NANO MICRO small

Supporting Information

for *Small,* DOI: 10.1002/smll.201803137

Acoustic Actuation of Integrin-Bound Microbubbles for Mechanical Phenotyping during Differentiation and Morphogenesis of Human Embryonic Stem Cells

Zhenzhen Fan, Xufeng Xue, Reshani Perera, Sajedeh Nasr Esfahani, Agata A. Exner, Jianping Fu, and Cheri X. Deng**

Supporting Information

Acoustic actuation of integrin-bound microbubbles for mechanical phenotyping during differentiation and morphogenesis of human embryonic stem cells

Zhenzhen Fan, Xufeng Xue, Reshani Perera, Sajedeh Nasr Esfahani, Agata A. Exner, Jianping Fu * and Cheri X. Deng*

Materials and methods

Cell culture

H9 human embryonic stem cell line (WiCell) was maintained using mTeSR1 medium (Stemcell Technologies) and lactate dehydrogenase-elevating virus (LDEV)-free hES cell qualified reduced growth factor basement membrane matrix Geltrex (Thermo Fisher Scientific) per manufacturers' instruction. The cell line was tested negative for mycoplasma contamination (LookOut Mycoplasma PCR Detection Kit, Sigma-Aldrich).

For differentiation assay, cells were seeded on glass bottom dishes (MatTek Corporation) coated with 1% (v/v) Geltrex solution as single cells at a density of 20,000 cells cm-1. ROCK inhibitor Y27632 (10 μM; Tocris) was added to prevent cell dissociation-induced apoptosis. On day 1 (after 24 h), cell culture medium was replenished with mTeSR1 medium without Y27632. To induce neuroepithelial cell differentiation, mTeSR1 medium was supplemented with TGF-β inhibitor SB 431542 (10 μM; Stemcell Technologies) and BMP4 inhibitor LDN 193189 (500 nM; Stemcell Technologies) and replenished daily. To form epiblast cysts, 3% (v/v) Geltrex was supplemented in mTeSR1 medium on day 1. To form amnion cysts, 3% (v/v) Geltrex was added in mTeSR medium 2 hours after cell seeding. Then, culture medium was replenished with mTeSR1 medium supplemented with 3% (v/v) Geltrex daily for both epiblast and amnion cyst formation.

Targeted microbubbles and attachment to cells

To formulate microbubbles, DPPC (1, 2-Dipalmitoyl-sn-Glycero-3-Phosphocholine), DPPE (1,2-Dipalmitoyl-sn-Glycero-3-Phosphoethanolamine), DPPA (1, 2-Dipalmitoyl-sn-Glycero-3-Phosphate), DSPE-PEG-biotin (1,2-distearoyl-phosphatidylethanol amine-methyl-poly ethylene glycol conjugate-2000 -biotin) (Avanti Polar Lipids, Inc.; Alabaster, AL) and mPEG-DSPE (Laysan Lipids, Arab, AL) were dissolved in chloroform in a 4:1:1:0.75:0.25 mass ratio. The solvent was then removed by evaporation, and the film was hydrated in PBS enriched with glycerol at 78°C for 30 min. Next, the vials were sealed, and air was replaced by octafluoropropane (C_3F_8). Bubbles were formed by mechanical agitation using the VialMix shaker (Bristol- Myers Squibb Medical Imaging, Inc., N. Billerica, MA). To separate MB, the content in the vial was transferred into 3ml syringe and centrifuged at 50g for 5 minutes. The lower potion was removed and upper foamy part was collected with 1 ml PBS. Bubble samples were stored at 4°C until use.

To form the RGD-microbubble complex, biotin-lipid microbubbles were first mixed with streptavidin solution (ThermoFisher, at concentration of 10mg/ml) for 20 min at room temperature, at a volume ratio of 20:1 to conjugate streptavidin onto microbubble shell. Then biotinylated Arg-Gly-Asp (RGD) peptides (Peptides International, at concentration of 2mg/ml) was mixed with the streptavidin linked microbubbles at the volume ratio of 2:21 and incubated for 20 min at room temperature to form RGD decorated microbubbles. At the end of the 20 min, bubbles flowed up near the surface of the solution. 2.5 µl was taken from the top layer of bubbles and mixed into 47.5 µl culture medium to form 50 µl of microbubble solution. To conjugate RGD-microbubbles to the cell surface, culture medium was removed, and then 50 µl of microbubble solution was added on the top of cells layer. Then the cell culture dish was flipped over and incubated for 10 min for the microbubbles floating up and conjugating with cells. After 10 min, the dish was flipped back, and the unbound microbubbles were gently washed away before experiment. The concentration of bubbles was

chosen to ensure that bubbles were sparsely distributed and a cell would have only one attached bubble, while many cells had no attached bubbles. This condition was chosen to avoid bubble-bubble interaction due to the secondary radiation force arising from the scattering of the incident ultrasound wave by a bubble. Thus we have maintained that bubbles were sparsely attached to cells in a monolayer and typically just one bubble for one cyst. The force on one bubble on a cell was predominately the primary acoustic radiation force.

