The Laryngoscope
Lippincott Williams & Wilkins, Inc.

CANDIDATE'S THESIS

Development and Maturation of the Pediatric Human Vocal Fold Lamina Propria

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Objective: **To identify characteristic patterns of maturation of the human vocal fold lamina propria as it develops into a mature structure.** ***Methods:*** **Histologic evaluation of sectioned true vocal folds from 34 archived larynges ages 0 to 18 years using hematoxylin-eosin, trichrome, Alcian blue pH 2.5, Weigert reticular, and Miller's elastin stain.** ***Location:*** **Pathology department at a tertiary care children's hospital.** ***Results:*** **At birth and shortly thereafter, there exists a relative hypercellular monolayer of cells throughout the lamina propria. By 2 months of age, there are the first signs of differentiation into a bilaminar structure of distinct cellular population densities. Between 11 months and 5 years, two distinct patterns are seen: 1) this bilaminar structure and 2) a lamina propria where there exists a third more hypocellular region immediately adjacent to the vocalis muscle (this region is similar to the superficial hypocellular region found just deep to the surface epithelium). By 7 years of age, all of the specimens exhibit this transition between the middle and the deeper layers according to differential density of cell populations. A lamina propria structure defined by differential fiber composition (elastin and collagen fibers) is not present until 13 years of age and then is present throughout adolescence.** ***Conclusions:*** **Using the classic adult model of fiber composition and density to differentiate the layered structure of the lamina propria of the human vocal fold may not adequately allow for a thorough description of the process of maturation and development. Rather, distinct regions of cell density are seen as early as 2 months postpartum, and the model of cellular distribution may serve better to describe the lamina propria as it develops. Cell-signaling processes that shape the formation of the lamina propria appear to produce layered populations of differential cell density that in turn will later produce differential fiber compositions. Early development therefore can be followed by evaluating the maturation of these differing cell populations. Future studies are needed to quantify these cell distribution patterns, to study the cell signaling processes that trigger this maturation, and to correlate these findings with mechanical modeling.**
Key Words: **Vocal cord, lamina propria, development, larynx.**

Laryngoscope, **115:4–15, 2005**

INTRODUCTION

Speech has played an essential role in the development of human civilization because it enabled (and continues to enable) communication and the verbal exchange of ideas. The field of laryngology arose as a product of research and thought from many diverse cultures as they sought to understand how exactly the human species was able to convert audible sound into complex forms of communication. An excellent review of the ancient history and the roots of laryngology is to be found in the chapter entitled "The Discipline of Voice: A Historical Perspective" by Drs. Donald S. Cooper and Hans Von Leden.[1] They describe the Chinese belief at least 2 millennia ago that exhaled air from the lungs during speech caused motion in the hyoid bone, the thyroid cartilage, and the tongue. As early as the fifth century BC in Greece, we find a clear understanding of the necessity of and the relationship between airflow and speech. Hippocrates (circa 460–370 BC) writes in the Peri Sarkon: "I have previously seen some who, trying to commit suicide, indeed do cut their throat, but nevertheless do live, but say nothing, unless someone grasps their throat."[2] Aristotle (circa 384–322 BC) furthered this thought by writing of the essential character of voice production as a mechanical interaction

Presented as a Candidate's Thesis (C.J.H.) to the American Laryngological, Rhinological and Otological Society.

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Editor's Note: This Manuscript was accepted for publication June 3, 2004.

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DOI: 10.1097/01.mlg.0000150685.54893.e9

between the airstream and the structures enclosing the airstream. Galen of Pergamon (circa 131–201 AD) looked closely at animal dissections of the airway and described the "membranous lips" of the vocal folds and the recurrent laryngeal nerve. He first describes the functional relationship of larynx to voice by noting that voice cannot be produced without a narrowing of the glottal passage.

The move from animal dissections to human cadaveric dissections would be critical to furthering the field of laryngology. This transition was slowed by Moslem and Christian tradition forbidding cadaveric dissection. With regards to the Christian custom, such prohibitions were enacted in part as a Papal response to the custom during the Crusades of boiling the bones of the fallen warriors so that they could be more easily transported home for burial. To stem this practice, Pope Boniface VIII issued a Papal Bull, *De Sepulturis*, forbidding such handling of corpses.[3] Perhaps incorrectly, this edict was interpreted to apply to medical dissections as well, and it was not until Monino dei Luzzi (1270–1326) published his seminal work entitled *Anatomy* from the medical school in Bologna, Italy that cadaveric dissections were performed.

Leonardo da Vinci (circa 1452–1519) used his incredible range of interests and the span of his knowledge of both medicine and hydraulics to the field of laryngology to produce some of the most accurate anatomic drawings of the human larynx, as well as to comment on the aerodynamically crucial character of the narrowing of the airway of the glottis for speech and song (Figs. 1 and 2). He writes "and this remains proved by what I have proved before, that the pipes of an organ are not made deeper or higher in pitch by mutation of the fistula (that is, the place where the voice is produced) in making it wider or narrower, but only by the mutation of the pipe to wide or narrow, or to long or short, as is seen in the extension or retraction of the trombone and also in pipes of fixed width or length where the sound is varied by introducing air with greater or lesser force."[4]

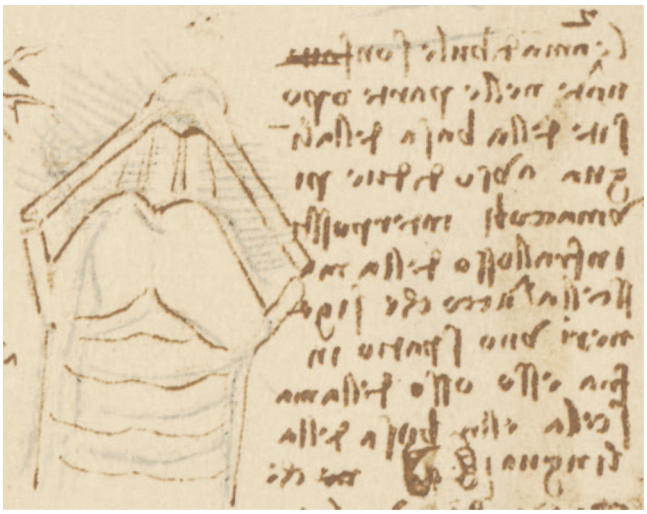

Fig. 1. Leonardo da Vinci's drawing of the human neck, laryx, and glottis, as taken from the Harvard University Countway Library Collection with permission.[4]

