



Establishment of a radiation-induced vocal fold fibrosis mouse model

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ABSTRACT

Post-radiation fibrosis of the vocal folds is thought to cause vocal impairment. However, the mechanism by which this occurs has been poorly documented, probably because of the lack of an appropriate experimental animal model. The purpose of this study was to establish a simple and reproducible mouse model of laryngeal radiation to investigate the development of vocal fold fibrosis over time. C57BL/6 mice individually placed in a lead shield were irradiated with a single dose of 20 Gy. At 1, 2, and 6 months after irradiation, larynges were harvested and subjected to histological examination and gene expression analysis. Irradiated vocal folds showed time-dependent tissue contraction and increased collagen deposition, with no significant difference in the changes in hyaluronic acid levels. Transcriptional analysis revealed upregulated expressions of *TGF-β1* and *iNOS* at 6 months, but downregulated expressions of *Acta2*, *Col1a1*, *Col3a1*, and *MMP8*. Moreover, elevated *TGF-β1* and reduced downstream gene expression levels indicated the existence of an inhibitory factor over the *TGF-β*/Smad pathway. Discrepancies in histological and transcriptional studies of collagen might suggest that radiation-induced vocal fold fibrosis could be caused by the elongated turnover of collagen. Overall, we established a mouse model of radiation-induced vocal fold fibrosis using a simple protocol. Further investigations are warranted to elucidate the pathogenesis of irradiation-induced fibrosis in vocal folds.

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1. Introduction

Approximately 930,000 new patients were diagnosed with head and neck cancer (HNC) globally, accounting for 5% of all cancers, with approximately 470,000 of these patients succumbing to the disease in 2020 [1]. Radiotherapy has become a major treatment option for HNC because of its high therapeutic success, including disease curability and the potential for larynx preservation. It is often used for definitive or palliative purposes, and according to Borras' report [2], approximately 80% of patients who are diagnosed with HNC receive radiotherapy.

Most patients can complete radiotherapy without interruptions, while receiving supportive care for acute radiation-related adverse effects (AEs). However, the management of late AEs after radiation-induced inflammation has not yet been established. Following

radiotherapy, patients often suffer from sequelae such as xerostomia, dysphagia, and hoarseness [3–7]. These late effects are usually irreversible and have negative impacts on the patients' quality of life; thus, recently attracting attention because of the need for urgent corrective measures.

Among radiation-related late AEs, hoarseness is thought to be caused by radiation-related fibrosis of the vocal folds [5,7]. In general, tissue fibrosis is characterized by the excessive accumulation of extracellular matrix (ECM), mainly collagen, resulting from the imbalance in ECM synthesis and degradation [8]. Fibrotic alterations stiffen the mucosa, causing the loss of their pliability and impairment of mucosal wave propagation, resulting in intractable dysphonia [5,9].

Once vocal fold fibrosis occurs, there is currently no established treatment, regardless of the cause, thus complicating the management of the condition. In the past few decades, many studies on vocal fold mucosal fibrosis have been performed. However, most of them have focused on fibrosis after surgical injury. Post-radiation

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fibrosis has rarely been documented, probably due to the lack of an appropriate experimental animal model. As the manner of fibrosis differs depending on the type of injury [10], a better understanding of the impact of irradiation on vocal fold tissues is warranted. Therefore, an appropriate experimental animal model, both simple and reproducible, is needed. Consequently, the purpose of this study was to establish, for the first time, a mouse model of radiation-induced vocal fold fibrosis and to investigate laryngeal histological and physiological changes over time. Once the model is established, it will contribute to the development of novel strategies for the prevention or resolution of radiation-induced vocal fold fibrosis.

2. Materials and methods

2.1. Animals

Thirty-eight 10- to 12-week-old and five 8-month-old C57BL/6 male mice were used in this study. The mice were purchased from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan). All animal experiments conformed to the Guidelines for the Proper Conduct of Animal Experiments of the Science Council of Japan and were approved by the Animal Committee of the Graduate School of Medicine, Kyoto University (approval number: MedKyo20137). Animal care was provided by the Institute of Laboratory Animals of the Graduate School of Medicine, Kyoto University.

