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Development of *In Vitro* Model to Elucidate the Mechanism of Cancer Development by Mechano-stress Induced by Sonic Stimulation

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Cancers are considered to be affected by mechano-stress caused by sonic stimulation such as vibration and vocalization during their development and malignant transformation, but no device has been available to enable such an investigation. We have developed an *in vitro* cell culture model that can reproduce mechano-stress by sonic stimulation. The device is fabricated by micro-stereolithography and can reproduce the 3D shape of the target tissue with a modeling resolution of 50 μm. Cells are cultured on a 50-μm-thick poly-dimethylpolysiloxane (PDMS) sheet, and sonic stimulation is applied directly through a micro-speaker set on the bottom of the device. To verify the function of this device, the HepG2 cancer cell line was cultured while being stimulated with vibrations from the micro-speaker. The results showed a significant change in ACTa1 gene expression, which is a gene with variable expression in various types of cancer. There are currently no reports on ACTa1 gene expression increasing only by the vibration stimulation of cancer cell lines. This result is the first data showing that mechano-stress induced by sonic stimulation may be involved in changes in the malignant potential of cancer.

1. Introduction

Mechano-stress caused by sonic stimulation may affect the development and malignancy of some cancers. As an example, head and neck carcinoma has a high incidence among singers and other professionals who overuse or stimulate their vocal cords. Therefore, a causal relationship between cancer and mechano-stress caused by sonic stimulation has attracted attention. [(1)] However, how sonic stimulation affects carcinogenesis and its mechanism of action are not clear. This is due to the lack of *in vitro* analysis methods. *In vitro* experiments are essential because a large amount of experimental data is required to elucidate the mechanism of carcinogenesis.

Several devices have been developed to apply mechano-stress to cultured cells *in vitro*.[(2–5)] However, the frequency of mechano-stress generated by these devices is 0.5–1 Hz since the mechano-stress is applied to cultured cells by stretching the elastic culture apparatus with linear actuators. This is because the target tissues of these devices are the heart, lungs, and blood

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vessels, and so forth, which are not subject to high-frequency stimulation. Therefore, it is difficult to provide high-frequency stimulation of 100 Hz to 1 kHz, such as that of the human voice, with these devices. Therefore, it is impossible to apply these systems to elucidate the function of sonic stimulation in cancer malignant transformation.

In this study, we have succeeded in developing an *in vitro* cell culture model that can reproduce mechano-stress induced by sonic stimulation. The cell sheet layer is made of highly flexible poly-dimethylpolysiloxane (PDMS) thin film. Furthermore, the cell sheet is in direct contact with the micro-speaker at the bottom of the device through a 50-μm-thick PDMS membrane. Therefore, high-frequency vibration stimuli emitted from the micro-speaker can be fed directly to the cells. This structure enables the reproduction of mechano-stress mimicking sonic stimuli such as vocalization. In addition, this cell sheet is mounted on a shape-determining frame. This frame is fabricated by micro-stereolithography. Because this method is capable of fabrication at a resolution of 50 μm, it is possible to reproduce the target tissue shape. Therefore, by mounting the cell sheet on this frame, it is possible to deform the cell sheet into an arbitrary 3D shape. This function will be necessary to imitate the 3D shape of the target tissue in detail when the target tissue is determined. Moreover, the device is made of highly transparent materials [PDMS and cycloolefene polymer (COP)] in the cell-cultured and observation areas. Thus, cells can be observed directly under a microscope without disassembling the device.

To demonstrate the feasibility of the developed system, we performed culture experiments using the HepG2 cancer cell line. We cultured HepG2 cells for 12 h with 500 Hz sonic vibration, which is the frequency range of human vocalization. As a result, a significant decrease in the expression of the ACTa1 gene, one of the cytoskeletal genes whose abnormal expression has been reported in various cancer types, was observed.[6–11] These results are the first data showing that mechano-stresses induced by sonic stimulation, such as vocalization, may be involved in changes in cancer malignancy.

