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Supporting Material

Improving Survival of Disassociated Human Embryonic Stem Cells by Mechanical Stimulation Using Acoustic Tweezing Cytometry

Di Chen, Yubing Sun, Cheri X. Deng, and Jianping Fu

Supporting Methods

Attachment of targeted microbubbles to cells

The cell-seeded 35-mm glass bottom dish was placed on a 37°C heating stage on an inverted microscope (Eclipse Ti-U; Nikon, Melville, NY). Cells were bound with targeted Arg-Gly-Asp peptide (RGD) coated microbubbles (RGD-MB) or acetylated low density lipoprotein (AcLDL) coated microbubbles (AcLDL-MB). To form RGD-MB, Targesphere™-SA microbubbles (Targeson, San Diego, CA) were mixed with biotinylated RGD molecules (Peptides International, Louisville, KY) for 20 min at room temperature, with a volume ratio of 5:1 between MBs (3×10^9 mL^{-1}) and RGD (2 mg mL^{-1}). As a negative control, AcLDL-MBs, without mechanical linkages to the intracellular actin cytoskeleton (CSK), were obtained by mixing MBs with AcLDL (Invitrogen, Carlsbad, CA) for 24 hr at 4°C with a volume ratio of 5:1 between MBs (3×10^9 mL^{-1}) and AcLDL (2.5 mg mL^{-1}). To conjugate MBs to disassociated hESCs, the culture medium from the cell-seeded dish was removed followed by immediate addition of 20 μL of the diluted (1:100 by volume in PBS) RGD-MB or AcLDL-MB solution. The petri dish was then flipped over for 10 min, so that attached cells on the glass bottom dish faced downward to facilitate attachment of MBs to the cells. The dish was flipped back, and unbound MBs were removed by a gentle wash with culture medium (Fig. 1b).

Cell culture and disassociation

hESC line H1 (WiCell) was cultured on mitotically inactive mouse embryonic fibroblasts (MEFs; GlobalStem) in growth medium with daily medium change. Growth medium contained DMEM/F12 (GIBCO), 20% KnockOut serum replacement (GIBCO), 0.1 mM β-mercaptoethanol (GIBCO), 2 mM glutamax (GIBCO), 1% non-essential amino acids (GIBCO), and 4 ng mL^{-1} human recombinant basic fibroblast growth factor (bFGF; GlobalStem). Cells were rinsed briefly with PBS and treated with TrypLE Select (Invitrogen) for 2 min to release MEFs. Cells were rinsed briefly again with PBS before all cells, including hESCs and remaining MEFs, were collected using a cell scraper (BD Biosciences). To remove contaminant MEFs, all cells were transferred onto a 60-mm tissue culture dish (BD Biosciences) coated with gelatin (Sigma) and incubated for 45 min. MEFs would attach to the dish while hESCs were still in the supernatant.

Single hESCs were seeded on vitronectin coated tissue culture plates immediately after disassociation and after 1 hr, MBs were added to bind the cells. Live cell imaging was then performed to monitor dynamics of morphology changes of hESCs.

Ultrasound system and generation of ultrasound pulses

A 10 MHz focused transducer (Olympus, Waltham, MA, USA), positioned at an angle of 45° was used for ATC experiments. The ultrasound (US) pulses applied in this study had fixed pulse duration (PD) of 50 ms, pulse repetition frequency (PRF) of 10Hz, total application time of 30 s, and acoustic pressure of 0.08 MPa. The transducer was driven by a waveform generator (Agilent Technologies 33250A, Palo Alto, CA) and a 75 W power amplifier (Amplifier Research 75A250, Souderton, PA). By applying US pulses, MBs conjugated to individual cells underwent a cyclic movement and exerted subcellular mechanical forces to live single cells.

Quantification of cell survival rate and cell area

A live cell imaging system was utilized to monitor dynamics of morphology changes of hESCs from 1 hour till 24 hour after the disassociated cells are seeded. The survival rate after 24 hours (Fig. 1e) is defined as the number of cells survived at t = 24 h divided by the total number of cells at t = 1h. Similarly, survival rate in Fig. 2b is defined as the corresponding number of survived cells at t = 24 h that were initially rounded divided by the total number of rounded cells at t = 1h with different number of microbubbles attached. Cells of interest were selected from the recorded image sequences using Image J (U. S. National Institutes of Health) and the cell area were estimated by tracing the boundary of the cell.

Characterization of ultrasound-driven microbubble kinetics

A high-speed camera (Photron FASTCAM SA1, San Diego, CA) was used for observation of microbubble activities during the 30 s of ultrasound application at a frame rate of 500 frames s^{-1}. Pixel intensity and its spatial gradient of the acquired images were used to track microbubbles using a customized algorithm refined from built-in circle tracking algorithms in Matlab (Mathworks). The displacement of a microbubble was

obtained from these images as a functional of time from its original location. The accumulative bubble displacement (Fig. 2c) was estimated as the summation of the bubble displacements sustained during all of the ultrasound pulses applied in the 30s treatment.

Supporting Discussion

Adhesion-mediated survival of hESCs

Our results indicated that integrin-mediated adhesion signaling might be important for promoting survival of single hESCs. Supporting this view, it has been nicely demonstrated that dissociation induced hESC apoptosis is not due to a loss of cell-ECM adhesion (1). Furthermore, removing Y27632 after cell spreading for 6-12 hrs does not affect survival of single hESCs (1). Integrin-mediated adhesion signaling trigged by binding of RGD-MB but not AcLDL-MB (Fig. 1e) may very likely suppress apoptosis signaling in hESCs. In addition, ATC stimulation may further strengthen integrin-mediated adhesion formation and thus further reduce apoptosis of single hESCs. As shown in Fig. 2d, integrin-mediated adhesion formation and strengthening by ATC stimulations further facilitated cell spreading of disassociated hESCs, which in turn increased cell adhesion to rescue the cells from hyper-activated actomyosin activities triggering downstream caspase-mediated apoptotic signaling pathways.

Supporting Movies

Movie S1. High speed recording of one-bubble case during ATC (first 3 s, 500 frame/s). Scale bar, 10 μm.

Movie S2. Time lapse live-cell images of hESCs for 24 hrs. Scale bar, 10 μm.

Movie S3. High speed recording of two-bubble case during ATC (first 3 s as shown, 500 frame/s). Scale bar, 10 μm.

Movie S4. High speed recording of three-bubble case during ATC (first 3 s as shown, 500 frame/s). Scale bar, 10 μm.

Movie S5. High speed recording of four-bubble case during ATC (first 3 s as shown, 500 frame/s). Scale bar, 10 μm.

Supporting References

1. Ohgushi, M., M. Matsumura, M. Eiraku, K. Murakami, T. Aramaki, A. Nishiyama, K. Muguruma, T. Nakano, H. Suga, M. Ueno, T. Ishizaki, H. Suemori, S. Narumiya, H. Niwa, and Y. Sasai. 2010. Molecular Pathway and Cell State Responsible for Dissociation-Induced Apoptosis in Human Pluripotent Stem Cells. Cell Stem Cell 7:225-239.