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Design and Characterization of a Dynamic Vibrational Culture System

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Abstract

To engineer a functional vocal fold tissue, the mechanical environment of the native tissue needs to be emulated *in vitro*. We have created a dynamic culture system capable of generating vibratory stimulations at human phonation frequencies. The novel device is composed of a function generator, a power amplifier, an enclosed loudspeaker and a circumferentially-anchored silicone membrane. The vibration signals are translated to the membrane aerodynamically by the oscillating air pressure underneath. The vibration profiles detected on the membrane were symmetrical relative to the center of the membrane as well as the resting position over the range of frequencies (60–300 Hz) and amplitudes tested (1–30 μm). The oscillatory motion of the membrane gave rise to two orthogonal, in-plane strain components that are similar in magnitude (0.47%), and are strong functions of membrane thickness. Neonatal foreskin fibroblasts (NFFs) attached to the membrane were subjected to a 1-h vibration at 60, 110 and 300 Hz, with the displacement at the center of the membrane varying from 1 to 30 μm , followed by a 6-h rest. These regimens did not cause morphological changes to the cells. An increase in cell proliferation was detected when NFFs were driven into oscillation at 110 Hz with a normal displacement of 30 μm . qPCR results showed that the expression of genes encoding some extracellular matrix proteins was altered in response to changes in vibratory frequency and amplitude. The dynamic culture device provides a potentially useful *in vitro* platform for evaluating cellular responses to vibration.

Keywords

Vocal Folds; Bioreactor; Vibration; Frequency; Displacement; Fibroblasts; Gene Expression; Extracellular Matrices

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1. Introduction

The human vocal folds, composed of a pliable lamina propria (LP) sandwiched between a stratified squamous epithelial layer and the vocalis muscle (Gray; 2000), are particularly adapted to sound production, capable of vibrating at frequencies well above 100 Hz (Titze; 1994). The dynamic motion of the vocal folds not only is indispensable for producing high quality sound but also contributes, at least in part, to the development and maturation of vocal folds. It has been postulated that the development of the vocal folds is shaped by the tasks that are required (Hartnick *et al.*; 2005). Newborn vocal fold LP is structurally and compositionally uniform, consisting of only one layer rich in ground substances, with sparse, immature, fibrous components homogeneously distributed throughout the LP. As the vocal folds develop, the amount of the fibrous components increases gradually and the ground substance decreases proportionally, thereby slowly altering the microstructure of the vocal fold LP (Sato *et al.*; 2001). Mature lamina propria exhibit a distinct layered structure consisting of the superficial lamina propria (SLP), the intermediate lamina propria (ILP), and the deep lamina propria (DLP).

While proper mechanical stimulations prompt cellular differentiation and tissue development and maturation, excessive mechanical stresses, as in voice overuse and abuse, result in the alteration of the structure, composition and mechanical properties of vocal fold LP. Such alterations are manifested in the form of nodules and polyps (Gray *et al.*; 1995). The vocal fold LP can also be accidentally scarred by surgical procedures in the head and neck region. Compared to the normal tissue, the scarred vocal fold exhibits reduced sensitivity, increased stiffness and disrupted ultrastructure, giving rise to compromised voice quality (Hirano; 2005). Although temporary relief can be achieved by rest or voice therapy, long-term, satisfactory treatments for vocal fold scarring do not exist. More severe vocal fold diseases, such as vocal fold tumors, require drastic surgical procedures in order to restore minimum sound production. An estimated 3–9% of the population has some type of voice dysfunction (Branski *et al.*; 2007).

The development of a tissue engineering methodology for the reconstruction of the vocal fold will not only provide an *in vitro* platform for the investigation of vocal fold diseases but also offer alternative treatments for vocal fold disorders. Successful engineering of vocal fold lamina propria relies on the strategic combination of viable cells, biomimetic matrices, and physiologically relevant biomechanical stimuli. Previously, work from the current authors has been devoted to creating various synthetic matrices that are chemically diverse, structurally complex and mechanically robust (Grieshaber *et al.*; 2009; Jha *et al.*; 2009; Jha *et al.*; 2010; Jha *et al.*; 2011). These scaffolding materials are suitable for vocal fold tissue engineering purposes. It is well-known that extracellular matrix (ECM) composition and the corresponding physical properties of many tissues are affected by mechanical stimuli, and that different regimens of mechanical stimuli can result in changes in ECM production and remodeling (Ingber; 1991; Ingber; 2006). Thus, to further improve the outcome of *in vitro* tissue growth, it is critical to simulate the mechanical environment experienced by the vocal fold fibroblasts *in vivo*.