2. Materials and Methods

2.1 Culture device

Figure 1(a) shows the developed culture device used for prototyping. This device consists of five parts: (1) the top frame with the culture layer, (2) the PDMS frame for cell sheet fixation, (3) the cell sheet for cell culture, (4) the bottom frame for the determination of the shape of the cell sheet, and (5) the micro-speaker. The top and bottom frames were created by micro-stereolithography (Smapri Sonic 4K LL, Hotty Polymer). The resin used for the frames was KT-0823-BK (Hotty Polymer). The top frame is equipped with a flow channel for medium inflow, through which the medium flows during culture experiments [Fig. 1(b)]. The top surface is covered with a high-transparency COP film (ZF16-100, ZEON), allowing the direct observation of the cell sheet. Furthermore, a convex structure is mounted on the lower layer, which distorts the cell sheet. This enables the reproduction of the contours of the future targeted tissue. The cell sheet used in this device is shown in Fig. 1(c). The cell sheet is formed from a 3 × 3 cm^2, 50-μm-thick PDMS sheet. To prepare the sheet, a mold (outer: 3.5 × 3.5 cm^2, inner: 3 × 3 cm^2, 50 μm

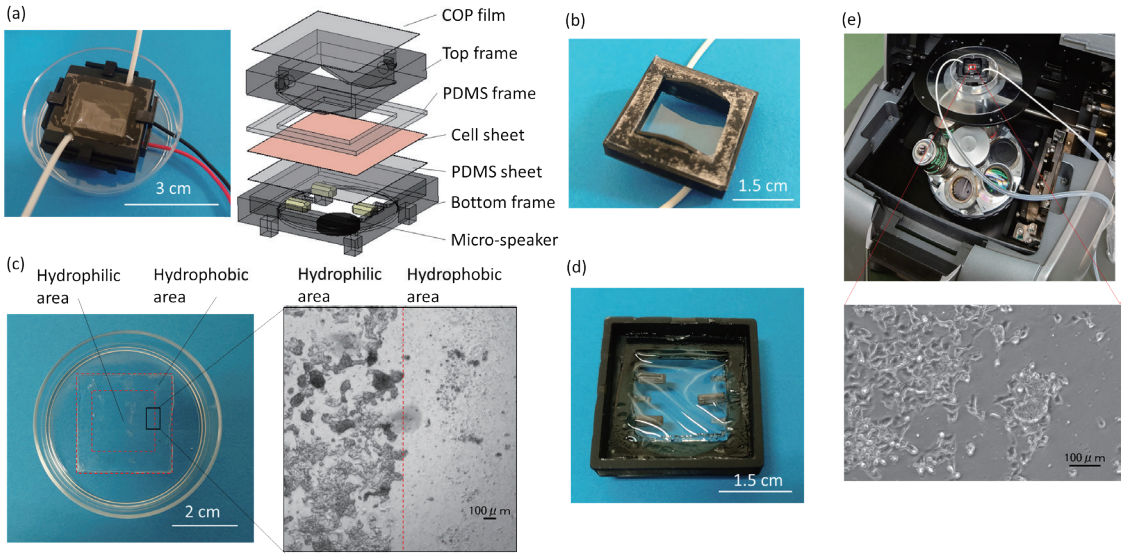

Fig. 1. (Color online) (a) Configuration of the developed device. (b) Construction of the top frame. (c) Construction of the cell sheet. (d) Construction of the bottom frame. (e) Device set on the microscope (upper) and microscopic image of the cultured cells (lower).

