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Supporting Information

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Materials and Methods

Matrigel preparation

Matrigel (Corning® Matrigel® hESC-Qualified Matrix, *LDEV-Free, cat# #354277) was diluted to a concentration of 0.1 mg/ml in cold Dulbecco's modified Eagle's medium/F12 (DMEM/F12; GIBCO) and then applied to glass bottom tissue culture polystyrene (TCPS) dishes (35 mm; 10mm glass diameter, MatTek Corporation cat# P35G- 1.5- 10-C) and 6-well plate glass bottom dishes (10mm glass diameter, MatTek Corporation cat# P06G-0-10-F). The coating was allowed to polymerize for 2 hours incubation at room temperature, or in 37^0C in incubator for 1 hour. Excess Matrigel-DMEM/F12 solution was aspirated before plating cells, and then dishes were washed with sterilized Dulbecco's phosphate buffered saline (D-PBS).

hESC culture

hESCs (H9, WiCell Research Institute, Madison, WI) were cultured on the synthetic surface PMEDSAH as described previously [33] with human-cell-conditioned medium (HCCM, MTI-Global Stem, Gaithersburg, MD, http://www.mti-globalstem.com) supplemented with 5 ng/mL of human recombinant basic fibroblast growth factor (FGF2; InvitrogenTM, Carlsbad, CA, http://www.invitrogen.com), and 1%antibiotic-antimycotic (Gibco). The hPSC culture medium was replaced every other day and all cell culture was performed in designated incubators at 37^0C in 5% CO2 and high humidity. Differentiated cells were mechanically

removed using a sterile pulled-glass pipet under a stereomicroscope (LeicaMZ9.5, Leica Microsystems Inc., Buffalo Grove, IL). Undifferentiated colonies were cut into small pieces and transferred them onto Matrigel coated glass dishes (~5-6 as small cell clusters for each plate), and let them to attach for overnight before experiments. (H9, NIH registration number #0062, WiCell Research Institute, Madison, WI, http://www.wicell.org)

Targeted microbubbles (MBs) and attachement to hESCs

For application of acoustic tweezing cytometry (ATC) in this study, monodisperse functionalized microbubbles (MBs), SIMB4-5 (Advanced Microbubble Laboratories, Boulder, CO), were used in this study. Biotin-tagged SIMB4-5 MBs have a peak diameter between 4 - 5 microns. Biotin-tagged SIMB4-5 MBs were first mixed with solution of streptavidin solution (10mg/ml) (ThermoFisher) for 20 min at room temperature, at a volume ratio of 20:1 to form streptavidin linked MBs. Then biotinylated Arg-Gly-Asp (RGD) peptides (Peptides International), at concentration of 2mg/ml, were added to the streptavidin MB mixture at the volume ratio of 2:21 and incubated for 20 min at room temperature to obtain RGD coated MBs. After 20 min, most MBs flowed to the surface of the solution, where 2.5 µl was taken from the top layer and mixed in 47.5 µl culture medium to obtain 50 µl of RGD-MB solution.

To attach the RGD-MBs to the adherent hESCs in a culture dish, the culture medium was first removed and 50 µl of RGD-MB solution was added immediately onto the monolayer of cells. The cell culture dish was flipped over and incubated for 10 min to allow RGD-MBs to float up towards the adherent cells for conjugation with the cells. Finally, the dish was flipped back and gentle washing was performed to remove unbound MBs before experiments.

Experimental setups and ATC application

Two experimental setups were employed in this study. For ATC induced neural induction experiments, a setup was used that allows high through-put and sterile application of ATC.

Here, an inhouse made single element US transducer with central frequency at 1 MHz was controlled using a programmable 3D manipulator. For ATC experiments, a 6-well plate was positioned above a water tank, with the bottom of the plate submerged in the water. The US transducer was set at a 45 degree angle to minimzie interferences from direct reflections and aimed at the cells at a distance of 54 mm (Rayleigh distance of the transducer). The position of the US transducer was antomatically controlled to apply US pulses at multiple locations to treat all cells within a cell in the plate with step size of 5 mm (half of the -6 dB beamwidth of the transducer), applying US pulses at location for 30 min for ATC application.

