Distribution and characteristics of slow-cycling cells in rat vocal folds

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Running title:

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This study was supported by a Grand-in-Aid of Ministry of Health and Welfare in Japan.

No financial interests, relationship and affiliations to disclose.

No conflict of interest to disclose.

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This is a pre-peer reviewed version of the following article: Distribution and Characteristics of Slow-Cycling Cells in Rat Vocal Folds, which has been published in final form at http://onlinelibrary.wiley.com/doi/10.1002/lary.25558/abstract;jsessionid=324C1E1E551CD4596BCE0E771E41578E.f02t03

Abstract

Objective: Stem cells are known to proliferate at a slow rate in adult organs, and thus slow-cycling cells exhibiting pluripotency are considered tissue-specific stem cells in some organs. Slow-cycling cells in the vocal fold (VF) have not been well documented. Here we sought to clarify the distributions and characteristics of slow-cycling cells in rat VFs. **Methods:** We applied double labeling technique to detect the distribution of slow-cycling cells. We injected the exogenous proliferation marker, 5-bromo-2'-deoxyuridine (BrdU) into Sprague-Dawley rats. After a chasing period, VFs were immunostained with antibodies to BrdU and the second endogenous proliferation marker, Ki-67. BrdU (+) Ki-67(+) cells were regarded as slow-cycling cells, and counted by VF regions. To reveal slow-cycling cells' characteristic, their immunophenotypes were histologically investigated and their kinetics in injured VFs were evaluated. **Results:** Most slow-cycling cells were detected in the basal layer of the epithelium. Slow-cycling cells in the epithelium displayed low positive ratio of E-cadherin and CK5, and high positive ratio of vimentin and CD31, compared with the other epithelial cells. The expression of S100A4 was low in slow-cycling cells of the lamina propria and the macula flava. FGFR1, HAS1, HAS2, and HAS3 were not detected in the slow-cycling cells. A time-dependent reduction of slow-cycling cells was observed in injured VFs. **Conclusion:** Most slow-cycling cells

resided in the epithelium, exhibiting various phenotypes in a relatively undifferentiated condition, and they are suspected to contribute to the tissue repair of the injured VFs.

Keywords:

vocal fold, label retaining cell, slow-cycling cell, stem sell, regenerative therapy

Level of Evidence: N/A

Introduction

The viscoelastic shear properties of the vocal fold (VF) are disrupted by injury, inflammation, or phonosurgical procedures, resulting in impaired VF oscillations. No optimal treatments for scarred VFs have been established, and various therapeutic approaches have been tried in attempt to restore scarred VF tissues.

In tissue engineering research, cells, growth factors and scaffolding materials have been widely used in experimental setups, and some restorative effects on injured VFs have been reported. Among these options, cell transplantation and growth factor administration are potent options, but some hurdles remain before their clinical use is possible. Because tissue-specific stem cells can be used in situ without cell transplantation, their use has attracted attention recently as a cell source for cell therapies.

Generally, adult tissue stem cells possessing the capacity for self-renewal and for the generation of multilineage cells are recruited into active proliferation during tissue regeneration and repair.[1] Tissue-specific stem cells have not been reported in mammalian VFs; however, should they exist, they may play a role in tissue homeostasis and the wound healing process. Various strategies have been used in attempts to identify the presence of tissue-specific stem cells in VFs. Yamashita et al. identified side population cells in the epithelium, the anterior and posterior macula flava (AMF and PMF), and the

lamina propria (LP) by their ability to exclude a DNA binding dye.[2] Sato et al. suggested stellate cells, a morphologically distinguished cell population in the MF, as potential tissue-specific stem cells in the VFs.[3] Hanson et al. reported human VF fibroblasts in LP are identical to mesenchymal stem cells, because VF fibroblasts derived from LP have the same cell surface markers as mesenchymal stem cells.[4] However, no consensus about the existence of VF-specific stem cells has been reached.

In the absence of a specific cell marker, identifying the stem cells by their slow-cycling properties has been recognized as a new approach in detecting tissue-specific stem cells in various organs. With this approach, 5-bromo-2'-deoxyuridine (BrdU),[5] 3H thymidine,[6] and histone H2B-green fluorescent protein (GFP)[7,8] have been used as DNA labeling that dilutes out over time during cell division. BrdU is a synthetic analogue of thymidine that becomes incorporated into the cell's DNA during the S phase of mitosis. After a certain chasing period, rapidly dividing cells lose the label, and infrequently dividing cells retain the label.

The duration of the chasing period is highly variable and depends on the growth kinetics of the tissues being studied. Moreover, label-retaining cells sometimes differentiate and lose their self-renewal ability during the chasing period,[9] and those cells should be excluded from the detection of slow-cycling cells as potential tissue-specific

stem cells.

