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Characterization of human vocal fold fibroblasts derived from chronic scar

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Abstract

Objectives/Hypothesis—*In vitro* modeling of cell-matrix interactions that occur during human vocal fold scarring is uncommon, as primary human vocal fold scar fibroblast cell lines are difficult to acquire. The purpose of this study was to characterize morphologic features, growth kinetics, contractile properties, α -smooth muscle actin (α -SMA) protein expression and gene expression profile of human vocal fold fibroblasts derived from scar (sVFF) relative to normal vocal fold fibroblasts (nVFF).

Study Design—*In vitro*.

Methods—We successfully cultured human vocal fold fibroblasts from tissue explants of scarred vocal folds from a 56-year-old female and compared these to normal fibroblast cells from a 59-year-old female. Growth and proliferation were assessed by daily cell counts and morphology was compared at 60% confluence for 5 days. Gel contraction assays were evaluated after seeding cells within a collagen matrix. Alpha-SMA was measured using western blotting and immunocytochemistry (ICC). Quantitative RT-PCR was used to assess differential extracellular matrix gene expression between the two cell types.

Results—sVFF were morphologically indistinguishable from nVFF. sVFF maintained significantly lower proliferation rates relative to nVFF on days 3–6 (day 3: $p = 0.0138$, days 4, 5, and 6: $p < 0.0001$). There were no significant differences in contractile properties between the two cell types at any time point (0h: $p = 0.70$, 24h: $p = 0.79$, 48h: $p = 0.58$). ICC and western blot analyses revealed increased expression of α -SMA in sVFF as compared with nVFF at passages 4 and 5, but not at passage 6 (passage 4: $p = 0.006$, passage 5: $p = 0.0015$, passage 6 = 0.8860). Analysis of 84 extracellular matrix genes using qRT-PCR revealed differential expression of 15 genes ($p < 0.01$).

Conclusions—nVFF and sVFF displayed differences in proliferation rates, α -SMA expression, and gene expression, whereas no differences were observed in contractile properties or morphology. Further investigation with a larger sample size is necessary to confirm these findings.

Keywords

vocal folds; fibroblasts; scar; voice disorders; gene expression; cell proliferation; polymerase chain reaction; RNA; collagen; human

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INTRODUCTION

Vocal fold scarring can result from laryngeal surgery, radiation therapy, vocal fold trauma, and idiopathic causes. It commonly involves a physical disruption of the vocal fold edge, impaired glottic closure, and disordered lamina propria extracellular matrix (ECM) translating to loss of tissue viscoelasticity, decreased vibratory potential, and moderate to severe dysphonia¹⁻³. Treatment modalities to prevent scar formation and/or to minimize subsequent detrimental effects have yet to be optimized^{2,4} and have been ascribed to poor understanding of the pathologic processes involved in the extracellular matrix (ECM) of the vocal fold lamina propria. The ECM is composed of collagen, elastin, hyaluronic acid, fibromodulin, decorin, and versican among other proteins. Vocal fold fibroblasts (VFF) produce a large proportion of the ECM and are key players in supporting the vocal fold lamina propria in health and disease⁵. Previous studies have shown that early recruitment of fibroblasts to the site of injury in the vocal fold is key to the wound healing process^{6,7}. Further, fibroblasts involved in the wound healing process in other parts of the body have been shown to undergo activation and manifest a phenotypic transition expressing α -smooth muscle actin (α -SMA) transiently⁸. These modified fibroblasts called myofibroblasts have been documented to be involved in wound healing and contracture as well as with the formation of a collagen-rich scar. Unfortunately, little is known regarding how human VFF from injured or scarred tissue behave as to date these have been unavailable to study. While VFF are used extensively in *in vitro* research of the vocal folds, the majority of these have been derived from previously normal human or animal vocal fold tissue (for example^{9,10}). *In vivo* canine, ferret, rat, mouse, porcine and rabbit models have all been utilized to investigate different facets of the injury and/or wound healing process in vocal fold mucosa (as reviewed in^{11,12}). While these studies have aided in determining pathways of scar formation and quantification of changes in the ECM, the next step would be to validate these findings with human tissue.

