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Evidence for Heterozygous Abnormalities of the Elastin Gene (*ELN*) Affecting the Quantity of Vocal Fold Elastic Fibers: A Pilot Study

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Summary

Objectives—To investigate the effects of a heterozygous elastin gene (*Eln*) abnormality (deletion of one *Eln* allele) on the structural characteristics of the vocal fold lamina propria using a mouse model of human disease.

Study Design—Cross-sectional between-subjects design.

Methods—Five mice, four with heterozygous *Eln* deletions (*Eln* +/-) serving as an animal model for the human disease supravalvular aortic stenosis and one normal wild-type control (*Eln* +/+) were used for this study. Vocal folds were obtained from each animal and stained for the protein elastin using histochemical methods. Descriptive data from qualitative visual inspection and quantitative data from microscopic digital image analysis were collected to determine the staining density of elastic fibers within the vocal fold lamina propria.

Results—Qualitative visual inspection revealed greater staining density (eg, a greater quantity) for elastic fibers in the *Eln* +/- animal. Quantitative measurements using digital pixel analysis of staining density revealed significant differences between mice with the two genotypes, confirming the qualitative findings.

Conclusions—Results suggest that *Eln* requires two functioning alleles for normal structural development of the vocal fold lamina propria. This pilot evidence supports the hypothesis of a structural etiology causing altered vocal function in humans with a similar genotype.

Keywords

Genes; Penetics; Voice; Larynx; Elastin; Elastic fibers

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This animal protocol was approved by the Institutional Animal Care and Use Committees of Texas Christian University and Washington University School of Medicine.

INTRODUCTION

Elastic fibers are part of the connective tissue scaffold that forms the vocal folds and elastic cartilage (epiglottis) of the larynx.¹ Together with collagen fibers they form the vocal ligament.²⁻⁴ It is believed that elastic fibers, as part of the vocal ligament, support tensile strain (longitudinal displacement) during vocal fold elongation, influence elastic recoil during phonation, and act in concert with collagen to provide a stiffness gradient (a transition area characterized by increasing viscosity from superficial to deep vocal fold sections) between the loose vocal fold cover and the rigid vocal fold body.^{2,5,6} In these roles, elastic fibers are responsible for both structural and biomechanical properties of the vocal fold tissue. A number of theories have been advanced to explain the role of the vocal ligament and the elastic fibers helping to form it; namely giving structural support to the vocal fold, allowing for elongation, limiting the extent of elongation, and supporting tensile stresses.^{1,2,4-7}

Most of the mature elastic fiber is composed of the protein elastin, whose production via fibroblasts is controlled by one specific gene, *ELN*, located in humans on chromosome 7 at the location q11.2 (in humans, *ELN* is denoted by upper case letters with italics; in mice, the gene is denoted *Eln* with italics but only the first letter capitalized). The remainder of the fiber is formed by microfibrillar proteins. Inherited heterozygous deletions, translocations, and mutations of *ELN* (any condition resulting in a heterozygous *ELN* abnormality will hence be denoted as *ELN*+/- or *Eln*+/- in this article) are known to cause the human disease supravalvular aortic stenosis (SVAS). This disease affects elastic fiber structure and function in various connective tissues of the body, such as blood vessel walls and skin. A related condition, Williams syndrome (WS), shares a genotypic trait with SVAS in that most individuals with WS have a heterozygous *ELN* abnormality. The published literature is consistent in anecdotally reporting hoarseness as a phenotypic characteristic of *ELN*+/- conditions, suggesting a possible link between vocal fold structural abnormality and altered biomechanical function.⁸⁻¹²

To date, only one study, reporting on vocal fold histology from a single child with WS, has provided evidence of vocal fold elastic fiber structural irregularities in *ELN*+/- conditions.⁸ Recently, however, we reported on a group of individuals with SVAS and WS who exhibited differences in vocal fold biomechanics, as measured indirectly via acoustics, compared with a normal control group.¹³ Subsequent auditory perceptual analyses confirmed the presence of altered voice quality in individuals with the *ELN*+/- genotype.¹⁴ However, because of the paucity of objective and subjective reports of the vocal fold structure in *ELN*+/- conditions, at the present time it is difficult to validly relate function (eg, altered voice quality) to structure (eg, altered vocal fold elastic fiber characteristics) as we know very little about the characteristics of the vocal fold lamina propria in *ELN*+/- conditions. The difficulty related to obtaining vocal fold tissue in living humans has led us and other authors to seek out animal models to better understand diseases that effect vocal fold structure and function.

