Received: 27 June 2024 | Revised: 28 August 2024 | Accepted: 15 September 2024

DOI: 10.1002/lio2.70018

ORIGINAL RESEARCH

Laryngoscope Investigative Otolaryngology

Histological characterization of rat vocal fold across different postnatal periods

Xumao Li MM | Xinsheng Lin MM | Xinqiao Xie MM | Xiangyu Chen MM | Yuhui Xie MM | Guangbin Sun MD, PhD

Department of Otorhinolaryngology-Head and Neck Surgery, Huashan Hospital, Fudan University, Shanghai, China

Correspondence
Guangbin Sun, Department of Otorhinolaryngology-Head and Neck Surgery, Huashan Hospital, Fudan University, No. 12, Middle Urumqi Rd, Shanghai 200040, China.
Email: sgb223@hotmail.com

Funding information
National Natural Science Foundation of China, Grant/Award Number: 82271154

Abstract

Objective: To evaluate the vocal fold histological characteristics during different postnatal periods in rats, especially older rats.

Methods: Sprague–Dawley rats aged 4 days, 4 and 12 weeks, and 12 and 24 months were used for the experiment. Five larynges were obtained for each age and cut into 5-μm consecutive sections. The expression of Ki-67 was assessed using immunohistochemistry to examine cell proliferation. Elastic van Gieson staining was used to detect the collagen and elastin concentrations. The cell type was determined using multicolor immunofluorescence.

Results: Ki-67 was not expressed in the macula flava (MF) of 12-week-, 12-month-, and 24-month-old adults. Collagen fibers in the lamina propria (LP) increased with age. The elastic fiber concentrations in the LP decreased significantly at 24 months ($p < .01$) but remained stable in the MF. All posterior MF cells showed strong glial fibrillary acidic protein and vimentin-positive reactions with weaker expressions of CD68 and α-smooth muscle actin (α-SMA). The myofibroblasts (α-SMA-positive) and macrophages (CD68-positive) in the LP of the 24-month-old rats were significantly the highest ($p < .01$).

Conclusion: The extracellular matrix in the LP increases with age, presenting as an increase in collagen with the loss of elastin, which may be due to myofibroblast proliferation. Moreover, the cellular properties or extracellular matrix components of the mature MF in rats are comparable to those in humans.

KEYWORDS
aged, histology, postnatal development, rat, vocal folds

1 | INTRODUCTION

Rats are commonly used as experimental laboratory models in basic research. They are ideal models for vocal fold-related research in laryngology because their characteristics are similar to those of humans.[1,2] Several studies have been conducted on rat vocal folds, including those on vocal fold scarring,[3] injuries and wound healing,[4–6] and anatomical structure.[7,8]

Fundamental knowledge of the microstructure of rat vocal folds is a meaningful prerequisite for animal studies. The structure of the rat larynx has been well studied,[9] and detailed information on the basic structure and epithelial distribution in the normal larynx of an

adult rat has been provided. The embryogenesis and development of the larynx have also been examined histologically. In rats, the larynx is identifiable at 17 embryonic days and well developed at birth.[7] During postnatal development from neonate to adult, the macula flava (MF) was observed as a mass of cells at the anterior and posterior of the vocal fold, and collagen type I and hyaluronic acid were denser in the MF.[10] Although these studies have examined the development of the rat larynx from the embryo stage to adulthood, none have specifically performed histological characterization across different postnatal periods, especially in aged rats.

Changes in aged vocal folds can cause presbyphonia in humans, negatively impacting communication and quality of life. Increased stiffness of the vocal fold mucosa, thyroarytenoid muscle atrophy, increased collagen deposition and decreased hyaluronan concentrations are observed in older adult vocal folds.[11,12] The clarification of the physiology of aged rat vocal folds is necessary, which may facilitate the development of presbyphonia treatments. However, data on changes during different postnatal periods (from neonatal to aged) in rats are rare. A study investigated changes in the expression and deposition of collagen and hyaluronan between young, adult, and elderly rat vocal folds.[13] However, this study did not differentiate between the various portions of vocal folds and did not investigate the distribution of elastin during different postnatal periods.

Therefore, this study aimed to establish a detailed histological description of the microstructures of rat vocal folds at different postnatal periods (neonate, pre-sexually mature, young, adult, and aged). Firstly, we investigated the proliferative state of the cells including epithelial and posterior MF cells. Secondly, we compared the changes in elastic and collagen fibers of the different portions of vocal folds including the lamina propria (LP) and the posterior MF area. We also observed the cell distribution of the posterior MF cells, macrophages, and myofibroblasts especially in aged rats, which, to our knowledge, has not been studied previously.