thick) fabricated by micro-stereolithography is placed on a glass slide (S9213, Matsunami), and PDMS (SYLGARD 184, Dow) is poured into the mold. The other glass slide is then inserted from the top and heated at 80 °C for 2 h to cure the PDMS in the shape of the mold. The cured PDMS sheet is removed, and a mask (outer shape 3 × 3 cm^2, inner shape 2.5 × 2.5 cm^2, thickness 100 μm) created by micro-stereolithography is placed on top of the sheet. The sheet surface is then irradiated with excimer UV (MDHD-1-150, M.D.COM Inc.) for 30 s to form a hydrophilic area on the PDMS sheet. This maintains the hydrophobicity of the area where the mask is placed, while the other areas become hydrophilic, allowing cell adhesion [Fig. 1(c)]. The hydrophobic part is the area where the PDMS frame is placed to fix the cell sheet. To prevent cells from entering this area, hydrophobicity is maintained by masking. The shape of the bottom frame is shown in Fig. 1(d). The bottom frame has several convex shapes, which allow the cell sheet to be deformed to reproduce the 3D shape of the target tissue. The deformation of the cell sheet by the top and bottom frames is observed using a laser microscope (LEXT 3000, Olympus). The bottom frame is equipped with a 50-μm-thick PDMS sheet, which is created using the same method described above, to prevent leakage of the culture medium. Furthermore, when the device is constructed, the center of the PDMS sheet, about 3 mm in diameter, is in direct contact with the micro-speaker placed at the bottom of the device. Therefore, vibration stimuli from the micro-speaker can be transmitted directly to the cell sheet through that contact point. The cell sheet and its upper and lower parts are all made of highly transparent materials (PDMS and COP) to directly observe the cells during the culture experiment [Fig. 1(e)].

2.2 Sonic vibration system

Sonic vibration stimulation is provided by a micro-speaker (MSI28-12R, SPL) placed at the bottom of the device. Sonic waves of 500 Hz input from a notebook PC (Vostro 14 3000, DELL) are amplified by a power amplifier (AE-386AMP-UNIV, Akizuki Denshi) and output from the micro-speaker to stimulate cultured cells on the device by sonic vibration. The amplitude and frequency of the cell sheet caused by sonic stimulation are measured using a laser displacement meter (LK-H020, Keyence).

2.3 Cell culture

The HepG2 cell line is obtained from the RIKEN BRC Cell Bank. The cells are cultured in Dulbecco's modified Eagle's medium (DMEM, Wako) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in a 5% CO_2 environment. The cultured cells are observed using a phase contrast microscope (BZ-9000, Keyence).

2.4 Sonic vibration experiment

Cell sheets prepared using the method described above are placed on a 6 cm dish, and 4 ml of 1.0×10^5 cells/ml HepG2 cell suspension is added and cultured for 4 days at 37 °C in a 5% CO_2 environment. After the complete adhesion of HepG2 cells on the cell sheet, the cell sheet is placed on the device to complete the device. Then, 1.5 ml of DMEM is added from the flow channel. The cells are cultured for 12 h at 37 °C in 5% CO_2 with sonic stimulation from the micro-speaker placed at the bottom of the device.

2.5 Gene expression analysis

After removing DMEM from the device, 2 ml of PBS(-) is added to wash the cultured cells. Then, 700 µl of Qiazol lysis buffer (QIAGEN) is added to obtain cell extracts. Total RNA is purified from the cell extracts according to the protocol of the miRNeasy Mini Spin Column Kit (QIAGEN). The concentration of purified total RNA is measured using a Qubit® 3.0 fluorometer (Life Technologies). Reverse transcription reactions are performed using 1 µg of purified total RNA. 50 ng/µl total RNA solution, 2.5 µM oligo dT_{20} primer (Invitrogen), 0.5 mM dNTPs (Invitrogen), 5 mM $MgCl_2$ solution (Invitrogen), 10× RT buffer (Invitrogen), 10 mM dithiothreitol (Invitrogen), 2 U/µl RNase inhibitor (Invitrogen), and 10 U/µl SuperScript III RT (Invitrogen) are mixed. The mixture is heated at 50 °C for 50 min and 85 °C for 5 min using a thermal cycler (T-100, BioRad). After the reaction, gene expression analysis is performed by real-time polymerase chain reaction (PCR). Analysis is performed for GAPDH, ACTB, and ACTa1 genes. Real-time PCR samples are prepared by mixing a 5 ng/µl post-reverse transcription reaction sample, 0.5 µM Fw primer, 0.5 µM Rev primer, and LuminoCt SYBR Green qPCR ReadyMix (Sigma). The Human Housekeeping Gene Primer Set (TaKaRa) is used

for GAPDH and ACTB. The primer sequence of ACTa1 used is shown in Table 1. Real-time PCR is performed on the real-time PCR samples by repeating 40 cycles of 95 °C for 10 s and 60 °C for 30 s using the Eco (illumina) real-time PCR system.

