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Vibration Stimulates Vocal Mucosa-like Matrix Expression by Hydrogel-encapsulated Fibroblasts

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Abstract

The composition and organization of the vocal fold extracellular matrix (ECM) provide the viscoelastic mechanical properties that are required to sustain high frequency vibration during voice production. Although vocal injury and pathology are known to produce alterations in matrix physiology, the mechanisms responsible for the development and maintenance of vocal fold ECM are poorly understood. The objective of this study was to investigate the effect of physiologically-relevant vibratory stimulation on ECM gene expression and synthesis by fibroblasts encapsulated within hyaluronic acid hydrogels that approximate the viscoelastic properties of vocal mucosa. Relative to static controls, samples exposed to vibration exhibited significant increases in mRNA expression levels of HA synthase 2, decorin, fibromodulin, and MMP-1, while collagen and elastin expression were relatively unchanged. Expression levels exhibited a temporal response, with maximum increases observed after 3 and 5 days of vibratory stimulation and significant downregulation observed at 10 days. Quantitative assays of matrix accumulation confirmed significant increases in sulfated glycosaminoglycans and significant decreases in collagen after 5 and 10 days of vibratory culture relative to static controls. Cellular remodeling and hydrogel viscosity were affected by vibratory stimulation and were influenced by varying the encapsulated cell density. These results indicate that vibration is a critical epigenetic factor regulating vocal fold ECM and suggest that rapid restoration of the phonatory microenvironment may provide a basis for reducing vocal scarring, restoring native matrix composition, and improving vocal quality.

Introduction

Voice is critical to quality of life and the primary professional tool for 25% of the US work force (teachers, singers, sales personnel etc) (Williams, 2003). Voice production (phonation) is a coordinated biological process involving the vocal folds, lungs, laryngeal muscles, and upper respiratory tract. The dynamic mechanical element of the phonatory system, the vocal folds transform aerodynamic energy from exhalation to acoustic energy through high frequency vibration. The human vocal folds consist of a squamous epithelium and lamina propria comprised primarily of fibroblasts and extracellular matrix (ECM) that are anchored to the thyroaretenoid muscle. The lamina propria has a heterogeneous, spatially organized ECM that is fundamental to the tissue biomechanics and in turn vocal quality (Hirano, 1981; Gray et al., 1999). Together, the epithelium and the superficial layer of the lamina propria form the *vocal mucosa*, the intermediate and deep layers of the lamina propria comprise the *vocal ligament*. Hyaluronic acid (HA) and proteoglycans (decorin, fibromodulin, and versican) in the mucosal matrix provide appropriate tissue viscosity for initiation and propagation of mucosal waves at frequencies ranging from 100-300 Hz during normal

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phonation (Pawlak et al., 1996; Butler et al., 2001; Chan et al., 2001; Hahn et al., 2005). The vocal ligament contains elastin and collagen fibers that provide flexibility and resistance to tensile stresses (Hammond et al., 1997; Gray et al., 2000; Hahn et al., 2006; Hahn et al., 2006).

The primary vibratory component of the vocal folds, the mucosa is the most common site of vocal injury and pathology. Damage to the mucosal layer may result from chronic vocal misuse, mechanical trauma, chemical or thermal injury, and surgical removal of cancerous or benign lesions. The subsequent wound healing response is characterized by conversion of the glycosaminoglycan (GAG)-based matrix to fibrous scar tissue (Benninger et al., 1996; Branski et al., 2005). These alterations in matrix composition are accompanied by increased tissue stiffness and viscosity (Thibeault et al., 2002; Rousseau et al., 2004), leading to dysphonia characterized by reductions in amplitude or absence of the mucosal wave, increased phonation threshold pressure, and increased effort to sustain phonation (Benninger et al., 1996; Rousseau et al., 2004). Scarring-induced dysphonia is currently treated by voice therapy and surgical augmentation procedures. Although effective in improving short term voice quality, these approaches have not consistently restored normal voice due to their inability to bring about regeneration of the native vocal fold tissue architecture / ECM composition.

