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The Effects of Decorin and HGF-Primed Vocal Fold Fibroblasts In Vitro and Ex Vivo in a Porcine Model of Vocal Fold Scarring

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Abstract

Objectives—Vocal fold injury can be irreversible, leading to vocal fold scarring, with permanent functional effects and no optimal treatment. A porcine model of vocal fold scarring was used to test effects of decorin and primed vocal fold fibroblasts in vitro using a cell migration assay and immunoblotting, and by using functional measurements of porcine larynges and excised porcine vocal folds.

Methods—In vitro: primary pig vocal fold fibroblasts (PVFFs) were subjected to cell migration assays (scratch) and treated with decorin 20 $\mu\text{g/mL}$, hepatocyte growth factor (HGF) 200 ng/mL , epidermal growth factor (EGF) 1 nM , or transforming growth factor- $\beta 1$ 10 ng/mL . Cells also underwent decorin dose response testing. Scratch assays were analyzed in MetaMorph[®] Imaging; cell lysates were processed for MMP-8 and type I collagen content. Eleven pigs underwent unilateral vocal fold stripping procedures. At day 3 postoperatively, subjects underwent superficial injection into the affected vocal fold either with decorin 20 $\mu\text{g/mL}$ or 1×10^6 HGF-primed fibroblasts. Larynges were harvested and either used for ex vivo laryngeal testing or for rheological testing.

Results—Scratch assay indicated significantly reduced cell migration in PVFFs treated with decorin or HGF. MMP-8 production was increased ($P < 0.01$) and collagen was decreased in cells treated with decorin at increasing doses. Viscoelastic measurements suggested somewhat increased stiffness for decorin treated samples. Ex vivo aerodynamic testing suggested improved vocal efficiency scores in decorin-treated larynges.

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Conclusions—Decorin has a noticeable effect on PVFF migration in vitro and appears to increase vocal fold stiffness but either does not change or slightly increases vocal efficiency.

Level of evidence: 5

Keywords

Molecular biology; laryngology

INTRODUCTION

Hoarseness affects millions of people yearly and can result in significant losses economically, socially, and psychologically given that we are living in a communication age. One of the most common causes for hoarseness is vocal fold scarring, a malady for which even modern phonosurgery has yet to treat optimally. This scarring can be caused by phonotrauma, inflammatory diseases, and even phonosurgical procedures. The delicate nature of the superficial lamina propria of the vocal fold is disturbed, and this translates into abnormal biomechanical properties of the vocal folds, affecting function.¹ Central to wound healing in the vocal folds is the fibroblast, as it generates most of the vocal fold matrix. The vocal fold has a proclivity for excess scar deposition when injured in some individuals. An ideal therapy would decrease scar deposition while restoring damaged tissue and function. Decorin and hepatocyte growth factor are two well-known proteins that have antifibrotic properties.¹ Decorin, a small leucine-rich repeat proteoglycan is a normal component of the superficial lamina propria.^{2,3} It has been shown to have antifibrotic properties in vivo in animal studies.⁴ Hepatocyte growth factor (HGF) has very strong antifibrotic properties and has been studied in vitro and in vivo in the vocal fold, confirming these properties in models of vocal fold scarring.⁵ The fact that decorin is already a native molecule in the lamina propria makes it potentially attractive for therapeutic use in repair and regeneration. Still, the clinical experience with added growth factors in many wound-healing applications is disappointing, likely due to the fact that the cells resident within an established scar are few and phenotypically limited.

Cell transplantation is being applied in many settings to overcome this cellular “arrest.” The concept behind priming of fibroblasts is harnessing the power of growth factors to change the phenotype of the fibroblast into a pro-synthetic or pro-regenerative phenotype.⁴ In addition, differentiated cells are much more readily available than stem cells, which carry some regulatory burdens, particularly in the United States, although this climate is changing. Last, fibroblasts are ideal, as they can be autologously harvested readily and present the ability to expand extensively. This study used a pig model of vocal fold scar to investigate both in vitro and in vivo the effects of decorin and fibroblasts primed with HGF as potential ways to treat vocal fold scarring. Cell migration and cytoskeletal machinery were examined in addition to functional measurements of excised laryngeal tissue.