A second setup was used for the purpose of videomiscroscopic imaging of US-driven MB activities (**Figure S1**). US pulses were applied from top (**Figure S1A**). Here a single element US transducer (1.25Mhz, Advanced Devices, Wakefield, MA), with center freqeuncy close to that of the first setup, was mounted from the top at 45 degree, with its active surface submerged in the cell culture medium and a distance of 9 mm (Rayleigh distance of this transducer) from the cells. The glass-bottom culture dish was placed on an inverted microscope (Nikon Eclipse Ti-U, Melville, NY) for videomicroscopic recording.

Both US transducers were calibrated in free field using a 40 μm calibrated needle hydrophone (Precision Acoustics HPM04/1, UK) prior to experiments. The transducers were driven by a waveform generator (Agilent Technologies 33250A, Palo Alto, CA) and a 75W power amplifier (Amplifier Research 75A250, Souderton, PA). The total application duration was 30 min, including three 10 min sessions with acoustic pressure ramped from 0.035 MPa, 0.045 MPa, and 0.055 MPa for each succesive session. The pulse repetition freqeuncy was 1 Hz, and duty cycle was 5 %, corresponding to a pulse length of 50 ms.

High-speed videomicroscopy of MBs and MB displacement

To capture MB dynamic activities driven by US pulses during ATC aplication, a high-speed camera (Photron FASTCAM SA1, San Diego, CA) with a frame rate of 1000 frames/s was

used. Videoes of MBs during ATC were taken in real time using a 40x objective. Recording was synchronized with the application of US pulses.

To track MB position and radius over time during ATC application from the recorded video, a customized algorithm refined from a built-in circle-tracking algorithm in MATLAB (Math Works) was employed. Displacement of an integrin-bound MB was determined as the distance of the MB traveled from its pre-US location during an US pulse. Accumulative displacement of MBs, such as the 5-pulse accumualtive displacement, which is the total displacement achieved during the first five US pulses, is calculated as the sum of the maximim MB displacement ahieved during each of the ultrasound pulses during ATC application.

Culture in neural induction medium

Stemdif™ SMADi neural Induction Kit (Stem Cell Tech # 08581) were added to the cells (with or without ATC treatement) for culture for either 6 hours, 24 hours, or 48 hours. At each of these time points, supernatant was aspirated out and plates were washed with PBS.

Inhibition experiments

PF-562271 (Sigma-Aldrich) (1 µM) was added to the culture medium including the cells 1 hour before ATC application to treat the cells for 30 min to inhibit focal adhesion kinase (FAK) phosphorylation. To inhibit the Rock pathway and the myosin-II heavy chain, Y27632 and blebbistatin (Sigma-Aldrich) was added to the media respectively at 10 µM 1 hour before applying ATC to treat the cells for 30 min.

Cell immunocytochemistry (ICC) analysis

hESCs on MatTek single glass bottom dishes and 6-well plate glass bottom dishes were washed with PBS for 5 min and the supernatant was aspirated out and 1 ml of Z-Fix solution

(Anatech LTD: cat# 170) was added for 10 min at room temperature. Next, we washed 3 times with 1 mL of PBS for 10 min at room temperature. Next, unmasking solution (PBS, 2N HCL, 0.5% TritonX) was added and after 10 min, was removed, and quenching solution (TBS, 0.1% Sodium Borohydride) was added and removed after 10 min, followed by addition of permeabilization solution (PBS, 0.02% TritonX) for 10 min. Then, 1 mL of 5% BSA in 1X PBS was added for incubation period of 30 min. Next, the cells in the dishes were incubated with primary antibodies overnight at 4˚C. Dishes were then washed with 1X PBS 3 times for 10 min at room temperature. Then they were incubated with secondary antibodies covered in foil for at least one hour at room temperature. Afterwards, the cells were washed twice in PBS, incubated in DAPI solution for 10 min and washed in 1X PBS for 10 min. Finally, 1 drop of ProLong® Gold Antifade Mountant was added onto each well, covered with a glass cover slip, and sealed with acrylic nail polish. Images were taken using a Nikon Ti Eclipse Confocal Microscope, 20x and 60x water immersion lenses. Images were taken with or without 3x digital zoom, ¼ frames per second, 512x512 image capture, 1.0 Airy Units, 2x line averaging, appropriate voltage and power settings optimized per antibody. No modification was done, except image sizing reduction, rotation, or gray scale change for figure preparation.