2 | METHODS

2.1 | Animals

Twenty-five larynges were obtained from male Sprague–Dawley breeder rats: five were 4 days old (neonate) and weighed 9.9 ± 0.7 g; five were 4 weeks old (pre-sexually mature) and weighed 108.4 ± 4.2 g; five were 12 weeks old (young) and weighed 357.4 ± 4.0 g; five were 12 months old (adult) and weighed 424.6 ± 6.3 g; and five were 24 months old (aged) and weighed 650 ± 9.3 g. The animal experimental protocol was approved by the Animal Care and Ethics Committee of the Department of Laboratory Animal Science, Fudan University.

2.2 | Tissue preparation

The rats were euthanized using an overdose intraperitoneal injection of 0.3% pentobarbital sodium, and the larynges were immediately excised. Whole larynges were preserved in 4% paraformaldehyde at 4°C for 24 h, decalcified, embedded in paraffin. The sections were made in the horizontal plane (parallel to the vocal fold) with consecutive slices (5-μm thick). Each slice contained the complete bilateral vocal folds, including LP and MF.

2.3 | Staining methods

Vocal fold tissue was examined using routine hematoxylin and eosin staining. Elastic van Gieson (EVG) staining was used to detect the collagen and elastin concentrations. The slides were examined under the Leica DM1000 LED Microscope (Germany) and digitalized by slide scan system KF-PRO-120 (Ningbo, Zhejiang, China) via SlideViewer v1.5.5.2 software (Ningbo, Zhejiang, China).

2.4 | Immunohistochemistry

To detect the proliferative state of the cells, we determined the expression of Ki-67 (proliferation marker) via immunohistochemistry (IHC). Antigens were retrieved using citrate buffer (pH 6.0). Block antigen with 3% BSA. Afterward, the tissue samples were incubated with the primary anti-Ki-67 (Abcam ab16667; 1:200) overnight at 4°C. After incubation with the secondary antibody horseradish peroxidase (HRP) goat anti-rabbit IgG (Beyotime A0208, 1:200) at room temperature (20–26°C) for 1 h, 3,3′-diaminobenzidine and hematoxylin staining were performed.

2.5 | Immunofluorescence

The cell type was determined using multicolor immunofluorescence. The glial fibrillary acidic protein (GFAP) is a Schwann cell specific marker of the MF cells.[14,15] Vimentin is positive for MF cells,[6,16] fibroblasts, myofibroblasts, and macrophages. The α-smooth muscle actin (α-SMA) is a marker for myofibroblasts. CD68 is a macrophage-specific marker.

Immunofluorescence was performed on 5 μm-thick sections of paraformaldehyde-fixed paraffin-embedded rat larynges. Antigens were retrieved using 10 mM citrate buffer (pH 6.0). The endogenous peroxidases were inhibited for 25 min using 5% hydrogen peroxide in methanol. Aspecific binding sites were blocked with 3% BSA. Rabbit monoclonal anti-GFAP primary antibody (Abcam ab68428; 1:1000) was incubated overnight at 4°C and detected using antimouse HRP-conjugated polymer secondary antibodies (Agilent Technologies) for 50 min at room temperature. HRP was visualized via tyramide signal amplification using AlexaFluor570-conjugated tyramides (Akoya Biosciences, FP1488001KT, 1:200 dilution). After incubation with a new citrate buffer, the same protocol was applied to the anti-vimentin primary antibody (Abcam ab8978; 1:1000), which was visualized with AlexaFluor520-conjugated tyramide (Akoya Biosciences, FP1487001KT, 1:200 dilution); anti-CD68 primary antibody (Abcam ab283654; 1:1000),

which was visualized with AlexaFluor690-conjugated tyramide (Akoya Biosciences, FP1497001KT, 1:200 dilution); and anti-α-SMA primary antibody (Abcam ab7817; 1:1000), which was visualized with AlexaFluor620-conjugated tyramide (Akoya Biosciences, FP1495001KT, 1:200 dilution). Finally, the cell nuclei were stained with 4′6-diamidin-2-phenylindol (DAPI, Beyotime, c1002). Sections were treated with an autofluorescent quenching solution (Servicebio G1221, Wuhan, China) and mounted in a fluorescence mounting medium (Servicebio G1401, Wuhan, China). The slides were digitized using a Pannoramic MIDI scanner (3DHistech).

