and incubated with the primary antibodies (goat anti-Oct4, 3 μg/mL, sc-8628, Santa Cruz, or mouse anti-E-cadherin, 5 μg/mL, clone 67A4, Chemicon). Normal goat IgG isotype and normal mouse IgG isotype (Sigma) and antibody diluent alone served as controls. For double and triple labeling procedures, primary antibody staining was visualized by incubation with Alexa Fluor 488 or 555-conjugated donkey anti-goat IgG or anti-mouse IgG (1:400 dilution, Molecular Probes). TUNEL staining was carried out using the ApopTag Plus FITC *in situ* kit (Chemicon) according to the manufacturer's instructions. AP staining was performed as previously described using Vector Red substrate (Vector Labs) (1; 8). Samples were mounted using Vectashield mounting medium (Vector Labs) containing DAPI.

Quantitative PCR (Q-PCR)

Total RNA was extracted and reverse transcribed as described previously (1; 4). An initial regular polymerase chain reaction (PCR) was performed for each gene of interest (GOI) to optimize the conditions and check the amplicon size. The conditions for Q-PCR reactions are: one cycle at 94°C for 90 seconds; followed by 40 cycles at 94°C for 10 seconds, 60°C for 30 seconds and 72°C for 30 seconds. Relative mRNA expression was determined by the ΔΔ-Ct method using the housekeeping gene β-actin levels as endogenous control. The primers used for β-actin: gcacagagcctcgccttt (forward), ggaatccttctgacccatgc (reverse); for Bcl-XL: atggcagcagtaaagcaagc (forward), cggaagagttcattcactacctgt (reverse); for Caspase-3: tgatgatgtggaagaacttagg (forward), acggctccgcacctgctgaggc (reverse).

Inhibition of stromal-derived factor 1 (SDF-1)

To investigate whether SDF-1 constitutes part of the extracellular cytokine/chemokine microenvironment contributing to hESC migration and re-aggregation, dissociated single hESCs were plated onto Matrigel-coated plates and cultured in MEF-CM supplemented with bFGF (12 ng/mL) at a density of 3000 cells/cm^2. Either anti-rhSDF-1 monoclonal antibody or control IgG (50 ng/mL for each, R&D Systems) was added into the culture media, and cell tracking was performed as described in the previous section for 20 h.

Passage and freeze-thaw of dissociated single hESCs

The procedure was completed within 30 min from dissociation to reseeding hESCs into the plate (or freezing them down). For dissociation, hESCs were washed twice with D-PBS ($Ca^{2+}Mg^{2+}$ free) after collagenase IV treatment, and incubated with either cell dissociation buffer (Invitrogen) or 4 mM of EGTA at 37°C for 10~15 minutes. After gently pipetting, double volume of spent MEFCM was added. Cells were centrifuged for 2 min at 750g and re-suspended in fresh MEFCM supplemented with bFGF (12 ng/mL).

To freeze the dissociated hESCs, we re-suspended cells at a concentration of 1~2 × 10^6 cells/mL per cryovial (Nalgene) in pre-cold cryopreservation solution, placed the cryovials in an isopropanol freezing container (Nalgene Mr. Frosty Freezing container), and froze them overnight at -80°C. The cryovials were transferred to liquid nitrogen next day. Cryopreservation solution was composed of 10% DMSO (Sigma), 40% FBS (HyClone) and 50% DMEM/F12 (Invitrogen), or 10% DMSO, 60% Knock-out serum replacement and 30% Knockout-DMEM (Invitrogen).

To thaw the dissociated hESCs, we placed cryovials on dry ice after removal from liquid nitrogen. Cryovial was immersed in a 37°C water bath until an ice crystal remained, 0.3 mL warmed KO-DMEM was added into the cells and then quickly removed the entire volume of cells into 9 mL of pre-warmed KO-DMEM. Cells were centrifuged at 750 g for 3 min, re-suspended in warmed MEFCM supplemented with bFGF (12 ng/mL) and reseeded in Matrigel-coated plates. The thawing and reseeding procedure was completed in approximately 15 min.

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II. SUPPLEMENTARY FIGURES

Figure S1

A

	0 h Cell number (%)	20 h Cell number (%)	
	Single cells	Single cells	Re-aggregates
Isotype	121 (100 %)	76 (63 %)	45 (37 %)
Antibody	123 (100 %)	100 (81 %)	23 (19 %)