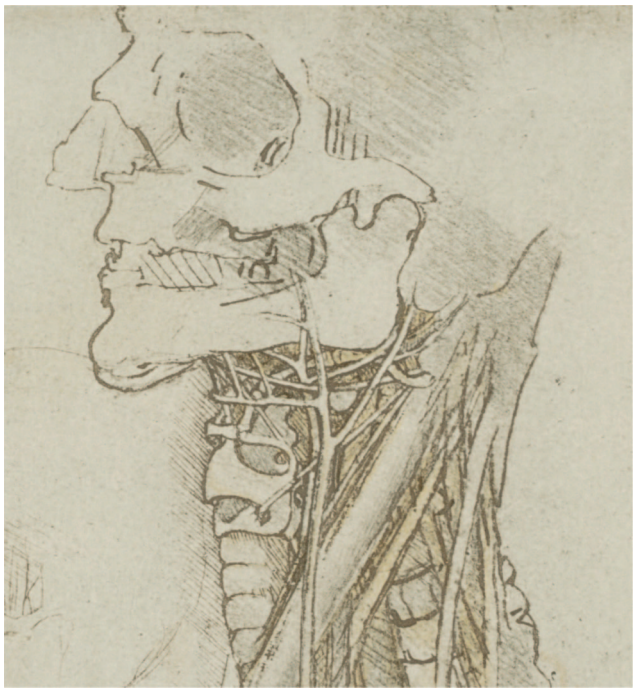

Fig. 2. Leonardo da Vinci's drawing of the human neck, laryx, and glottis, as taken from the Harvard University Countway Library Collection with permission.[4]

Although acknowledging the many contributions over the following centuries that provided meaningful insights into the structure and function of the human vocal folds, the next seminal work that moved the field of laryngology forward was produced by the French anatomist A. Ferrein (1693–1796), who provided an exhaustive work on the principles of voice production using both animal and human larynges. He conceived of the vocal folds as a vibrating string composed of several layers and described them thus: "The edge of each vocal fold is a band, the width of a line, covered with a very fine membrane." [5]

Ferrein was perhaps the first to begin to delineate a distinct, layered structure of the vocal folds, although he himself gave little mention as to how these different layers might uniquely alter mechanical properties of phonation. This "thin membrane" would be further described as a "mucus membrane" in the early 19th century by the British doctor J. Bishop, who built on Ferrein's work to further characterize the anatomy and layered structure of the vocal folds with regards to mechanical function. He writes that "the true vibratory surface of the glottis is the mucus membrane. The vocal cords confer onto it the tension, resistance, position, and probably other conditions necessary for vibration."[6] The exact line of demarcation between the mucus membrane and the vocal cords was not elucidated and would remain the subject of much debate for years to come.

Future studies of the human vocal folds would focus not only on their internal layered structure as a source of different acoustic patterning but also on the effects wrought by the extrinsic musculature of the larynx. In 1866, the French physician E. Fournie writes that "the

voice is a sound produced by a particular reed having walls modifiable under the influence of muscular action; the vibratory part being furnished by the mucous fold which limits the borders of the glottis. The vibrations are occasioned by the passage of air through the glottis. For the membranes to vibrate, one tenses them on a frame like the skin of a drum, and they will emit their vibrations transmitting them through the air. They produce a more high pitched shrill sound according to how small and taut they are."[7] What remained to be studied was how the internal micro-anatomy of the human vocal folds was designed to accommodate and to respond to these extrinsic changes in stress and subsequent physical deformation.

A turning point in the field of laryngology came with the publication of Hirano's seminal work in 1975 entitled *Phonosurgery. Basic and Clinical Investigations*.[8] Hirano and his colleagues examined the developing larynges and characterized the growth of the human vocal cord with time (Table I). Hirano also focused closely at the histologic structure of the human vocal fold and described in detail the laminar structure. Hirano divided the layers into a surface epithelial layer followed by a trilaminar lamina propria structure (superficial lamina propria [SLP], middle lamina [MLP], and deep layer of the lamina propria [DLP]).[8–10] He further divided these structures into the "cover" that included the epithelium and the SLP, the "body" that included the DLP and the vocalis muscle, and the "transition zone" that included the MLP (Fig. 3). It is important to note here that this layering was the product of anatomic description and *not* that of functional consideration. Alternative definitions of the cover and body have been used throughout the literature. For example, Hammond et al.[11] have described the cover as consisting of epithelium, the SLP, and most of the MLP, with the "body" consisting of the remainder of the MLP, the DLP, and the vocalis muscle. Dikkers[12] describes the conus elasticus as composed of dense fibrous tissue that in turn consists of collagen, the elastin fibers, the DLP, and the MLP. In his description, the body comprises the vocalis muscle and the conus elasticus, whereas the cover comprises the epithelium and the SLP.[12] Titze[13] attempted to address this controversy by describing two systems, a two- and a three-layered system. In the three-layered system, the "mucosa" consists of the epithelium and the SLP, the ligament consists of the MLP and DLP, and the muscle refers to the thyroarytenoid muscle. In a two-layered scheme, the body is defined as the DLP and the vocalis muscle, whereas the cover is defined as the epithelium, the SLP, and the MLP. Clearly, a consensus is lacking concerning the exact boundaries of the cover and the body with regards to the relationship between anatomy, biomechanics, and differential properties of viscoelasticity (Table II).

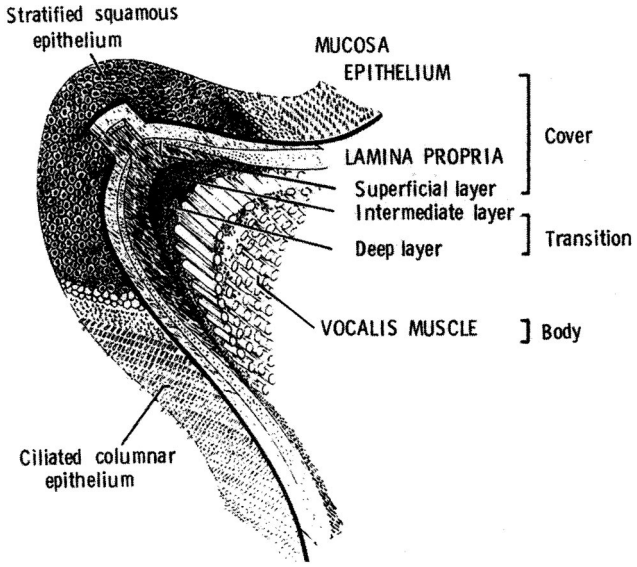

Fig. 3. Dr. M. Hirano's depiction of the layered structure of the human vocal fold. Reprinted with permission.