2.2. Irradiation

Mice were irradiated with a single dose of 20 Gy using a ^{137}Cs γ -ray source (Gammacell 40 Exactor; Nordion, Ottawa, Canada). Thick 3 cm lead shields with 3 cm holes were employed to avoid

whole-body irradiation and to expose the larynges to higher irradiation (Fig. 1A). This used radiation dose was determined in our preliminary experiments following the survival of only one of four mice irradiated with 30 Gy and none of the four mice irradiated with 40 Gy. Mice were placed in the supine position after being anesthetized with an intraperitoneal injection of 5.0 mg/kg butorphanol (Meiji Seika Pharma Co., Ltd., Tokyo, Japan), 4.0 mg/kg midazolam (Astellas Pharma Inc., Tokyo, Japan), and 0.3 mg/kg medetomidine (Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan).

2.3. Histological examination

The larynges from 4 to 5 mice were harvested before irradiation and at 1, 2, and 6 months after irradiation (Fig. 1B). The mice that were not exposed to irradiation were the control group. Non-irradiated age-matched mice, 6 months after irradiation, were the old control group to determine if age had an effect on study results.

Prior to euthanasia, mice were anesthetized as described above and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. The larynges were dissected and post-fixed for 3 h in 4% PFA. Subsequently, the larynges were placed in graded sucrose solutions (10–20–30% sucrose in PBS) for cryoprotection. They were embedded in the optimum cutting temperature compound (Sakura Finetek, Torrance, CA, USA) and frozen using liquid nitrogen. Serial 7- μm coronal sections were obtained using a cryostat (CM 1850, Leica Instruments, Nussloch, Germany).

To detect the presence of collagen fibers, Masson's trichrome staining was performed, while Alcian blue staining (pH 2.5) with and without hyaluronidase digestion at 37 °C for 1 h was used to determine the presence of hyaluronic acid (HA). HA in lamina

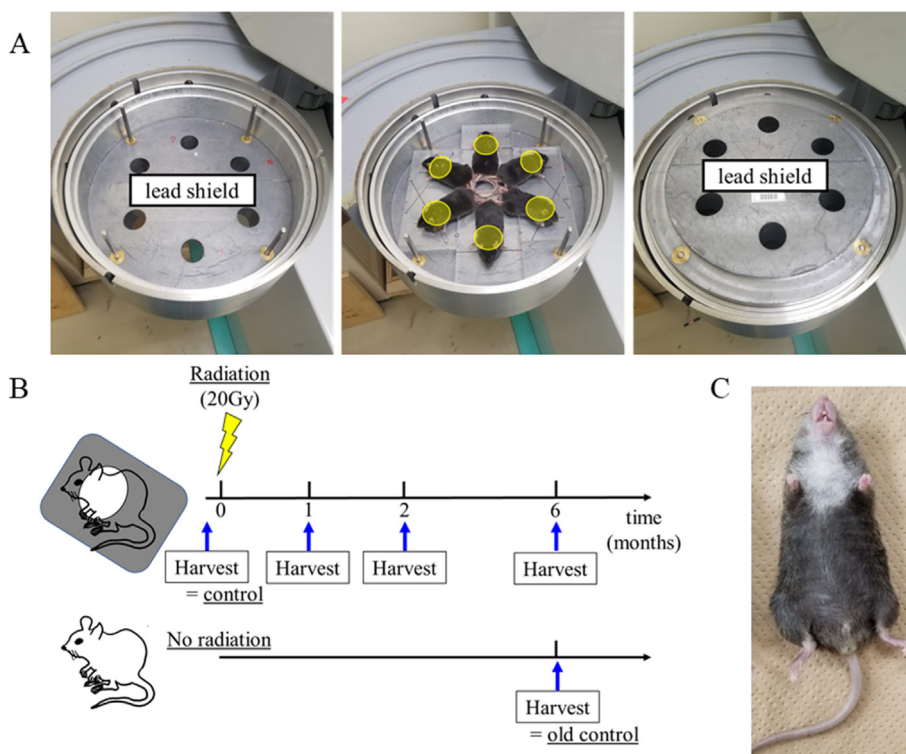


Fig. 1. Irradiation procedure and appearance of an irradiated mouse.