For ICC fluorescence imaging, all primary antibodies were used as following with a working volume of 1 mL in 5% BSA in PBS, unless noted otherwise: Oct4 (SantaCruz: goat, 1:500, cat# sc8629), Pax6 (D3A9V) (CST: rabbit, 1:200 cat#60433), Pax6 (SantaCruz: mouse, 1:500 cat#81649), Sox1 (CST: rabbit, 1:100, cat#4194), β-catenin (E-5) (SantaCruz: M, 1:500, cat#SC7963). For secondary antibodies, all antibodies were used at a concentration of 1:1,500 with a working volume of 1.5 mL in 5% BSA in PBS. DAPI stain was used for DNA. Donkey anti-Mouse IgG Secondary Antibody, Alexa Fluor® 488 (TFS: cat# R37114). Donkey anti-Rabbit IgG Secondary Antibody, Alexa Fluor® 594 (TFS: cat# A-21207).

Image analysis and fluorescent intensities from ICC

The fluorescence intensity of Oct4, Pax6, or Sox1 in each cell was quantified from the ICC fluorescence staining images using ImageJ. DAPI images were used as the template to identify cell nuclei in an image and create a binary image as a mask. Then this mask was applied to OCT4, PAX6, or SOX1 fluorescence images respectively, in order to extract the fluorescence intensity within the nuclear for each channel. Next the total fluorescence intensity inside the nuclear was measured in each channel. All the images obtained in our experiments were analyzed and histograms of the fluorescence signals were generated.

Extraction and purification of total RNA

All groups with cells were washed with PBS and 1000µl of Trizol Reagent (Invitrogen, Carlsbad, CA) and 20 µl of Glycogen were added to the plates, and RNAs were collected after vigorous pipetting, and collected in 1.5 ml tubes and stored at -80^{0}C till they were used. 200 µl Chloroform was added to this solution followed by centrifugation (13,000 g-15 min). Aqueous phase containing RNA was separated and 500 µl isopropanol was added and stored at 20ºC overnight. Then, the manufacturer's RNA Clean-up protocol, RNeasy Mini-Kit (Qiagen, Valencia, CA), with the optional On-column DNAse treatment was followed. RNA quality and concentration were checked using a Synergy NEO HTS Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT).

Reverse-transcription PCR (RT-qPCR) analysis

Reverse transcription from 2.5 µg of the total RNA in a 20 µL reaction into cDNA was performed using SuperScript™ VILO™ Master Mix (ThermoFisher Cat#11755050). The synthesis of first-stranded cDNA was carried out in the PCR tube after combining SuperScript VILO, RNA, and DEPC-treated water, in the first cycle at 25ºC for 10 min, incubating at 42ºC for 60 min, and terminating the reaction at 85ºC for 5 min. Quantitative PCR was performed triplicate for for each sample using TaqMan probes (Applied Biosystems) and

TaqMan Universal PCR Master Mix (Applied Biosystems) on 7900 HT Fast Real Time PCR system (Applied Biosystems). Relative quantification of Nanog, Oct4, Sox2, Pax6, Sox1, Nestin, BetaIII tubulin, AP2A1, and AP2A2 gene expression data were normalized to the GAPDH expression and calculated using the 2-ΔΔCT expression level[34] .

List of primers used in qPCR. All primers were purchased from ThermoFisher Life Technologies.