MATERIALS AND METHODS

Cell Culture

Primary pig vocal fold fibroblasts were derived from pig vocal fold explants. Pig vocal fold tissue was dissected under magnification to separate muscle from lamina propria and epithelium and LP; epithelium tissue was minced into 1- to 2-mm pieces in 6- or 12-well plates. Explants were grown in Fibroblast Growth Media[®]-2 (FGM, Lonza Walkersville, Walkersville, MD) to which 5% nonessential amino acids, 5% L-glutamine, 5% penicillin–streptomycin, 10% fetal bovine serum, and a Bullet Kit (with 0.5 mL h-FGF, 0.5 mL Gentamycin–Amphotericin B, 0.5 m Insulin) was added. Pig vocal fold fibroblasts (PVFFs) were passaged and were used in experiments from passages 3–8 to avoid effects of replicative senescence.

Scratch Assay

PVFFs (passages 3–8) were plated on uncoated plastic six-well plates. Once 70% to 80% confluence was reached, cells were serum starved for 24 hours. A midline vertical scratch was formed using a rubber policeman with a blunt surface.⁶ Photographs were then taken with a Sony CCD-IRIS camera attached to an Olympus CK2 microscope using Xclaim[™] Video Player (ATI Technologies, Inc., Sunnyvale, CA) at 2× magnification. Immediately afterward, either plain media (without FGF), Hepatocyte growth factor (HGF, Sigma-Aldrich, St. Louis, MO) at 200 ng/mL, Decorin at 20 µg/mL (R&D Systems[®], Minn-eapolis, MN), transforming growth factor-beta (TGF-B1, Peprotech, Inc., Rocky Hill, NJ) at 10 ng/mL, and epidermal growth factor 1 nM (EGF, BD Biosciences, San Jose, CA) were applied for 24 hours. Photographs were again taken at the 24-hour point and were then analyzed using MetaMorph[®] Imaging Software (Molecular Devices Corporation, Sunnyvale, CA). Experiments were run in at least triplicate and wound distances were measured in triplicate before and after treatment and compared within and between treatment groups. These cells were then lysed with RIPA buffer and cell material was collected using a cell scraper; specimens were then stored at –80°C until used for immune blotting.

Western Blot

Proteins contained within cell lysates were separated on and transferred to nylon membranes by standard methods. Membranes were prepared for immunodetection by first blocking with nonfat dry 5% milk for 30 minutes at room temperature and washed three times at 10 minutes each with 1× TBS Tween 20. The membrane was placed and sealed in Kapad with primary antibodies (dilutions of 1:100–1:1,000 depending on the antibody—collagen I, fibronectin, matrix metalloproteinase-8 [MMP- 8], zyxin, vinculin) diluted with 1% nonfat dry milk and left to shake by a RotoMix 50800 at 4°C overnight. The pad was opened and washed with TBS Tween 20 three times, transferred to a second Kapad and secondary antibody (diluted at 1:2,000 in TBS–Tween 20) was added and shaken for 1 hour at room temperature. The membrane was again washed three times and 1:1 diluted detect reagent (Amersham Biosciences, Arlington Heights, IL) was added to the membrane for 1 minute. The membrane was placed into an autoradiographic film cassette and exposed for between 1

and 7 minutes. Finished images were scanned using Adobe Photoshop® CS3 and optical density was measured after conversion of images to grayscale.

Confocal Fluorescence Microscopy

Eight-chamber slides were either left uncoated, coated with decorin 5 µg/mL, collagen 5 µg/mL, or combination of decorin and collagen 5 µg/mL each for 30 minutes in a 37°C incubator. PVFFs were then trypsinized and plated and incubated for 24 hours, and one slide was treated with decorin 20 µg/mL for an additional 24 hours. Cells were then fixed with 2% paraformaldehyde for 30 minutes, and then permeabilized with Triton-X 100 (1%) buffer for 30 minutes. Slides were blocked with 5% bovine serum albumin-phosphate-buffered saline (BSA-PBS) for 15 minutes and then incubated with a different primary antibody in each chamber: antipaxillin, antizyxin, and anti-RhoA-C, anti Rac, anti-cdc42 (all Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or PBS (control) for 1 hour (all 1:50 dilution), with propidium iodide added at the same time as the primary antibody. The slide was then washed with PBS-Tween 20 (0.05%) and incubated with fluorescent-tagged antibody Alexa 488 (either antirabbit or antigoat depending on the antibody) at (1:150) for 30 minutes and then washed at least six times with PBS-Tween 20. The slides were then mounted with gelvatol or similar mounting media and coverslipped and left to dry for at least 12 hours. Images were then taken on an Olympus IX70 confocal microscope using the Fluoview program.