Several groups have reported various dynamic vibratory devices that are promising for vocal fold tissue engineering purposes (Titze *et al.*; 2004; Wolchok *et al.*; 2009; Kutty & Webb; 2010). These bioreactor systems utilized electromagnetic voice coil actuators for the generation of vibrational stimulus. The vibratory signals were transferred to the samples via moving bars (Titze *et al.*; 2004) or sample holders (Wolchok *et al.*; 2009). As a result, cells may be subjected to undesirable mechanical agitation and fluid perturbation, and the difficulty associated with overcoming the system's inertia limited the frequency range achieved. The primary goal of this study was to establish a dynamic culture system capable of generating vibrations at frequencies more relevant to human phonation without causing cell death. The bioreactor described in this study creates the vibration electromagnetically and transfers the energy aerodynamically, thus minimizing the number of moving components. The vibrations of the speaker cone induce pressure variations, which in turn creates an acoustic wave that is propagated through the air and translated to the silicone membranes in the T-75 flasks. The elastomeric nature of the silicone membrane ensures that the vibratory signals are transmitted to the attached cell monolayers. Laser Doppler Vibrometry (LDV) was used to systematically characterize the amplitude, frequency and modes of membrane motion in response to different driving signals. The strains imposed on the membrane were modeled using an incompressible Neo-Hookean constitutive model. Finally, neonatal foreskin fibroblasts (NFFs) plated on the flexible silicone membrane were subjected to various regimens of vibration. The effects of vibration on cell proliferation, cell morphology and the expression patterns of genes related to several important ECM molecules were systematically investigated.

2. Materials and methods

2.1. Bioreactor design

The dynamic culture system consists of a two-tiered acrylic stage (30 cm × 43 cm × 12 cm, W × L × H), 6 parallel, custom-modified T-75 cell culture flasks, a series of plastic tubes, a loudspeaker, a function generator and a power amplifier (Figure 1A). The loudspeaker (YD110-85 × 02, R: 8 Ω, Power: 30 W, Nanjing, China) is enclosed in a wooden box with two circular holes at the front and back ends. The front hole is connected to plastic tubing through a Y-shaped splitter, and the second hole on the back remains open for air influx. The speaker is driven by a function generator (Agilent 33220A, Agilent Technologies, Santa Clara CA) via a power amplifier (Pyle pro PT-2400, Brooklyn, NY). The vibrating air stream from the loudspeaker is equally divided by several three-way polypropylene connectors and is delivered to the modified T-75 flasks using rigid polyvinyl chloride (PVC) (medical grade, Tygon S-50-HL, Saint-Gobain, Akron, OH) hoses. Inside each T-75 flask are two pre-installed vibration tubes covered by collagen I-coated silicone membranes (Thickness: 0.020", SFM-C, Flexcell, Hillsborough, NC). The same PVC tube is used to effectively secure and seal the membrane, creating a small vibration chamber (12 mm inner diameter and 15 mm deep), where the cell culture medium is contained. The overall bioreactor design consists of 6 replaceable sterile T-75 culture flasks that are anchored on the stationary acrylic stage through side screws and built-in anchors. The entire assembly fits easily into a commercial incubator (37°C, 5% CO₂) (Figure 1B), while the electronic

components remain outside. The connecting tube is fed through the vent hole at the back/top of the incubator.

2.2. Mechanical testing

Tensile measurements were performed using a Rheometrics Mechanical Analyzer (RSA III, TA Instruments, New Castle, DE) at ambient temperature. Collagen-coated silicone membranes were cut into dumbbell-shaped samples according to the ASTM D412-06a standard. The initial grip separation was 12 mm and the stretching speed was 100 mm/min. Young's modulus (kPa) was calculated as the slope of the initial linear portion of the stress-strain curve, and the stress and strain at the breaking point were also recorded. The hysteresis was determined by calculating the area between the extension and retraction curves.

2.3. Bioreactor characterization

A Laser Doppler Vibrometer (LDV) was employed to characterize the vibration induced on the silicone membrane. In a typical single-point measurement, the laser was focused perpendicularly to the membrane so that the reflected beam was returned to the vibrometer (PDV-100, Polytec, Germany) and detected by the photo detector. Measurements were performed on the membrane with or without the cell culture medium at frequencies varying from 0 to 400 Hz and an output voltage up to 10 V. To map vibration across the membrane, the laser was positioned at normalized radii of 0, 1/2, 1/4, 3/4 and 1, and the corresponding displacement was measured. The results reported were an average of measurements from 4 membranes from two separate flasks. The overall three dimensional (3D) vibrational profiles of the silicone membrane were captured using a 3D Scanning Vibrometer (PSV-400, Polytec, Germany). The 3D vibrometer consists of 3 synchronized scanning laser heads oriented triangularly towards the membrane. Additionally, a reference laser was focused on a fixed point of the membrane and was used as the reference signal. Images were captured using a high speed digital camera placed perpendicularly to the membrane.

2.4. Cell culture

Primary human neonatal foreskin fibroblasts (NFFs) were purchased from ATCC (PCS-201-010, Manassas, VA) and cultured at 37°C with 5% CO₂ in fibroblast basal media (ATCC, PCS-201-030), supplemented with a fibroblast low-serum growth kit (ATCC, PCS-201-041) and 1% penicillin/streptomycin (Gibco, Carlsbad, CA). The modified T-75 flasks were sterilized under a germicidal UV lamp for 6 h before collagen I-coated silicone membranes were affixed on the vibration tubes. The bioreactor was assembled just before cell seeding. The trypsinized NFFs (passage 4–5) were seeded on the membranes at a density of 50 000 cells/cm² and were cultured under static conditions for 24 h before the initiation of the dynamic vibration. The NFFs were subjected to 1-h vibration at a pre-determined frequency and center displacement (Table 1), followed by a 6-h rest. Cells cultivated in an identical bioreactor housed in a separate incubator without any vibration were used as the static controls.