The ECM composition and biomechanical properties of many human tissues are regulated by their mechanical environment during both developmental morphogenesis and adult homeostasis (Grodzinsky, 1983; Wang and Thampatty, 2006). Using 3D culture systems and bioreactors, physiologically relevant mechanical stimulation has been shown to elicit tissue-specific ECM expression and increases in corresponding mechanical properties relative to static controls. Examples include application of cyclic strain to smooth muscle cells (Kim et al., 1999) and fibroblasts (Webb et al., 2006) and dynamic compression / hydrostatic pressure to chondrocytes (Kim et al., 1994; Mauck et al., 2000) and annulus fibrosus cells (Gokorsch et al., 2005; Reza and Nicoll, 2008). While the effects of cyclic strain / compression have been widely investigated, the response of cells to vibratory stimulation has been relatively less explored. Tanaka and coworkers reported increased expression of osteocalcin and MMP-9 by osteoblasts cultured in collagen gels subjected to various vibratory regimes relative to static controls (Tanaka et al., 2003). Titze and coworkers observed significant increases in mRNA expression of proteoglycan / glycosaminoglycan (GAG) genes in tracheal fibroblasts cultured under vibratory stimulation within 3D, porous, elastomeric Tecoflex substrates (Titze et al., 2004).

Our long term hypothesis is that rapid restoration of the vibratory microenvironment using mechanomimetic scaffolds will facilitate vocal mucosa-specific matrix deposition by transplanted or endogenous cells, and will ultimately lead to improved functional outcomes by reducing fibrotic scarring during the acute stage of wound healing. Towards this end, we recently reported the preparation of injectable hyaluronic acid (GMHA) based hydrogels with rheological properties that approximate native human vocal mucosa (Kutty and Webb, 2008). However under static conditions, fibroblasts encapsulated within GMHA hydrogels synthesized a matrix rich in both collagen and s-GAGs. The objective of this study was to investigate the ability of high frequency vibration to stimulate vocal mucosa-specific gene expression and matrix accumulation by human fibroblasts encapsulated in mechanomimetic GMHA hydrogels.

Materials & Methods

Materials

Sodium hyaluronate (HA) ($MW = 1.4 \times 10^6$) was purchased from Genzyme (Cambridge, MA). Polyethylene glycol diacrylate (PEGDA) ($MW = 258$) and tetrabutylammonium bromide, triethylamine, glycidyl methacrylate, 4-hydroxyproline, chloramines-T, and p-dimethyl amino benzaldehyde were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dichloromethane (HPLC grade) was obtained from Acros organics (NJ, USA). Acetone was purchased from Fisher Chemical (Fair Lawn, NJ). 2-hydroxy-1-[4-(hydroxyethoxy) phenyl]-2-methyl-1-propanone (I-2959) was obtained from Ciba Specialty Chemicals (Basel, Switzerland). A 10% w/v stock solution of I-2959 was prepared in 70% ethanol. Adult normal human dermal fibroblasts (NHDF, CC-2511) obtained from a 39-year-old female were purchased from Biowhitaker (Rockland, ME, USA). DMEM F-12 50/50, 1X with L-glutamine, 15mM HEPES / trypsin EDTA (0.05% trypsin/0.53mM EDTA in HBSS), Dulbecco's Phosphate-Buffered Saline with calcium and magnesium (PBS), and penicillin / streptomycin stock solution were obtained from Mediatech (Herdon, VA, USA). Bovine growth serum was purchased from Hyclone (Logan, UT, USA).

Synthesis of methacrylated HA (GMHA)

Methacrylated HA (GMHA) was synthesized by reacting sodium hyaluronate (HA) solubilized in double distilled water with a 20-fold molar excess of glycidyl methacrylate in the presence of excess triethylamine and tetrabutylammonium bromide as previously described (Leach, 2003). GMHA was precipitated in acetone, dialyzed against double distilled water for 48 hrs, and recovered by lyophilization. An ~8% degree of methacrylation was calculated from the $^1\text{H-NMR}$ (D_2O) spectra based on the ratio of peak integrals derived from the methacrylate and HA carbohydrate protons, as previously described (Nettles et al., 2004).

Cell culture

Adult normal human dermal fibroblasts (NHDF) were routinely cultured in 175 cm^2 T-flasks using DMEM F-12 50/50, 1X with L-glutamine and 15mM HEPES enriched with 10% v/v bovine growth serum and 50 U/ml penicillin and 50 $\mu\text{g/ml}$ streptomycin. Culture medium was changed once every two days and cells were passaged weekly. All experiments were performed with cells from the 4th-6th passages. For hydrogel encapsulation, monolayers of fibroblasts (ca. 95% confluence) were trypsinized, centrifuged, resuspended, counted in a hemacytometer, and adjusted to a final concentration of 1.6×10^7 cells/ml.