With regards to histopathologic examination of newborn vocal folds, Tucker et al.[14] have commented on the general similarities between neonatal epithelium and that described for the adult. They note there are distinct ciliary distribution patterns that change over time and with maturation. With regards to the lamina propria in particular, Sato et al.[15] have reported that there exists a uniform, nonlayered lamina propria with no vocal ligament. The maculae flavae (the regions at the anterior and posterior ends of the membranous vocal folds) are thought to be responsible for the development of the vocal ligament[16] and are composed of fibroblasts, ground substance, elastin fibers, reticular fibers, and collagenous fibers.[16] Hirano et al.[17] also supported the notion that there is no defined layered structure and no vocal ligament seen in the true vocal fold in the newborn. Exactly when and by what signal mechanism the layered structure develops remains to be elucidated.

Ishii et al.[18] performed scanning electron microscopy on pediatric cadaveric larynges and noted the development of superficial and deep structures by 10 years of age and a defined, layered lamina propria structure by 17 years of age. Ishii et al. noted that there was no discernible SLP at ages 3 or 5 but that a SLP structure was identified

TABLE I.
Dr. M. Hirano's Characterization of the Development of the Human Vocal Cord in Terms of the Length of theMembranous fold (M), the Cartilaginous fold (C), and the Ratio of the Two (M/C).[17]

Age	Overall Length Vocal Fold (mm)	Membranous Vocal Fold (mm)	Cartilaginous Vocal Fold (mm)	M/C
Newborn	2.5–3.0	1.3–2.0	1.0–1.4	1.1–1.8
Adult males	17–21	14.5–18	2.5–3.5	4.7–6.2
Adult females	11–15	8.5–12	2.0–3.0	3.3–4.5

TABLE II.
Different Systems of Defining the Cover/Body of the Lamina Propria of the Vocal Fold

	Cover	Body	Transition
Hirano[A9,10,19]	Epithelium, SLP	Vocalis muscle	MLP, DLP
Titze[A13]	Epithelium, SLP, MLP	DLP, Vocalis muscle	
Hammond[A11]	Epithelium, SLP, MLP	MLP, DLP, Vocalis muscle	
Dikkers[A12]	Epithelium, SLP	Conus elasticus, Vocalis muscle	

Varying descriptions and definitions of the layered structure of the human vocal fold as seen in thepublished English literature.

SLP = superficial layer of the lamina propria; MLP = middle layer of thelamina propria; DLP = deep layer of the lamina propria.

by age 12. Here again, there were an abundance of young larynges but a paucity of adolescent larynges to document the process of maturation (there were no laryngeal specimens from children ages 5–12 years). The authors note the previously established theory that the development of the vocal fold occurs late in comparison with other laryngeal structures.[19] They write that the period between 5 and 10 years of age corresponds to a "period of pediatric hoarseness" where there still exists an immature lamina propria structure; however, there are no larynges studied within this age group either to document this supposition or to characterize when the SLP as an identifiable structure develops. Gray et al.[20] have also studied the development of the human vocal fold and note that the density of collagen and elastin fibers varies with age, whereas the density of collagen fibers does not. However, the pediatric specimens were obtained from the ages of 3 to 8 months, and the remaining larynges represented the adult and geriatric population. There has also been some preliminary work regarding the role that hormones play in the development of the human vocal fold; the presence of estrogen and progesterone receptors has been identified.[21] Here, as elsewhere, the majority of specimens examined were adult and geriatric larynges, and the focus was on the process of aging rather than that of maturation. The exact timing of this development and maturation remains to be carefully elucidated (Table III), and this is the focus of the current research study.

MATERIALS AND METHODS

Case Selection

Laryngeal tissue was obtained from autopsy cases in which permission had been given for examination purposes and after Institutional review board approval had been granted. Thirty-four archived larynges from autopsies performed from 1975 to 2003 were examined. The cases ranged in age from newborn to 18 years. When possible, at least two cases from each sex for each of these age groups were examined. The cases were selected after a review of the clinical history and autopsy diagnosis. Pertinent data including postmortem interval and history of prolonged intubation was noted before selection of cases. Patients who had been intubated for more than 24 hours were excluded from this study.

Tissue Submission

These archival laryngeal (vocal cord) tissues were divided into the right and left halves, where possible. Larynges that were already sectioned as part of the earlier autopsy examination and lacked anatomic completeness were not included. The vocal folds were closely examined to avoid specimens containing a mucosal abnormality (i.e., ulceration, polyp, denudation, or other abnormality). The portion of the larynx that includes the vocal folds was submitted for paraffin embedding and processing. The portion submitted included a centimeter of tissue above the vocal fold and a few centimeters below the folds. During embedding, care was taken to ensure that the tissue was embedded correctly to provide an intact and continuous vocal fold surface and underlying lamina propria and vocalis muscle, for optimal evaluation of the layers beneath the epithelium.

Slide Preparation and Staining

Glass slides were prepared and cut at 4 microns thickness. Slides were stained with hematoxylin-eosin (H&E) by the automated stainer.

Every case was stained with the following special stains:

1. H&E: the hematoxylin-metal complex acts as a basic dye, staining nucleic acids in the nucleus and the cytoplasm blue, brown, or black. Eosin is an acid aniline dye that stains the more basic proteins within cells (cytoplasm), and in extracellular spaces (collagen), pink to red. Cartilage and mucus will stain light blue.
2. Elastic (Millers elastic stain): 1% acidified potassium

TABLE III.
Development of the Superficial Lamina Propria (SLP) and the Vocal Ligament.

Study Author	Development of SLP (years)	Development of VL (years)	Development of the Trilaminar Structure (years)
Hirano[10]	Entire LP represents SLP at birth	4	Puberty
Ishii[27]	>10	3–5	>10
Sato[23]	Entire LP represents SLP at birth		

Different published theories as to the timing of the development of the human vocal ligament andmicrolayered structure of the lamina propria according to standard definitions using models ofdifferential elastin and collagen fiber deposition.

permanganate with 1% oxalic acid stained with Victoria blue 4R, new fuchsin, and crystal violet. Elastic fibers and most cell granules will stain black.
3. Alcian blue pH 2.5: used to identify glycosaminoglycans.
4. Weigert Reticular fiber stain: reticular fibers are impregnated with a silver salt and appear as sharp black. Collagenous fibers stain purple.
5. Trichrome stain: a staining sequence involving iron hematoxylin, acid fuchsin, and light green. This stain distinguishes cellular from extracellular components. Collagen fibers stain an intense green. Black or brown nuclei: mucus and ground substances take on varying shades of green. Cytoplasm stains red. Elastic fibrils, erythrocytes, and nucleoli stain pink.

RESULTS

Overall, 34 pediatric larynges were examined. (Table IV delineates the age and sex distribution for the cases examined. Table V delineates the cause of death, whether the patient was intubated and for how long, and when the specimen was obtained.