2.6 | Statistical methods

Image analysis was performed using ImageJ (Fiji) software (National Institutes of Health, Bethesda, MD, USA) for Ki-67 values as percentage positive cells of the epithelium area. Cells were counted by the ImageJ software based on the criteria provided in the reference.[17] Digitized EVG staining slides of each species were examined. The posterior MF area and three different random locations in the LP were chosen for measurement by the ImageJ software. We set the Image Type to RGB Stack, selected the Red panel and set the same threshold to avoid cell nuclei while capturing elastic fibers. The percentage elastic fiber area was calculated as follows:

$$\textit{percentage elastic fiber area} = (\textit{elastic fiber pixel area} \div \textit{visual field pixel area}) \times 100$$

The proportion of the measurement area accounted for by the collagen area was also calculated. We set the Image Type to HSB Stack, selected Saturation panel and set the same threshold to capture collagen.

The digitized multicolor immunofluorescence slides were examined, and cell counts were performed using ImageJ software with the same threshold. The number of macrophages (CD68-positive) and myofibroblasts (α-SMA-positive) per section of the LP, excluding the anterior and posterior MF, was determined. The percentage of positive cells was calculated for five slices from each animal (4- and 12-week-old, and 12- and 24-month-old rat), and the data were averaged for statistical analysis.

In this study, every vocal fold data of each laryngeal slice was analyzed ($n = 10$ for each subgroup). Data analyses were performed using SPSS Statistics software (version 20.0; IBM Corp, Armonk, NY, USA). Normality (Shapiro–Wilk test) and homogeneity (Levene's test) tests were performed. If the data conformed to the normal distribution and homogeneity of variance, a paired t-test or one-way ANOVA was used to analyze the Ki-67 values and collagen and elastic fiber concentrations. Otherwise, the Wilcoxon or Kruskal–Wallis test was used. The p-values <.05 denoted statistical significance.

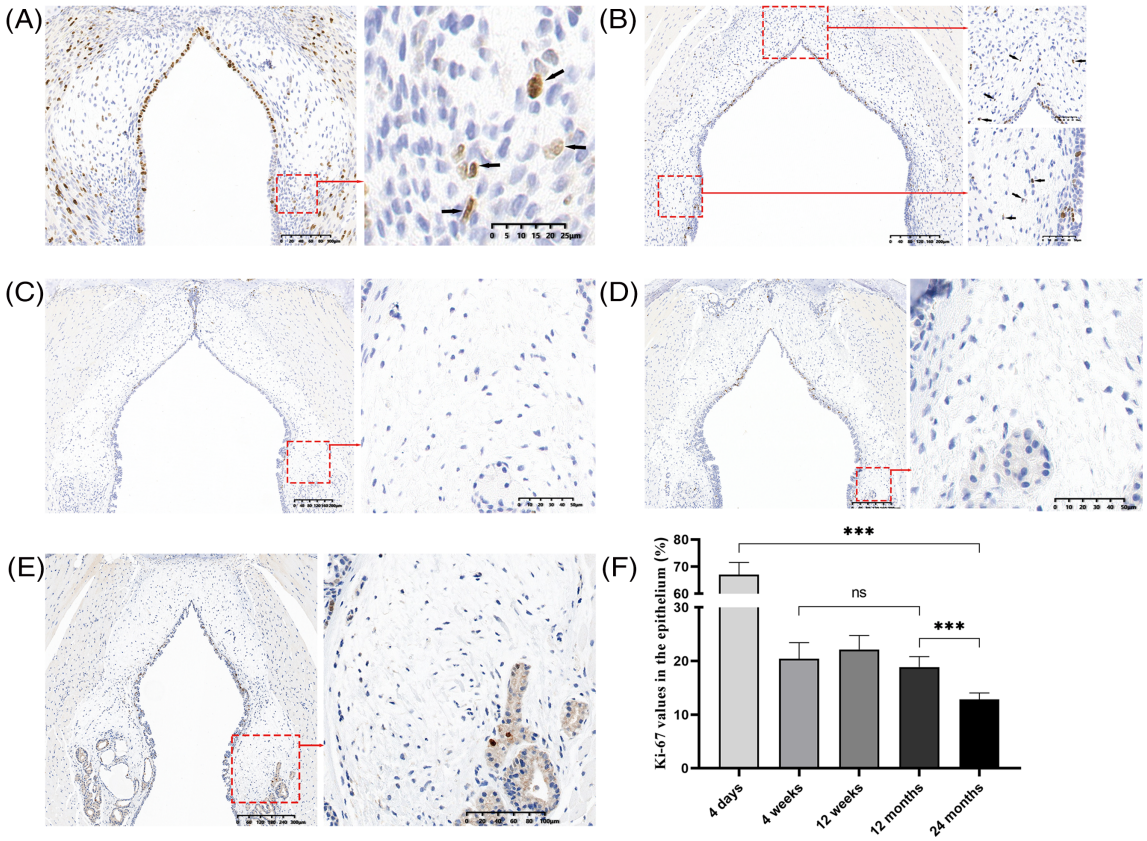

FIGURE 1 (A–E) Representative immunohistochemistry of Ki-67 of 4 days, 4 and 12 weeks, and 12 and 24 months, respectively (Black arrow: Ki-67 positive cells in the macula flava); (F) Ki-67 values in the epithelium area (***p < .01, ns p > .05).