(A) Schematic of the irradiation setup. Each anesthetized mouse was placed in a lead shield with 3 cm holes that blocked radiation except to the neck. Another slab of lead was placed over animal. Circled areas represent the estimated radiation fields. (B) Irradiation and tissue collection schedule. Pre-irradiated mice = control group. Non-irradiated age-matched mice 6 months after irradiation = old control group. (C) Typical mouse 6 months after irradiation. Irradiated area was replaced with white or gray fur.

propria (LP) was digested by hyaluronidase. Digital images were captured using an all-in-one microscope (BZ-X710; Keyence, Osaka, Japan). We then determined the thickness of the LP of the vocal folds, the collagen content in LP, and the HA content in LP using a BZ-X analyzer (Keyence).

2.4. Immunohistochemistry

Identical samples to those used for histological examinations were employed. For antigen retrieval, sections were soaked in 10 mM citric acid (pH 6) at 95 °C for 20 min. After permeabilization with 0.2% Triton-X in PBS and blocking with 1% bovine serum albumin in PBS, the sections were incubated overnight at 4 °C with primary antibodies, 1:500 dilution of a mouse monoclonal anti- α -smooth muscle actin (SMA) (Dako, M0851, Carpinteria, California) and 1:200 dilution of a rabbit monoclonal anti-vimentin (Cell Signaling, D21H3, Danvers, MA). The sections were then incubated for 1 h at 25 °C with the following secondary antibodies: 1:500 dilution of Alexa 488-labeled donkey anti-mouse IgG (Invitrogen, Carlsbad, CA) and 1:500 dilution of Alexa 568-labeled donkey anti-rabbit IgG (Invitrogen). Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen). Omission of the primary antibodies served as a negative control.

2.5. Quantitative reverse transcription polymerase chain reaction (RT-PCR)

To examine gene expression in the vocal folds, the larynges of 3 to 5 mice were harvested at the same time points as for histological evaluations. The mucosa of the vocal folds was dissected using a 29G needle under a stereomicroscope. RNA was extracted using the PicoPure RNA Isolation Kit (Arcturus, Mountain View, CA, USA), following the manufacturer's protocol. DNase treatment was carried out with an RNase-Free DNase Set (Qiagen, Valencia, CA, USA). The quantity of RNA from each sample was evaluated using a NanoDrop spectrophotometer (Thermo Fischer Scientific, Waltham, MA). Reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative reactions were performed using a StepOnePlus Real-Time PCR System (Applied Biosystems). Each quantitative PCR was performed in triplicate.

mRNA expression levels of *TGF- β 1*, *iNOS*, *Acta2*, *Col1a1*, *Col3a1*, *MMP1a*, and *MMP8* were determined. The primers were synthesized by Hokkaido System Science Co., Japan, and the primer sequences are listed in [Supplementary Table 1](#). The relative fold change was calculated using the $\Delta\Delta C_t$ method. Based on a previous report [11], *Sdha* was used as a housekeeping gene.

2.6. Statistical analysis

All data are represented as the mean with standard error of mean (SEM). Statistical analyses were performed using JMP 15 (SAS Institute, Cary, NC, USA) software. To compare the difference at each time point between groups, one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test was performed. Statistical significance was set at $P < 0.05$.

3. Results

3.1. General condition of mice after irradiation

Mild-moderate dermatitis was temporarily observed on the animals in the acute phase after irradiation. The irradiated area was replaced with white or gray fur after 6 months. This revealed that irradiation was uniformly distributed, and the larynges were fully

exposed to irradiation ([Fig. 1C](#)). Although one mouse died of unknown cause 3 months after irradiation, the remaining 27 mice survived until sample collection (survival rate: 96.4%).

3.2. Histological results

The thickness of each LP was evaluated by measuring the maximum distance between the thyroarytenoid muscle and the free edge of the vocal fold following Masson's trichrome staining. LP thickness gradually decreased with time and was significantly reduced at 6 months after irradiation ($P = 0.035$) ([Fig. 2A and B](#)). The effect of age-related changes in the LP was small, as a similar thickness was observed between the control group and the old control group. Post-irradiation collagen deposition in the LP increased in a time-dependent manner, which became significant at 6 months ($P < 0.001$) ([Fig. 2C](#)). The old control group showed no age-dependent increase in collagen density. Alcian blue staining showed that the % area of HA in the LP was unchanged after irradiation and following non-irradiated aging ([Fig. 2D and E](#)).