Porcine Model of Vocal Fold Scarring

Eleven random farm animal pigs ages 12–14 weeks (Wally Whippo, Enon Valley, PA) were used for the *in vivo* and *ex vivo* of the experiment. Animals were first sedated with xylazine 2 mg/kg and ketamine 20 mg/kg intramuscularly (IM). Animals were intubated with 5-0 or 6-0 endotracheal tubes and administered isoflurane 1.5% to 2% inhalational anesthetic for maintenance of general anesthesia. A Dedo laryngoscope was used to visualize the endolarynx and a 0° endoscope was used to photodocument the presurgical appearance of the larynx. One vocal fold (usually right) was surgically stripped with a 3-mm cup forceps and photodocumentation was again performed. Lidocaine 4% topical anesthetic was applied to mitigate any potential laryngospasm on emergence. On day 3 after vocal fold stripping, the same procedure for visualization was carried out, and now with the aid of an assistant holding the rigid telescope, the affected vocal fold was injected superficially with either 0.5 mL of saline, 0.5 mL of decorin 20 µg/mL, or a 0.5-mL suspension of 1×10^6 fibroblasts primed with HGF using an orotracheal injector with a 27-gauge needle (Medtronic Xomed, Jacksonville, FL) (Figs. 1 and 2). The first primed fibroblast suspension injected was stained with India ink for proof of concept (Fig. 3). Postinjection photodocumentation was performed. Lidocaine 4% topical anesthetic was again applied and the subject was recovered in standard fashion. On day 30 after treatment, animals underwent the laryngoscopy procedure for photodocumentation and were euthanized and larynges harvested.

Rheology

A custom-built linear simple shear rheometer was developed based on work previously published to study linear viscoelastic shear properties of the vocal fold tissue.⁷ The system consisted of an EnduraTEC Electro-Force Mechanical Testing system (Model ELF 3200, Bose, Minnetonka, MN) configured horizontally with fixturing to allow parallel plated shear testing. Vocal fold samples from harvested porcine laryngeal specimens were dissected and vocalis muscle was removed leaving epithelium with attached lamina propria. Specimens were subjected to linear shear tests with amplitude of 0.1 mm at frequencies ranging from 1–150 Hz. The fidelity of the data acquired during the tests was high over the entire range. The elastic modulus, viscous modulus, dynamic viscosity, and tan delta for the specimens were calculated.

Ex Vivo Larynx Experimental Setup

The sample population consisted of six porcine larynges. The larynges were stored frozen until immediately before use. They were dissected according to the procedure described by Jiang and Titze.⁸ Bilateral sutures were used on larynges 368, 1178, and 400 to prevent supraglottic tissue from resting on the vocal folds. External bilateral pressure on the thyroid cartilage laminae was required on larynges 399, Normal, and 1178 to properly adduct the vocal folds.

Apparatus

The larynges were mounted to an excised larynx phonation system according to Jiang and Titze,⁸ which simulated the physiological characteristics of the respiratory system by delivering heated and humidified air at controlled airflow rates. The trachea was fastened to the excised larynx phonation system using a metal pull clamp, and two bilateral three-pronged micromanipulators were inserted into the arytenoids to achieve vocal fold approximation. The laryngeal prominence of the thyroid cartilage was sutured to a medial micromanipulator to precisely control vocal fold tension.

Pressurized air from a building source was heated and humidified using two Concha Therm III humidifiers (Fisher & Paykel Healthcare Inc., Laguna Hills, CA). Airflow was measured using an Omega airflow meter (model FMA-1601A; Omega Engineering Inc., Stamford, CT) and the rate of airflow was manually controlled with a valve. Air pressure measurements were collected directly below the larynx with a Heise digital pressure meter (901 series; Ashcroft Inc., Stratford, CT). Acoustic measurements were taken by a Sony microphone (model ECM-88; Sony Electronics Inc., New York, NY). The microphone was positioned at a 45° angle to the vertical axis of the vocal tract and 10.0 cm from the glottis to reduce any acoustic noise produced by turbulent airflow. The acoustic signal was amplified using a Symetrix pre-amplifier (model 302; Symetrix Inc., Mountlake Terrace, WA). A National Instruments data acquisition board (model AT-MIO-16; National Instruments Corp., Austin, TX) and customized LabVIEW 8.2.1 software (National Instruments Corp.) was used to record airflow, pressure, and acoustic data simultaneously and continuously on a personal computer at sampling rates of 1 kHz, 10 kHz, and 44 kHz. All trials were conducted in a triple-walled, sound-attenuated room to reduce background noise and stabilize humidity and temperature levels.