Bioreactor construction

A vibratory bioreactor module comprising a modified T-75 tissue culture flask and a voice coil actuator (BEI Kimco) was constructed partly as explained previously (Figure 1A) (Titze et al., 2004). A sinusoidal waveform from a function generator (BK Precision, CA) was fed to the input terminal of a reed switch. The reed switch was connected at the collector terminal (+5V from a PC) of a NPN transistor, the base terminal of which was connected to the output from a custom Labview program (National Instruments) and an analog output board running on a PC. The variables controlled by the Labview software were the total number of work days, active vibration intervals (*on* and *off* times) and inactive (rest) time per day. The output from the reed switch stimulated the voice coil which ultimately controlled the vibration actuator bar. The voltage level of the sinusoidal wave was set so as to maintain 1mm vibratory amplitude at the actuator bar which was verified using a digital stroboscope before every experiment.

Bioreactor cell culture

Tecoflex film fabrication—The cell encapsulated hydrogel samples were crosslinked to elastomeric Tecoflex films mounted on “U” shaped plastic frames (40mm × 15mm). Tecoflex SG-80A (Thermedics) was dissolved in methylene chloride (5% w/v) and 11 ml added to 100 mm diameter glass dishes to form thin films by solvent casting. After overnight evaporation, residual solvent was removed by heating at 60 °C under vacuum for 48 hours. The Tecoflex films were subjected to oxygen plasma treatment for 8 mins at 300 mTorr to generate radicals on the film surface (Suzuki, 1986) and immediately immersed in PEGDA (MW = 258) for 30 mins at room temperature, with the expectation that limited graft copolymerization (Chang, 2008) would introduce free vinyl groups on the film surface. The films were rinsed extensively with water and then sterilized by immersion in 70% ethanol for 30 mins, rinsed three times with sterile water, and briefly air dried prior to gel photopolymerization. Although optimization and characterization of the Tecoflex surface modification process was not extensively explored, GMHA hydrogels polymerized in contact with unmodified films delaminated within minutes of immersion in media, while gels on treated films remained stably adherent throughout the 10 day time period of these studies.

Hydrogel photopolymerization and vibratory culture—4% w/v GMHA macromer solution was prepared in sterile PBS. A fibronectin-derived cell adhesion peptide, (GRGDS, Bachem, PA, USA) was conjugated to acrylate-PEG-NHS (MW=3400, Nektar) as previously described (Hern and Hubbell, 1998). GRGDS-PEG-acrylate was prepared as a stock solution in PBS (10 μmol/ml) and sterile filtered. For cell encapsulation studies, the final concentrations of GMHA, acrylate-PEG-GRGDS, and NHDF were 2 %, 2.5 μmol/ml, and 4×10^6 cells/ml, respectively, unless noted otherwise. Cell-macromer-peptide suspensions (200μl) containing 0.1% w/v I-2959 initiator were crosslinked on modified Tecoflex films by exposure low intensity UV illumination (365 nm, 10 mW / cm², Black-Ray B100-AP, Upland, CA) for 6 ½ minutes (Figure 1B). N = 4 hydrogel samples were mounted between the support pins in the bioreactor flask and the frames cut to allow the samples to move freely in the axial and longitudinal directions. The hydrogel samples were allowed to equilibrate overnight before the vibratory stimulus was applied. The samples were then stimulated by a 100Hz, 5.3Vrms sinusoidal waveform from a function generator in a 2sec on / 2sec off regimen for 4hrs / day. N = 4 fibroblast encapsulated hydrogel samples were cultured in a Petri dish under static conditions as controls.

Encapsulated fibroblast viability, morphology, and proliferation within the 2% w/v GMHA hydrogels

Viability assessment—After 5 and 10 days in culture, hydrogel samples (N = 4 per time point) from both the vibratory and static groups were collected, rinsed three times with sterile PBS, and stained with 1 μM fluorescein diacetate (FDA, Molecular Probes, Eugene, OR) and 2.5 μM propidium iodide (PI, Molecular Probes). The samples were once again rinsed with sterile PBS, visualized, imaged by fluorescence microscopy (Zeiss Axiovert 200), and the number of live and dead cells manually counted to calculate percent viability.