Recent studies have validated the mouse as a viable animal model for translational investigations of vocal fold cartilaginous and muscular structures.¹⁵⁻¹⁷ Abdelkafy et al¹⁸ recently published findings demonstrating similarities between the mouse and human vocal folds with relation to the distribution of collagen and hyaluronic acid, and the changes that take place in these substances during aging. As in human vocal folds, collagen fibers were present in young and old mice. Furthermore, similar patterns with respect to aging of collagen fibers were found. More recently, Thomas et al¹⁶ published findings demonstrating similarities between laryngeal cartilaginous structure in the mouse and human. In addition, we have recently confirmed the presence and identified the location of elastic fibers within

the vocal fold lamina propria of mice, demonstrating this animal model as viable for investigating genetic connective tissue disorders that influence vocal production, such as SVAS.¹⁹ Further studies are needed to determine if this animal model holds the potential for testing theories of biomechanical function of the vocal fold extracellular matrix proteins.

It has been confirmed that the vocal folds of wild-type mice homozygous for *Eln* (*Eln* +/+) have elastic fibers within the vocal fold lamina propria.¹⁹ Whether altered elastic fiber quantity and structure are characteristic of the vocal folds in *Eln* +/- conditions, and whether this condition influences macroscopic vocal fold structure and/or laryngeal development remains to be elucidated more clearly. Knowledge of these mechanisms would further our understanding of vocal fold physiological processes, which might facilitate translational application (eg, from the laboratory bench to clinical application). *Eln* +/- mice have been developed to study the effects of elastin haploinsufficiency in an animal model.²⁰ The purpose of this preliminary study was to use *Eln* +/- mice to investigate the effect of this genotype on vocal fold lamina propria structure by measuring the staining density of elastic fibers in *Eln* +/- and *Eln* +/+ mice. In addition to potentially providing information on the influence of a specific gene on vocal fold structure, findings from this study hold potential to establish a connection between vocal fold structure and biomechanical function within a specific genetic abnormality that occurs in humans. The research question addressed was: Are levels of elastin within the vocal folds of *Eln* +/- mice different than those of an *Eln* +/+ control? Based on findings from published work showing that the extracellular matrix in blood vessel walls are affected by this genotype, our hypothesis was that the density of elastic fibers stained in *Eln* +/- mice would be less than that of the *Eln* +/+ control.

METHODS

Animals

As this was a preliminary pilot study conducted without the benefit of previous reports of vocal fold elastic fibers in mice with the targeted genotype, a small sample size was used. A total of five mice were used for this study. All mice were male and were obtained from the laboratory breeding colony of genetically altered *Eln* knockout mice of the second author (RPM) at Washington University in St. Louis. The procedures used in the initial establishment of this line have been previously reported, using mice from the C57Bl/6 background.²⁰ Four of these mice were genotyped and confirmed as *Eln* +/- (heterozygous), whereas the fifth mouse was genotyped as *Eln* +/+ (homozygous). The study used mice across the mature age range, as age was not a controlled factor in this experiment. This was in part because of the unavailability of multiple mice in the same age range during the time frame of the study and presented a study limitation. Two of the *Eln* +/- mice were mature (eg, sexually mature adult) young animals aged 22 weeks, whereas two of the *Eln* +/- and one *Eln* +/+ were mature older animals, as well as littermates, aged 77 weeks.

Procedures

All procedures were approved by an Institutional Animal Care and Use Committee at Texas Christian University and Washington University in St. Louis. Animals were sacrificed humanely. Laryngeal tissue was harvested immediately after sacrifice and fixed in a solution of 10% buffered formalin. After fixation, laryngeal tissue was dehydrated in graded concentrations of ethanol, rinsed with xylene, and then immersed in paraffin for embedding. Tissue was then sectioned into six micron-thick segments using a rotary microtome, cut in a coronal plane from anterior to posterior exposing cross-sections of the vocal fold tissue. Tissue segments were mounted on standard slides, and the paraffin was removed from the sections using ethanol and xylene. Five consecutive vocal fold segments from each animal were used for subsequent analyses.

Histochemical methods were used to identify specific constituent elements of the vocal fold extracellular matrix, specifically the protein elastin. Identification of elastin was accomplished using a Hart's elastin stain on the five vocal fold segments from each mouse larynx. In this protocol, elastic fibers stained black, whereas muscle and epithelium stained yellow. Digital image processing was used to capture still images of vocal fold sections for subsequent analysis. An Olympus CX31 microscope (Olympus Imaging America, Center Valley, PA) and Lumenera Infinity 2.0 (Lumenera Corp., Ottawa, Ontario, Canada) digital microscope camera were used to obtain 10× images of the five consecutive vocal fold sections from each animal.