At birth and shortly thereafter, there appears to be a relatively hypercellular monolayer within the lamina propria (Fig. 4). Within this cell layer, there is tremendous activity, with an apparently single cell population haphazardly arranged. The cells exist in both spindle and plumper, rounder form. There are trace reticular as well as elastin fibers scattered throughout this monolayer.

By 2 months of age, there are already visible signs of cellular differentiation and a movement toward a bilaminar structure (Fig. 5). There is a hypocellular superficial layer followed by a deeper layer with cells that are plumper and less spindly in shape than were the cells seen within the first few days of life. The superficial hypocellular layer lacks any traceable mucin; the deeper, more cellular layer has more mucin than in the superficial layer but less than that seen in the earlier samples. This sample shows one of the tissue abnormalities seen frequently in archival tissue. There is pseudoepitheliomatous hyperplasia consistent with chronic inflammation. These changes, however, do not obscure the findings of the beginnings of a bilaminar structure.

By 11 months, a three-layered structure is beginning to be noted according to cell densities within the different layers in approximately 20% of the samples (Fig. 6). There still exists a superficial hypocellular layer just below the epithelial cover followed by a deeper, more hypercellular layer. Now, however, one can see signs of a deeper, more hypocellular region (just superficial to the vocalis muscle) reminiscent of the superficial layer in terms of type of cell population as well as in terms of cell density. (Of note, this is not representative of the classic trilaminar structure as defined by differential elastin and collagen fiber compositions within the middle and deep layers. In fact, there appear to be both elastin and collagen fibers interspersed within these layers.) The remaining 80% of the specimens continue to exhibit the characteristic pattern of a hypocellular superficial layer followed by a deeper more cellular region (Fig. 7). The relatively hypocellular superficial region approximates 50% of the LP followed by a deeper more cellular layer. Again, these cells are plumper than the cells seen in younger specimens.

By 7 years, all of the specimens examined clearly contain a three-layer lamina propria structure as defined by distinct regions of differential cell populations and cell density (Fig. 8). The cellular structure of the superficial layer remains hypocellular. The middle layer is denser in cell population; there are almost two distinct regions within this layer, a superficial layer where there is greater cellularity and a slightly deeper layer of greater collagen and elastin deposition. The deeper layer, however, returns to a less cellular layer. Although there is a distinction between middle and deep layers, it is not the classic vocal ligament that we are seeing at this stage of development. This does not appear until the early adolescence.

By the age of 11 and 12, the regions of cellular distinction have apparently matured to the point where fiber deposition has occurred and regions can be seen according to these fibers. There exists now the classic pattern of a hypocellular superficial layer followed by a middle layer of predominantly elastin fibers and a deeper layer of predominantly collagen fibers (Fig. 9). Both the middle and deep fibrillar structure is arranged in a fashion that the fibers are oriented parallel to the direction of the deeper vocalis muscle fibers themselves. This pattern continues to be visible in the older specimens up until the age of 17, which represented the oldest sample collected.

It must be noted that the number of cases reviewed in this study are small. The difficulty in obtaining pediatric larynges, and the decline in number of autopsies has unfortunately contributed to a slump in obtaining larger numbers of pediatric larynges. Another point to be emphasized is that our study of pediatric vocal cord histology is a dynamic, evolving project, and we have started staining the larynges with various immunoperoxidase markers to identify cell populations (e.g., smooth muscle actin, vimentin, CD68, etc.) and broadened our study to quantify different cell populations in the different layers in an attempt to understand the histology and the functional correlates better. The projected study will include quantification of fibroblasts, myofibroblasts, histiocytes, and density of blood vessels in each layer after immunohistochemical staining.

Although we have used formalin-fixed, archival tissue, we are not too concerned about possible deleterious effects on the tissue caused by long-term storage. Collagen and elastin have been shown to be very resistant to changes occurring with long-term storage. Montes et al.[22] have showed that collagen and elastin have been well

TABLE IV.
Age and Sex Characteristics of Archival Larynges Examined.

Case Age	Male	Female
0–2 years	4	2
3–5 years	2	3
6–8 years	2	1
9–11 years	4	1
12–14 years	4	4
15–17 years	1	4
18 and older		

TABLE V.
Description of Archival Larynges with Regards to Age, Sex, Cause of Death, Data Regarding whetherthe Child was Intubated and for How Long, and the Time of the Postmortem Autopsy (PMA).

Case	Age	Sex	Cause of Death	Intubation (yes/no/unknown)	PMA	Time of PMA (hours)
1	12 year	male	Cerebral Palsy	Yes, several hours	2001	4
2	2 day	female	Asystole	No	2001	21
3	15 year	male	Cystic fibrosis	Yes, several hours	2001	11
4	1 day	male	Cardiac anomaly	Yes, several hours	2001	45
5	15 year	female	Cerebral palsy	Yes, several hours	2002	45
6	1.5 year	male	Cardiac anomaly	No	2001	72
7	8 year	male	Neuroblastoma	Yes, several hours	1999	6
8	13 year	female	HIV	Yes, several hours	2000	60
9	12 year	male	Pulmonary failure	No	2000	5.5
10	17 year	female	SLE	No	2001	20
11	10 year	male	Seizures	No	2002	76.5
12	11 month	male	Cardiac anomaly	No	2002	48
13	3 year	male	Lymphangiomatosis	No	2002	33
14	13 year	female	Cardiac anomaly	No	2003	48
15	3 month	female	SIDS	No	1985	20
16	7 year	female	Intracranial thrombosis	No	2003	22
17	10 year	female	Seizures	No	2003	18
18	2 month	female	Liver failure	Yes, several hours	1985	5
19	10 day	male	SIDS	No	2001	11
20	5 year	female	Liver failure	No	2003	5
21	13 year	male	Hunter syndrome	Yes, several hours	1987	6
22	11 year	female	Cystic Fibrosis	Yes, several hours	1983	Unknown
23	3 year	female	Cardiac Anomaly	Yes, several hours	1986	10
24	8.5 year	male	Retinoblastoma	No	1985	10.5
25	6 year	male	Wiskott-Aldrich syndrome	Yes, several hours	1982	Unknown
26	2 year	female	Waterhouse-Friederichson syndrome	No	2003	11
27	5 year	female	Cystic fibrosis	Yes, several hours	2003	2
28	10 year	male	Cystic fibrosis	No	1975	17
29	13 year	female	Cystic fibrosis	No	1977	4
30	13 year	male	Heterotaxy	Unknown	1991	18
31	17 year	female	Heterotaxy	Unknown	1992	16
32	14 year	female	Marfan's syndrome	No	2003	38
33	17 year	male	Noonan's syndrome	Yes, several hours	2003	18
34	9 year	male	Sepsis	No	2003	40

preserved in 2000-year-old Egyptian mummies. Several studies on various different tissues have shown that the effects of long-term storage on tissue immunoreactivity (antigenicity and reaction to immunohistochemistry) can be variable.