3 | RESULTS

Ki-67 was expressed in the MF regions of the 4-day- and 4-week-old rats, but not in those of the 12-week-, 12-month-, and 24-month-old rats (Figure 1). In addition, vocal fold epithelial cells were proliferated at different ages (Figure 1). The Ki-67 values in the epithelium were analyzed (Figure 1F). The expression of Ki-67 was the highest at 4 days ($p < .01$), and it decreased significantly at 24 months ($p < .01$). No significant differences were observed at 4 weeks, 12 weeks, and 12 months ($p = 0.17$).

EVG staining revealed that the elastic fibers were blue and black, the collagen fibers were red, and the background was yellow (Figure 2). Collagen fibers in the LP area were arranged in parallel in the 4-week-, 12-week- and 12-month-old rats, but disordered in the 24-month-old rats. The collagen concentrations increased in the LP and posterior MF with age ($p < .01$). The elastic fiber concentrations of the MF were significantly higher than those of the LP ($p = .02$). Notably, the elastic fiber concentrations of the LP decreased significantly at 24 months ($p = .01$), whereas they remained stable in the posterior MF.

Immunofluorescence-positive reactions revealed a strong positive reaction of GFAP and vimentin in the posterior MF cells of all different periods, with weaker expressions of CD68 and α-SMA (Figure 3). Moreover, immunofluorescence revealed the absence of apparent MF cell aggregation in the posterior LP of the neonatal rats.

The number (CD68-positive) of cells increased in the LP area of the 24-month-old rats (Figure 4); the percentage of cells was higher than that at the other time points (Table 1). In addition, the myofibroblasts (α-SMA-positive) in the LP area of 24-month-old rats were significantly the highest (Table 1).

4 | DISCUSSION

The rat is an important animal model for studying the pathophysiology of vocal folds. Embryogenesis and the development of the rat larynx have been well-analyzed in previous studies.[7–10] However, histological characterization across different postnatal periods was seldom studied, especially in aged rats. In our study, we investigated cell proliferation using IHC and the distribution of elastic and collagen fibers using EVG staining during different postnatal periods. In addition, we investigated the cell distribution, especially in the posterior MF cells, macrophages, and myofibroblasts using immunofluorescence.