Confocal analysis of morphology—After 1, 3, 5, and 10 days in culture, samples (N = 4 per time point) from both the vibratory and static groups were fixed in 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100, and stained with Alexa 594-phalloidin (Molecular Probes). Samples were visualized and imaged using a Zeiss Confocal LSM510 microscope.

Fibroblast proliferation—N = 4 samples each from the vibratory and static groups were collected after 1, 5, and 10 days in culture, rinsed twice with sterile PBS, snap-frozen in an

ethanol-dry ice bath, and stored at -80°C . DNA solubilization and quantitation were based on modifications of previously described methods (West et al., 1985). Briefly, the samples were subjected to 3 freeze-thaw cycles in 1.4 ml of 10mM EDTA, pH 12.3. Once the hydrogels were completely dissolved, the pH was neutralized by the addition of 100 μl 1M potassium phosphate (monobasic) solution. Total DNA content was measured using the Picogreen DNA-binding dye (Molecular Probes) and a fluorescence microplate reader (Tecan GENios, excitation: 485nm, emission: 535nm). DNA content was converted to cell number based on a DNA standard curve prepared from serial dilutions of NHDF and compared to the cell number at the end of 1 day.

Gene expression

The hydrogel-encapsulated fibroblast samples ($N = 4$ samples per time point) from both the vibratory and static groups were harvested from culture after 1, 3, 5, and 10 days and stored in 1ml Trizol reagent (Aldrich) at -80°C . The frozen hydrogel samples were thawed, homogenized, centrifuged at 13,000g for 15mins at 4°C , and the supernatant removed and collected in RNase-free tubes. Total RNA was isolated according to the manufacturer's protocol and finally redissolved in 20 μl RNase-free water. The samples were treated with RNase-free DNase I (Turbo, Ambion) to eliminate genomic DNA contamination. Total RNA concentration and purity were determined by UV spectrophotometry. Sample volumes containing 1 μg of total RNA were reverse transcribed to cDNA in 20 μl reactions using the Retroscript cDNA Synthesis Kit (Ambion) and the Rotorgene 3000 light thermal cycler (Corbett Research, Mortlake, NSW, Australia). Realtime PCR was performed using custom-designed sense and antisense primers (Table I) and the QuantiTect SyBr Green PCR kit (Qiagen). Briefly, after an initial heat activation at 95°C for 15 minutes, cDNA products were amplified through 35 cycles (denaturation- 94°C -15s, annealing- 54°C -20s, extension- 72°C ,-20 s). Melt curve analysis was performed at the end of all reactions to verify formation of single products. Amplification efficiencies were tested using serial dilutions of cDNA covering four orders of magnitude and found to range from 0.94-1 for all products. Relative expression levels of target genes were quantified by the $\Delta\Delta\text{C}_\text{T}$ method using $\beta 2$ microglobulin ($\beta 2\text{M}$) as an internal control and 1 day static samples as reference condition (calibrator) (Livak and Schmittgen, 2001). Mean $\beta 2\text{M}$ Ct values did not significantly vary among experimental conditions or time points. Controls in which either cDNA template or the RT enzyme was not included were routinely performed to ensure the absence of cDNA and genomic DNA contamination, respectively. Data for 3 and 5 day groups represent the combined results of two independent experimental replicates.

Encapsulated fibroblast ECM production

To quantify ECM production by the encapsulated fibroblasts, $N = 4$ samples / time point from both the vibratory and static groups were collected from the study after 5 and 10 days of culture. The samples were digested using 1ml hyaluronidase (40U/ml) per hydrogel under constant agitation for 4hrs. Following digestion, the samples were transferred to 2 ml tubes, and stored at -80°C .

Sulfated GAG synthesis (DMMB Assay)—Sulfated GAG (s-GAG) accumulation was measured using the 1,9-dimethylmethylene blue (DMMB) assay as previously described (Yang et al., 2005). Briefly, 20 μl of sample was added to 200 μl of the DMMB reagent stock solution (16 mg DMMB+5 ml 95% ethanol+3 ml formic acid+25.6 ml NaOH+966.4ml distilled water, pH 3.5) in a 96-well plate. DMMB dye binding to s-GAGs was measured by absorbance at 525 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT) and was quantified relative to a linear standard curve prepared from chondroitin-4-sulfate standards (0-100 $\mu\text{g}/\text{ml}$). The absorbance of cell-free 2% w/v GMHA hydrogels

(background) was subtracted from the absorbance values of vibratory / static groups. For data analysis, s-GAG values were normalized to total DNA values.