2.5. Cell morphology

At the end of the experiment, the medium was removed, and cells were rinsed two times with Dulbecco's Phosphate Buffer Saline (DPBS, Gibco) before being fixed in a paraformaldehyde solution (4% in DPBS, Electron Microscopy Sciences, Hatfield, PA) for 20 min. Cells were then washed two times with the washing buffer (0.05% Tween 20 in DPBS, Fisher, Pittsburgh, PA), permeabilized with 0.1% Triton X-100 (in DPBS, Fisher) for 5 min, rinsed twice with the washing buffer, blocked with 1% bovine serum albumin (BSA, in DPBS, Jackson ImmunoResearch, West Grove, PA) for 30 min and washed a final time with the washing buffer before staining. To visualize the cell morphology, the fixed cells were stained overnight with Alexa Fluor 488 Phalloidin (1:1000, Invitrogen, Carlsbad, CA) and Draq5TM (1:2000, Alexis Biochemicals, Axxora LLC, San Diego, CA). Shortly before imaging, silicone membranes containing the fixed and stained cells were carefully removed from the vibration chamber and mounted on microscope slides with Gel Mount (Biomed, Foster City, CA). Images were acquired using an LSM510 Axiovert confocal microscope (Zeiss, Thornwood, NY).

2.6. Metabolic activity

The metabolic activity of the cells before and after vibration was analyzed using Cell Titer Blue (Promega, Madison, WI). After a 24 h pre-culture and 1 h before vibration, 100 μ L of cell titer blue was added to the vibration chamber. One hour later, 100 μ L of the medium was aliquoted to a 96-well plate, and the solution fluorescence was recorded (excitation: 550 nm; emission: 590 nm) using a microplate reader (Perkin Elmer, Waltham, MA). The experiment was repeated 5 h after vibration so that the total rest time of 6 h was maintained. The metabolic activity ratio, defined as the fluorescent signal after the vibration divided by that before the vibration, was plotted with respect to the vibration conditions.

2.7. Gene expression analysis

Quantitative polymerase chain reaction (qPCR) was implemented to analyze the expression of ECM-related genes. Upon completion of the dynamic culture, cells were rinsed two times with DPBS and trypsinized with 100 μ L of trypsin (ATCC) for 2 min. After the addition of a trypsin neutralizing solution (100 μ L, ATCC), 600 μ L of DPBS was added and the cell suspensions were collected. Cells were collected after a 3-min centrifugation at 2,000 rpm. The cell pellets were re-dispersed in DPBS and centrifuged again at 3,000 rpm for 4 min. The excess liquid was aspirated and the cell pellets were stored at -80°C . The total RNA was extracted using a Qiagen RNeasy kit (Waltham, MA) following the manufacturer's instructions. The RNA concentration was measured using a ND-1000 spectrophotometer (Nanodrop, Nanodrop Technologies, Wilmington, DE). Two micrograms of RNA from each sample were purified with gDNA elimination and were reverse transcribed into cDNA using a QuantiTect Rev Transcription kit (Qiagen). Quantitative polymerase chain reaction was performed on an Applied Biosystems 7000 real-time PCR system using 25 μ L reaction volumes of SYBR green reactions (Applied Biosystems, Carlsbad, CA). Primers specific for glyceraldehydes-3-phosphate dehydrogenase (GAPDH), collagen type I (Col I), fibronectin (FN), matrix metalloproteinase 1 (MMP1), hyaluronan synthase 3 (HAS3), tissue inhibitor of metalloproteinase 1 (TIMP1), hyaluronidase 1 (HYAL 1) and CD44 are listed in Table 2.

GAPDH was used as the housekeeping gene, and the relative gene expression was calculated with respect to GAPDH and static controls, employing the $2^{(-C_t)}$ method. The validity of the primers was verified by running a traditional PCR on the sample cDNA. The PCR amplification was performed for 35 cycles using GoTaq Green master mix (Promega), and a MyGene Series Peltier thermal cycler (MG96G, Long Gene, Hangzhou, China). The cycle conditions were as follows: initial denaturation at 94°C for 3 min, denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were electrophoresed on 1.2% agarose gels with ethidium bromide (Invitrogen, Carlsbad, CA), and the images were captured with an Alpha Imager (Alpha Innotech Corporation, Santa Clara, CA).

2.8. Statistical analysis

Unless otherwise specified, quantitative data are the result of an average of at least three separate repeats, and the error bars are standard deviation of the mean (SEM). Statistical significance was analyzed using a two-tailed, equal variance Student's t-test, where a p-value of <0.05 was considered to be statistically different.

3. Results

3.1. Bioreactor characterization

The dynamic culture system is composed of an enclosed loudspeaker, a function generator, a power amplifier, parallel vibration chambers and a series of connectors and hoses. Two vibration tubes were introduced to each T-75 flask through the holes on the bottom of the flasks (Figure 1A). The silicone membrane covering the vibration tube is elastomeric, having an average Young's modulus of 1100 ± 80 kPa and a strain-to-break of $250 \pm 10\%$. Cyclic loading/unloading curves at a strain of 100% under uniaxial tension revealed a large hysteresis in the first cycle, followed by perfectly overlapping loading/unloading curves for subsequent cycles without any permanent deformation (data not shown).