Experimental setup and ultrasound system

As described before, the cell culture dish was plated on the stage of an inverted microscope (Nikon Eclipse Ti-U, Melville, NY). A single element planar transducer (center frequency 1.25 MHz, 7 mm diameter, Advanced Devices, Wakefield, MA) was mounted at a 45° degree facing down with its active surface submerged in the medium and 9 mm (Rayleigh distance) away from the cells. The 45° angle was used in order to avoid direct interference of incoming ultrasound wave with reflected waves inside the cell culture. 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), to generate ultrasound pulses with desired parameters. In this study, a single 50 ms ultrasound pulse with acoustic pressure in the range of 0.02 to 0.05 MPa was employed. Since cell stiffness was expected to be different for different cells at different stages, in order to obtain a similar level of bubble displacements to maintain similar signal to noise ratio in all measurements, a range of acoustic pressure was used.

The transducer was pre-characterized in free field using a computer-controlled 3D manipulation system with a 40 μm calibrated needle hydrophone (Precision Acoustics HPM04/1, UK). At its working distance (9 mm), a chart between input voltage set on the waveform generator and the output acoustic pressure was measured and generated (Supplementary Table 1). The -6 dB beam width of this transducer at 9 mm is 3.54 mm.

Before each experiment, the transducer was aligned with the field of view of the microscope using a small metal wire. During the alignment, the transducer was connected to a pulser/receiver. By monitoring the reflected acoustic signal from the small metal wire, the transducer was positioned at 9 mm (reflected signal was received at 12 μs) away from the bottom of the dish. Before each measure, an input voltage was set up in the waveform generator to generate desired acoustic pressure at 9mm, based on the calibration results (Supplementary Table 1). Similar ultrasound transducer calibration and alignment procedure was described in our previous publication[20].

High-speed videomicroscopy of microbubble dynamics and cell stiffness calculation

To capture microbubble dynamics driven by ultrasound pulses, a high-speed camera (Photron FASTCAM SA1, San Diego, CA) with a frame rate of 1000 frames/s was used. The movie was taken under a 40x objective to obtain enough resolution. And the recording was synchronized with the application of ultrasound pulse. To track microbubble position and radius change over time, a customized algorithm refined from a built-in circle-tracking algorithm in MATLAB (Math Works) was employed.

The primary acoustic radiation force is calculated using the following equation[16]:

$$F_P = \frac{2\pi {P_A}^2 D R_0}{\delta_{tot} \rho_0 c \omega_0 T}$$

where P_A is the acoustic pressure, R_0 is the bubble radius, δ_{tot} is the total damping constant ($\delta_{tot} = 0.16$), ρ_0 is the medium density (1000 kg/m^3), c is the sound speed in media (1500 m/s), $\omega_0 = 2\pi f_0$ and f_0 is the resonance frequency (1.25MHz). D is the duration of each pulse, and T is the pulse repetition period. Since we are calculating primary acoustic radiation force during ultrasound pulse, so D/T=1.

Effective cell stiffness is defined as the following, with the unit of nN/μm.

$$\text{Cell stiffness} = \frac{\text{Primary acoustic radiation force}}{\text{Max bubble displacement}}$$

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) for 30 min at room temperature and then permeabilized with 0.3% Triton X-100 (Sigma-Aldrich) for another 30 min. Then cells were incubated in 10% goat serum (Thermo fisher Scientific) for 1 h to block nonspecific binding, followed by primary antibody solutions and secondary antibody solutions (1:400) for 1 h, respectively. 4,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific) was used to counterstain cell nucleus. AlexaFluor 555 conjugated phalloidin (Invitrogen) was used to label actin microfilaments. All primary antibodies are listed in Supplementary Table 2.

Image analysis

To quantify OCT4 and TFAP2A fluorescence intensity, DAPI nuclear staining images were used to create binary masks and the total fluorescence signals of corresponding OCT4 or TFAP2A immunostaining images within the binary masks were quantified as nuclear intensity of OCT4 or TFAP2A. The fluorescence intensity of p-MYOSIN and ACTIN was quantified using the total fluorescence intensity of each cyst subtracted by background intensity.

Statistics

All experiments are repeated at least 3 times. In all cases, error bars denote SEM. Statistical analysis was conducted using independent, two-sided Student's t-test in Excel (Microsoft). $P < 0.05$ was considered statistically significant.

Supplementary Table 1. Transducer calibration result.

Input voltage at waveform generator (mV)	2.1	5.6	9.0	12.5	15.9	19.4	22.8	26.3	29.7
Output acoustic pressure at 9mm (MPa)	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.1

Supplementary Table 2. List of antibodies used in immunocytochemistry.

Protein	Vendor	Catalog number	Dilution
p-MYOSIN	Cell Signaling Technology	3671S	1:200
OCT4	Cell Signaling Technology	2840S	1:200
PAX6	Santa Cruz Biotechnology	sc-81649	1:100
TFAP2A	Santa Cruz Biotechnology	sc-12726	1:100

References

[20] Z. Fan, R. E. Kumon, J. Park, C. X. Deng, J. Control. Release **2010**, 142, 31.