Collagen synthesis (hydroxyproline analysis)—Collagen accumulation by the encapsulated fibroblasts in both the vibratory and static groups was quantified by the hydroxyproline assay as previously described (Kothapalli, 2009). Briefly, hyaluronidase-digested gels were homogenized, centrifuged (10,000g, 10 min), and digested with 1 ml 0.1 N NaOH for 1 hour at 98 °C. After centrifugation, the supernatant was mixed with an equal volume of 12 N HCl, hydrolyzed (110 °C, 16 hours), dried with N₂ gas overnight, and reconstituted in 20 µl sample volumes. Standards (4-hydroxyproline, 0-5 µg/ml) and samples (20 µl) were mixed with 250 µl chloramine T solution (1.41g chloramine-T in 10 ml distilled water, 10 ml n-propanol, and 80 ml OH-Pro buffer (50 g citric acid, 120 g sodium acetate, 24 g NaOH, dissolved in 1.2 L distilled water containing 12 ml glacial acetic acid and 300 ml n-propanol, pH 6). Samples were mixed with 250 µl of p-dimethyl amino benzaldehyde solution (15 g in 60 ml n-propanol and 26 ml 70% v/v perchloric acid), heated to 60 °C for 15 min, and the absorbance measured at 558 nm. Total collagen content was calculated based on the estimation that hydroxyproline makes up about 13.2% of the total collagen. Total collagen values were normalized to total DNA values.

Rheological characterization

To investigate the effect of the vibratory regimen and neo matrix synthesis on the mechanical properties of the 2% w/v GMHA hydrogels, two cell-loaded hydrogel groups (4×10⁶cells/ml and 2×10⁶cells/ml) and acellular hydrogels were photopolymerized and exposed to vibratory stimulation for 10 days. The hydrogel samples (vibratory and static controls) from both the cell-loaded and acellular experiments were collected after 10 days in culture, rinsed two times with sterile PBS, and immediately subjected to rheological testing under conditions similar to those used to investigate the rheological properties of human vocal mucosa (Chan and Titze, 1999). Rheological characteristics as a function of mechanical stimulus and presence / absence of cells were investigated in a stress controlled 'ARES' Rheometer using parallel plate geometry. The hydrogel samples were placed in the gap (0.8mm) between the stationary upper plate and the rotating lower plate, and the temperature was maintained at 37±0.1°C by a Peltier water cooling system. In order to determine the region of linearity, strain sweep tests were performed at fixed oscillation frequency (1Hz). Rheological tests were performed in the linear strain region (strain amplitude ~0.01rad), over a frequency range of 0.01 – 15Hz, covering 25 frequencies over 3 decades. The dynamic viscosities of the 2% w/v GMHA hydrogels tested were calculated by dividing the observed values of the loss moduli by the corresponding angular frequencies tested.

Statistical analysis

All data sets are presented as mean ± standard deviation. All data sets were statistically compared by ANOVA using Tukey's method for post hoc testing with P<0.05 considered significant.

Results

Encapsulated fibroblast viability, morphology, and proliferation in 2% w/v GMHA hydrogels

Fibroblasts were encapsulated within 2% w/v GMHA hydrogels containing immobilized RGD cell adhesion peptides by photopolymerization and cultured under static or vibratory conditions for up to 10 days. Encapsulated fibroblast viability was significantly greater in the static controls after 5 and 10 days in culture than in the corresponding vibratory samples

(Figures 2). Cell viability was uniform throughout the bulk of the hydrogel constructs. Fibroblasts in the static controls exhibited increased clustering and aggregation in various areas throughout the hydrogel constructs relative to the vibratory samples at the same time points (data not shown). Confocal microscopy was performed to visualize fibroblast morphology within the 3D hydrogel networks. Encapsulated fibroblasts exhibited spreading within 3 days (data not shown) and development of stellate morphology at 5 days in the static controls (Figure 3A) and the vibratory samples (Figure 3B). Stellate cell morphology persisted for the entire duration (10 days) of the experiments in the static (Figure 3C) and the vibratory samples (Figure 3D). The cell number (DNA content) of the hydrogel constructs at 5 and 10 days is shown in Figure 4. Relative to the initial cell number measured at 1 day, significant increases in cell number were observed in 5 day static controls and 10 day static and vibratory groups. Although cell proliferation was observed in the 10 day vibratory group (relative to 1 day), cell numbers measured in vibratory samples were significantly lower than corresponding static controls at both time points assayed.