The objective of this study was to characterize the morphologic features, growth kinetics, contractile properties, alpha-smooth muscle actin protein expression and gene expression profile of human vocal fold fibroblasts (hVFF) derived from scar (sVFF) relative to normal vocal fold fibroblasts (nVFF). Based on findings from previous studies, it was hypothesized that sVFF would exhibit increased contraction, greater α -SMA expression and greater proliferative rates than nVFF. With regards to ECM analysis, we hypothesized that there would be an overexpression of collagens I and III, as well as TGF- β , as these have been implicated in deposition of scar tissue and fibrosis.

MATERIALS AND METHODS

Fibroblast Isolation and Culture

Vocal folds of a 56-year-old female undergoing laryngofissure with bilateral mucosal grafts to replace severely scarred vocal folds were used for this study. Diagnosis for vocal fold scarring was made by the attending laryngologist after a full examination which included videostroboscopy and high speed imaging. Further, images were reviewed by five blinded clinicians who each have over 7 years experience in voice. Descriptions of vibratory function included “severe stiffness”, “lack of a discernable mucosal wave”, “abnormal tissue”, and “fibrotic” representing vocal fold scarring. The donor’s prior laryngeal surgical history included multiple extensive biopsies of both vocal folds with pathology revealing hyperkeratosis, parakeratosis, and chronic inflammation. Vocal fold samples were received immediately after excision and were placed in phosphate buffered saline (PBS) for transport. The University of Wisconsin Madison Health Sciences Institutional Review Board (IRB) approved the protocol for tissue procurement and informed consent was received from the donor.

Normal vocal folds obtained from a 59-year-old female were also used in this study. Larynx and vocal folds were normal with no evidence of disease, and were received immediately following excision. These primary fibroblasts have previously been characterized elsewhere^{10,13}.

For primary cell culture, true vocal fold tissue was cut into small pieces and suspended in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 0.01 mg/mL streptomycin sulfate and 1× NEAA (all from Sigma). Cells were grown on uncoated plastic tissue culture dishes (Focal) at 37°C in 5% CO₂-humidified atmosphere. After 17 days, the adherent confluent sVFFs were trypsinized and passaged. Cell culture conditions for nVFFs were the same as for sVFFs as described in Chen and Thibeault¹⁰.

Morphology

Passage 4, 5, and 6 nVFF and sVFF were each seeded on a 10 cm culture dish (BD Biosciences, Durham, NC) at a density of 300,000 in DMEM with 10% FBS, 1% penicillin/streptomycin, and 1% non-essential amino acids (Sigma, St. Louis, MO). Cells were allowed to reach 60% confluence at which point photos were taken daily for five days via the Olympus CKX41 inverted light microscope (Olympus, Japan) at a magnification of 40X. Photos of the cells were printed and blinded assessments were then made to identify whether the photos belonged to nVFF or sVFF as well as passage identity by two raters.

Growth Characterization

Passage 5 cells were seeded at a density of 1.5×10^4 cells per well each in a 24 well plate. The growth curves of the two cell lines, nVFF and sVFF, were determined by cell counts of parallel quadruplicate wells on a daily basis for 6 days. ANOVA was performed between days 1 to 6 using Fisher's Protected Least Significant Difference for post-hoc analysis and statistical significance was defined by $p < 0.05$.

Western Blot

nVFF and sVFF at passages 4, 5, and 6 were seeded into nine sterile uncoated 10mm plates (three for each passage) and allowed to grow until they reached 90% confluence. Cells were then washed with PBS and total cell protein was extracted using the M-PER protein extraction kit (Pierce, Rockford, Illinois) according to the manufacturer's instructions. Total protein was quantified using BCA assay method (Pierce). Protein aliquots were stored at -80°C. For each protein sample, 1.5 µg of total protein was characterized electrophoretically on NuPAGE 10% Bis-Tris gels (Invitrogen, Carlsbad, California) under reducing conditions using NuPage MOPS SDS buffer. Protein was transferred onto a polyvinylidene fluoride (PVDF) membrane using an XCELL Blot Module (Invitrogen). MagicMark XP Western protein standard (Invitrogen) was used to validate bands in the range of 20 to 220kd. Membranes were probed for α-SMA with an α-SMA antibody mouse clone 1A4 (Sigma, St. Louis, Missouri) and GAPDH using a mouse anti-GAPDH antibody (Sigma, St. Louis). Membranes were blocked overnight using blocking solution in 4°C and then incubated with a 1:1000 dilution of mouse monoclonal α-SMA antibody for 1 hour at room temperature (RT) and a 1:20,000 dilution of anti-GAPDH antibody. Bound antibodies were detected using the WesternBreeze anti-mouse chemiluminescent kit (Invitrogen). Chemiluminescent signal was captured using the LAS-4000 (GE Healthcare, Piscataway, NJ). Densities of the bands were measured using ImageJ (NIH). Differences between densities of α-SMA expression between cell lines were analyzed with ANOVA. Post-hoc analysis was conducted using Fisher's Protected Least Significant Difference Tests. Statistical significance was defined by $p < 0.05$.