3.1.1. Vibration measurements—LDV was employed to characterize the vibrations precisely without direct contact with the membrane using the Doppler effect in combination with a high precision interferometer. The single-point LDV measurements were carried out with the bioreactor assembly positioned in the same fashion as the dynamic cell culture experiments (Figure 1B) without the cells or the culture medium. When the refractive index of water was taken into consideration, LDV measurements conducted in the presence of cell culture medium did not deviate significantly from those obtained in the air (data not shown). The sinusoidal input signals from the function generator were captured by the membrane with high fidelity, as confirmed by the detected velocity profile (Figure 2A). At a driving frequency of 110 Hz and an output voltage (from the power amplifier) of 3 V, the center of the membrane moves longitudinally at a maximum velocity of +13.7 mm/s. The frequency spectrum, obtained from the fast Fourier transform of the time signals, reveals that the predominant signals are centered at the driving frequency of 110 Hz (Figure 2B–D). Harmonic signals at 220 and 330 Hz are also present, but their amplitudes are at least one order of magnitude lower than those at the fundamental frequencies. At the driving frequency, the normal displacement at the center of the membrane (w_0) was 19.7 μm ,

whereas the displacement at 220 and 330 Hz were 0.3 μm and 0.2 μm , respectively. A peak vibration acceleration of 9.43 m/s^2 (0.96 g) was detected at 110 Hz.

Figure 3A shows w_0 as a function of the output voltage and the driving frequency. At a given output voltage, as the excitation frequency varied from 0 to 400 Hz, w_0 went through several local maxima at 26, 110, 218 and 314 Hz. In general, there was an inverse relationship between the displacement and the peak frequency. As the output voltage increased, w_0 increased accordingly, but the positions of the resonance peaks remained unchanged. Similar vibrational pattern was detected on thinner silicone membranes (thickness: 0.005"). Although displacement maxima occurred at the same frequencies as the thick membranes, the respective normal displacement was much higher (data not shown). Taking into account the fundamental frequencies of children (300 Hz) and adults (125 Hz, adult male, females around 210 Hz) (Brown *et al.*; 1991), we chose to study the cellular responses to the vibrational stimulations at frequencies of 110 Hz and 300 Hz. The frequency range was further expanded to include 60 Hz so that a frequency-dependent cellular response, if any, can be extrapolated. At these frequencies, at a first approximation, w_0 is a linear function of the output voltage (Figure 3B). At any given output voltage, the normal displacement at 110 Hz is significantly larger than 60 and 300 Hz. Figure 3C shows the normalized displacement as a function of the position on the membrane. The highest displacement was detected at the center of the membrane, and the detected vibration amplitude decreased as the laser moved outwards towards the edge of the membrane. The normalized displacement at a normalized radius between 0 and 0.5 is greater than 0.8. In other words, the middle half of the membrane experiences the highest displacement that does not vary significantly with the distance from the center. On the other hand, there is a large variation in displacement at the outer half of the membrane, with the normalized displacement dropping from 0.81 at a normalized radius of 0.5 to 0.2 at the edge of the membrane. This graph also shows that the vibration profile of the membrane is independent of the applied frequency.

3D scanning LDV was employed to visualize the pattern of vibration across the entire membrane surface. 3D LDV permits simultaneous measurements of the membrane deformation at high frequencies using 3 sensor heads with a high spatial resolution, providing accurate results with the separation of out-of-plane and in-plane vector components. In order to accommodate the bulky 3D LDV components, the bioreactor had to be set up outside the incubator with a minimal number of connectors and tubes. Thus, results obtained from the 3D measurements are qualitative in nature. Figure 4 is a screen capture of the velocity profile in the z-direction across the surface of the silicone membrane that was driven into motion at 110 Hz and 8.5 V. The instantaneous velocity values are color-coded. Note that the pattern of vibration for two orthogonal lines (blue and red) across the membrane overlaps perfectly, confirming that symmetrical vibration centered at the middle of the membrane was generated. Commensurate with the single-point measurements, the highest velocity was detected at the center of the membrane, with the lowest one at the edge. Examination of the animated 3D membrane deflection in slow motion (Movie, Supporting Information) clearly shows that the vibration introduced to the membrane is highly

symmetrical, with the in-plane deformations much smaller in magnitude than the out-of-plane displacements.

3.1.2. Strain calculation—Strains generated during membrane vibration were modeled statically using an incompressible Neo-Hookean constitutive model with the deformation being symmetric relative to the center of the membrane, as proven experimentally. The total deformation occurred in two steps: a biaxial pre-strain (σ^*) applied prior to the cell seeding, and the deformation due to the applied air pressure (p) (Figure 5A). The cells seeded on the surface of the membrane experience only the strains due to the applied pressure. Therefore, the strains reported are the Lagrange strains due to the deformation caused by the pressure only. The total deformation of the membrane was calculated using Abaqus®, a finite element analysis software suite.