A study by Dwork et al.[23] on archived tissue, some as old as 50 years, showed extremely good results, with immunostaining for senile plaques and neurofibrillary tangles. In another study, Bertheau et al.[24] showed that the immunohistochemical detection of some nuclear, cytoplasmic, and membrane antigens could be affected by storage as short as 3 months. However, we have obtained adequate positive internal control staining in our samples that indicate the tissue has retained antigenicity, and any cases that do not show good internal positive controls will not be used for our future study into cell population and density.

Variability of results within various age groups cannot be commented on for the following reason: the sample size is very small and to make conclusions regarding different age groups would perhaps be fallacious and, therefore, as described earlier, this is an evolving study, and we will collect more samples, analyze them, and then draw conclusions including interage variability, using software programs to make quantifications and reproducible data available.

DISCUSSION

The development of the lamina propria within the human true vocal fold bears important consequences with

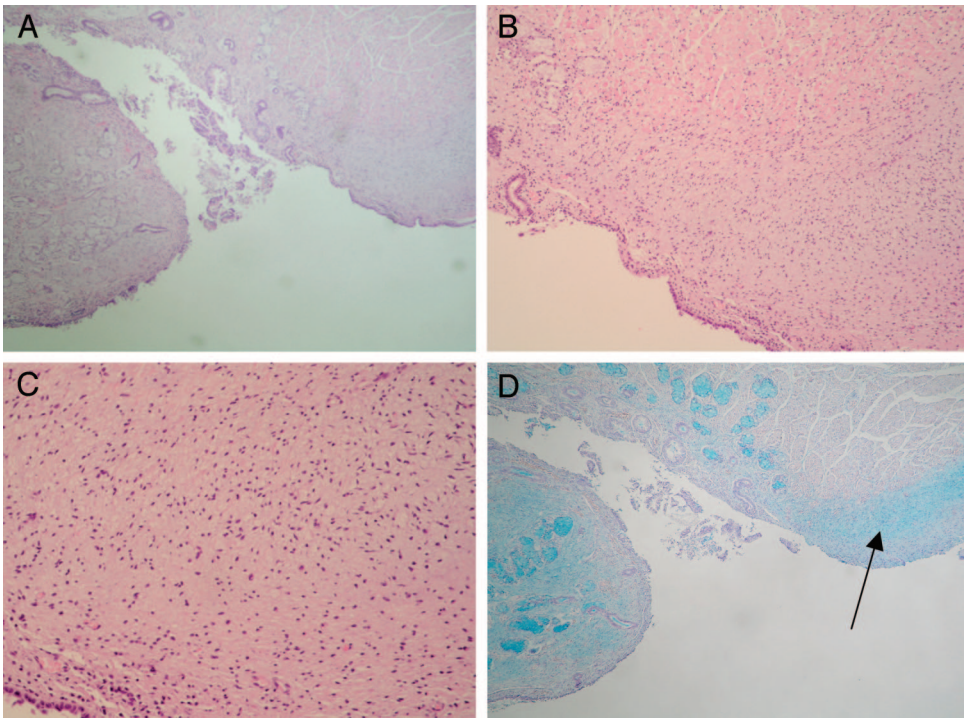

Fig. 4. Four micrometer section of 2-day-old female true vocal cord at (**A**) ×40 magnification using hematoxylin-eosin stain, (**B**) ×10 magnification using hematoxylin-eosin stain, (**C**) ×20 magnification using hematoxylin-eosin stain, and (**D**) ×40 magnification using Alcian blue stain. Note the staining of the mucin glands with the Alcian blue stain demonstrating adequate positive controls. (**D**) Alcian blue highlights the "mucin-rich" nodular central area (arrow).

regards to the ability to phonate and to engage in complex speaking and singing tasks. A baby can be heard to audibly cry at birth but does not require a wide dynamic range to make him or herself understood. It has been posited that the development of the vocal cord is shaped by the tasks that are required.[25,26] In this light, it is logical to assume that a semimature vocal apparatus that would allow vocal inflection to connote mood and emotion would be required soon after a child began to speak and perhaps even before formal language had developed. If the cover/body is used as a model for mucosal wave propagation along a stiff vocal ligament, then one would expect some form of division within the lamina propria at an early age. These hypotheses appear to be supported by the data provided within the body of this research. The cellular monolayer within the lamina propria begins to differentiate by the second month of life. There is a paring of cells in the superficial layer of the lamina propria. This suggests some process of cell signaling and further supports the theory of selective apoptosis, although this mechanism remains to be elucidated. It is clear that the mechanical tasks that the vocal folds are asked to engage in and accomplish increase in complexity over time, and it is one hypothesis that this mechanical stimulation prompts cellular differentiation.

The classic model for the layered structure of the human vocal fold was predicated on what was seen in the adult vocal fold, and this was a reasonable and rational decision process. The process of development toward the adult model was then described according to the terms used and established by the adult lamina propria.[8,10] Thus, elastin and collagen fiber deposition and direction with respect to the direction of the vocalis muscle fibers were the characteristics required to distinguish between the various layers. This was one of the primary reasons why elastin stains were used as the chief stain to distinguish between the MLP and DLP. Review of the vascular surgery literature provided a means of understanding the different mechanical properties of elastin and collagen (i.e., different stress/strain patterns) that would allow for proper functioning within a given environment.[27,28]

On reflection, however, it would seem logical that differential fiber deposition would of necessity follow a period of maturation whereby cell signaling had produced differential cell distribution. With the advent of molecular biology, this cell distribution patterning may be of fundamental importance in understanding not only how but, also, why the mature vocal fold structure develops. Past seminal studies have looked to the vascular literature to find models of stress/strain patterning according to protein fiber concentration to explain how the vocal fold's mechanical functions can be explained histologically.[27–29] (NB, the study of vascular flow is predicated on how physical structures adapt to changing applications of mechanical forces, and this field of study represented an attractive parallel model to study the mechanical dynamics of airflow through the glottis.) If we use the same motif today and look once again to the vascular literature now not to explain the mechanics of fiber composition but, rather, to explain the molecular basis of anatomic development, we find models whereby mechanical stress patterns produce regions of selective apoptosis as triggered by this environmental stimuli.[30] By looking for distinct regions of cell populations and cell density, we therefore may well be able to identify regions where later fiber composition will vary according to the mechanical stresses that are placed on these regions and the subsequent cell signaling that occurs.