3.3. Immunohistochemical results

Fibroblasts and myofibroblasts are the main contributors of fibrosis through their production of collagen. In the general course of fibrotic change, the proliferation of fibroblasts and differentiation into myofibroblasts can be observed [12]. Herein, immunohistochemistry of vimentin and α -SMA was performed to examine the population of these cells. Vimentin is a marker of fibroblasts, and α -SMA is a marker of myofibroblasts in the LP of vocal folds [13]. LP cells that were positive for vimentin but not α -SMA were counted as fibroblasts and those that were positive for both were counted as myofibroblasts. LP cells mainly consisted of fibroblasts, and these did not change after irradiation. Myofibroblasts in the LP were few and did not increase throughout the post-irradiation period ([Fig. 3](#)).

3.4. Quantitative RT-PCR results

The most abundant forms of collagen in fibrotic tissues are types I and III [14]. Collagen deposition occurs if collagen synthesis exceeds degradation [8,14,15]. *Col1a1* and *Col3a1* are precursors of collagen synthesis, while *MMP1* and *MMP8* have been reported to play an important role in degrading collagen in vocal folds [16,17]. We examined mRNA expressions of *Col1a1*, *Col3a1*, *MMP1a*, the mouse homologue of *MMP1* [18], and *MMP8* by quantitative PCR. Expression levels of *Col1a1*, *Col3a1*, and *MMP8* were significantly reduced after irradiation ([Fig. 4](#)). *MMP1a* expression was not detected at any time point in the present study.

TGF- β 1 is the master regulator of fibrosis and stimulates myofibroblast differentiation, leading to the high-level synthesis of collagen [19]. *TGF- β 1* is responsible for the production of nitric oxide (NO), which can combine with superoxide and cause DNA damage, which is related to chronic inflammation and fibrosis [20]. To analyze these key factors in fibrosis, we examined the mRNA expressions of *TGF- β 1*, *iNOS*, and *Acta2*, a myofibroblast marker. *TGF- β 1* expression gradually increased after irradiation. In parallel with *TGF- β 1*, *iNOS* expression also increased significantly 6 months after irradiation. However, *Acta2* expression was significantly downregulated following irradiation.

4. Discussion

Herein, we developed an animal model of radiation-induced vocal fold fibrosis and investigated alterations in ECM synthesis over 6 months after irradiation. Immunohistochemistry and