Immunocytochemistry

Cells were studied using immunofluorescent staining with antibodies directed against α -SMA (Sigma). Passage 4 nVFF and sVFF were seeded in quadruplicate into sterile Permax 4 chamber slides (Sigma) at a density of 1000 cells/well in DMEM-10% FBS and incubated until they reached 50% confluence. nVFF at 70% confluence, treated with TGF- β at a concentration of 10 ng/mL over 48 hours in serum-free media, served as a positive control. Cells were washed with PBS and then fixed in 4% paraformaldehyde for 10 minutes at RT. Cells were washed three times with 0.1% Triton X-100 (Sigma) in PBS for 5 minutes each. Serum blocking was conducted for 60 minutes using 5% normal goat serum in PBS. Primary antibody (mouse anti- α -SMA, Sigma) was diluted to the appropriate concentration in blocking solution (1:250) and cells were incubated with this antibody for 90 minutes. Cells were once again washed three times with the Triton/PBS solution for 5 minutes each. Species-specific secondary antibody (Alexa 488 labeled goat anti-mouse, Invitrogen) was diluted to the 1:200 in PBS and cells were incubated with secondary antibody for 60 minutes in a dark chamber. Slides were washed with 1% Triton X-100 in PBS (PBST) at the end of secondary incubation and then were quickly rinsed with dH₂O. Chambers and gaskets were carefully removed and slides were mounted with Vector aqueous anti-fade fluorescent mounting medium with DAPI. Images were captured using a Nikon Eclipse E600 Epi Fluorescence inverted microscope (Japan) at 200x magnification and subsequently analyzed qualitatively for presence of green fluorescence indicative of α -SMA by two raters who were naïve to the purpose of the study.

Collagen Gel Contraction Assay

sVFF (passage 6) and nVFF (passage 5) were cultured in medium (10% FBS, 1% AA, 1% P/S) and trypsinized, counted, and 480,000 cells were seeded in wells each containing 1.5 mL collagen gel lattices (Cell Biolabs, San Diego, CA). After polymerization and a culture period of 2 days, the contraction assay was completed. Stressed matrices were released from the surrounding brim of the wells. Pictures of free-floating collagen gel lattices were taken at full optical zoom using the white light setting on a BioDoc-It imaging system (UVP, Upland, CA) immediately after release as well as 24h and 48h later. After blinding for cell type and time point, areas of collagen gel size were analyzed with ImageJ (National Institutes of Health, Bethesda, MD) and normalized to areas of the well. Degree of contraction was evaluated by determining the area of the gel matrix at each time point. The assay was run in triplicate for each cell type, and a cell-free collagen matrix was used as a comparison control. Mean gel area was compared across each time point and group using an ANOVA and Fisher's Protected Least Significant Difference tests with statistical significance assigned at $p < 0.05$.

Gene Expression by Quantitative RT-PCR

sVFF (p5) and nVFF (p5) were trypsinized and total RNA was extracted according to a standard protocol (Invitrogen, Carlsbad, CA). RNA samples were assessed for purity by ultraviolet spectrophotometry; A₂₆₀/A₂₃₀ ratio was over 1.8, and A₂₆₀/A₂₈₀ ratio was greater than 2.0. Template cDNA prepared from total RNA of sVFF and nVFF was characterized in technical triplicates using the Human Extracellular Matrix & Adhesion Molecule PCR Array with the RT² SYBR Green/Fluorescein PCR master mix (SABiosciences, Frederick, MD) on the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Data generated from the arrays were analyzed by an Excel-based analysis template (SABiosciences, Frederick, MD). Gene expression was calculated from the number of cycles by using standard curves, and the results were normalized to three stable housekeeping genes (B2M, GAPDH, ACTB). Fold-change ($2^{(-\Delta\Delta Ct)}$) was calculated based on normalized gene expression ($2^{(-\Delta Ct)}$) in the sVFF divided by the normalized gene expression

($2^{(-\Delta\text{Ct})}$) in the nVFF. P-values were calculated based on a Student's t-test of the replicate $2^{(-\Delta\text{Ct})}$ values for each gene in the nVFF group and sVFF group. As we were examining multiple genes, we chose a more stringent p-value for statistical significance of $p < 0.01$ to control for Type I error.