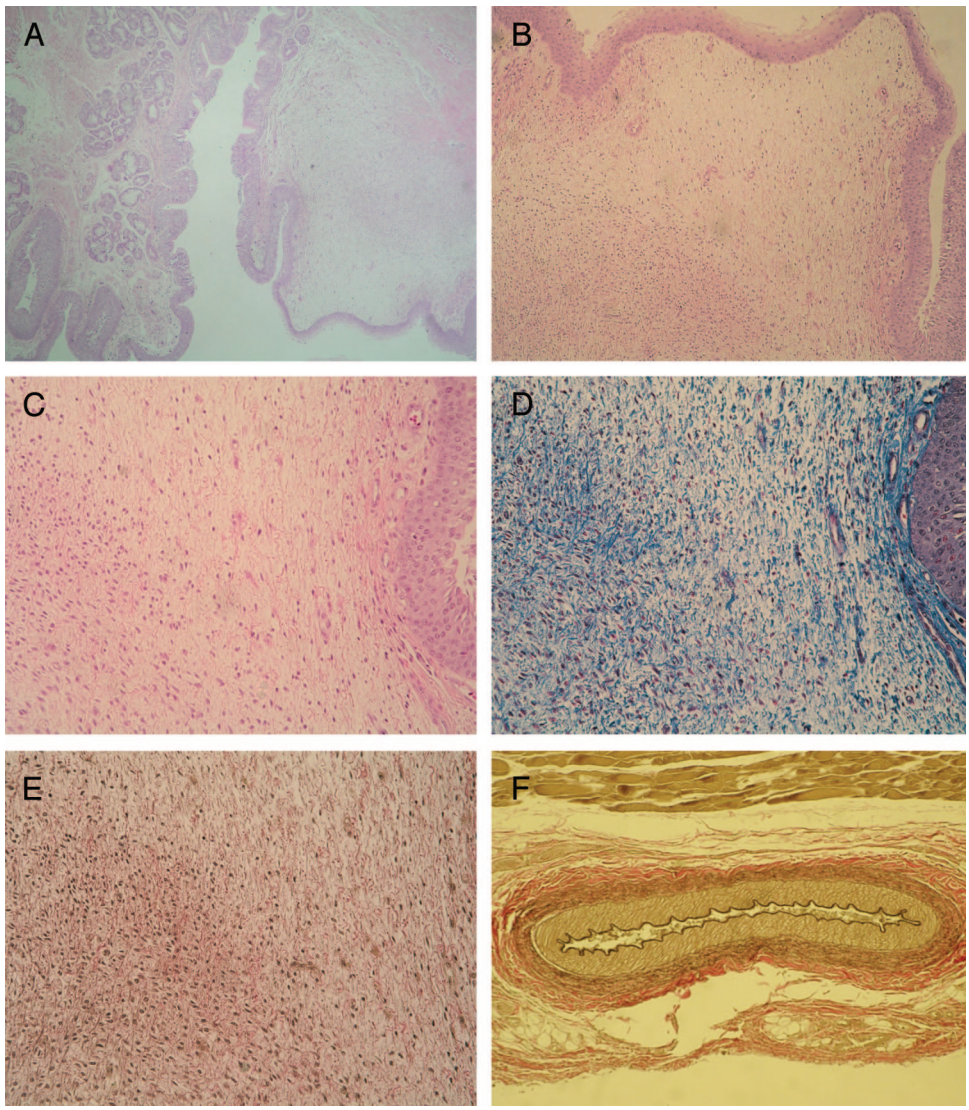

Fig. 5. Four micrometer section of 2-day-old female true vocal cord at (**Ab**) ×40 magnification using hematoxylin-eosin stain, (**B**) ×10 magnification using hematoxylin-eosin stain, (**C**) ×20 magnification using hematoxylin-eosin stain, (**D**) ×10 magnification using trichrome staining, (**E**) ×100 magnification using elastin staining, and (**F**) positive control for staining of elastic laminae of wall of blood vessel with elastin stain.

If we then describe the development of the pediatric vocal fold in terms of a layered cellular (rather than fiber) composition, then it becomes more difficult to define periods of time in which the vocal ligament and a mature cover/body develop. If the vocal ligament is defined by the adult definition of a combination of the middle and deep layers where there is an organized distribution and orientation of elastin and collagen fiber deposition,[8] then we do not see this in full maturation until the teenage years. Even at this time point, the distinction between the elastin and collagen fiber concentrations to form discrete layers is qualitative at best, and both fibers can be seen to be interspersed throughout both the middle and deep layers. However, there is a clear demarcation between a middle and DLP by 7 years of age when we look at regions of cell population and density. What remains is to use markers of proliferation and, in turn, of apoptosis to better quantify this description of cellular distribution. It may well be that as we understand the functional demands placed on the developing larynx (i.e., what it demands of the human larynx to cry, to coo, to begin to speak and implement language), then we will be able to formulate a more functional description of the layered structure of the human vocal cord that makes sense with the histologic structures seen and describes the changes in mechanical properties that are evident. As was described above in the introductory section, even in the adult notion of the lamina propria structure and the true vocal cord anatomy in toto, there is still debate over the precise layered structure and where appropriate divisions lie (i.e., between the cover and the body).[8,12,13] Further studies are needed to bind anatomic and histologic studies with mechanical data to make sense with what we know to be the functional demands on the human voice at distinct periods of time. Whereas initial studies may be descriptive in terms of defining anatomy, a more complex understanding is imperative to couple anatomy first to biochemistry as we understand the cellular nature of the various layers and then to molecular biology where we can begin to understand why and how these layers are triggered to develop. To answer these questions, we will need to replace qualitative analysis that is essential in the beginning with quantitative analysis that will

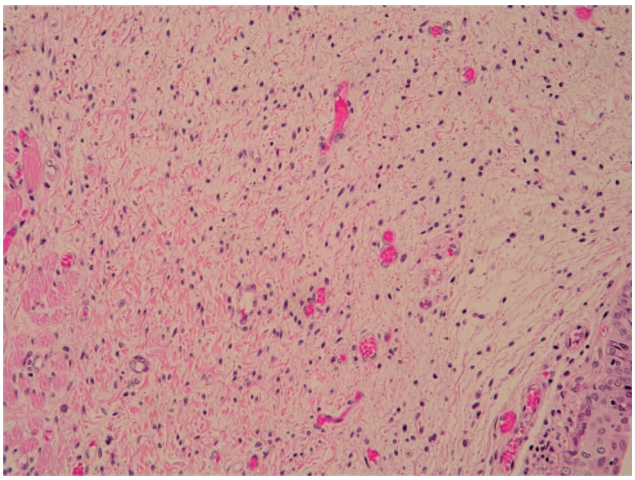

Fig. 6. Four micrometer section of 11-month-old female true vocal cord at ×200 magnification using hematoxylin-eosin stain.

allow for an understanding of the underlying molecular processing as well as allow for mechanical modeling where close quantitative description and analysis is essential.