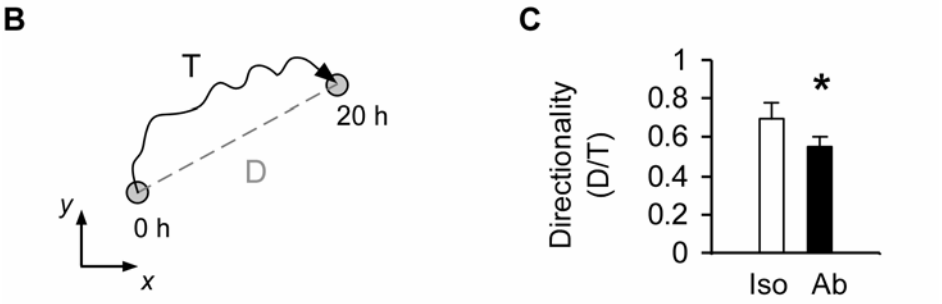

Figure S1. SDF-1 contributes to the communication of dissociated single hESCs
(A) In comparison to isotype control, the addition of a SDF-1 neutralizing antibody to the culture medium results in a 2-fold decrease in cell re-aggregation (cell separation distances: 30-70μm). Data were pooled from 3 independent experiments.
(B) The schematic illustrates how D/T ratios are obtained. D/T represents the ratio of the direct distance [D] from the start point to the end point (dashed line) divided by the total length of cell trajectory [T] (5).
(C) Quantification of the D/T directionality ratio of individual hESCs (cell separation distances: 30~70 μm) in the presence of either isotype control antibody (Iso) or neutralizing antibody against SDF-1 (Ab). Data are mean ± SD, n = 3 independent experiments (~40 cells per group); * $p < 0.05$.
For all panels in this figure, cell movements of dissociated single hESCs were tracked at 5 min intervals over a span of 20 h. The separation distance of cells was measured at the starting time point (0 h).

Figure S2

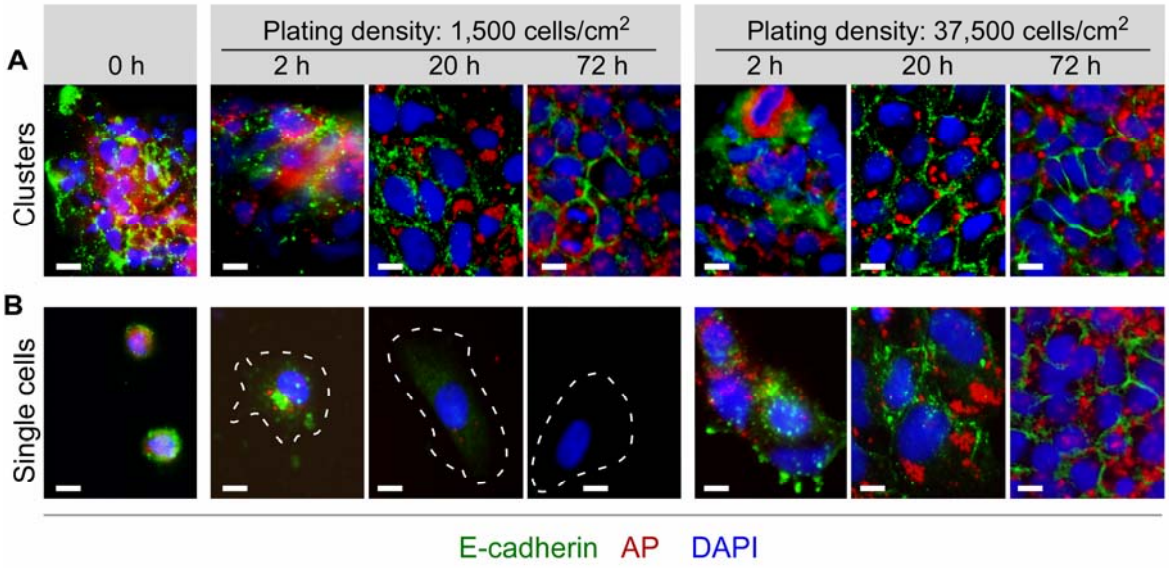

Figure S2. Cell re-aggregation facilitates the preservation of E-cadherin expression and survival of hESCs

(*A*) Multicellular clusters of hESCs preserve E-cadherin expression (green) and AP activity (red, in the cytosol). Typical hESC colonies are observed 72 hours post reseeding in both plating densities. Nuclei were counterstained with DAPI (blue). Scale bars, 10 μm.

(*B*) Dissociated single hESCs fail to preserve E-cadherin (green) and AP activity (red). At low plating density (1500 cells per cm^2) and lack of cell re-aggregation, E-cadherin and AP in single hESCs diminish gradually. After 72 hours, 98~99% of single hESCs undergo cell death or differentiation (determined by negative for AP staining). In contrast, at high plating density (37,500 cells per cm^2), single cells are able to re-aggregate and E-cadherin is detected on cell-cell contacts 2 hours post reseeding and thereafter. Consequently, typical hESC colonies are observed 72 hours post reseeding. Scale bars, 10 μm.