Experimental Procedure (Ex Vivo)

The vocal folds were kept hydrated by frequent application of 0.9% saline solution. Once mounted, ascending and descending sweeps of subglottal pressure were used to induce sustained phonation. Typical sweeps increased subglottal pressure to 20 cm H₂O from 0 cm H₂O in a period of 10 seconds. Phonation threshold pressure (PTP) and vocal efficiency were determined for each sweep. Twenty sweeps were performed per larynx.

Data Analysis

Scratch data was analyzed using analysis of variance (ANOVA) for in-between group differences. Individual one- and two-tailed t-tests for samples with unequal variances were used to compare between two groups. Optical density of protein bands on immunoblotting was converted to absolute intensity and normalized by log transformation into relative intensity compared to control groups in each set of experiments. ANOVA to look for overall differences and individual t-tests were performed to compare between two treatment groups. Graphs were designed using Microsoft Excel®.

Data for *ex vivo* measurements were analyzed on a personal computer using custom Labview 8.2.1 software in conjunction with custom MATLAB R2006a software (Mathworks, Natick, MA). PTP was interpreted as the subglottal pressure at the time of phonation onset. To determine phonation onset, the acoustic data were decomposed into the sinusoidal components using a fast Fourier transform (FFT) and phonation onset was defined when a dominant fundamental frequency was identifiable. One-way ANOVA on ranks analyses were conducted on the aggregate PTP and vocal efficiency data across different larynges. Linear regressions were calculated for vocal efficiency as a function of airflow, pressure, and sound pressure level (SPL).

RESULTS

In Vitro Scratch Assay

PVFF monolayers showed significantly decreased gap width or cell migration with the application of decorin 20 µg/mL (Fig. 4) ($P < .00001$) after 24 hours. Two tailed *t*-tests comparing decorin, EGF, and HGF to TGF-β1-treated monolayers were highly significant ($P < .00001$, $P < .00001$, and $P < .0000001$).

Western Blotting

Optical density measurements of bands showed a significant difference based on dose for levels of MMP-8 ($P < .01$). There was a trend in decreasing collagen production until application of 20 µg/mL and then an increase in collagen production ($P = \text{NS}$). Actin cytoskeletal associated proteins vinculin and zyxin and the ECM protein fibronectin did not exhibit any significant change in response to increasing doses of decorin (Figs. 5, 6, and 7).

Confocal Fluorescence Microscopy

Comparison of PVFFs grown on untreated plates versus PVFFs grown on a combination of collagen and decorin matrix (5 µg/mL each) showed a mild increase in staining of the protein zyxin in cells grown on matrix. Distribution of Rho and Rac small GTPases was

consistent with the stress fiber and lamellipodia localization known from previous studies in other cell types (Figs. 8, 9, and 10A and B).⁹

Rheological Properties

The elastic shear modulus (G') (Fig. 11) remained relatively constant and frequency independent until 150 Hz, which would be more typical of phonation. Normal curves showed an increasing tissue stiffness and final drop off between 150 to 200 Hz. Two of the decorin treated specimens also followed this pattern (decorin #2 and #3), whereas the first decorin treated specimen had higher tissue stiffness inherently. The primed fibroblast-treated specimen showed decreased tissue stiffness starting at 100 Hz, which is consistent with a previous study using a recellularized scaffold.⁷ The saline-treated specimen did not greatly vary from the normal specimens in tissue stiffness.

The viscous shear modulus (G'') (Fig. 12), did not show a great deal of variability across the frequencies except at approximately 100 Hz where the primed fibroblast specimen and two normal specimens showed a decrease in G'' indicating a loss in viscous or liquid-like behavior.

The dynamic viscosity (η') (Fig. 13) showed a steady decrease until the end of the frequency sweep near 200 Hz with an increase in frequency with the primed fibroblast specimen and one decorin specimen displaying a much lower dynamic viscosity than the rest. The other two decorin treated specimens and the saline-treated specimen followed a pattern very similar to normal tissue. This also corresponds well to previously published reports.^{7,10,11}

Ex Vivo Laryngeal Results

The means and standard deviations of PTP and vocal efficiency are presented in Tables I and II. The distributions of PTP and vocal efficiency per larynx are illustrated in Figures 14 and 15. One-way ANOVA on ranks indicated that the differences between the median values among larynges for PTP and vocal efficiency were statistically significant ($P < .001$, $P < .001$), and multiple comparison procedure results are presented in Tables III and IV. Linear regression analysis indicated that vocal efficiency was dependent upon SPL but not glottal flow ($R^2 = .006$, $P = .104$) or subglottal pressure ($R^2 = 0.037$, $P < .001$).