The total deformation is described by the deformation gradient $\mathbf{F}$, defined as

$$\mathbf{F} = \mathbf{I} + \nabla \mathbf{d} \quad (1)$$

where $\mathbf{I}$ is the identity tensor, $\mathbf{d}$ is the displacement vector, ∇ is the gradient operator, and boldface indicates a vector (lowercase letter) or a tensor (capital letter). The total deformation is composed of two steps, with the total deformation given as

$$\mathbf{F} = \mathbf{F}^{(2)} \mathbf{F}^{(1)} \quad (2)$$

where the superscripts (1) and (2) denote the biaxial pre-strain and the applied pressure, respectively. The Lagrange strain tensor ($\mathbf{E}$) is defined as

$$\mathbf{E} = \frac{1}{2} (\mathbf{F}^T \mathbf{F} - \mathbf{I}) \quad (3)$$

To compute the strain due to the applied pressure only, equation (3) is applied using $\mathbf{F}^{(2)}$, the deformation gradient due to the applied pressure only.

The membrane was modeled as an axisymmetric body subjected to the loading shown in Figure 5A. Briefly, the deformation was calculated in two steps, just as the actual deformation was performed. Dimensionless variables were used throughout the calculation. All lengths were normalized with respect to the radius (6.71 mm), while forces were normalized with respect to the shear modulus of the silicone membrane. Prior to deformation the membrane was 0.0757 thick and had a length of 0.89286. A biaxial stretch of $\lambda = 1.12$ was imposed by applying a uniform radial traction σ^* at the outer edge of the membrane. The second deformation consisted of a uniform normal pressure applied to induce the desired displacement at the mid-plane of the center ($r=0$) of the membrane. The traction that induced the biaxial pre-strain was retained for this step. Both normal and radial displacements were constrained to be zero at the bottom outer corner of the membrane.

The membrane was modeled as a continuum with axially symmetric, 8 noded, reduced integration elements (element CAX8R in Abaqus®). The complete membrane used 8

elements through the thickness, with 89 along the radial direction, with an aspect ratio of 0.94 prior to deformation. The strain energy density for a Neo-Hookean material has the

form $U = C_{10} \left(\bar{I}_1 - 3 \right) + \frac{1}{D_1} (J - 1)^2$, where U is the strain energy density, C_{10} and D_1 are moduli; $\bar{I}_1$ is the first invariant of the deviatoric part of the left Cauchy-Green deformation tensor (i.e. $\left(\mathbf{B} - \frac{1}{3} \text{trace}(\mathbf{B}) \mathbf{I} \right)$ where $\mathbf{B} = \mathbf{F}\mathbf{F}^T$) and J is the Jacobian (i.e. the determinant of the deformation gradient tensor $\mathbf{F}$). Because the loading condition is one of imposing a prescribed displacement at the center of the membrane, the value of the modulus C_{10} (twice the shear modulus) can be chosen arbitrarily (unity was used). The applied pressure required to obtain the intended central displacement can then be interpreted as representing the ratio of the applied pressure to the modulus C_{10} . The modulus D_1 was taken to be 1×10^{-9} to model the material as essentially incompressible. The applied dimensionless radial traction σ^* was 1.2378 while the dimensionless pressure required to deform the membrane to 30 μm was 1.34×10^{-3} .

The strains were calculated directly from the deformation gradients that were output from Abaqus®. The radial dependence of the in-plane strain components (E_{rr} and $E_{\theta\theta}$) for a center membrane displacement of 30 μm at the top, mid-plane, and bottom of the membrane are shown in Figure 5B. Here, ‘top’ refers to the surface on which the cells are seeded, and ‘bottom’ refers to the surface on which the air pressure is applied. At the center of the membrane, the in-plane strains are equal, as expected. They vary slowly with radial distance, and monotonically through the thickness. At the center of the membrane, where the majority of the cells are attached (see below), the strains have a value of 4.7×10^{-4} . Although a non-linear analysis and constitutive model were used, the strains are essentially proportional to the center displacements over the range of center displacements used in the experiments. The large strains and changes in strains at the outer edge of the specimen are due to the approximations involved in the boundary conditions applied there. These approximations do not affect the solution far from the edge, where the cells are attached to the membrane.

3.2. Dynamic cell culture

In the current investigation, NFFs were used in place of primary vocal fold fibroblasts (VFFs) because undamaged and disease-free vocal folds from live donors are virtually impossible to obtain. This difficulty is further compounded by the relatively short replicative life span of VFFs (Chen & Thibeault; 2009). The extensive phenotypic and functional overlaps between these two types of fibroblasts (Bae *et al.*; 2009) motivates us to explore the use of vibrational stimulations to coax NFFs into producing vocal fold like ECM. The bioreactor provides a biomimetic, *in vitro* platform for assessing the effects of physiologically relevant, biomechanical cues on the morphology, phenotype, proliferation, and expression of matrix-related proteins by NFFs.