3. Results and Discussion

3.1 Cell sheet on device

The developed device deforms the cell sheet according to the shape of the top and bottom frames. This enables the reproduction of the shape and contours of the target tissue. However, when cells are seeded on the sheet after the device is constructed, it is likely that cell adhesion will be unequally distributed owing to the 3D deformation of the cell sheet. In this study, cell sheets were placed on a flat 6 cm dish, cell suspension was added, and cells were seeded on the cell sheets. Figure 2(a) shows the HepG2 cell line incubating for 24 h at the cell sheet. As shown in Fig. 2(a), this method succeeded in seeding cells uniformly on the sheet. A cell sheet was then placed on the device. This enabled the uniform seeding of cells on the cell sheet on the device, regardless of the shape of the cell sheet. Figure 2(b) shows the deformation of the cell sheet on the device. As shown in Fig. 2(b), the top frame formed a slope of about 10° from the bottom of the cell sheet. Additionally, the cell sheet was lifted by about 300 μm according to the convex structure of the bottom frame. Because this device is a prototype, the 3D shape formed by the frame is a simple structure. The resolution of the micro-stereolithography technology used in this study was less than 50 μm. In addition to using rigid resins (HS-830, Hotty Polymer), it is also possible to fabricate the frame using flexible resins. Additionally, by mixing rigid and flexible resins, it is also possible to mold resins with arbitrary elasticity. Thus, it is possible to reproduce the contour, shape, and elasticity of various tissues in detail.

3.2 Sonic vibration stimulation

The developed device can culture cells while applying mechano-stress using a micro-speaker. In this study, cells were cultured for 12 h while being subjected to 500 Hz sonics, which is the frequency range of human vocalizations. The amplitude of the cell sheets was measured using a laser displacement meter (Fig. 3). In this experiment, the amplitude was measured at the center of the cell sheets where the micro-speaker was in direct contact through the PDMS sheet. The cell sheets were subjected to vibration stimulation with an amplitude of approximately 60 μm and a frequency of 500 Hz. This experiment verified only the effect of sonic with a frequency of

Table 1
Sequences of primers used in this study.

Name	Sequence (5'–3')
ACTa1 Fw	AGGTCATCACCATCGGCAACGA
ACTa1 Rev	GCTGTTGTAGGTGGTCTCGTGA

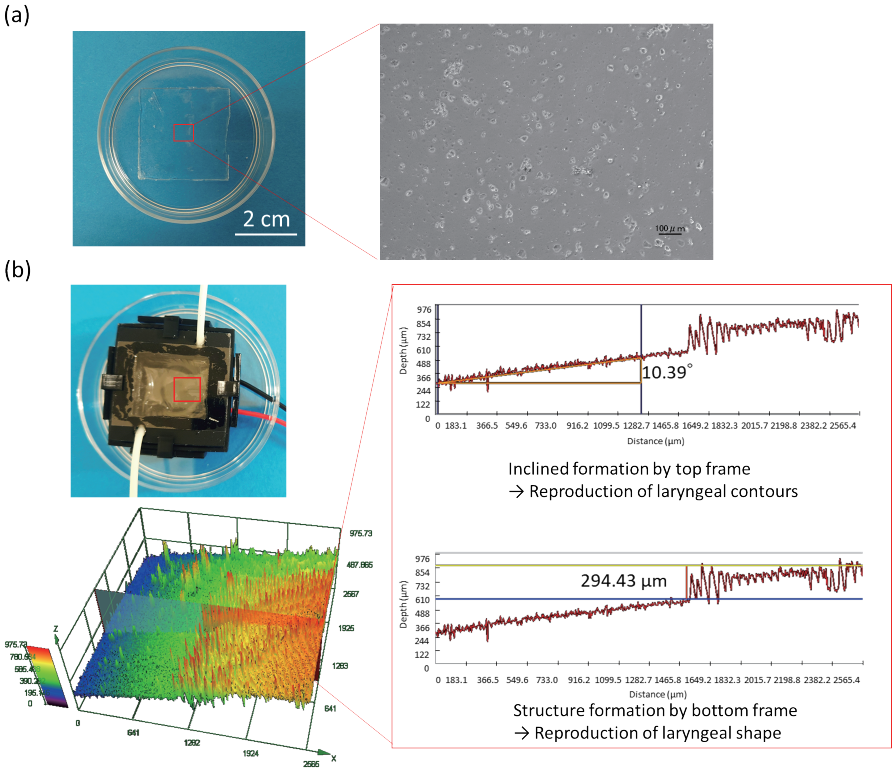

Fig. 2. (Color online) (a) Cell sheet at 24 h after seeding HepG2 cell line. (b) Deformation of the cell sheet.