Analyses

Microscopic visual inspection of the slides was performed to identify the margins of the membranous vocal fold for qualitative visual analysis and quantitative digital image measurement. Qualitative descriptive analysis focused on elastic fiber distribution patterns within the vocal fold and the overall morphology of the vocal fold tissue. For each vocal fold section (five sections per animal), quantitative analysis measured the staining density of elastic fibers contained within the vocal fold lamina propria. This was accomplished using two software packages: *Adobe Photoshop CS4* (Adobe Systems, San Jose, CA) and *Image J* (National Institutes of Health, Bethesda, MD, <http://rsb.info.nih.gov/ij/>). Photoshop was used to crop each image to a window of 223×349 pixels, which contained the superior to inferior vocal fold margins, epithelial vocal fold cover, lamina propria, and thyroarytenoid muscle (see Figure 1). This process resulted in 25 vocal fold images, where each contained the same vocal fold section and 77,827 total pixels. *Image J* was then used to convert the color image to red-green-blue stack (see Figure 2—shown in grayscale). This conversion created a binary image (elastin-stained pixels shown in red, others in white), where the threshold for coloring a pixel red or white is set by manual adjustment of two sliders. For this study, threshold adjustment was normalized for all 25 images by setting the white threshold slider (upper slider) to the “0” position and the red threshold slider (lower slider) to the “164” position before measurement. These threshold settings resulted in elastic fibers being highlighted red (see Figure 3—shown in grayscale, where elastic fibers are black), whereas other tissue or spaces were colored white (white to light gray in Figure 3). Using the particle analysis function of *Image J*, the area fraction (AF) was then measured, which represented the percentage of pixels in the image that were highlighted red (eg, a ratio of total red pixels, representing elastin, to total pixels, representing all tissue and space within the image).

RESULTS

Figures 4–6 illustrate representative examples of stained vocal folds from the wild-type *Eln* +/+, younger, and older *Eln* +/- mice, respectively. Qualitative analysis using visual inspection of the slides revealed the appearance of greater staining (ie, more abundant) of elastin within the vocal folds of the *Eln* +/+ mouse compared with the *Eln* +/- mice. Table 1 shows the mean and standard deviation of the AF measures for the *Eln* +/+ mouse and the pooled data for the *Eln* +/- mice. AF measurements were greater in the *Eln* +/+ animal, suggesting greater density of elastic fibers stained in the vocal fold of that animal compared with the *Eln* +/- mice. The confidence intervals of the two measures did not overlap, suggesting a significant difference. Figure 7 shows a box and whisker plot of the data from the groups, illustrating the difference between the AF measures and the location of the respective medians (dark horizontal line within each box).

Because of the small sample size and unequal group sizes, a nonparametric Wilcoxon rank-sum test was applied to the AF data to test for differences between the two groups (+/+ and +/-). The Wilcoxon rank-sum test is a nonparametric alternative to a *t* test and assesses

whether two samples come from the same distribution (the null hypothesis) using ranks. It is valid for small sample sizes with uneven group sizes.²¹ Results revealed a significant difference (Wilcoxon $W=210.0$, $P=0.001$) between the AF measures for the *Eln* $+/+$ and *Eln* $+/-$ animals, indicating that elastic fibers were stained with significantly greater density within the vocal folds of the *Eln* $+/+$ mouse compared with the *Eln* $+/-$ mice.

DISCUSSION

The purpose of this study was to investigate the effect of an abnormal heterozygous *Eln* genotype (*Eln* $+/-$) on the staining density of vocal fold elastin and the morphological structure of the vocal folds by comparing the vocal fold tissue of *Eln* $+/-$ and *Eln* $+/+$ mice. *Eln* $+/-$ mice served as an animal model of the human disease SVAS, which shares the same genotype. Although this was a small pilot study, results revealed significant differences in the staining density of elastic fibers between these two genotypes. Specifically, the *Eln* $+/+$ mouse manifested significantly more vocal fold elastin within the lamina propria compared with the *Eln* $+/-$ mice (eg, compare Figure 4 to Figure 6).

These findings are supportive of a previous study that used qualitative analyses of elastic fiber quantity and structure in the vocal fold of one human with a related genotype resulting in WS.⁸ Additionally, the results of this study provide evidence for a structural etiology of altered vocal characteristics in humans with *ELN* $+/-$ genotypes, including those with nonsyndromic SVAS and WS. We have previously reported on altered acoustic profiles and auditory-perceptual characteristics of individuals with *ELN* $+/-$ genotypes compared with control groups.^{13,14} In humans, this genotype can cause a rough voice quality and, in SVAS, an abnormally low fundamental frequency in adult males. We have previously put forth the hypothesis that altered vocal function in this genotype was because of an abnormal quantity and possibly structure of elastic fibers within the vocal fold lamina propria, which the present study supports.