Gene Symbol	Assay ID	UniGene ID
NANOG	Hs02387400_g1(FAM-MGB)	Hs.635882
POU5F1 (OCT 3/4)	Hs03005111_g1(FAM- MGB)	Hs.249184
SOX2	Hs01053049 (FAM-MGB)	Hs.518438
PAX6	Hs00240871_m1(FAM-MGB)	Hs.270303
SOX1	Hs01057642_s1(FAM-MGB)	Hs.202526
NESTIN	Hs04187831g1(FAM-MGB)	Hs.527971
BETA III TUBULIN (TUBB3)	Hs00801390_s1(FAM-MGB)	Hs.511743
AP2A1	Hs00900330_m1(FAM-MGB)	Hs.467125
AP2A2	Hs00392195_m1(FAM-MGB)	Hs.19121
GAPDH	Hs02786624_g1(FAM-MGB)	Hs.544577

Statistical Analysis

Statistical analysis was performed using Graphpad Prism 8.0. Results are presented as Mean ± SEM. Kruskal-Wallis (for nonparametric comparison among multiple groups) followed by Dunn's test (to compare the mean rank with every other group) was performed for data that are not normally distributed. One way ANOVA followed by Tukey's test was used for data that follow a normal distribution. Mann Whitney two tail test was used to compare data in two groups. A p-value < 0.05 was considered statistically significant.

References

[33] L. G. Villa-Diaz, H. Nandivada, J. Ding, N. C. Nogueira-de-Souza, P. H. Krebsbach, K. S. O'Shea, J. Lahann, G. D. Smith, Nat Biotechnol 2010, 28, 581.
[34] T. D. Schmittgen, K. J. Livak, Nat Protoc 2008, 3, 1101.

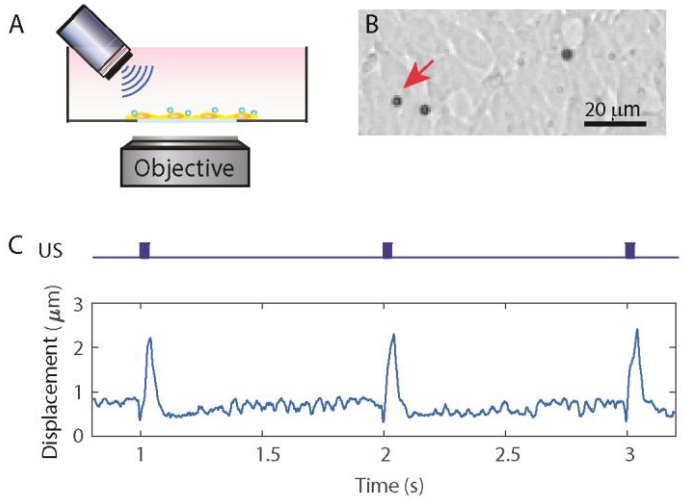

Figure S1. ATC setup with real-time videomicroscopy to record dynamic activities of microbubbles driven by ultrasound (US) pulses. (A) Exerimental setup for ATC application to treat cells with attached microbubbles (MBs) via integrin. (B) Example of brigh field image of adherent cells with integrin-bound MBs. (C) US pulses used in ATC application and an example of measured displacement of an integrin-bound MB during three US pulses. Pulse repetition frequency was 1 Hz, pulse duration was 50 ms, and acoustic pressure was 0.035 MPa.