Gene expression

Fibroblast mRNA expression levels of several ECM-related genes relevant to human vocal mucosa were examined after 1, 3, 5, and 10 days culture under vibratory and static conditions. In general, fibroblasts exposed to vibratory stimulation exhibited increased expression of HA Synthase2 (Figure 5A), decorin (Figure 5B), fibromodulin (Figure 5C), and MMP-1 (Figure 5D) relative to static controls for corresponding time points at 1, 3, and 5 days. Within sample groups exposed to vibration, significant temporal increases in HA synthase 2 (3 and 5 days), fibromodulin (3 day), and MMP-1 (5 day) were observed relative to the 1 day time point. Interestingly, expression levels of all 4 genes in vibratory sample groups were significantly reduced at 10 days relative to 3 and 5 days. The dramatic increases observed in decorin and fibromodulin expression are at least partially attributable to the extremely low baseline expression levels of the static 1 day reference condition. In contrast, expression levels of type I collagen and elastin were relatively insensitive to vibratory stimulation. Relative to static controls at all time points, collagen and elastin expression levels were reduced in samples exposed to vibratory stimulation, although the differences were significant only at the 5 day time point (Figure 5E and F).

Matrix expression

Consistent with the significant upregulation of proteoglycan gene expression at 1, 3, and 5 days, sGAG accumulation (normalized to cell number) in the hydrogel constructs at 5 and 10 days was significantly higher in the vibratory groups than in the static controls (Figure 6). Also, sGAG levels in the vibratory sample groups significantly increased between 5 and 10 days. Total collagen accumulation (normalized to cell number) measured in the hydrogel constructs at 5 and 10 days was significantly lower in the vibratory group relative to the static controls (Figure 7). In addition, total collagen levels measured in the vibratory groups significantly decreased between 5 and 10 days.

Rheological characterization of acellular and cell-loaded 2% w/v GMHA hydrogels

For acellular hydrogels maintained under static and vibratory conditions, dynamic viscosities calculated over the range of tested frequencies (0.01-15Hz) were almost identical (Figure 8). Cell-loaded hydrogels maintained under vibratory culture conditions exhibited significantly decreased dynamic viscosity at all frequencies tested relative to static controls (Figure 9). However, when the encapsulated cell number was reduced by 50%, the dynamic viscosity of static controls and vibratory samples was approximately equal after 10 days in culture (Figure 10).

Discussion

In contrast to the complex layered organization and variable matrix composition observed in the adult, newborn vocal folds are homogeneous structures (Hirano, 1983; Hirano and Sato, 1993). Recent analyses of pediatric human vocal folds from subjects ranging from postnatal to adolescence indicate that the vocal folds undergo extensive development and maturation into the early teens (Hartnick et al., 2005; Boseley and Hartnick, 2006). Consistent with the recognized contributions of externally applied mechanical forces in the development and homeostatic maintenance of many soft connective tissues, these observations suggest that the complex mechanical forces imposed during voice development may be an important epigenetic mechanism regulating tissue composition and mechanics. Vocal injury and pathology are accompanied by alterations in both matrix composition and vibratory function. On this basis, we hypothesize that rapid restoration of phonation and the vibratory microenvironment will support regeneration of the physiological matrix composition and viscoelastic properties, leading to improved vocal performance. Conceptually, this approach is analogous to early mobilization strategies for tendon/ligament healing that improve recovery of structure and function.

As a 3D, *in vitro* culture model of the vibratory microenvironment, human dermal fibroblasts were encapsulated within methacrylated HA (GMHA) hydrogels that approximate the dynamic viscosity of native tissue using a cytocompatible photocrosslinking methodology. (Bryant et al., 2000; Williams et al., 2005) The samples were subjected to physiologically relevant high frequency vibration (100Hz sine wave, 1mm amplitude). A 2sec *on* / 2 sec *off* regimen was chosen to simulate the alternation of inhalation and vocalization. The 2 hr / day (net vibration *on* time) approximated the average vocalization period for a classroom teacher in a 6-7 hr working day. (Masuda et al., 1993; Titze et al., 2007)