RESULTS

Morphology and Growth Characterization

nVFF and sVFF were compared over passages 4-6 for morphology. Both cell lines demonstrated a typical spindle shape and did not deviate from this standard at any of the passages (Figure 1), nor did they differ from each other. No significant difference in growth rate was seen on days 1 and 2. nVFF were found to have faster proliferation rates than sVFF with significantly different proliferative rates on days 3-6 (Day 3: $p = 0.0138$, Days 4, 5 and 6: $p < .0001$) (Figure 2).

Western Blot Analysis and Immunocytochemistry

To explore differences in α -SMA expression in nVFF and sVFF we conducted Western Blot and immunocytochemical analysis. While both cell lines differentially expressed α -SMA, this difference was more apparent in western blot densitometric analyses which indicated that sVFF had significantly greater expression at passages 4 and 5 compared to nVFF ($p = 0.006$ and $p = 0.0015$ respectively). There was no difference in α -SMA expression between sVFF and nVFF at passage 6 ($p = 0.8860$) (Figure 3). Subjective assessment of ICC images (Figure 4) showed sVFF (B) had stronger α -SMA staining in cytoplasmic myofilaments than nVFF (A).

Fibroblast-Populated Collagen Lattice Contraction

Following release of the collagen lattice from the bottom of the wells, nVFF and sVFF each contracted within 24 hours. At 24 hours, collagen contracted 58 percent with sVFF, 59 percent with nVFF, and 14 percent for the unpopulated collagen matrix. There were no significant differences in collagen lattice contraction for sVFF relative to nVFF at any of the time points ($p = 0.70$, $p = 0.79$, $p = 0.58$ for 0h, 24h, and 48h respectively), although both were significantly different from control at 24 and 48 hours following release of the collagen matrix ($p < 0.001$) (Figure 5).

Gene Expression Analysis

Using a real-time PCR array, we investigated the differences in expression of 84 genes encoding proteins important in extracellular matrix homeostasis and cell adhesion. All genes were detected in both cell types; however, two genes within nVFF and one gene within sVFF showed relatively high average threshold cycles meaning that their relative expression levels were low. For nVFF, ITGAM and SELE were minimally expressed, whereas for sVFF, NCAM1 was minimally expressed. Fifteen ECM and adhesion molecule genes were significantly differentially expressed between sVFF and nVFF at $p < 0.01$ (Table I). sVFF had down-regulated expression of NCAM1 (–87.11 fold change), COL11A1 (–40.71 fold change), ITGB3 (–13.98 fold change), ITGA2 (–9.05 fold change), ITGA3 (–8.16 fold change), MMP10 (–6.57 fold change), CLEC3B (–6.35 fold change), PECAM1 (–5.64 fold change), ITGB4 (–3.37 fold change), TNC (–2.66 fold change), and TIMP1 (–2.46 fold change). The following genes were found to be upregulated in sVFF relative to nVFF: LAMA1 (65.52 fold change), THBS2 (13.19 fold change), MMP3 (11.62 fold change), THBS1 (4.04 fold change).

DISCUSSION

Effective and long-term clinical strategies for managing vocal fold scarring have yet to be developed. Recent research has focused on the use of animal models to explore tissue engineering scaffolds and cellular-based therapies for vocal fold scarring. Animal models are the current standard for exploring vocal fold scarring etiology and treatment given the inherent barriers in procuring vocal fold tissue from humans. In this study, we were successful in deriving fibroblasts from chronically scarred human vocal folds and examined several phenotypic, genotypic, and protein expression properties of these cells relative to vocal fold fibroblasts derived from a normal human donor of the same age. We report with one biological sample of sVFF that these cells grew slower than nVFF, although they displayed similar morphology and contractile properties. Western blot and ICC analyses revealed elevated α -SMA in sVFF. Genotypically, we report on 15 differentially expressed genes between sVFF and nVFF.