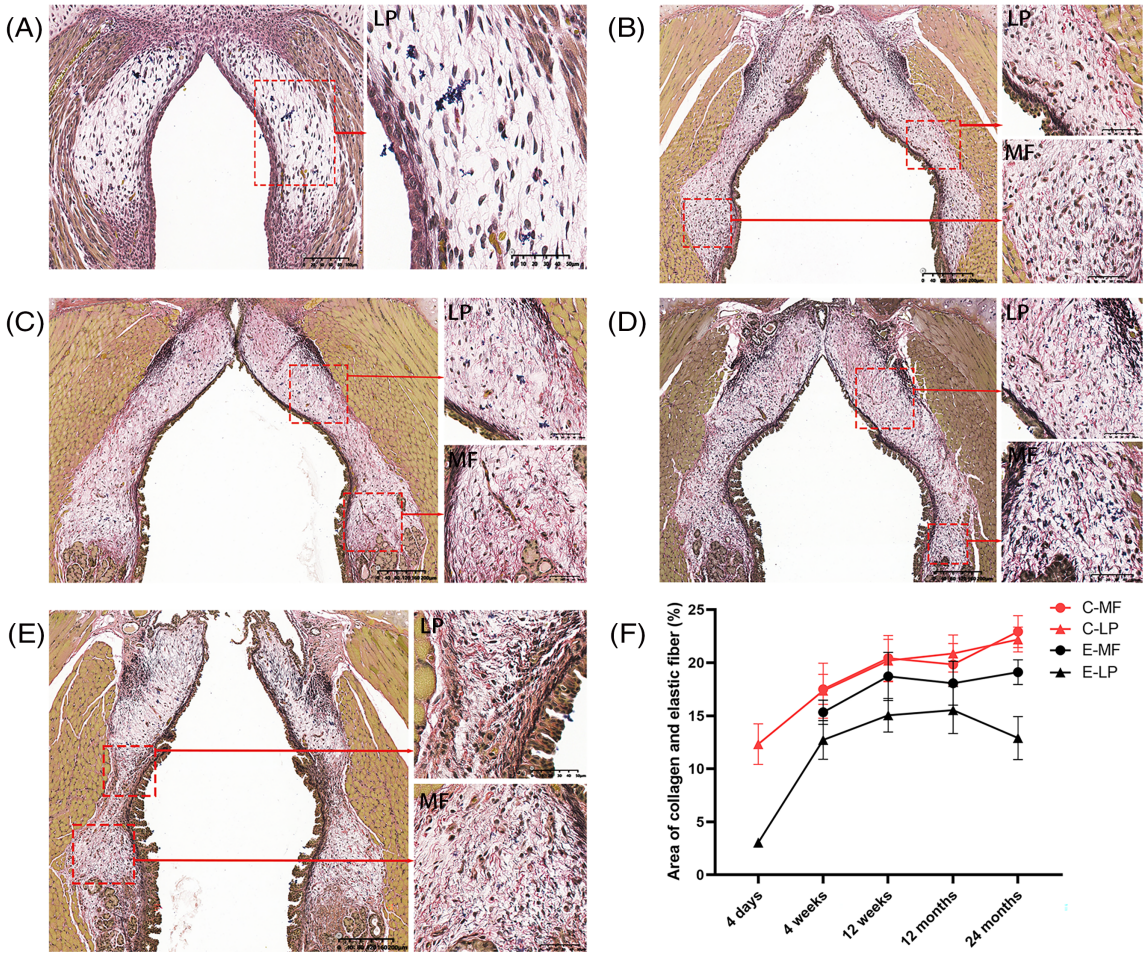

FIGURE 2 (A–E) Representative Elastic van Gieson staining of 4 days, 4 and 12 weeks, and 12 and 24 months, respectively; (F) collagen and elastic fiber levels in different postnatal periods. (LP, lamina propria; MF, macula flava; C, collagen fiber; E, elastic fiber).