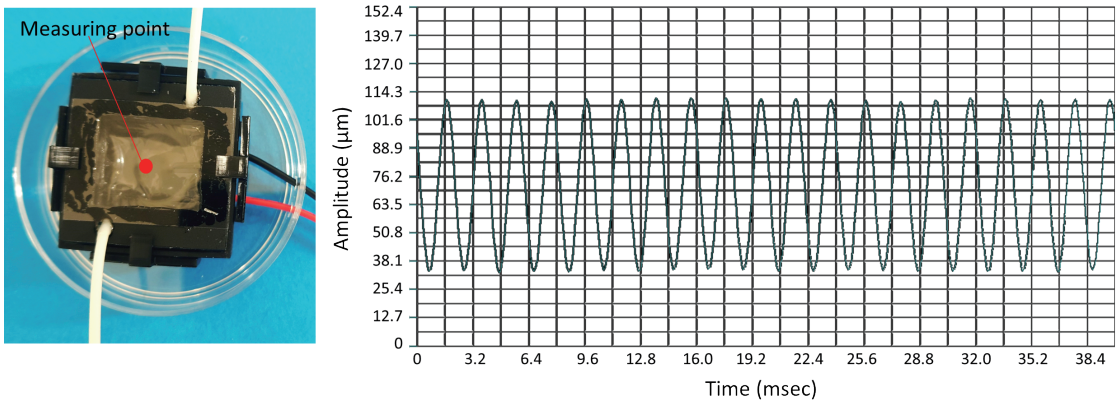

Fig. 3. (Color online) Amplitude of the cell sheets. This graph shows the distance between the surface of the cell sheet and the head of the laser displacement meter at the time of measurement.

500 Hz and did not verify the effects of sonics at other frequencies. Therefore, it is necessary to verify the effects of sonics at other frequencies. We plan to elucidate the effects of sonic frequency and intensity on cell culture.

3.3 Expression changes in cultured cells stimulated by sonic waves

The purpose of this study is to develop an *in vitro* model in which cells can be cultured while subjected to mechano-stress by sonic stimuli. We conducted an experiment to determine whether any expression changes occur in cultured cells while mechano-stress caused by sonic stimulation is applied. The cells were cultured for 12 h under the conditions described above while being stimulated with sonic vibrations by a micro-speaker. RNA was extracted from the cells after culture and analyzed for gene expression by quantitative real-time PCR (qRT-PCR). Recently, it has been reported that actin filaments function as mechanosensors of tensile force applied to cells and that the application of tensile force stabilizes actin filaments and promotes stress fiber aggregation.[12,13] Therefore, we selected the actins ACTB and ACTa1 as targets for expression analysis. The results are shown in Fig. 4, indicating the expression levels of genes in cells with vibration stimulation relative to those of genes in cells without vibration stimulation. GAPDH was used as the reference gene. The graph shows the results of qRT-PCR performed on each sample in triplicate, and error bars in the graph indicate standard deviation (SD). A *t*-test was performed between the sample groups with and without vibration stimulation. Interestingly, vibration stimulation did not significantly change the expression level of ACTB but significantly decreased that of ACTa1. The amount of decrease was smaller when the vibration amplitude was reduced by half. These results suggest that ACTa1 is suppressed by vibration stimulation at a wavelength of 500 Hz in an intensity-dependent manner. ACTa1 has variable expression in various cancers, including head and neck cancer.[6–11] In head and neck cancer, decreased ACTa1 expression has been reported to be associated with a more severe prognosis for cancer patients.[6,7] It has also been reported that in gastrointestinal cancers such as colon and pancreatic cancers, the suppression of ACTa1 expression increases the invasion and metastatic potential of