Morphological comparative assessments of nVFF and sVFF at passages 4, 5 and 6 did not show any distinguishable characteristics. These findings mirror similarities reported between normal lung fibroblasts and those derived from idiopathic pulmonary fibrosis (IPF)¹⁴. In contrast, Kumai et al.¹⁵ examined morphologic differences in nVFF and sVFF from ferrets and reported sVFF to be more elongated and to have fewer processes than nVFF. In that study, sVFF were isolated from vocal fold scar tissue that had been induced two weeks prior to dissection and morphological analysis was based on passage 3 cells. The lack of differences in morphologic characteristics in the present study may result from the presumption that human scar tissue obtained for the present study represents a more chronic condition. Others have reported morphologic differences in keloid or hypertrophic scar versus normal dermal fibroblasts^{16,17}. One possible explanation of our disparate findings may be that we determined morphological differences based on blinded judgments of printed images from light microscopy whereas other studies have reported on differences found in light and electron microscopy under potentially unblinded conditions.

Following tissue damage and the subsequent inflammatory reaction, activated fibroblasts begin to migrate, proliferate, and differentiate into subpopulations. Myofibroblasts, which express α -SMA, are considered a contractile activated fibroblast, and are mainly responsible for scar contraction and excessive ECM deposition. By ICC and western blot, sVFF showed greater expression of α -SMA compared with nVFF, indicating that a proportion of the sVFF we studied were phenotypically myofibroblasts. While some nVFF displayed myofibroblast phenotype via expression of α -SMA, this has been shown to be a normal finding during cell cycle progression and differentiation of fibroblasts in culture^{18,19}. Similar investigations of IPF fibroblast phenotype relative to fibroblasts isolated from normal lung tissue using confocal microscopy and flow cytometry have revealed greater proportions of α -SMA-positive IPF fibroblasts compared to normal lung fibroblasts, suggesting that the majority of IPF fibroblasts were myofibroblasts^{14,20}. In a ferret model, Kumai et al.²¹ measured α -SMA-positive nVFF and sVFF in monoculture by flow cytometry and immunohistochemistry and also reported significantly greater α -SMA expression in sVFF. While it is generally believed that myofibroblasts undergo apoptosis after epithelialization occurs, it has been suggested that this mechanism fails to work in certain fibrotic diseases (e.g., liver cirrhosis) and in keloids or hypertrophic scars leading to persistence of myofibroblasts and the expansion of the extracellular matrix^{22,23}. It is possible that a similar mechanism is responsible for vocal fold scarring in humans.

Results of a growth and proliferation assay demonstrated that sVFF grew significantly slower than nVFF. Our concurrent finding that sVFF may phenotypically be myofibroblasts supports this finding. Ramos et al.²⁰ observed a marked decrease of growth rate in

fibroblasts derived from lung tissue of patients with IPF when compared with lung fibroblasts derived from normal subjects at passages 5-8. Based on the theory that myofibroblast differentiation *in vitro* results in reduced telomerase activity, they speculated that decreased expression of telomerase may have accounted for the reduced proliferative capacity of IPF-derived fibroblasts. We did not measure telomerase expression in the current study, however, this theory warrants further investigation. In contradiction, comparing nVFF and sVFF from ferrets, Kumai et al.¹⁵ observed faster proliferation of sVFF relative to nVFF in monoculture conditions. Others have found no difference in growth kinetics between scar-derived and normal dermal fibroblasts under standard culture conditions *in vitro*^{24,25}. These disparate findings likely reflect differences in methodologies employed for the various studies, including tissue origin, cell passage number, and growth medium.