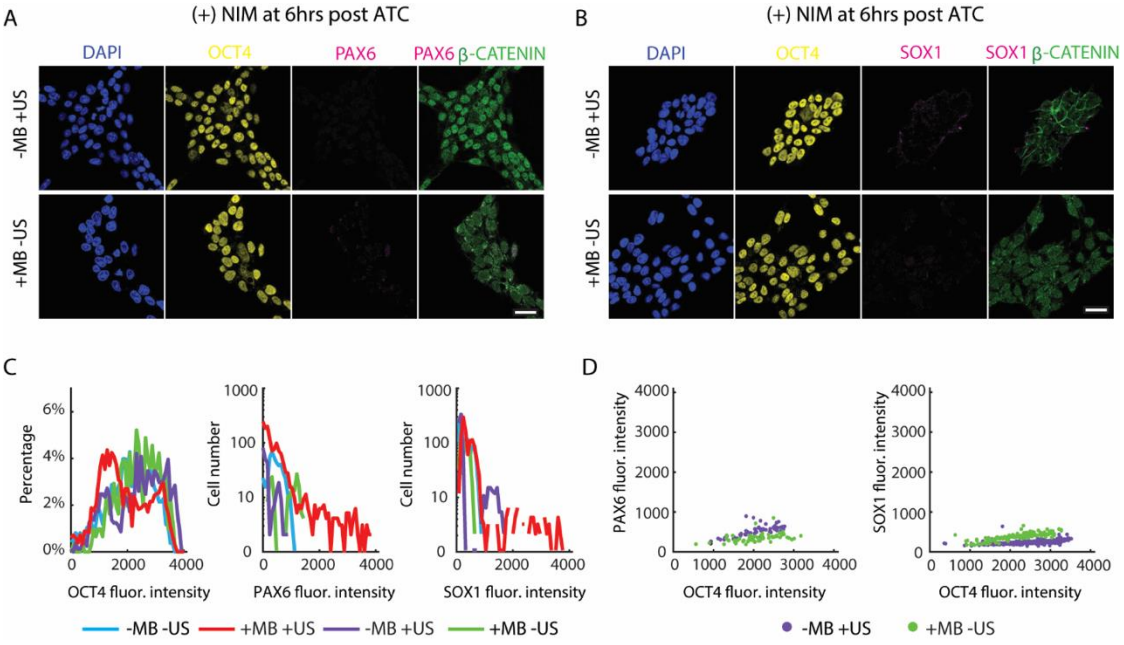

Figure S2. hESCs in control groups without microbubbles (MB) (-MB+US+NIM) or without ultrasound (US) (+MB-US+NIM) exhibited no changes in PAX6 and SOX1 expression after 6 hour culture in neural induction medium (NIM). (A-B) Representative confocal immunocytochemistry (ICC) fluorescence images for hESCs in –MB+US+NIM and +MB-US+NIM group respectively. Application of US pulses was for 30 min followed by 6 hour culture in NIM. Scale bar 50 µm. (C) Histograms of OCT4, PAX6, and SOX1 intensity in hESCs in four groups (-MB-US+NIM, +MB+US+NIM, -MB+US+NIM, +MB-US+NIM). A total of 907 cells (-MB+US+NIM) and 651 cells (+MB-US+NIM) from 4 independent experiments were analyzed for OCT4. A total of 228 cells (-MB+US+NIM) and 163 cells (+MB-US+NIM) from 2 independent experiments were analyzed for PAX6. A total of 679 cells (-MB+US+NIM) and 488 cells (+MB-US+NIM) from 2 independent experiments were analyzed for SOX1. (D) Scatter plots of PAX6 vs. OCT4 and SOX1 vs. OCT4 for the hESCs in the control groups -MB+US+NIM and +MB-US+NIM respectively.