Additional reports of connective tissue disorders in humans with an *ELN* $+/-$ genotype also support the results found in the animal model used for this study. A number of abnormalities in elastin distribution and structure have been reported in tissue other than vocal folds. These include hypercellular growth in the blood vessel walls, premature aging of the skin, and joint laxity.^{22,23} Histological investigation of the skin in WS has revealed reduced elastin distribution and abnormal elastin structure, together resulting in subtle changes in skin texture and pliability.²⁴ Similar findings relative to elastin distribution have been shown in the arterial walls.²⁵

Further understanding of the role of *ELN* and more thorough understanding of the roles and epigenetic influences of vocal fold elastic fibers may have significant clinical and basic science implications. Heritable connective tissue disorders caused by *ELN* $+/-$ abnormality (eg, SVAS and WS) hold the potential to provide unique and important insights into the epigenetic activities that influence vocal fold development, physiology, and biomechanics. It has been demonstrated that this genotype negatively impacts the formation of mature elastic fibers in humans and mice. As such, it allows for the selected alteration of a single gene and gene product for studying the interactive effect on the surrounding interstitial tissue environment, as well as formation of vocal fold elastic fibers themselves. During normal development and maintenance of vocal fold tissue, the characteristics of these interactive relationships are currently unknown. Knowledge of these interactions would be beneficial not only for foundational knowledge of vocal fold physiology but also holds translational potential for clinical application, as genetic therapies are already being applied to animal models in an attempt to influence the expression of genes within vocal fold tissue. The information gathered from this research also holds prospective value for broader areas of

voice science. These include information that might increase our understanding of the genes and epigenetic forces involved in vocal function and/or vocal traits, and how gene products interact with each other during laryngeal maturation, tissue maintenance, and injury.

LIMITATIONS OF THE STUDY

Although this study found significant differences in the staining density of elastin within the vocal folds of *Eln* +/- and *Eln* ++ mice, a number of methodological limitations necessitate guarded generalizations being drawn from the study. First, as this was a pilot study, a small sample was used so that the reported data are from five animals only, with one wild-type as a control (unequal groups). Additionally, the *Eln* +/- mice were from two age ranges (younger mature and older mature), and it is not clear at this point if the *Eln* +/- genotype results in aging effects on vocal fold elastic fibers. Larger and more complex studies will be needed to validate the preliminary findings reported in this investigation. Furthermore, the findings from this study relate only to vocal fold proteomic structure and not vocal fold biomechanics in the mouse, as biomechanics of the mouse vocal folds have not been studied. In addition, there is little evidence that all sounds (eg, ultrasonic high-frequency sounds) emitted by mice are produced by their vocal folds. Thus, at this stage, we do not know if the mouse is an adequate model for studying vocal fold biomechanics, although the unique *Eln* +/- mouse may present the opportunity to investigate the above questions.

SUMMARY

This small pilot study found that mice with *Eln* +/- genotypes manifested significantly less elastin in the vocal fold lamina propria compared with an *Eln* ++ mouse. These findings are consistent with altered elastic fiber concentrations in other connective tissues of humans with an *ELN* +/- genotype and support the *Eln* +/- mouse as a viable animal model to study vocal consequences of a specific genotype in humans (SVAS, WS) and potentially study genetic influences on vocal fold development and physiology. Larger studies will need to be conducted in the future to further validate the findings from the present study.

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FIGURE 1.
Original 10× color image of mouse vocal fold showing elastin stained black with muscle yellow, via Hart's elastin stain.

FIGURE 2.
Same image converted to red-green-blue stack to allow adjustment of threshold for isolation of elastic fibers.

FIGURE 3.
Same image with red threshold adjusted to isolate elastic fibers for measurement of AF
(percentage of stained pixels).

FIGURE 4. Representative example of a vocal fold section from a homozygous (*Eln* $+/+$) wild-type mouse. Elastin stained black. 10 $\times$ image magnification.

FIGURE 5. Representative example of a vocal fold section from a younger heterozygous (*Eln*^{+/-}) mouse. Elastin stained black. 10× image magnification.

FIGURE 6. Representative example of a vocal fold section from an older heterozygous (*Eln*^{+/-}) mouse. Elastin stained black. 10× image magnification.

FIGURE 7.

Box and Whisker plot showing median (dark horizontal bar) and interquartile range (box edges) of the data from the *Eln* +/- and *Eln* +/- mice.

TABLE 1AFs (Percentage of Stained Pixels), SDs, and 95% CIs for the $+/+$ Animal and $+/-$ Animals

Group	Mean AF, %	SD	95% CI
Eln $+/-$	3.22	0.72	2.88%–3.56%
Eln $+/+$	6.32	1.91	3.95%–8.69%

Abbreviations: SD, standard deviation; CI, confidence interval.