The importance of gaining a clearer insight into the chronology of the development of the mature structure of the lamina propria exists on several dimensions. First, as we understand more about the signaling that underlies normal development, we also gain insight into aberrations from the normal pathway that in turn lead toward pathology. If the mechanism holds true that there is some form of signaling in the form of targeted cell death that allows for a layering of different cell populations and densities, then aberrations in this mechanism may be responsible in part for the development of laryngeal pathology. Moreover, if there are signaling pathways that direct the composition within the different layers of the lamina propria, then these same pathways may be involved in the healing process after local tissue injury. There has been some initial work looking at the role that growth factors such as hepatocyte growth factor (HGF), transforming growth factor B1, epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF) play with regards to proper wound healing and the development of scar within the lamina propria.[31,32] It may be interesting to see how these factors influence the earlier process of development and maturation.

Studying the development of the layered structure of the human vocal fold not only holds the promise of understanding laryngeal pathology, but it also affords the potential of providing means of surgical therapy. Many of the contemporary phonosurgical procedures have been designed as subepithelial dissections sparing the SLP to as great an extent as is possible.[33–37] Defining the appropriate age where these adult phonosurgical procedures can be applied to children to treat lesions such vocal fold nodules and cysts has been complicated by lack of data to support when such a trilaminar structure exists. Our data would suggest that all else being equal, phonosurgical excisions can be accomplished by means of subepithelial flap elevation and SLP preservation as early as 7 to 8 years of age. It remains true that these patients must be carefully chosen for their ability to undergo preoperative voice therapy and peri- and postoperative treatment regimens; nevertheless, in the appropriate patient, such procedures can yield dramatic effects.

The limitations of this study stem from the use of archival larynges for purposes of analysis. As has been shown in other fields of otolaryngology such as the study

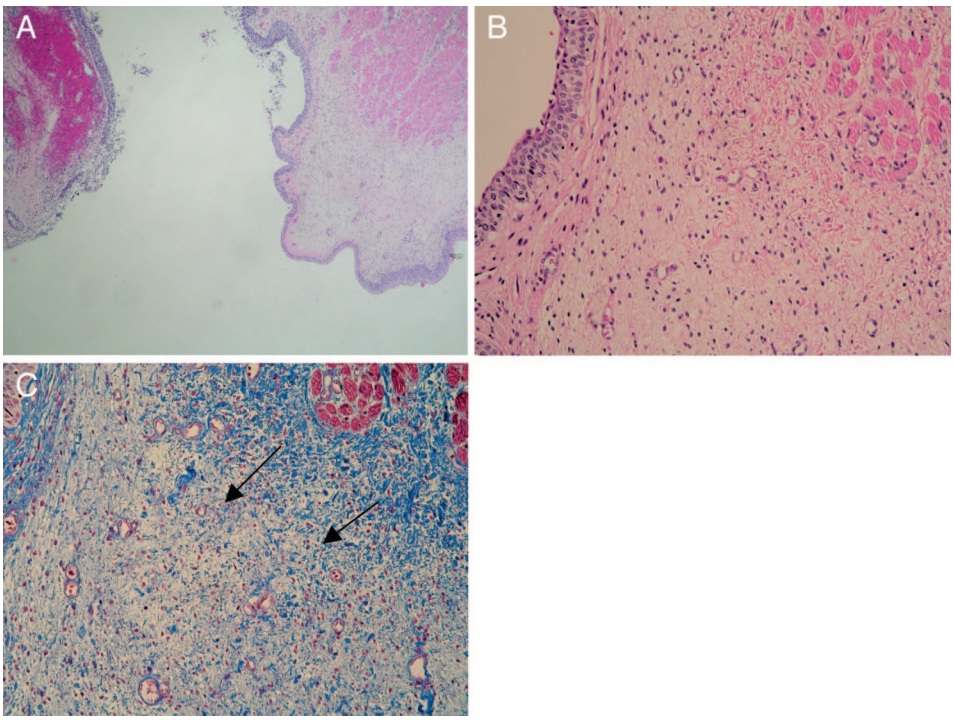

Fig. 7. Four micrometer section of 3-year-old female true vocal cord at (**A**) ×40 magnification using hematoxylin-eosin stain, (**B**) ×100 magnification using hematoxylin-eosin stain, and (**C**) ×100 magnification using trichrome stain. Note red nuclei highlighting plump fibroblasts (arrows).

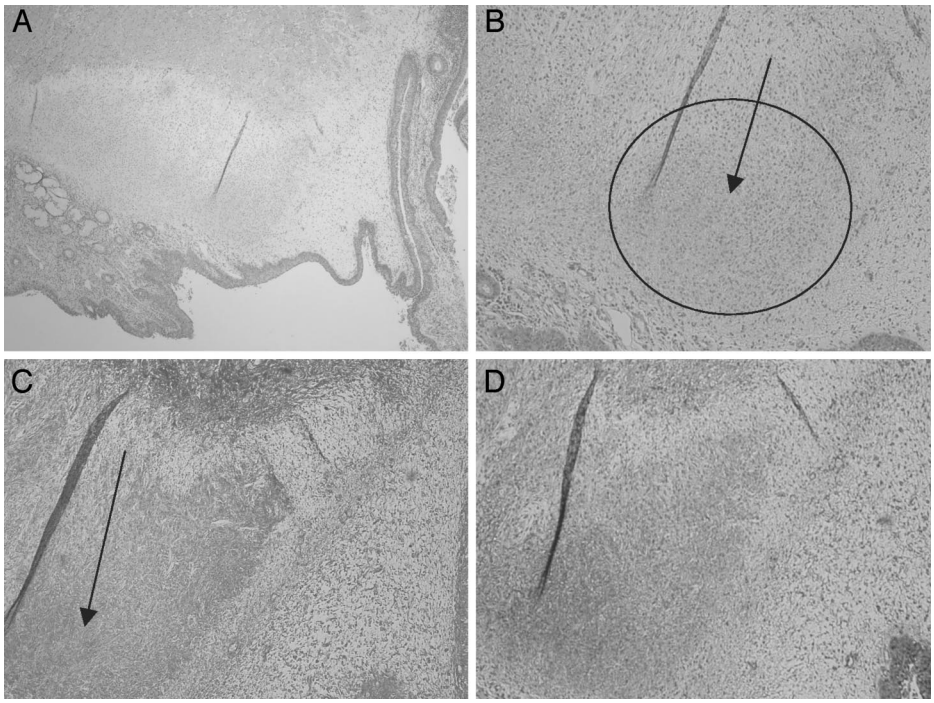

Fig. 8. Four micrometer section of 7-year-old male true vocal cord at (**A**) ×40 magnification using hematoxylin-eosin stain, (**B**) ×100 magnification using hematoxylin-eosin stain (note a "nodular" configuration of cellular central area [oval]), and (**C**) ×100 magnification using trichrome stain. Circle shows an area of concentrated fibroblasts (arrow) and collagen. (**D**) ×100 magnification using elastin stain.