DISCUSSION

Vocal folds, especially in mammalian species, are both delicate and strong, because of an evolution of phonatory functions in addition to protective and respiratory functions. This duality between delicacy and strength exists because of the highly specialized microarchitecture of the vocal folds that contain a complex interplay of fibroblasts, proteins, and proteoglycans, allowing the vocal folds to remain supple yet withstand a vibratory frequency in the human of up to 250 times a second. Any trauma, be it phonotrauma or surgical trauma, will likely disrupt the balance within the extracellular matrix (ECM). Disruption of this balance will lead to scarring in the vocal fold, with loss of important ECM components and subsequent loss in function.^{11–13} The process of vocal fold wound healing can be divided essentially into three phases: acute, which encompasses the first 2 weeks after injury; subacute, which encompasses approximately 2 weeks to 3 months; and any time

after 3 months would be considered chronic. This study looked at the beginning of the subacute period of vocal fold wound healing, which includes a period where collagen is laid down and is just beginning to be remodeled.

Decorin is one member of a large family of small leucine-rich repeat proteoglycans. Decorin, biglycan, and fibromodulin have all been proposed to have a role in vocal fold ECM.³ Initially, decorin was thought to be only a structural molecule, but research over the last decade has proven that it has many signaling functions and has a role in the control of cell adhesion and proliferation. It not only serves to help space collagen fibrils appropriately but regulates collagen fibrillogenesis, causing a decrease in the diameter of fibrils in addition to lowering the amount of fibrils.¹⁴ Decorin is one component of the ECM that is significantly decreased within vocal fold scar.¹⁵ As such, replacing lost decorin early in the scarring process may mitigate detrimental functional effects and help regenerate lost tissue or stabilize the existing microarchitecture. In fact, it appears to be able to bind type I collagen and TGF- β simultaneously.¹⁶ HGF can serve to regulate proteoglycan synthesis in interstitial fibroblasts and is thus attractive in its therapeutic potential to combat vocal fold scarring; in fact, this has been demonstrated *in vitro* and *in vivo*.⁵ However, it appears effects are short lived, and there is no lasting regeneration of tissue. Cell therapy can potentially offer that missing piece; application of cells, especially if “primed” or treated prior to use, can help create a synthetic and regenerative environment in the damaged vocal fold. This is why we chose to look at HGF-primed fibroblasts in addition to decorin.

The *in vitro* scratch assay supported decorin’s effect on inhibition of cell migration even in PVFFs. TGF- β 1 had an overwhelming effect on gap closure as we would expect and the difference between the two groups was highly significant. This effect could be explained by inhibition of the interaction between integrins, which mediate cell adhesion, and matrix protein ligands, which decorin binds; decorin acts as a competitive inhibitor of matrix proteins. Cell detachment is also seen, which likely adds to the decreased cell migration in closure of the wound.¹⁶ Decorin 20 μ g/mL, HGF 200 ng/mL and EGF 1 nM all had a similar effect in decreasing cell migration of PVFFs (or preventing it), although decorin appeared to have the greatest effect; all gap distances were not significant when compared to control but were all significant when compared to PVFFs treated with TGF- β 1. EGF typically increases individual cell locomotion but is associated with decreased directional persistence, which leads to cell scattering. This could be understood as lack of directional movement toward the wound, but effects are highly dependent on the matrix.¹⁷ One could infer that addition of decorin may decrease an influx or migration of fibroblasts or myofibroblasts into a wound; with less cells in the wound, less ECM proteins such as collagen I are produced, potentially reducing scar development. Alternatively, the presence of too much decorin may delay wound healing by the decreased migration of fibroblasts and myofibroblasts. These cells were only treated for 24 hours, however, so it is unclear what effect on migration would be seen at 48 hours or in longer term wound healing and what functional results would actually manifest.

Decorin also interacts with important enzyme regulators of the ECM known as MMPs. MMPs can cleave decorin and by doing so, actually may release sequestered TGF- β back into the ECM to perpetuate the wound healing process and establish homeostasis in the

ECM, a balance between degradation and regeneration. MMP-8, otherwise known as neutrophil MMP, has as its substrates collagen I, II, and III.¹⁸ This MMP was chosen because of its participation in very early wound healing and the fact that there is *in vivo* evidence of its increase during phonotrauma.¹⁹ Our experiments confirmed that MMP-8 was indeed increased in the presence of increasing doses of decorin, likely as part of a feedback mechanism. There are several other MMPs that are upregulated during vocal fold scarring,²⁰ but because of use of a porcine model, we were limited in antibodies available that were compatible with this model. The trend for decreased collagen until an increase in collagen is seen also suggests that there may be a biphasic response to decorin dosing in the ECM, further highlighting its modulatory role in the ECM.