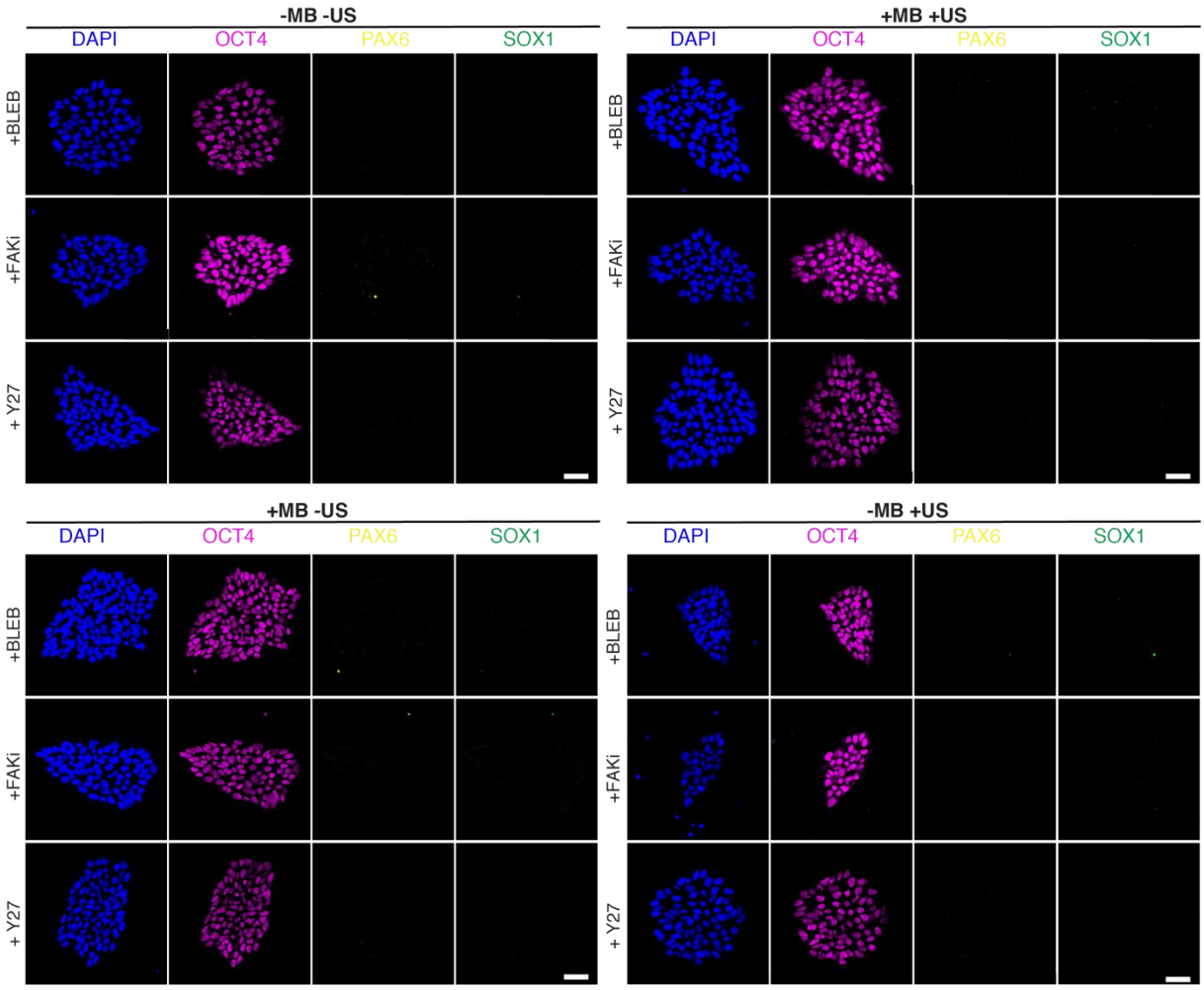

Figure S3. Inhibition of FAK, myosin II and RhoA/ROCK signaling abrogated ATC effects on hESCs. The cells were pretreated 1 hour before ATC using Blebbistatin (myosin II activity inhibitor), or FAK inhibitor, or Y-27632 (RhoA/ROCK signaling inhibitor), and then treated in four conditions: -MB-US (A), +MB+US (B), +MB-US (C), -MB+US (D) for 30 minutes, followed by 6 hours culture in NIM. Scale bar 50 μm.