3.2.1. Cell morphology and metabolic activity—Figure 6 shows the morphology of NFFs cultured under static and dynamic culture conditions. In general, cells preferentially clustered in the middle area of the membrane. Taking into account the weak function of displacement with respect to radius within the middle half of the membrane (Figure 3C), it is

reasonable to assume the majority of the cells on the membrane are subjected to vibrations with a normalized displacement of 0.8–1. NFFs readily attach and spread on the collagen-coated silicone membrane, adopting a spindle-shaped morphology, both under static (Figure 6A) and dynamic conditions (Figure 6, B–D). NFFs cultured at 300Hz/1 μ m exhibited a more stellate morphology with smaller overall cell body (Figure 6D) as compared to cells cultured under other conditions. With or without vibration, cells attached to the silicone membrane had developed distinct stress fibers traversing the entire cell body. No preferential cell alignment was observed under the conditions investigated. Membrane relaxation prior to imaging inevitably subjected cells to compressive forces that may have altered the appearance of the cells and stress fibers. Figure 7 shows that NFFs cultured at 110 Hz and 30 μ m were metabolically more active than cells cultured under static or other dynamic conditions.

3.2.2. Gene expression—Using qPCR, we assayed the effects of vibratory stresses on the relative expression of seven ECM components: collagen type I (Col I), fibronectin (FN), MMP1, TIMP1, CD44, hyaluronidase 1 (HYAL1), and HA synthase 3 (HAS3) (Table 2). The specificity of the PCR primers was confirmed by melting curves (data not shown) and PCR reactions (Figure S1) which show the single band at the predicted size of the DNA band for each gene product.

In initial gene expression experiments, w_0 was 1 μ m and the driving frequency was varied. qPCR results are shown in Figure 8A. NFFs cultured at 60 Hz exhibited significantly ($p<0.05$) higher expression of Col I than those cultured at 300 Hz. Stimulation at 110 Hz did not induce statistically significant changes in Col I expression relative to the static control. Vibration at 60 Hz led to down-regulation of the mRNA level of MMP1 ($p<0.05$). TIMP1 expression was decreased by 30% at 300 Hz ($p<0.05$) compared to static culture. Next, w_0 was varied while stimulation frequency was maintained at 60 Hz (below the phonation frequency, Figure 8B). Col I expression at 1 μ m was significantly higher (1.28 fold, $p<0.05$) than that at 10 μ m. A displacement of 5 μ m enhanced the expression of HYAL1 significantly relative to 1 μ m displacement. Similar experiments were carried out at a vibration frequency of 110 Hz (Figure 8C). Vibration at 110 Hz/30 μ m led to a >20% ($p<0.05$) reduction in the mRNA level of Col I relative to the static controls. The same vibration condition caused a 10% ($p<0.05$) reduction in gene expression of MMP1. Cells expressed a higher level of CD44 at 30 μ m ($p<0.05$) than at 1 μ m.

4. Discussion

Vocal folds vibrate actively and regularly at frequencies well above 100 Hz at strains up to 30%. As such, it differs from other connective tissue in terms of structure, composition and mechanical properties in order to facilitate wave propagation during normal phonation, while at the same time sustaining large mechanical deformation and resisting high impact collision. Differences in the magnitudes of forces in the superficial and deeper layers of the lamina propria are believed to induce the expression of different genes in the different layers (Sato & Hirano; 1995), giving rise to a unique gradient structure across the thickness of the LP.

We have designed and fabricated a dynamic culture system that is capable of delivering high frequency vibrations to the cultured cells. The vibrations were generated aerodynamically by an enclosed loud speaker that propagates a sinusoidal pressure wave. The alternating air pressure, in turn, drove an elastomeric silicone membrane into motion. The culture system can accommodate six T-75 flasks at one time, each containing two parallel vibration chambers. The vibration waveform can be delivered to 12 samples simultaneously without any cross-flask or cross-chamber perturbations. The vibration profiles induced on the silicone membranes are highly axisymmetric, and the displacement profile is independent of the excitation frequencies, with the normal displacement decreasing monotonically from the middle to the edge. Consequently, the strain profile is the same regardless of the driving frequency and depends only on the center displacement w_0 . The linear relationship between w_0 and the output voltage, as well as the direct correlation between w_0 and the driving frequency permit facile adjustment of the dynamic culture conditions imposed on the cultured cells. The in-plane normal strain that the cells experience remains fairly constant across the membrane. At a normal center displacement of 30 μm , the maximum strain that occurs, excluding edge effects, is the strain at the center, 0.047%.

NFFs cultivated on the oscillating silicone membrane maintained their fibroblastic morphology and oriented randomly across the membrane. The lack of preferential cell alignment implies that NFFs do not perceive any directionality in stress; thus they are unable to reorganize their cytoskeleton to orient the cell body along the direction of minimal stress (Benhardt & Cosgriff-Hernandez; 2009; Dan *et al.*; 2010). The vibrations applied did not induce any physiological trauma to the cells as evidenced by their uncompromised metabolic activities as compared to the static cultures. Most intriguingly, a considerable increase in cell number was observed after 1 h vibration at 110 Hz with a w_0 of 30 μm . At other frequencies investigated, cell proliferation was inhibited, but the overall cell number did not decrease. This effect appears to be limited to the early stages where ECM accumulation is at a minimum and the cells are more proliferative than synthetic.