Analysis of both vital dye staining and DNA content revealed that vibratory stimulation reduced cell viability/number relative to static controls. Exposure to vibration also reduced cell-cell aggregation observed here and previously in GMHA matrices maintained under static conditions. (Kutty and Webb, 2008) The reduction in cell viability may be attributable to vibration-induced physical trauma. Several studies have reported strain-induced apoptosis in smooth muscle cells and fibroblasts (Mayr et al., 2000; Sanchez-Esteban et al., 2002). A reduction in cell density has been observed between adult and newborn vocal mucosa, although the mechanisms responsible are unknown (Hirano et al., 1989; Hirano et al., 1999). Collectively, these results suggest that mechanical forces imposed during phonation may select for a cell population adapted to high frequency vibration. Alternatively, cell death could result from differences in the mechanical forces experienced in the bioreactor relative to native tissue. However, this is considered unlikely because the present bioreactor design does not include stresses originating from collisions with the opposite vocal fold and it was operated at the low end of the physiological frequency range with corresponding sub-physiological acceleration forces (Titze et al., 2004).

The ECM of native vocal mucosa ECM exhibits relatively high levels of GAG/ proteoglycans and relatively low levels of fibrous matrix proteins such as collagen and elastin. Vocal fold injury results in increases tissue viscosity and stiffness, reduces or eliminates mucosal wave vibration and substantially changes matrix composition during the acute wound healing response, decreasing levels of HA, decorin, and fibromodulin, as well as increasing levels of collagen (Rousseau et al., 2003; Thibeault et al., 2003; Thibeault et al., 2004). HA, decorin, and fibromodulin are all associated with scarless healing and their depletion following injury may contribute to vocal fold scarring. Sustained elevation of HA is observed during scarless fetal wound healing and HA has been shown to inhibit fibroblast

collagen synthesis and deposition (Longaker et al., 1991; Croce et al., 2001). Both decorin and fibromodulin bind collagen and inhibit fibrillogenesis (Vogel and Trotter, 1987; Hedlund et al., 1994). Furthermore, both proteoglycans bind and sequester transforming growth factor beta-1, a potent fibrotic cytokine that stimulates collagen synthesis during scarring and fibrotic disease (Yamaguchi et al., 1990; Hildebrand et al., 1994; Gharaee-Kermani and Phan, 2001).

In the present study, vibratory stimulation of GMHA-encapsulated fibroblasts stimulated a pattern of ECM-related gene expression and matrix synthesis that is consistent with the biochemical composition of native vocal mucosa. Significant increases in HA synthase 2, decorin, and fibromodulin gene expression were observed, as was increased total accumulation of sulfated GAGs. In contrast, increases in MMP-1 expression and decreases in collagen and elastin expression were observed, indicating a net increase in fibrous matrix catabolism. This was confirmed by measurements of collagen accumulation based on hydroxyproline content. These results suggest that early restoration of phonatory vibration may counteract the changes in matrix composition observed following vocal injury.

Several interesting similarities and differences may be observed between the present studies and the previous study by Titze et al (Titze et al., 2004). Despite considerable differences in experimental design (scaffold structure and mechanical properties; vibration regime; and fibroblast tissue origin), both studies reported that vibration produced significant increases in HA synthase 2, decorin, and fibromodulin and significant decreases in collagen relative to static controls in both studies. At 6-24 hours, both studies observed approximately 1.5 to 3 fold changes in expression levels. While Titze's study examined a single time point, the present study demonstrated a substantial temporal pattern to the induction of gene expression, with relative expression levels of HA synthase 2, decorin, and fibromodulin increasing to 10 to 50 fold differences at 3 and 5 days. In addition, substantial reductions in gene expression were observed at 10 days. This result may reflect cellular adaptation to vibratory stimulation due to downregulation or desensitization of receptors/signaling intermediaries responsible for vibratory mechanotransduction. Although few studies have examined adaptive responses to mechanical stimulation, Tranquillo recently reported that a regimen of cyclic strain incorporating steadily increasing strain amplitude produced significant improvements in mechanical properties relative to continuous application of strain at fixed amplitude (Syedain et al., 2008).