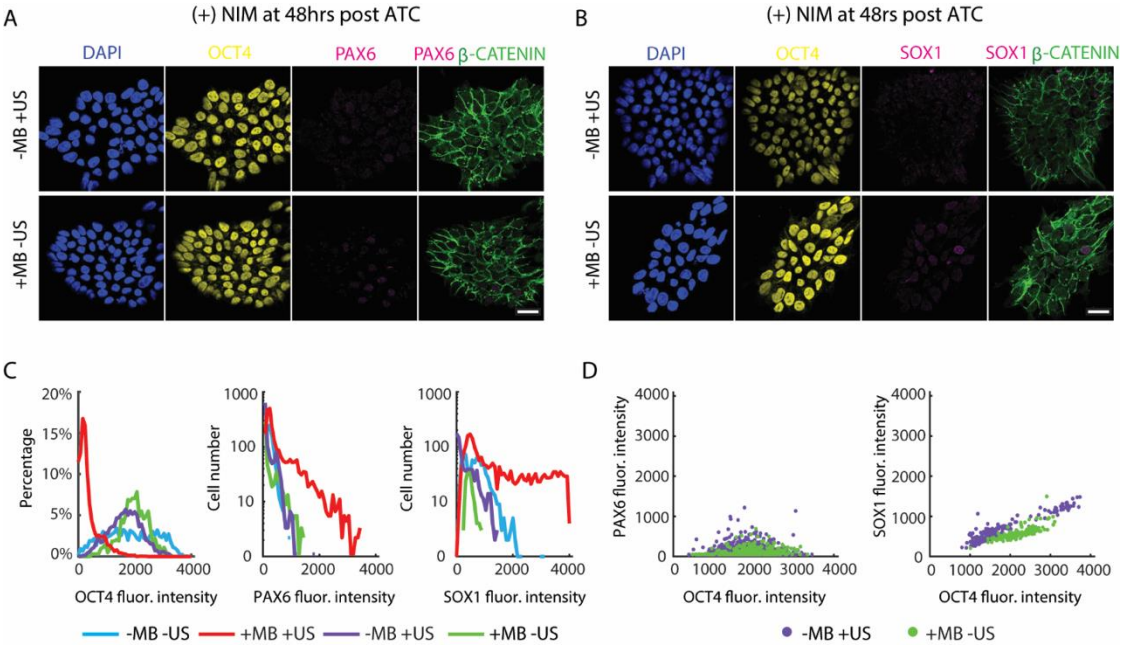

Figure S4. hESCs in control groups exhibited no changes in PAX6 and SOX1 without ATC (-MB+US+NIM, +MB-US+NIM) after 48 hour culture in neural induction medium (NIM). (A-B) Representative immunocytochemistry (ICC) fluorescence images of cells after application of ultrasound (US) pulses for 30 min and 48 hours culture in NIM. (C) Histograms of OCT4, PAX6, and SOX1 fluorescence intensity in four groups (-MB-US, +MB+US, -MB+US, +MB-US) resectively. A total of 1730 cells (-MB+US), and 632 cells (+MB-US) from 4 independent experiments were analyzed for OCT4. A total of 991 cells (-MB+US), and 493 cells (+MB-US) from 2 independent experiments were analyzed for PAX6. A total of 739 cells (-MB+US), and 139 cells (+MB-US) from 2 independent experiments were analyzed for SOX1. (D) Scatter plots of PAX6 vs OCT4, and SOX1 vs OCT4 from cells in the the control groups -MB+US and +MB-US respectively. Scale bar 50 µm.

Supplementary Movie S1: dynamic movement of integrin-bound microbubbles during ATC application.

Supplementary Table S1: Results of statistical analysis for ATC induced changes at 6 h after ATC application. Kruskal-Wallis followed by Dunn's test was performed to test the results for the 4 experimental groups (NIM+) for each marker. Mann-Whitney test was performed for the results in the 2 groups (NIM-) for each marker.

At 6hours			
4 groups with NIM+ (+MB +US; -MB +US; +MB –US; -MB –US)	OCT4 intensity	PAX6 intensity	SOX1 intensity
	P=0.249	**P<0.0001**	**P<0.0001**
+MB+US vs. –MB+US		P<0.0001	P<0.0001
+MB+US vs. –MB-US		P<0.0001	P<0.0001
+MB+US vs. +MB-US		P<0.0001	P<0.0001
-MB+US vs. +MB-US		P>0.9999	P>0.9999
-MB+US vs. –MB -US		P=0.3317	P=0.0907
+MB-US vs. –MB-US		P>0.9999	P>0.9999
2 groups with NIM- (+MB +US; -MB –US)	OCT4 intensity	PAX6 intensity	SOX1 intensity
	P=0.594	**P=0.058**	**P=0.806**

Supplementary Table S2: Results of statistical analysis for ATC induced changes at 48 h after ATC application. Kruskal-Wallis followed by Dunn's test was performed to test the results for the 4 experimental groups (NIM+) for each marker. Mann-Whitney test was performed for the results in the 2 groups (NIM-) for each marker.