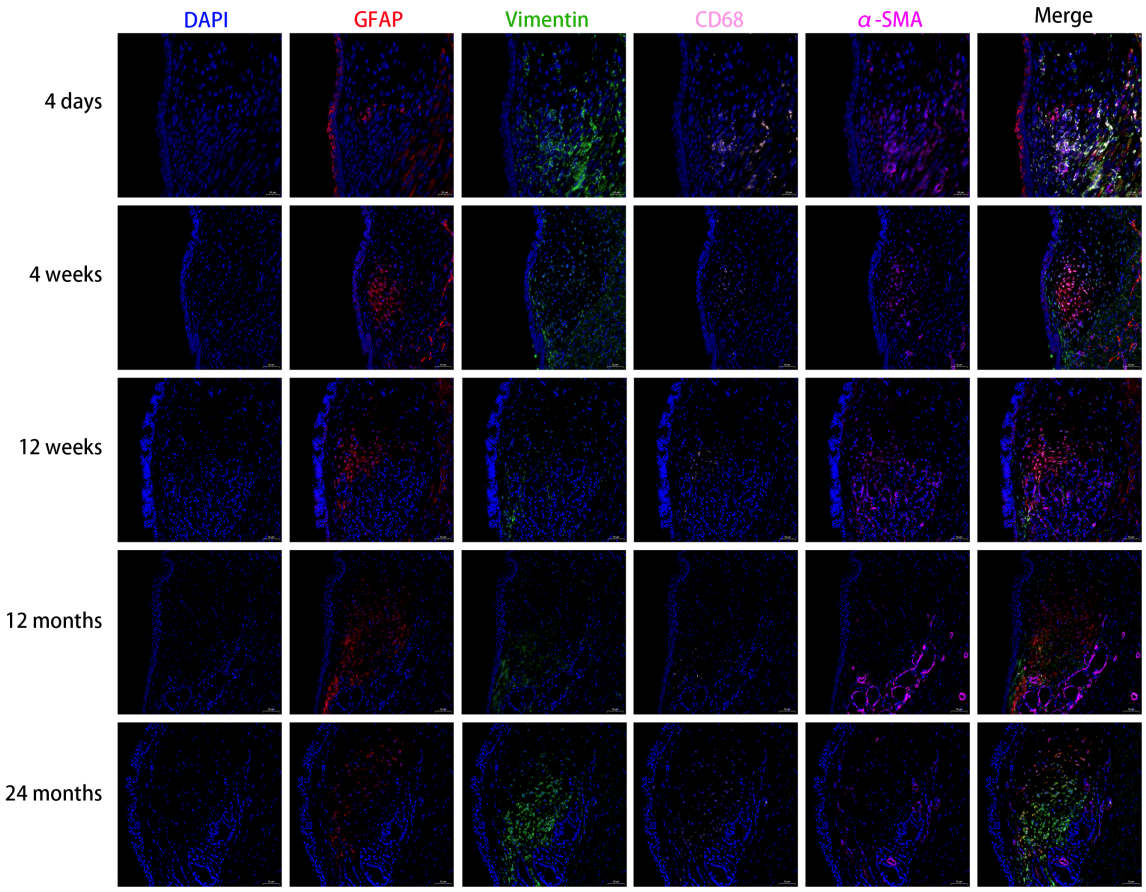

FIGURE 3 Representative immunofluorescence of GFAP, vimentin, CD68, and α-SMA in the posterior macula flava: Blue stains the nucleus. Red is marked with GFAP protein staining. Green is marked with vimentin protein staining. Pink is marked with CD68 staining. Purple is marked with α-SMA staining. α-SMA, α-smooth muscle actin; GFAP, glial fibrillary acidic protein.

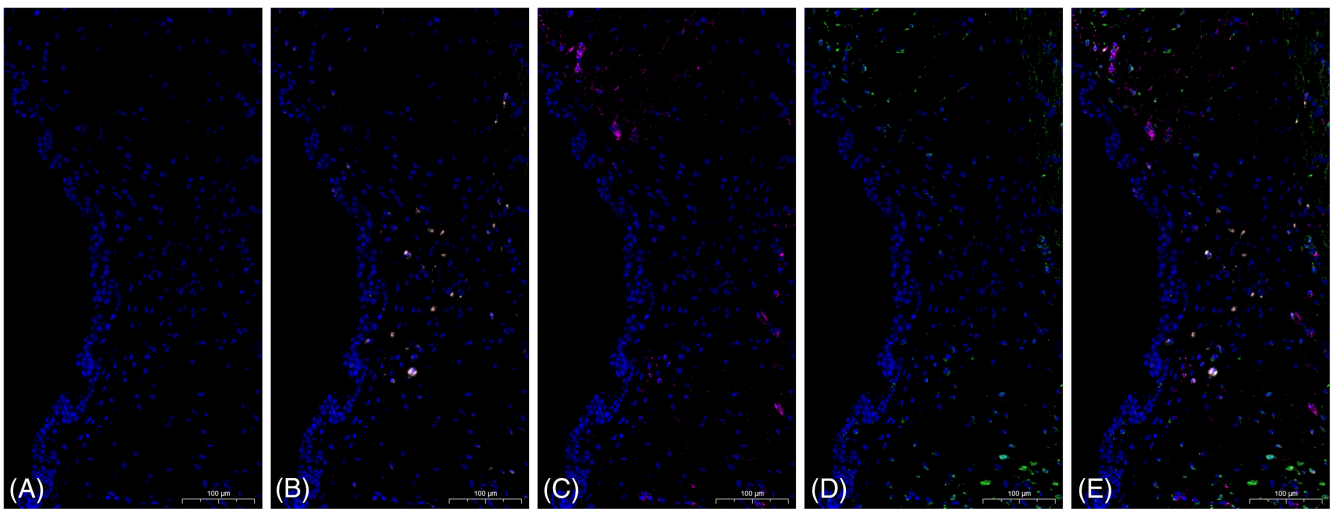

FIGURE 4 Representative immunofluorescence of CD68, α-smooth muscle actin (α-SMA), and vimentin in the lamina propria of 24 months rat: (A) DAPI; (B) CD68 positive marker; (C) α-SMA-positive marker; (D) vimentin positive marker; (E) merge.

Ki-67 is a nuclear protein associated with the cell cycle. It is expressed during the G1, S, G2, and M phases of cell division but not during the G0 phase. Vocal fold epithelial cells actively proliferate, especially during acute wound healing. This is prominent on day 1 after injury.[6] In our study, the decreased expression of Ki-67 at 24 months indicated diminished self-renewal of the epithelium. Notably, Ki-67

TABLE 1 Percentage [minimum; maximum] of the macrophages (CD68-positive) and myofibroblasts (α-SMA-positive) in the LP of 4 and 12 weeks, and 12 and 24 months rat.