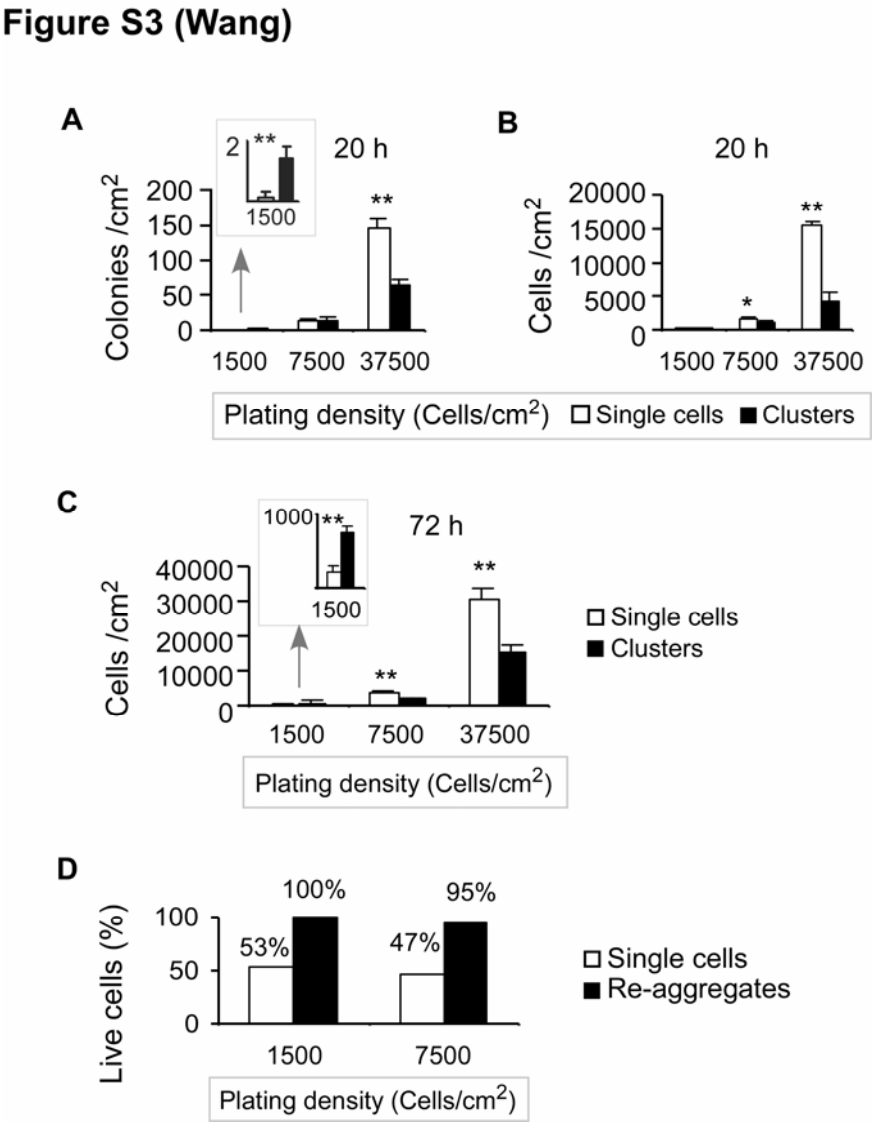

Figure S3. Motility-induced cell re-aggregation facilitates the survival of dissociated hESCs
(*A-C*) Survival and proliferation of dissociated single hESCs are related to the plating density. hESCs were plated as single cells or multicellular clusters in the same density as indicated. The number of colonies was monitored daily. Colonies and undifferentiated cells per cm^2 were quantified on unprocessed tile-scan images with Leica AF6000 software, and further verified after AP staining. Data are mean ± SD of four independent experiments; ** $p < 0.01$ versus cluster group at the same plating density.
(*D*) The percentage of survival cells during the course of 20 h post reseeding. Time-lapse movies were analyzed with cell tracking function of Volocity software. Data are pooled from four independent experiments; error bars indicate SD based on 160-298 cells per group.