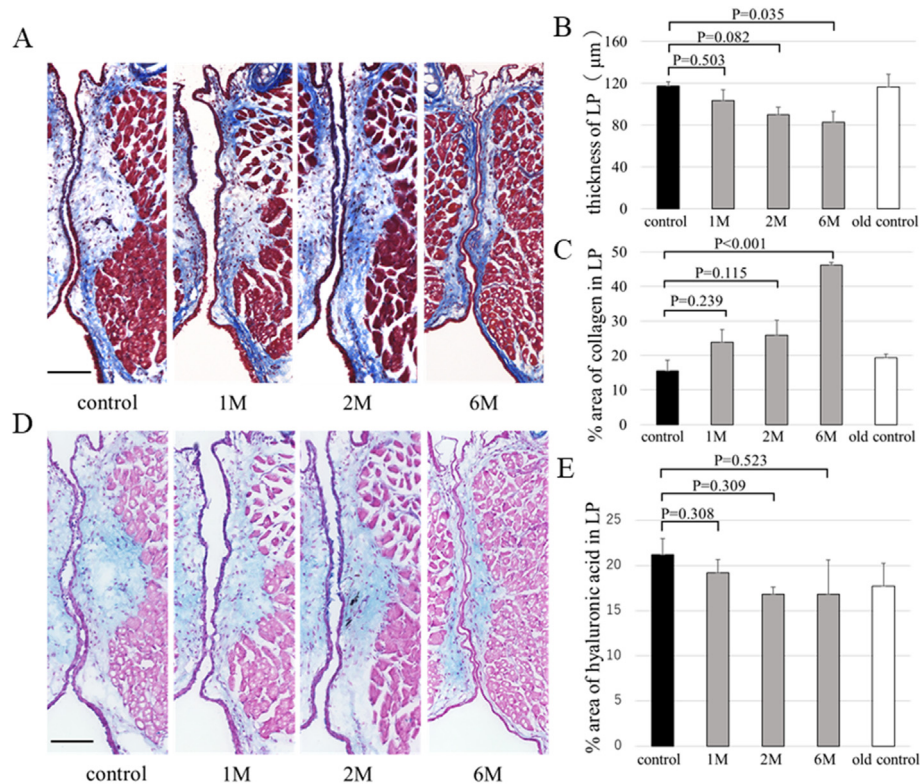


Fig. 2. Histological findings of irradiated vocal folds.

(A) Representative images of Masson's trichrome staining at each time point. Scale bar; 100 μm. M = month. (B) Analysis of regions with collagen fiber in lamina propria (LP) (%). (C) Analysis of LP thickness. (D) Representative images of Alcian blue staining at each time point. Scale bar; 100 μm. (E) Analysis of regions covered by hyaluronic acid in LP (%). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

histological evaluation demonstrated a time-dependent increase in collagen deposition in the LP and tissue contraction throughout the observation period. However, transcriptional analysis revealed downregulated expression of *Acta2*, *Col1a1*, *Col3a1*, and *MMP8*, which indicated decreased collagen turnover.

Our experimental model demonstrated histological/transcriptional changes with low mortality. In the design of radiation therapy protocols, the radiation field and the number of fractionations and doses are important factors. Radiotherapy is usually used in a fractionated schedule in clinical practice. Previous reports, in which irradiation of animal larynges was performed, also applied fractionated schedules [5,21,22]. However, animal models with a single dose of irradiation are frequently used to investigate irradiation impacts on different organs [23–27]; therefore, we adopted a single 20 Gy dose exposure. The greatest advantage of a single irradiation protocol is its simplicity. Furthermore, both single and fractionated schedules can cause similar histological changes [26]. Moreover, a previous study on whole-body irradiation indicated the possibility of lethal damage to the bone marrow [5]; therefore, lead shields were applied for focused irradiation herein, which enabled protection of other organs. With this protocol, the larynges were robustly irradiated with low mortality and no systemic complications. This model is expected to be applied in other studies, such as post-irradiation dysphagia and salivary secretion disorder experiments.

To assess the changes over time and long-term effects of irradiation on vocal fold mucosa, we harvested the larynges at various time points up to 6 months after irradiation. In previous reports [5,6,21], the effects of irradiation on vocal folds were evaluated once between 1 and 3 months after irradiation; however, radiation-induced fibrosis has been reported to occur 4–6 months after

irradiation in various organs [28–30]. In our experimental model with multiple evaluation time points, collagen deposition in the LP of the vocal folds became significant at 6 months after irradiation, which was consistent with previous studies [28–30]. Nonetheless, a long-term investigation is required to clarify the pathogenesis of radiation-induced fibrosis in vocal folds.

Our results indicated that radiation-induced injury to the vocal folds is likely to persist and worsen over time, while previous studies on post-surgical injury fibrosis of vocal folds in rodents revealed scar maturation in 2 months [31,32]. Moreover, HA, which strongly contributes to the viscoelasticity of vocal folds, did not change much after irradiation, as in a previous report [5], whereas it is thought to decrease in post-surgical injury vocal folds [32,33]. These results indicate a unique fibrosis mechanism of the vocal folds after irradiation, which is different from that after surgical injury. In contrast, the LP of the vocal folds became thinner after irradiation. This suggests that the absolute amount of HA decreased, which resulted in the thinning of the LP [34]. This may mean that the decrease in HA in the LP after irradiation is smaller than that after surgical injury.