The small GTPases Rho, Rac, and Cdc42 are integral parts of the cytoskeletal machinery. Rac is important in regulation of lamellipodia and filopodia that assist in membrane ruffling and forward movement of cells. Rho is intimately involved and bound to stress fibers that develop from actin polymerization and myosin activity, an intermediate step of cell motility involved with movement at the rear of a cell. Confocal microscopy confirmed localization of these GTPases in PVFFs grown on decorin matrix with Rho staining along stress fibers and Rac staining in the edge of the membrane in lamellipodia. Knowing these GTPases are involved is important in that smaller doses of decorin may actually stimulate activity of these GTPases, which may lead to increased migration in some cases.²¹

Viscoelasticity of the treated porcine vocal fold specimens displayed characteristics similar to that described in the literature.⁷ A definite trend in decreased stiffness was seen in the one specimen treated with primed vocal fold fibroblasts with all three rheological measures, G' , G'' , and η' . An expected decrease in dynamic viscosity was seen with higher frequency due to well-known effects of shear thinning in the vocal fold.⁷ The effect of exogenous decorin on vocal fold viscoelasticity has not been clearly elucidated; there appeared to be no difference or somewhat increased elastic modulus indicating increased stiffness but essentially no difference in dynamic viscosity from normals. This is the first study to investigate the viscoelastic response of the vocal fold to exogenous decorin. These data have to be interpreted cautiously, however, due to the small sample size in our experiments. An increase in stiffness in decorin treated vocal folds may be due to actual increased collagen binding, or release of TGF-B1 by the action of upregulated MMPs in the presence of increased soluble decorin. The interactions are understandably complex and require further investigation in relation to the vocal fold. Increased stiffness would be seen as a detriment and would likely result in poorer phonatory quality; however, this stiffness related to increased decorin exposure could provide some resistance or protective effect as well. An effect such as this might hold benefit in disorders of vocal fold tone such as vocal fold atrophy. However, these are only hypotheses at this time, requiring further testing, given the shorter time frame of this study; results from this study cannot be extrapolated to more long-term wound healing or eventual functional results in the vocal fold as of yet.

PTP is defined as the minimum subglottal pressure to initiate vocal fold oscillation,²² causing the transduction of aerodynamic energy into acoustic energy. Titze^{22,23} predicted that PTP is dependent upon the tissue and configurational properties of the larynx. Because of this dependence, PTP can be considered a biological indicator of the “ease of

phonation.”²³ The PTP was measured in the six larynges, and the results suggest the order from greatest to least ease of phonation as follows: Normal, 1179, 399, 368, 400, 515, and 1178. The trend seems to be for the decorin treated vocal folds to display decreased PTP though sample 515 had much increased PTP. Given the small sample size, it is possible this was an outlier due to the short length of trachea distal to the cricoid that was attached in this specimen, affecting its overall dynamics. There were no scarred untreated specimens to detect an improvement from no treatment; all results were compared to normal vocal folds, making this comparison more stringent.

The efficiency of an energy transducer is broadly defined as the amount of output to a given amount of input. Accordingly, vocal efficiency is the amount of radiated acoustic power (sound pressure) to aerodynamic power (subglottal pressure multiplied by glottal airflow).²³ The vocal efficiency was measured in six larynges, and the results suggest in order from most to least efficient: 1179, 515, Normal, 400, 399, 1178, and 368. Larynges 400, 399, and 1178 did not have statistically significant differences in median vocal efficiency. It is interesting to note that two of three decorin treated specimens had normal vocal efficiency, but again, specimen 368 appeared to be an outlier, or it could be representative of the actual effect, but the study is naturally underpowered to make any definite conclusions. Increased vocal efficiency in decorin treated vocal folds, if possible, seems to be an interesting phenomenon considering the results of viscoelasticity testing, which suggested increased stiffness in decorin treated vocal folds. Again, this increased stiffness could potentially be advantageous; it is not feasible to assess this in the porcine model where phonatory recordings would be difficult to interpret, let alone perform.