To gain a better understanding of the role of vibrational stimulations in the maintaining of tissue homeostasis, we determined the gene expression of important ECM-related proteins. Col I and HA are two of the major structural components of VF LP. While collagen fibers provide tensile strength to the vocal fold LP (Ishii *et al.*; 1996; Gray; 2000), HA contributes to the maintenance of optimal tissue viscosity and stiffness (Chan *et al.*; 2001). Col I is the most differentially expressed gene under the dynamic culture conditions and the expression level is strongly dependent on the frequency and amplitude. In general, lower frequency and lower amplitude promote higher Col I expression. A one-hour vibration at 110 Hz with a normal center displacement of 30 μm led to >20% reduction in Col I mRNA level compared to the static culture. On the other hand, the mRNA levels for CD44, a transmembrane receptor of HA, and HYAL1, an enzyme responsible for HA degradation, could be differentially modulated by varying the vibration amplitude at a frequency of 110 Hz and 60 Hz, respectively. Consequently, CD44 and HYAL1 could possibly indirectly modulate HA production in the culture. Collectively, the ability to modulate the gene expression of Col I and HA related proteins underscores the role of high frequency vibration in defining the tissue structure and viscoelasticity.

The ECM structure and composition is controlled not only by the protein synthesis but also by its degradation. MMPs are secreted as a latent complex, which must be activated following secretion for proteolytic activity and can be inactivated by TIMPs. MMP and TIMP work synergistically to regulate matrix degradation and remodeling, thus tissue homeostasis (Clark *et al.*; 2008). Our results show that their activities can also be modulated by vibration stimulations. MMP1 and TIMP1 are more sensitive to frequency changes than amplitude differences. At a w_0 of 1 μm , vibration at 300 Hz resulted in a statistically lower TIMP1 expression (close to 30% decrease), while 60 Hz led to a slight decrease of MMP1 expression.

When anchored on the elastomeric silicone membrane, NFFs established well-defined stress fibers so that they are mechanically compliant to the substrate on which they are cultured (Engler *et al.*; 2006; Buxboim *et al.*; 2010). Under such conditions, NFFs experience the same levels of shear stresses as the top plane of the membrane itself. The shear forces introduced to the cell membrane could cause changes in intracellular pressure. Further, the acceleration induced by vibration can be perceived by the cells (Takeuchi *et al.*; 2006). These factors combined lead to conformational changes of transmembrane proteins, such as mechanosensitive ion channels or integrin receptors (Wang *et al.*; 1993). Finally, mechanical loading modulates ECM protein production by releasing growth factors in an autocrine or paracrine manner (Skutek *et al.*; 2001; Gupta & Grande-Allen; 2006). Future study is required to ascertain which pathways are involved in order to obtain a better understanding of vibration-induced mechanotransduction.

Several groups have explored the effects of high frequency vibratory stimulations on cell functions. Tanaka *et al.* (Tanaka *et al.*; 2003) revealed that osteoblasts, entrapped in collagen gels, are more sensitive to low amplitude, broad frequency strain, and that this kind of strain could sensitize osteoblasts to high amplitude, low frequency strain. Desmoulin *et al.* subjected bovine caudal intervertebral discs to free axial vibration for 10 to 60 minutes at 0 to 0.5 g and 0 to 200 Hz. They discovered that the expression of extracellular matrix genes was significantly upregulated at high amplitudes (>0.4 g) in as little as 10 minutes. Titze *et al.* (Titze *et al.*; 2004) showed that 20% axial strain and vibration at 100 Hz for 6 hours resulted in a significant increase in fibronectin, MMP-1, HA synthase 2, and CD44 (with respect to static control) in tracheal fibroblasts cultured within 3D, porous, elastomeric Tecoflex substrates. In a recent study, Webb and co-workers (Kutty & Webb; 2010) investigated the effects of physiologically relevant vibratory stimulations on gene expression and protein production by fibroblasts encapsulated within photocrosslinkable HA hydrogels. Their results show that vibrational stimulations significantly increased the mRNA levels of HA synthase 2, decorin, fibromodulin and MMP-1, but did not lead to significant changes in collagen and elastin expressions. It is generally agreed that cells are dynamic entities, and their structures and functions can change in response not only to the mechanical load but also to the substrate stiffness. A direct comparison of our data with results accumulated from these studies is not possible due to the differences in the mode, amplitude, frequency and duration of vibration, as well as the types of cells and the substrates on which the cells were cultured.

This study represents our initial effort in validating the bioreactor and quantifying the cellular responses. Cells were cultured as a monolayer without preferential alignment on a membrane that is stiffer than the native vocal fold tissue (Min *et al.*; 1995). Cells were subjected to a small amplitude vibration for a short period of time and the stimulatory effects observed were at the transcriptional level. We acknowledge that the dynamic culture device reported in this study has several limitations. First, the degree of pre-strain introduced to the silicone membrane during bioreactor assembly cannot be readily adjusted. Second, the vibration stage cannot be accommodated to a standard microscope for *in situ* visualization purposes. Third, the level of strain imposed on the cells was 2 orders of magnitude lower than that experienced by vocal fold fibroblasts in the native tissue. Finally, the current geometry does not permit the introduction of collision that plays an important role in vocal fold biomechanics. We are currently designing a more adaptable, modular device that overcomes the aforementioned limitations. Future studies will concern the effect of vibratory stimulations on cells cultured in 3D, biomimetic matrices that exhibit mechanical properties similar to that of the vocal fold tissue.