TGF-β1 expression is usually upregulated during the pathogenesis of fibrosis. *TGF-β1* stimulates the differentiation of fibroblasts into myofibroblasts [8] and induces collagen synthesis via the *TGF-β*/Smad pathway [19,35]. Moreover, *TGF-β1* induces NO production by *iNOS*, leading to DNA damage and chronic injury after irradiation [20]. A previous report revealed an increased expression of *TGF-β1* in the skin at 6 months after irradiation [23], and upregulated expressions of *TGF-β1* and *iNOS* at 6 months after irradiation herein indicated prolonged chronic inflammation. However, our experiment exhibited the following contradictory results: despite upregulated expressions of *TGF-β1* and *iNOS*, downregulated

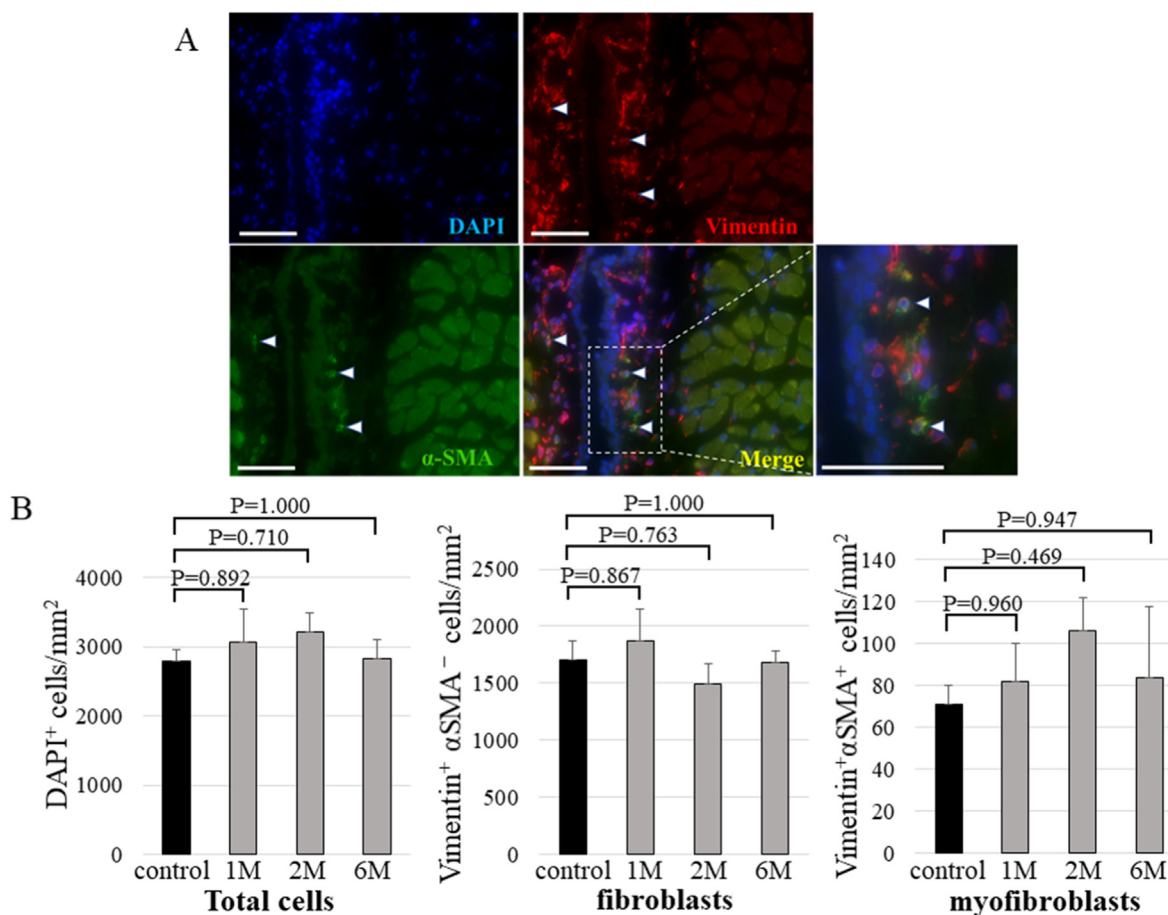


Fig. 3. Immunohistology of irradiated vocal folds.