Although this is the first study looking at the biomechanical and aerodynamic properties of decorin treated vocal folds, there are a few notable limitations to this project. Use of the porcine model and PVFFs made obtaining biochemical markers (antibodies, etc.) more difficult than it would be using a rat or rabbit model. The choice of a porcine model of vocal fold scarring was made because of the relative microarchitectural similarity and size comparability of the pig vocal fold to the human vocal fold to perform a standard vocal fold stripping procedure and relatively accurate and consistent superficial vocal fold injection with available equipment. In addition, intersubject variability, including any coughing (despite the use of laryngotracheal anesthetic) or phonotraumatic behaviors of the pigs, would have been difficult to monitor or predict and add an element of uncertainty in the vocal fold wound healing process. This variable would also potentially affect retention of any treatment via the vocal fold injection; the same volume was delivered, but as we know in human patients, this is no guarantee that the entire volume will be retained. We did not specifically test for cell viability in the HGF-primed fibroblast-treated vocal folds, which would have affected any results, although our few specimens seemed comparable to what was found in the literature in which cell viability was tested.⁷

CONCLUSIONS

In conclusion, this study investigated the potential of soluble decorin and HGF-primed fibroblast injections as therapies for vocal fold scarring. *In vitro* data indicated that decorin and HGF both significantly slow down cell migration and that decorin appears to increase

synthesis of MMP-8 and appears to decrease type I collagen production or availability. Although there were a small number of specimens, viscoelastic data and *ex vivo* laryngeal data suggest that injectable decorin treatment and HGF-primed fibroblasts independently may help clinically with ameliorating vocal fold scarring and helping preserve normal vocal fold biomechanics.

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Fig. 1. Superficial injection of right vocal fold using orotracheal injection on day 3 after vocal fold stripping. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 2.
Postinjection appearance of vocal fold. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 3. Proof of concept of superficial injection of HGF-primed vocal fold fibroblasts. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 4.

Scratch assay results on pig vocal fold fibroblasts. Fibro-blasts were plated to 70% to 80% confluence, subjected to serum starvation, and monolayers were scratched in the midline with a rubber policeman. Cells were then treated with plain media (control), decorin 20 $\mu\text{g/mL}$, EGF 1 nmol, HGF 200 ng/L, and TGF- β 1 10 ng/mL. Scratch closure by pixels was measured at 24 hours after treatment using MetaMorph[®] Imaging Software ($P < .00001$) All experiments were done at minimum in triplicate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 5. Optical density of MMP-8 protein content on Western blot from PVFFs treated with varying doses of decorin. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 6.

Optical density of type I collagen protein content on Western blot from PVFFs treated with different growth factors (HGF-200 ng/mL, TGF- β 1-10 ng/mL, decorin-20 μ g/mL, and EGF 1nmol). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 7.

Western blots showing protein level in response to varying doses of decorin. Definite trends in type I collagen and significant change in MMP-8 levels ($P < .01$) were seen with increasing doses of soluble decorin. No significant trend was seen with either vinculin or zyxin.

Fig. 8.

Pig vocal fold fibroblasts grown on quiescent media with no matrix stained for zyxin, a zinc binding phosphoprotein that concentrates at focal adhesions and along the actin cytoskeleton. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 9. PVFFs grown on a matrix of collagen and decorin (both 5 $\mu\text{g/mL}$). There is increased staining along actin fibers compared to the untreated cells (left). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 10.

(A) PVFFS grown on decorin matrix ($5 \mu\text{g/mL}$) stained for small GTPase Rho, and (B) shows staining for small GTPase Rac. are important in inducing cytoskeletal change during cell migration. Anti-Rho labels actin stress fibers (long stranding, white block arrows), whereas Anti-Rac labels for lamellipodia (red circle) involved in membrane ruffling in the front of cells, which is a step during cell movement [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 11.

Elastic shear (storage) modulus (G') of porcine vocal folds-normal, scarred and treated with decorin 20 $\mu\text{g/mL}$, treated with HGF primed fibroblasts or sham (saline) treated. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 12. Viscous shear (loss) modulus (G'') of porcine vocal folds-normal, scarred and treated with decorin 20 $\mu\text{g/mL}$, treated with HGF primed fibroblasts or sham (saline) treated. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 13.

Dynamic viscosity (η') of porcine vocal folds-normal, scarred and treated with decorin 20 $\mu\text{g/mL}$, treated with HGF primed fibroblasts or sham (saline) treated. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 14.

Boxplot of phonation threshold pressure (PTP) by the larynx. The lower and upper edges of the box represent the 25th and 75th percentiles, and the line dividing the box represents the median. Whiskers below and above the box represent the 5% and 95% percentiles. Statistical outliers are graphed as points. One-way ANOVA on ranks indicated that the differences between the median PTP of each larynx were statistically significant ($P < .001$).

Fig. 15.