5. Conclusions

A dynamic culture system capable of non-destructively imposing vibratory stimulations to cells at human phonation frequencies was constructed and characterized. The oscillating air pressure induced by the loudspeaker was transmitted to an elastomeric membrane with high fidelity. The vibration pattern generated on the membrane is highly axisymmetric and reproducible, independent of the driving frequency. The normal displacement is variable across the membrane, while the in-plane strains are relatively constant over approximately central 76% of the membrane. At the tested frequencies (60–300 Hz), there is a linear correlation between the normal displacement and the driving voltage. NFFs subjected to various vibrations remained attached to the membrane and. Certain vibrations were conducive to cell proliferation while others led to differential expression of genes related to ECM proteins. Our results underscore the importance of mechanical environment in modulating cellular function. While the bioreactor cannot replicate completely all the complexities of the native vocal folds, it does provide a platform that permits examination of the influence of substrate vibration on the cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A): Schematic of the aerodynamically-driven vocal fold bioreactor. 1: T-75 flask; 2: vibration tube; 3: anchoring tube; 4: washer; 5: PDMS membrane; 6: culture medium; 7: acoustic pump; 8: power amplifier; 9: function generator; 10: connecting tube; 11: acrylic stage. (B): A photograph showing 6 parallel vibration devices assembled inside an incubator. Insert shows the vibration chamber filled with the cell culture medium and the PDMS membrane being secured by a piece of tygon tubing.

Figure 2.

Vibration characteristics of the PDMS membrane at driving frequency of 110 Hz and an output voltage of 3 V. Data were collected using a single-point LDV with the laser pointing at the center of the membrane. (A): Velocity profile as a function of time; (B): Velocity vs frequency; (C): Acceleration vs frequency, (D): Normal displacement vs frequency.

Figure 3.

Characterization of membrane vibration using a single-point LDV. (A): Frequency scan at a fixed output voltage; (B): Voltage scan at a fixed applied frequency. The normal displacement was measured by positioning the laser at the center of the membrane. (C): Normal displacement as a function of the position on the membrane.

Figure 4. Characterization of membrane vibration using 3D LDV. The PDMS membrane was driven into vibration at 110 Hz and 8.5 V. (A): Screen capture of the z-velocity profile; (B): Screen shot showing two orthogonal lines (red and blue) on the membrane having a perfectly overlapping velocity profile.

Figure 5.

(A): Schematic of the membrane model used in Abaqus® showing the presence of biaxial pre-stretch (σ^*) prior to the applied air pressure (a) and the presence of biaxial stretch and air pressure (p, b). (B): In-plane strain profiles for 30 μ m center displacement. ‘Top Surface’ refers to the surface on which the cells are seeded, while ‘Bottom Surface’ refers to the surface to which the air pressure is applied.

Figure 6.
Effects of dynamic vibration on cell morphology, as revealed by F-actin/Draq5 staining.
(A): static control, (B–D): dynamic culture at 60 Hz/10 μm (B), 110 Hz/30 μm (C) and 300 Hz/1 μm (D).

Figure 7. Cellular metabolic activity assessed by cell titer blue. The metabolic activity ratio is defined as the absorbance after vibration divided by that before the vibration. The results reported are the average of three repeats $\pm$ SEM.

Figure 8.

Effects of vibrational stimulations on the gene expression of dynamically cultured NFFs. The relative gene expression was normalized to GAPDH and the static controls, employing the $2^{(-Ct)}$ method. (A): Fold change with respect to frequency variations at a constant center displacement of 1 μm; (B) Fold change with respect to the change in center displacement at a frequency of 60 Hz; (C) Fold change with respect to center displacement at a constant frequency of 110 Hz. *, #: $p < 0.05$

Table 1

Dynamic culture conditions employed.

$f(\text{Hz})$	$w_0 (\mu\text{m})$			
	1	5	10	30
60	x	x	x	
110	x		x	x
300	x			

Table 2

Specific primers used for Real-Time PCR

Gene	GenBank #	Forward Primer	Reverse Primer	Product Size (bp)
Col I	NM000089	AACAAATAAGCCATCACGCCTGCC	TGAAACAGACTGGGCCAATGTCCA	101
FN	NM002026	ACCTACGGATGACTCGTGCTTTGA	CAAAGCCTAAGCACTGGCACAACA	116
MMP1	NM002421	GGGAGATCATCGGGACAACCTC	GGGCCTGGTTGAAAAGCAT	72
CD44	NM001001392	TGCCGCTTTGCAGGTGTAT	GGCCTCCGTCCGAGAGA	66
TIMP1	NG012533	TTTCTTGGTTCCCCAGAATG	CAGAGCTGCAGAGCAACAAG	99
HYAL1	NM000088	TAACCCTGCCAGTTTCTCCATCCA	AGCCAGGGTAGCATCGACATTGA	125
HAS3	NM005329	TGTGCAGTGTATTAGTGGGCCCTT	TTGGAGCGCGCGGTATACTTAGTT	177
GAPDH	BC023632	GAAATCCCATCACCATCTTCCAGG	GAGCCCCAGCCTTCTCCATG	120