(A) Representative immunofluorescent images of vocal folds. The LP was immunostained against α -SMA (green), vimentin (red), and counterstained with DAPI (blue). Arrowheads indicate myofibroblasts. Scale bar; 50 μ m. (B) Total number of cells, fibroblasts, and myofibroblasts were counted. Each count was determined respectively as cells positive for DAPI, cells positive only for vimentin, and cells positive for vimentin and α -SMA. *P* values in comparison to the control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

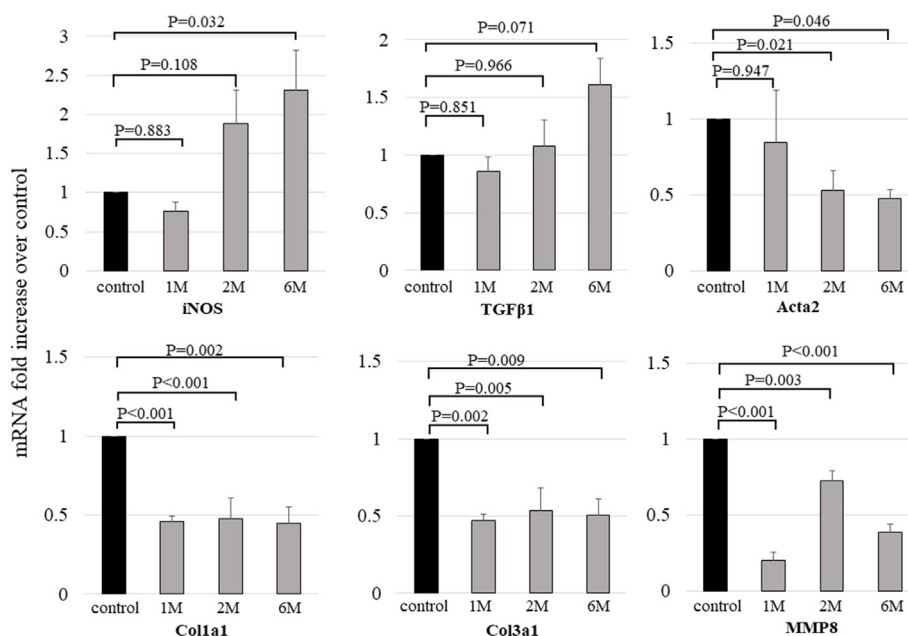


Fig. 4. Quantitative RT-PCR analysis of mRNA levels after irradiation. *P* values in comparison to the control group.

expressions of *Acta2*, which is downstream of the TGF- β /Smad pathway, *Col1a1* and *Col3a1*, collagen synthesis genes, and *MMP8*, a collagen degradation gene were observed. A possible explanation for the discrepancy between TGF- β 1 and its downstream gene is that irradiation could block cellular responses to TGF- β by down-regulating TGF- β receptor II [36]. The observed unchanged number of fibroblasts and myofibroblasts per unit area, reduced *Acta2*, and increased TGF- β 1 levels are consistent with the aforementioned hypothesis. These results suggest the existence of an inhibitory mechanism against the TGF- β /Smad pathway, which leads to differentiation into myofibroblasts. Another discrepancy is between collagen deposition and the reduced expressions of *Col1a1* and *Col3a1*. Decreased expression of *MMP8* may be another key factor. With the downregulated expression of collagen synthesis-related genes and degradation-related gene, the contraction of LP may have contributed to the increased density of collagen, as these findings indicated that the unchanged amount of collagen would be compressed in the thinning LP. Moreover, it has been reported that collagen crosslinking by irradiation increases resistance to degradation in collagenase [37]. Therefore, we suggest that collagen deposition could result from the relative excess of collagen synthesis over degradation and the difficulty in degrading irradiated collagen. This can be described as a decreased collagen turnover.

The present study has some limitations. Although the dose of irradiation was fixed at 20 Gy, previous reports showed that the expression level of TGF- β 1 in the lung and skin was altered with radiation dose [38,39]. Therefore, another protocol could lead to different outcomes. The yield of the extracted RNA from larynxes was too small to determine various gene expressions using quantitative PCR. To clarify the mechanisms of fibrotic alteration in the irradiated vocal fold mucosa at the molecular level, further comprehensive studies, such as single-cell RNA sequencing, are warranted.

In this study, we established a mouse model of radiation-induced vocal fold fibrosis using a simple protocol of a single 20 Gy dose exposure. Collagen deposition increased significantly at 6 months after irradiation, but HA content remained constant. This shows that the mechanism of vocal fold fibrosis after irradiation could be different from that observed after surgical injury. It is thought that radiation-induced vocal fold fibrosis could result from the reduced turnover of collagen.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrc.2022.02.065>.

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