Boxplot of vocal efficiency by larynx. The lower and upper edges of the box represent the 25th and 75th percentiles, and the line dividing the box represents the median. Whiskers below and above the box represent the 5% and 95% percentiles. Statistical outliers are graphed as points. One-way ANOVA on ranks indicated that the differences between the median vocal efficiency of each larynx were statistically significant ($P < .001$).

TABLE I
Mean and Standard Deviation of Phonation Threshold Pressure (PTP) and Phonation Threshold Flow (PTF) Values Among Larynges.

Larynx	n	PTP (cm H ₂ O)		PTF (mL/sec)	
		Mean	SD	Mean	SD
Normal	20	4.494908	0.658674	164.7452	22.53905
* ₃₆₈	23	7.643604	0.389018	366.1332	36.94571
† ₃₉₉	26	6.349153	0.778461	438.4125	84.37456
† ₄₀₀	22	8.577089	1.026505	296.9684	126.5985
† ₁₁₇₈	24	12.89756	1.300886	221.433	103.5852
* ₁₁₇₉	22	4.657816	0.67227	111.8231	43.8491
* ₅₁₅	45	13.417	1.57508	30.2252	4.97838

* Treated with Decorin 20 µg/mL.

† Treated with 1×10^6 HGF primed fibroblasts (primed with HGF 200 ng/mL for 24 hours).

The number of sweeps is denoted as the sample size n.

TABLE II

Mean and Standard Deviation of Vocal Efficiency (VE) and Sound Pressure Level (SPL) Values Among Larynges.

Larynx	n	VE (%)		SPL (dB)	
		Mean	SD	Mean	SD
Normal	61	9.81126E-06	1.03715E-05	78.47319357	4.68438743
*368	70	2.81257E-06	4.05724E-06	76.312466	4.031302496
†399	88	4.32319E-06	4.28229E-06	77.91705957	4.445584905
‡400	65	4.02784E-06	3.52379E-06	79.81344466	2.841794801
†1178	72	2.58667E-06	1.07784E-06	77.70926526	3.628442516
*1179	76	2.38461E-05	2.46583E-05	81.58512604	6.941782704
*515	45	9.0438E-06	3.7419E-06	102.51800	2.2960800

The number of points evaluated is denoted as the sample size n.

TABLE III

Multiple Comparison Procedure (Dunn's Method) Results of One-Way ANOVA on Ranks of PTP Data Across Larynges.

Comparison [‡]	<i>P</i> < .05
L 1178 versus L Normal	Yes
L 1178 versus L 1179	Yes
L 1178 versus L 399	Yes
L 1178 versus L 368	Yes
L 1178 versus L 400	No *
L 400 versus L Normal	Yes
L 400 versus L 1179	Yes
L 400 versus L 399	Yes
L 400 versus L 368	No *
L 368 versus L Normal	Yes
L 368 versus L 1179	Yes
L 368 versus L 399	No *
L 399 versus L Normal	Yes
L 399 versus L 1179	No *
L 1179 versus L Normal	No *
L 1178 versus L 515 7.631 0.558 Do Not Test	No *
L 515 versus L Normal	Yes
L 515 versus L 1179	Yes
L 515 versus L 399	Yes
L 515 versus L 368	Yes
L 515 versus L 400	No *

Differences that were not statistically significant are denoted by asterisks.

* KEY: 368,515,1179 treated with decorin 20 µg/mL.

399,400,1178 treated with 1×10^6 HGF primed fibroblasts.

TABLE IV

Multiple Comparison Procedure (Dunn's Method) Results of One-Way ANOVA on Ranks of Vocal Efficiency Data Across Larynges.

Comparison [‡]	<i>P</i> < .05
L 1179 versus L 368	Yes
L 1179 versus L 1178	Yes
L 1179 versus L 399	Yes
L 1179 versus L 400	Yes
L 1179 versus L Normal	No *
L Normal versus L 368	Yes
L Normal versus L 1178	Yes
L Normal versus L 399	Yes
L Normal versus L 400	No *
L 400 versus L 368	Yes
L 400 versus L 1178	No *
L 400 versus L 399	No *
L 399 versus L 368	Yes
L 399 versus L 1178	No *
L 1178 versus L 368	Yes
L 1179 versus L 515	No *
L 515 versus L 368	Yes
L 515 versus L 1178	Yes
L 515 versus L 399	Yes
L 515 versus L 400	Yes
L 515 versus L Normal	No *

Differences that were not statistically significant are denoted by asterisks.

* KEY: 368, 515, 1179 treated with decorin 20 µg/mL.

399, 400, 1178 treated with 1×10^6 HGF primed fibroblasts.