	Different postnatal periods				
Cell type	4 weeks	12 weeks	12 months	24 months	*p*
Macrophages (CD68-positive)	16.80 [13.68; 20.88]	14.98 [12.27; 18.73]	11.41 [9.86; 13.27]	36.29 [32.18; 39.82]	<.01
Myofibroblasts (α-SMA-positive)	22.47 [19.23; 23.53]	12.39 [9.84; 14.21]	8.42 [6.80; 10.70]	31.73 [29.89; 35.90]	<.01

was not expressed in the MF area of the 12-week-, 12-month- and 24-month-old rats. These results suggest that the MF cells of adult rats are G0-phase cells, which is consistent with the reports of previous studies involving adult humans.[14]

The extracellular matrix of vocal folds comprises fibrous proteins, interstitial proteins, and glycosaminoglycans. The density and spatial arrangement of these elements, especially elastic and collagen fibers, are important for the biomechanical properties and vibratory function of the vocal folds.[18] Voice disorders in older adults considerably impact communication and quality of life.[19] In humans, collagen I and III increase while elastin decreases in the LP of older adult vocal folds.[12] An animal experiment revealed that collagen was dense in the vocal folds of adult and aged rats.[13] However, collagen in the LP or the MF was not quantified in this research. EVG staining revealed collagen fibers in the LP area with arrangement disorders in aged rats. The collagen concentrations increased in the LP area of aged rats, whereas the elastic fiber concentrations decreased significantly.

The anterior and posterior MF is a special structure comprising dense cells and a fibrous matrix at one end of the vocal folds.[20] In the older adults, scanning electron microscopic analysis showed a higher concentration of fibers in the posterior MF than in the other region.[21] The results of this study indicated that the MF remains functional even in the senile larynx. In our study, we investigated the collagen and elastic fiber concentrations in the posterior MF. The collagen concentrations increased with age in the posterior MF. The elastic fiber concentrations remained stable in the MF, whereas they decreased significantly at the LP of aged rats. Our findings align with those of previous studies to some-extent. The MF may remain functional in the vocal folds of aged rats.

Pierre Fayoux analyzed the characteristics and development of the MF in human vocal folds.[20] The posterior MF appeared between 13 and 15 weeks of amenorrhea and matured between 33 weeks of amenorrhea to birth. However, our study revealed the absence of apparent MF cell aggregation in the posterior LP of the vocal folds of neonatal rats, and the MF was matured at 4-week-old rats. However, the maturation of the MF was unclear in this research. The development of MF in vocal folds of rats from birth to 4 weeks needs to be addressed in further research.

In our study, the posterior MF cells of all different postnatal periods demonstrated a strong positive GFAP and vimentin reaction, which is in agreement with previous studies.[2,16] In addition, we found that the posterior MF cells can weakly express CD68 and α-SMA, which can be used for MF cells identification in vitro. The increased fibrous matrix in the LP of the vocal folds in older individuals may be largely attributed to production by fibroblasts. However, Carneiro et al.[22] confirmed no statistical difference between the concentrations of fibroblasts in the LP of the older adult and control groups. Myofibroblasts are largely responsible for synthesizing collagen I in wounds and fibrosis.[23] In 2009, Kumai et al.[24] reported that myofibroblasts were involved in vocal fold fibrosis. In the present study, the concentration of myofibroblasts (α-SMA-positive) was the highest in the LP area of 24-month-old rats. Thus, increased myofibroblasts may contribute to excess fibrous matrix in the LP of older adult vocal folds.

5 | CONCLUSION

In the present study, we established a detailed histological description of the structures of rat vocal folds across different postnatal periods. The extracellular matrix in the LP increases with age, presenting as an increase in collagen with the loss of elastin, which may be due to myofibroblast proliferation. Moreover, cellular properties or extracellular matrix components of mature MF in rats are similar to those in humans.

FUNDING INFORMATION

The study was sponsored by the National Natural Science Foundation of China (Grant No. 82271154).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

ORCID

Xumao Li https://orcid.org/0000-0001-6019-8109
Xinqiao Xie https://orcid.org/0000-0003-4639-1724
Guangbin Sun https://orcid.org/0000-0002-8686-9553

REFERENCES

1. Tateya T, Tateya I, Sohn JH, Bless DM. Histologic characterization of rat vocal fold scarring. *Ann Otol Rhinol Laryngol*. 2005;114(3):183-191.
2. Toya Y, Riabroy N, Davis CR, et al. Interspecies comparison of stellate cell-containing macula flavae and vitamin a storage in vocal fold mucosa. *J Anat*. 2014;225(3):298-305.
3. Suzuki R, Kawai Y, Tsuji T, et al. Prevention of vocal fold scarring by local application of basic fibroblast growth factor in a rat vocal fold injury model. *Laryngoscope*. 2017;127(2):E67-E74.
4. Mizuta M, Hirano S, Ohno S, et al. Expression of reactive oxygen species during wound healing of vocal folds in a rat model. *Ann Otol Rhinol Laryngol*. 2012;121(12):804-810.
5. Ujvary PL, Blebea CM, Maniu AA, Pop S, Sarpataki O, Cosgarea M. Vocal fold injury models in rats: a literature review on techniques and methodology. *J Med Life*. 2022;15(3):336-343.