The capacity of fibroblasts to organize and contract collagen by tractional structuring is most important in early wound healing to facilitate rapid epithelialization. The fibroblast-collagen-matrix contraction model is a way to examine mechanisms that contribute to wound healing, as the mechanical forces generated by fibroblasts that lead to wound contraction can be large enough to cause considerable scarring. The contraction assay used in our study involved a two day period of mechanical loading, whereby isometric tension developed. Within this paradigm, cells have been found to form large extensions and stress fibers that collapse and disappear when matrices are released²⁶. Upon release of the collagen matrices in our study, both nVFF- and sVFF-populated matrices contracted; however, there was no difference in contraction between the two cell types. A similar finding has been reported within the dermal literature with comparison of burn hypertrophic scar and normal skin fibroblasts²⁷. Data from matrix metalloproteinase 3 (MMP3)-deficient mice has shown that MMP3 is critical for initiating wound contraction in the early stages of wound-healing²⁸. We found that MMP3 was significantly upregulated in sVFF, although this finding does not explain the absence of differential contraction seen between sVFF and nVFF. Boemi et al.²⁹ characterized differences between scar and dermal cultured fibroblasts at passages 3-8 and found more α -SMA within cytoplasmic stress fibers, reduced expression of the collagen integrin receptor α -2, and reduced collagen contraction in scar fibroblasts relative to dermal fibroblasts. They speculated that a decreased level of α -2 integrin, a collagen-binding cell surface receptor, was responsible for their finding of reduced lattice contraction in scar fibroblasts. We also found that the genes encoding several integrin subunits (ITGA2, ITGA3, ITGB3, and ITGB4) were underexpressed in sVFF, suggesting that cell-cell and cell-matrix interaction and communication may have been compromised in the *in vivo* scar environment. It has been shown that dermal fibroblasts during the early stages of wound-healing express mainly α -2 and α -5 subunits of integrins associated with an upregulation of the β -1 subunit³⁰. In spite of genotypic differences in MMP3 and integrin subunit α -2, sVFF and nVFF demonstrated similar contractive properties, suggesting that there are other mechanisms responsible for this finding.

Matrix metalloproteinases (MMPs) are involved in the breakdown of ECM in tissue remodeling and disease processes. We determined that the gene encoding MMP 3 was significantly upregulated for sVFF versus nVFF. MMP 3, or stromelysin, degrades fibronectin, laminin, and collagens III, IV, IX, and X. Although normal dermal scars *in vivo* contain little message and subsequent protein for fibronectin, it has been reported that cultured fibroblasts derived from these scars have fibronectin messenger RNA and rates of biosynthesis *in vitro* similar to those of keloid fibroblasts³¹. In both cases, fibronectin biosynthesis is significantly greater than found in normal dermal tissue, indicating that the fibronectin regulatory pathway in scar fibroblasts is likely influenced by the tissue environment. Overall, our finding of upregulation of MMP 3 in sVFF is perplexing, as one would expect a downregulation given what has been reported in the literature; however, our contradictory results may be explained by culture conditions. In the dermal fibroblast

literature, expression of MMP 3 showed statistically significant decreases with increasing cell density, such that expression was lower in confluent compared with preconfluent cells and in postconfluent versus confluent cells³². While care was taken to ensure equivalent cell counts for RNA extraction and cDNA synthesis between cell types, cells were trypsinized at 80-90% confluence, representing a pre-confluent state, which may have affected MMP3 expression. Future studies should consider confluence as a potentially relevant factor in differential gene expression with care taken to ensure equal confluence across comparison cell types.

Several limitations in the present investigation related to both methodology and analysis warrant discussion. First, serial passaging and freeze-thaw cycles endured by both nVFF and sVFF may have impacted phenotype, as it has been demonstrated that vascular smooth muscle cells in culture show disassembly of actin stress fibers after passage 3 and a reduction in α -SMA protein expression after freezing-thawing³³. Further, vocal fold fibroblasts have been found to undergo replicative senescence beyond passage 5³⁴. This study evaluated cells at passages 4-6, leading to the possibility that there may have been significant differences in collagen contraction and even more robust differences in expression of α -SMA between nVFF and sVFF at earlier passages or had the cells not been frozen.

A major limitation of the present study is our lack of replicates as we were only able to compare one biological sample of sVFF to one biological sample of nVFF. Confirmation and replication of our results with additional biological samples is necessary. Just as there is variation among normal cell lines, it is unlikely that all vocal fold scars would exhibit the same *in vitro* behavior, particularly with differences in donor age, smoking history, reflux history and etiology of the scar. For instance, it is known that for normal fibroblasts, including nVFF, proliferation is inversely related to donor age^{10,35}. Other differences in nVFF have included decreases in relative expression of fibronectin, collagen I, collagen VI, procollagen I, and elastin, as well as shifts in morphology from a typical spindle shape to wider, shorter cells at earlier passages with increasing donor age¹⁰. Histologic analysis of the normal tissue and vocal fold scar used in this study may have revealed *in vivo* characteristics of these tissues, such as collagen composition and fiber alignment, potentially lending support to our *in vitro* findings. Given the qualitative ICC and western blot findings of elevated α -SMA in sVFF relative to nVFF, it would be beneficial to quantify these findings using flow cytometry to determine specific proportions of α -SMA-expressing myofibroblasts for both cell types. For this reason, further investigation comparing primary sVFF to myofibroblasts is also warranted.