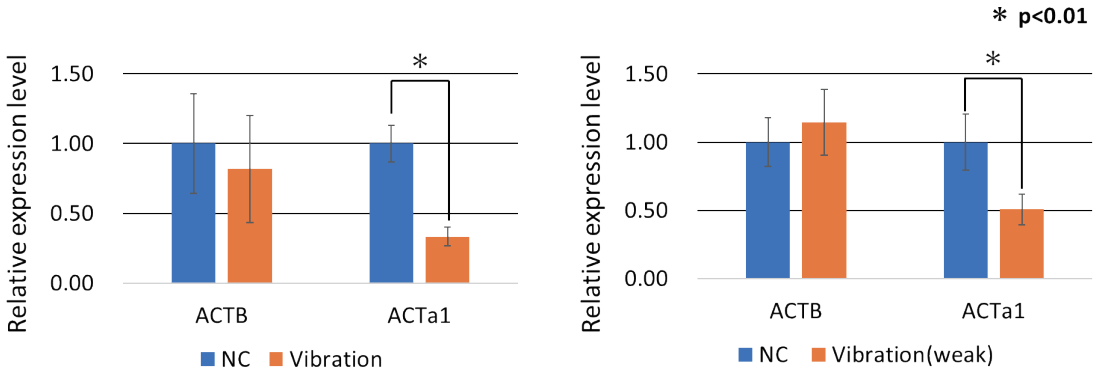

Fig. 4. (Color online) Result of the vibration stimulation experiment. These graphs show the gene expression level of the actin family ACTB and ACTa1. The right graph shows the experimental result with vibration stimulation at half intensity. The graph shows the results of qRT-PCR performed on each sample in triplicate, and error bars in the graph indicate SD. A t-test was performed between the sample groups with and without vibration stimulation. *p*-value less than 0.01 was considered to be statistically significant.

cancer.[8,9] Conversely, in prostate cancer, an increased expression of ACTa1 increases cancer metastatic potential,[10] and for oral squamous cell carcinoma, patients with high ACTa1 expression have a lower 5-year survival rate.[11] Changes in ACTa1 expression are closely related to carcinogenesis, but there are no reports of changes in ACTa1 expression in cancer cell lines induced by mechano-stress. Thus, these results are considered as the first to demonstrate the possibility of inducing changes in the expression of cancer-related genes and altering the degree of malignancy of cancer by simply applying sonic vibrational stimuli to cancer cells. However, this verification was performed only with 500 Hz vibration stimulation, and there was little variation in intensity. In addition, although this experiment performed a short incubation time of 12 h, gene expression variation should be investigated when longer incubations are performed. To investigate the relationship between the endogenous mechano-stress induced by sonic stimulation and cancer development in detail, it is necessary to perform stimulation experiments in a wide ranges of frequencies, intensities, and incubation time and investigate gene expression changes.

4. Conclusions

In this report, we developed an *in vitro* cell culture model that can reproduce mechano-stress induced by sonic stimulation to investigate the effects of sonic stimulation on cancer malignant transformation. The developed device reproduces the contour and 3D shape of the target tissue and enables cell culture while applying mechano-stress induced by sonic stimulation. By applying sonic waves from a micro-speaker set at the bottom of the device, the cell sheet can be vibrated at a high frequency of up to kHz. When the HepG2 cancer cell line was cultured on the device with vibration stimulation, a decreased expression of the ACTa1 gene was observed. This result indicates that the vibration stimulation of cancer cells affects their degree of malignancy. We are currently examining details of the signal transduction pathway of the decrease in ACTa1 and the malignant transformation of cancer caused by the vibration stimulation. We will develop an *in vitro* model that mimics not only the shape but also the tissue structure by further developing this device to enable the cultivation of not only monolayer cultures but also multilayer cell structures. This will enable us to develop a complete mimetic model of laryngeal tissue, which is especially affected by mechano-stresses such as vibration and vocalization, and to elucidate the developmental mechanism targeting laryngeal cancer.

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