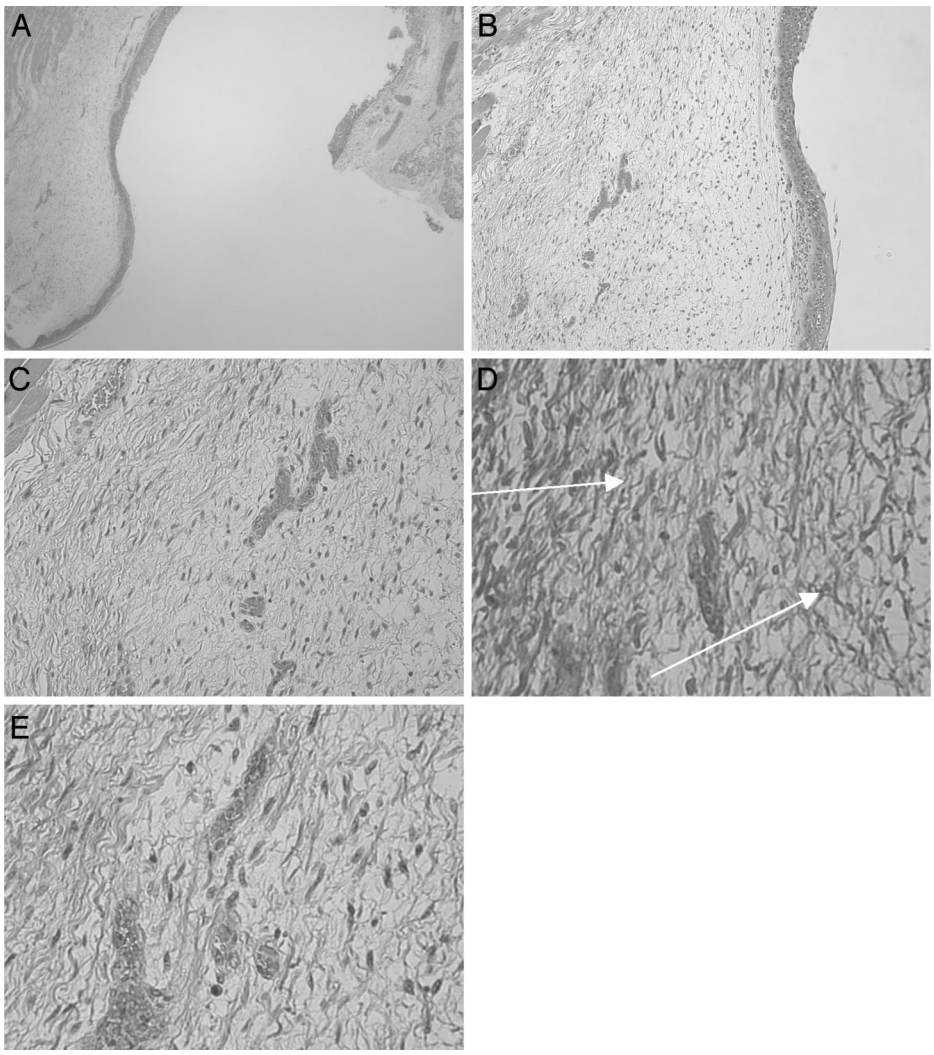

Fig. 9. Four micrometer section of 13-year-old female true vocal cord at (**A**) ×40 magnification using hematoxylin-eosin staining, (**B**) ×100 magnification using hematoxylin-eosin staining, (**C**) ×200 magnification using hematoxylin-eosin staining, (**D**) ×200 magnification using trichrome staining (note delicate collagen fibrils [arrow]) and (**E**) ×200 magnification using elastin staining; note thin and elongate elastic fibers (arrow).

of the pattern of spread of laryngeal cancer, the study of archival tissue has an important role in research.[38,39] Nevertheless, the number of specimens, although comparatively large with regards to other studies, still is far too small to allow for the changes that might be seen according to the ethnicity of the patient, the language spoken, the dialect of a particular language, as well as according to geography where the patient came from. Although there are samples from both sexes, the numbers are too small to allow for adequate characterizations according to sex. This study has, however, allowed greater insight into particular periods in childhood where critical transitions can be seen. Further studies are planned to look at more samples within these particular points of maturation to understand and characterize cell populations in distinct regions that will later produce the classic, layered-fiber composition pattern. Moreover, because much of development in other systems and presumably even of the larynx and lamina propria itself as it ages is guided by hormonal signaling, it will be important to move beyond preliminary studies[21] and to note how such signaling affects the lamina propria structure as it develops.

Other limitations stem from the use of archival tissue regarding processing artifacts. Although care was taken to avoid using specimens that had been intubated for prolonged periods or which either showed signs of laryngeal pathology or trauma, nevertheless, there still exists the possibility of tissue artifacts. Some of the archived tissue had been stored for several decades and, with time, greater tissue artifacts can be seen. Future studies will be directed toward acquiring more recent specimens and, when possible, examining fresh rather than archival tissue. In this fashion, further studies such as electron microscopy can be accomplished. Finally, the results of our research suggest a pattern of development and maturation, but these need in the future to be coupled with biomechanical models that demonstrate how the maturing lamina propria structure develops different mechanical properties that mirror the changes in histology.

The future directions needed to carry on from this initial work will be to continue to develop and to characterize a larger library of pediatric cadaveric larynges, taking into account the different ethnic and linguistic backgrounds from where the specimens came. Molecular studies are needed to help uncover the cell signaling that promotes this cellular differentiation and maturation. By developing quantitative methods of describing the process of maturation, we can in turn use these methods to address the classic definitions of the adult vocal fold structure and to attempt to unravel the controversies that still exist regarding how best to describe the mature structure. (Pediatric laryngology is in its nascency and looks to its adult counterpart for much of its clinical underpinnings; however, with the study of archival pediatric larynges, the tools exist potentially to bridge the fields and to help answer some of the basic questions that are essential to both disciplines.) We can then explore whether there are indeed two or three layers biologically and functionally; at the same time, we can move toward developing mathematical models that describe mechanical function using the histologic and molecular data that is available. As we understand the process of development and the signaling pathways that facilitate maturation, we hope to be able to improve on the diagnostic and therapeutic arsenal that currently exists within the field of pediatric as well as adult laryngology.

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