At 48hours			
4 groups with NIM+ (+MB +US; -MB +US; +MB –US; -MB –US)	OCT4 intensity	PAX6 intensity	SOX1 intensity
	P<0.0001	**P<0.0001**	**P<0.0001**
+MB+US vs. –MB+US	P<0.0001	P<0.0001	P<0.0001
+MB+US vs. –MB-US	P<0.0001	P<0.0001	P<0.0001
+MB+US vs. +MB-US	P<0.0001	P<0.0001	P<0.0001
-MB+US vs. +MB-US	P=0.2187	P=0.1843	P>0.9999
-MB+US vs. –MB -US	P>0.9999	P=0.1405	P>0.9999
+MB-US vs. –MB-US	P=0.6174	P>0.9999	P>0.9999
2 groups with NIM- (+MB +US; -MB –US)	OCT4 intensity	PAX6 intensity	SOX1 intensity
	P<0.0001	**P=0.920**	**P=0.198**

Supplementary Table S3: Results of statistical analysis for ATC induced changes at 6, 14, and 48 h after ATC application observed in RT-qPCR analysis. One way ANOVA followed by Tukey's test was performed for each marker.

Gene expression- Fold Change- At 6hours					
4 groups with NIM+ (+MB +US -MB +US +MB –US -MB –US)	Nanog **P=0.0167**	Oct4 (**P=0.9492**) Sox2 **P=0.9447** Pax6 (**P=0.9677**) AP2A1 (**P=0.961**) AP2A2 **(P=0.4844)**	Sox1 **P=0.0191**	TUBB3 **P=0.0116**	Nestin **P<0.0001**
	Groups has difference (+MB+US vs. –MB-US -MB+US vs. +MB+US +MB-US vs. +MB+US)	No significant difference among groups	Groups has difference (+MB+US vs. –MB+US +MB+US vs. –MB-US +MB-US vs. +MB+US)	Groups has difference (+MB+US vs. –MB+US +MB+US vs. –MB-US +MB-US vs. +MB+US)	Groups has difference (+MB+US vs. –MB+US +MB+US vs. –MB-US +MB-US vs. +MB+US)

Gene expression- Fold Change- At 24 hours				
4 groups with NIM+ (+MB +US -MB +US +MB –US -MB –US)	Nanog **(P=0.0001)**	Oct4 (**P=0.777**) Sox2 **P=0.1237** AP2A1 (**P=0.0.6446**) AP2A2 (**P=0.6946)**	Pax6 **(P=0.0001)** Sox1 **(P=0.0003)** Nestin **P<0.0001**	TUBB3 **P<0.0001**
	Groups has difference (+MB+US vs. –MB-US -MB+US vs. +MB+US +MB-US vs. +MB+US)	No significant difference among groups	Groups has difference (+MB+US vs. –MB+US +MB+US vs. –MB-US +MB-US vs. +MB+US)	Groups has difference (-MB –US vs. +MB-US -MB –US vs. +MB +US -MB +US vs +MB-US -MB+US vs +MB+US +MB-US vs +MB+US)

Gene expression- Fold Change- At 48 hours				
4 groups with NIM+ (+MB +US -MB +US +MB –US -MB –US)	Nanog **P=0.0053**	Oct4 (**P=0.8201**) AP2A1 (**P=0.961**) AP2A2 (**P=0.4844)**	Sox2 **(P=0.0097)** Pax6 **(P=0.001)** Sox1 **(P=0.0063)** Nestin **(P=0.0005)**	TUBB3 **P=0.0022**
	Groups has difference (+MB+US vs. –MB-US -MB+US vs. +MB+US +MB-US vs. +MB+US)	No significant difference among groups	Groups has difference (+MB+US vs. –MB+US +MB+US vs. –MB-US +MB-US vs. +MB+US)	Groups has difference (–MB-US vs. –MB+US -MB-US vs. +MB+US -MB+US vs. +MB-US +MB-US vs. +MB+US)