Due to the critical impact of mucosal viscoelasticity on vocal function, GMHA hydrogels in the present study were designed to approximate native tissue properties and should maintain those properties during remodeling and matrix synthesis. Rheological analysis of acellular hydrogels confirmed that exposure to vibratory stimulation did not significantly alter GMHA dynamic viscosity. The reductions in dynamic viscosity initially observed in cell-loaded samples following vibratory culture suggest that matrix degradation may occur more rapidly than functional organization of newly synthesized matrix. In the present study, this could be overcome by a reduction in cell number that probably altered the kinetics of remodeling. However, this would be likely to delay new tissue formation and might not be sufficient for *in vivo* applications, where remodeling is likely to be further accelerated by the presence of endogenous inflammatory cells. The results reported here indicate that cell density could be an important variable in transplantation-based approaches to vocal fold regeneration and suggest that it may be necessary to adjust scaffold mechanical properties to initially exceed physiological values.

Conclusion

Exposure of GMHA-encapsulated fibroblasts to physiologically relevant vibratory stimulation produced a pattern of ECM-related gene expression and matrix synthesis consistent the composition of native vocal mucosa. This supports the concept that rapid restoration of phonatory vibration may provide a means to reduce vocal scarring and restore physiological matrix composition, mechanical properties, and vocal quality. This hydrogel/bioreactor system may also provide an *in vitro* model for studying vibratory mechanotransduction and associated vocal pathology. Although little is known about the mechanisms involved in vibration-induced signaling, the induction of GAG/proteoglycans with anti-scarring properties described herein suggests that direct application of vibration or activation of associated cellular signaling pathways may provide a potential source of therapeutic strategies with broad applicability for reducing scar formation and fibrotic disorders.

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Figure 1. Vibratory bioreactor assembly (A) and cell encapsulated 2% w/v GMHA hydrogel crosslinked on an elastomeric Tecoflex film (B). Scale bars = 10 mm.

Figure 2. Viability of fibroblasts encapsulated in 2% w/v GMHA hydrogels and cultured under static and vibratory conditions for 5 and 10 days. # indicates significant differences relative to the corresponding static control at the same time point.

Figure3.
Confocal analysis (200 μm depth) of fibroblast morphology and spreading within 2% w/v GMHA hydrogels. Samples were stained with Alexa 594-phalloidin and imaged after culture under static (A,C) and vibratory (B,D) conditions for 5 (A, B) and 10 days (C,D). All scale bars = 200 μm .

Figure 4. Fibroblast proliferation within 2% w/v GMHA hydrogels under vibratory and static conditions. * indicates significant differences relative to the corresponding cell number at 1 day. # indicates significant differences relative to the corresponding static control at the same time point.

Figure 5. mRNA expression levels of HA Synthase 2 (A), Decorin (B), Fibromodulin (C), MMP-1 (D), Collagen (alpha 1 type I) (E), and Elastin (F) in a time course study. * indicates significant difference relative to the 1 day time point of the corresponding group. # indicates significant differences relative to the corresponding static control at the same time point. ** identifies significant differences observed among other experimental groups.

Figure 6. Picograms of sulfated-GAGs per encapsulated cell in the 2% w/v GMHA hydrogels after 5 and 10 days of static and vibrational culture. # indicates significant differences relative to the corresponding static control. ** indicates a significant difference among experimental groups.

Figure 7. Picograms of collagen per encapsulated cell in the 2% w/v GMHA hydrogels after 5 and 10 days of culture. # indicates significant difference relative to the corresponding static control. ** indicates a significant difference among experimental groups.

Figure 8. Dynamic viscosity of acellular 2% w/v GMHA hydrogels maintained for 10 days under static and vibratory conditions.

Figure 9. Dynamic viscosity of cell-loaded (4×10^6 cells/ml) 2% w/v GMHA hydrogels cultured for 10 days under static and vibratory conditions.

Figure 10. Dynamic viscosity of cell-loaded (2×10^6 cells/ml) 2% w/v GMHA hydrogels cultured for 10 days under static and vibratory conditions.

Table 1

Target Genes and Primers for Real Time RT-PCR Analysis

Target gene	Sense primer	Antisense primer
Collagen, type I, alpha 1	tgctggtgctcctggtactc	tccagaggacattgtttgc
Elastin	gcctacaccacagggaac	accgaactttgctgctctt
HA Synthase 2	cataaagaaagctcgcaacacg	cacacttcgtcccagtgtc
Decorin	gtgtcagccggattgtttc	gcaggtgtgaaaggaggag
Fibromodulin	tgataaggtgggcaggaagg	tggttgtgtcgcagatggag
MMP-1	tgctgaaacctgaaggtga	cttgcaaatctggcgtgta
Beta-2-microglobulin	tgtgctcgcgctactcttc	cggatggatgaaaccagac