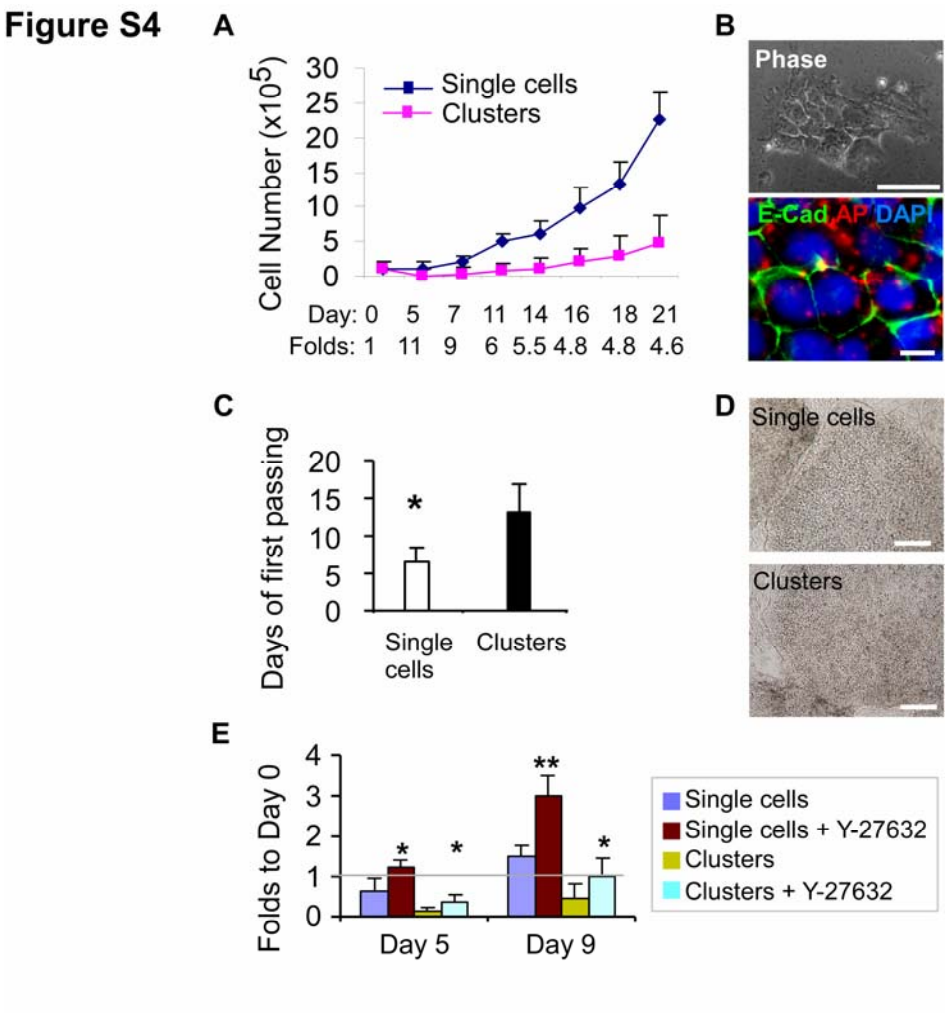

Figure S4. Motility-induced cell re-aggregation improves the recovery of dissociated hESCs after freeze-thawing

(*A*) Dissociated single hESCs show higher proliferation capacity in comparison to multicellular clusters after thawing. Paired vials of hESCs were cryopreserved as single cells or clusters (control) in liquid nitrogen for 1-12 months, thawed and seeded at a density of 250,000/cm^2. Folds represent the ratio of the total cell number of single cells group over the total cell number of cluster group at the indicated times. Error bars indicate SD based on 24 independent experiments using 3 cell lines (H1, H9 and CA1). **(*B*)** Dissociated single hESCs form colonies (phase contrast image) and re-establish E-cadherin (green) mediated cell-cell cohesion 3 days after thawing. Scale bars, 10 μm. **(*C*)** The average time for cryopreserved single hESCs to achieve colony confluence after thawing is approximately 2-fold shorter than those of cluster controls (mean ± SD; n = 21 independent experiments; ***$p < 0.05$* versus control). **(*D*)** Colonies derived from cryopreserved single cells and clusters are identical after the second passage. Phase contrast images; scale bars, 200 μm. **(*E*)** Addition of ROCK inhibitor Y-27632 augments the recovery of cryopreserved hESCs after thawing. Data are mean ± SD; n = 9 independent experiments using three different hESC cell lines (H1, H9 and CA1); ***$p < 0.05$*, ** $p < 0.01$ versus control.

III. SUPPLEMENTAL MOVIE LEGENDS

Movie S1. Time-lapse movie of a single hESC in the absence of neighbor cells as shown in Figure 1*B*. Images were acquired at 5 min intervals for 41 h.

Movie S2. Time-lapse movie of hESCs plated in the presence of neighbor cells (< 70 μm) as shown in Figure 1*C*. Images were acquired at 5 min intervals for 8 h.

Movie S3. Time-lapse movie of asymmetric collective movements/colony expansions as shown in Figure 1*E*. Images were acquired at 5 min intervals for 49 h.

Movie S4. Time-lapse movie of hESC re-aggregation as shown in Figure 2*A*. The movie was recorded at 2 min intervals for 2 h immediately after plating (37,500 cells/cm^2).

Movie S5 and S6. Time-lapse movie of hESCs in the presence (Movie S5) or absence (Movie S6) of Rock inhibitor Y-27632 (10 μM) as shown in Figure 4*A* upper and lower panels. Plating density was 1500 cells/cm^2. Images were acquired at 5 min intervals for 20 h.