While the data described herein are interesting, the real value of performing such *in vitro* experiments is not to describe the basal phenotype, but rather to investigate mechanistic complexities underlying this phenotype, which cannot be assayed *in vivo*. Therefore, future studies will aim to examine the effects of various molecules on sVFF *in vitro*, including cytokines, chemokines, and growth factors, with the goal of expanding the scope of current human models of vocal fold scarring. Assessing cellular changes under vibratory conditions may help to illuminate genotypic and phenotypic responses associated with the types of mechanical forces present within the laryngeal microenvironment. This model may provide a better approximation of the *in vivo* environment from which these cells were isolated.

CONCLUSIONS

In comparing a single sample of fibroblasts derived from vocal fold scar to fibroblasts derived from normal tissue, this study provides the first data to suggest that human sVFF and nVFF display differences in proliferative capacity, α -SMA expression levels, and gene

expression. Results also indicate no difference in morphology or contractile properties. Given visually significant differences in tissue phenotype observed with videostroboscopy, the inherent *in vitro* differences reported herein were not as pronounced as had been expected. In all likelihood, this relates to the complexities of the *in vivo* environment, such that *in vitro* models can be viewed as approximations of what actually occurs in the living organism. Additional biological replicates are necessary to further characterize the *in vitro* properties of human vocal fold scar; however, these preliminary data will inform future *in vitro* studies investigating the use of fibroblasts as a therapeutic target in the treatment of vocal fold scarring.

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FIGURE 1. nVFF (A) and sVFF (B) at passage 5. Both cell types exhibit spindle shape typical of fibroblasts (40X magnification). No differences can be noted between nVFF and sVFF.

FIGURE 2.

Growth curves of nVFF and sVFF at passage 5. Means and standard deviations are the results for four replicated. Statistical significance is observed between groups on days 3-6 (Day 3: $p = 0.0138$, Days 4,5 & 6: $p < .0001$) as indicated by the asterisk.

FIGURE 3.

Representative Western blots showing α-SMA expression and GAPDH for nVFF and sVFF at passages 4, 5, and 6. Graphs contain corresponding densitometric analysis of α-SMA which was normalized to GAPDH for each passage. Significance in upregulation was observed at passages 4 and 5 for sVFF ($p = 0.0006$ and $p = 0.0015$ respectively).

FIGURE 4.

IHC images showing α -SMA expression in passage 5 nVFF (A) and sVFF (B) at 200x magnification. Green is indicative of α -SMA expression in representative cells whereas blue (DAPI) highlights the nucleus of the cell. sVFF exhibited greater expression of α -SMA than nVFF.

FIGURE 5.

Contraction of fibroblast-populated collagen lattice with no cells (control), sVFF and nVFF. Each lattice (three replicates for each condition) was measured upon initial release of the collagen matrix, at 24 and 48 hours. Each point on the graph represents the average and standard deviation for three geometrical areas measured at each time point. Difference in contraction between sVFF and nVFF was not significant ($p = 0.70$, $p = 0.79$, $p = 0.58$) for any of the time points measured.

Table 1

Significant differential gene expression between sVFF and nVFF

Symbol	Description	T-TEST p-value	Fold Up- or Down- Regulation
CLEC3B	C-type lectin domain family 3, member B	0.003577	−6.35
COL11A1	Collagen, type XI, alpha 1	0.007822	−40.71
ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	0.007131	−9.05
ITGA3	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	0.005330	−8.16
ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	0.002898	−13.98
ITGB4	Integrin, beta 4	0.002550	−3.37
LAMA1	Laminin, alpha 1	0.000055	65.52
MMP10	Matrix metalloproteinase 10 (stromelysin 2)	0.000191	−6.57
MMP3	Matrix metalloproteinase 3 (stromelysin 1, progelatinase)	0.000020	11.62
NCAM1	Neural cell adhesion molecule 1	0.001942	−87.11
PECAM1	Platelet/endothelial cell adhesion molecule	0.000927	−5.65
THBS1	Thrombospondin 1	0.008209	4.04
THBS2	Thrombospondin 2	0.005910	13.19
TIMP1	TIMP metalloproteinase inhibitor 1	0.007131	−2.46
TNC	Tenascin C	0.002945	−2.66