6. Tateya I, Tateya T, Lim X, Sohn JH, Bless DM. Cell production in injured vocal folds: a rat study. *Ann Otol Rhinol Laryngol*. 2006;115(2):135-143.
7. Alli O, Berzofsky C, Sharma S, Pitman MJ. Development of the rat larynx: a histological study. *Laryngoscope*. 2013;123(12):3093-3098.
8. Hernández-Morato I, Pascual-Font A, Ramírez C, et al. Somatotopy of the neurons innervating the cricothyroid, posterior Cricoarytenoid, and Thyroarytenoid muscles of the Rat's larynx. *Anat Rec*. 2013;296(3):470-479.
9. Smith G. Structure of the normal rat larynx. *Lab Anim*. 1977;11(4):223-228.
10. Tateya T, Tateya I, Munoz-del-Rio A, Bless DM. Postnatal development of rat vocal folds. *Ann Otol Rhinol Laryngol*. 2006;115(3):215-224.
11. Malmgren LT, Fisher PJ, Bookman LM, Uno T. Age-related changes in muscle fiber types in the human thyroarytenoid muscle: an immunohistochemical and stereological study using confocal laser scanning microscopy. *Otolaryngol Head Neck Surg*. 1999;121(4):441-451.
12. Branco A, Todorovic Fabro A, Gonçalves TM, Garcia Martins RH. Alterations in extracellular matrix composition in the aging larynx. *Otolaryngol Head Neck Surg*. 2015;152(2):302-307.
13. Ohno T, Hirano S, Rousseau B. Age-associated changes in the expression and deposition of vocal fold collagen and hyaluronan. *Ann Otol Rhinol Laryngol*. 2009;118(10):735-741.
14. Sato K, Umeno H, Nakashima T. Vocal fold stem cells and their niche in the human vocal fold. *Ann Otol Rhinol Laryngol*. 2012;121(12):798-803.
15. Tateya T, Tateya I, Surles RL, Tanumihardjo S, Bless DM. Roles of vitamin a and macula flava in maintaining vocal folds. *Ann Otol Rhinol Laryngol*. 2008;117(1):65-73.
16. Sato K, Umeno H, Nakashima T. Vocal fold stellate cells in the human macula flava and the diffuse stellate cell system. *Ann Otol Rhinol Laryngol*. 2012;121(1):51-56.
17. Grishagin IV. Automatic cell counting with ImageJ. *Anal Biochem*. 2015;473:63-65.
18. Hirano M. Cover-body theory of vocal cord vibration. *Speech Science*, College-Hill Press; 1985:1-46.
19. Roy N, Stemple J, Merrill RM, Thomas L. Epidemiology of voice disorders in the elderly: preliminary findings. *Laryngoscope*. 2007;117(4):628-633.
20. Fayoux P, Devisme L, Merrot O, Chevalier D, Gosselin B. Histologic structure and development of the laryngeal macula flava. *Ann Otol Rhinol Laryngol*. 2004;113(6):498-504.
21. Pessin ABB, Martins RHG, Gushiken LFS, Pellizzon CH. Sectorial analysis of the fibrous matrix of vocal folds in the elderly. *J Voice*. 2022;36(3):309-315.
22. Carneiro NS, Pessin ABB, Rodrigues SA, Pellizon CH, Xavier CM, Martins RHG. Concentration of fibroblasts in the vocal fold of elderly. *J Voice*. 2022;S0892-1997(22):00345-00349.
23. Kumai Y. Pathophysiology of fibrosis in the vocal fold: current research, future treatment strategies, and obstacles to restoring vocal fold pliability. *Int J Mol Sci*. 2019;20(10):2551.
24. Kumai Y, Kobler JB, Park H, Galindo M, Herrera VL, Zeitels SM. Modulation of vocal fold scar fibroblasts by adipose-derived stem/stromal cells. *Laryngoscope*. 2010;120(2):330-337.

How to cite this article: Li X, Lin X, Xie X, Chen X, Xie Y, Sun G. Histological characterization of rat vocal fold across different postnatal periods. *Laryngoscope Investigative Otolaryngology*. 2024;9(5):e70018. doi:10.1002/lio2.70018