Ki-67, a second proliferation marker that labels cells in the late G1, S, G2, and M phases, has been used to identify label-retaining cells that maintain proliferative capacity.[5]

This double labeling technique has been successful in detecting putative stem cells in many organs, including cochlear stem cells,[5] stomach stem cells,[10] endometrial epithelial stem cells,[11] and mammary epithelial stem cells.[12] Although Leydon et al. have already reported label-retaining cells in the mouse VF with BrdU injection,[13] they didn't apply any second proliferation marker. The double labeling technique applied in this study is expected to detect putative stem cells with higher specificity, and will be helpful to explore their characteristics.

In the present study, using this approach coupled with immunohistochemical analyses, we identified the location of slow-cycling cells in naive and injured rat VFs, and we investigated their characteristics.

Material and Methods

Animals

Eighteen Male 13-wk-old Sprague-Dawley rats were purchased (Japan SLC, Shizuoka, Japan). All experimental protocols were approved by the Animal Research Committee of

the Kyoto University Graduate School of Medicine. Animal care was provided by the Institute of Laboratory Animals of Kyoto University.

BrdU Labeling and Vocal Fold Injury

All rats were intraperitoneally administered 1% BrdU (Sigma-Aldrich, St. Louis, MO) at a dose of 50 mg/kg/day using a 22-ga. needle for 7 consecutive days, according to previous report.[13]

For the detection of the optimal chasing period, twelve rats were sacrificed and their larynges were harvested at 1, 5, 10 and 15 days post-injection. (n = 3 per condition). At the time of sacrifice, rats were euthanized with inhalative sedation with diethyl ether, followed by an intracardiac injection of sodium pentobarbital (200 mg/kg). Additionally, we investigated the characteristics of slow-cycling cells with the specimen obtained from the same animals.

VF injuries were created as described.[14] Rats underwent anesthesia induction with diethyl ether, followed by maintenance using an intraperitoneal injection of ketamine hydrochloride (90 mg/kg) and xylazine hydrochloride (9 mg/kg). The anesthetized rats were placed on an operating platform in a near-vertical position, and a 1-mm-dia. steel wire laryngoscope was inserted to facilitate VF visualization. A 2.7-mm-dia. 30° rigid

endoscope (A70961A, Olympus, Tokyo) connected to an external light source and video monitor was used for surgical monitoring. The VF mucosa was incised using 20-ga. scissors (AU-1286-ED, DORC, Zuidland, Netherlands). Rats were incised their right VF mucosa at 7 days (n = 3) or 9 days (n = 3) after the BrdU injection. At 10 days post-injection, rats were sacrificed and their larynges were harvested and subjected to histological analyses.

Immunohistochemistry

The larynges were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 4 h at 4°C and dehydrated in 30% sucrose. The specimens were embedded in frozen OCT compound (Sakura Finetek Japan, Tokyo) and frozen quickly with liquid nitrogen. Serial 7-μm axial sections were obtained with z cryostat (CM 1850 Kryostat, Leica Instruments, Nussloch, Germany).

Slides with specimens were incubated at 99°C in citrate buffer (pH 6.0) for 15 min for antigen retrieval, then permeabilized in 0.2% triton-X for 10 min and blocked in 5% skim milk (Nakalai Tesque, Kyoto, Japan) for 1 h.

Specimens were incubated with primary anti-BrdU antibody at 4°C overnight, then incubated with primary anti-Ki-67 antibody at room temperature (RT) for 3 h. After

incubating with secondary antibodies, specimens were carefully washed and another primary antibody incubation and secondary antibody incubation were performed to characterize them. To stain nuclei, 2 mg/mL 4', 6'-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen, Carlsbad, CA) was used. All antibodies and working dilutions in this experiment are shown in Table 1.

Images were obtained using a confocal laser-scanning microscope (TCS SPE, Leica Microsystems, Wetzlar, Germany) and a fluorescent microscope (Biorevo, BZ 9000, Keyence, Osaka, Japan)

Cell Counts and Statistical Analyses

To analyze the localization of slow-cycling cells, we subdivided naive VFs into five regions: the basal layer of the epithelium, the supra-basal layer of epithelium, the LP, the AMF and the PMF. Injured VFs were subdivided into four regions: the epithelium, LP, AMF, and PMF, as it was difficult to identify the basal layer of the epithelium after injury. At each region, eight sections were examined for naive VFs, and four sections were examined for injured VFs. BrdU/Ki-67-double-positive cells were counted, and the averaged numbers were used for analyses.

Multiple comparisons were performed using the Tukey-Kramer method. P-values

less than 0.01 were considered significant. All statistical results were obtained using JMP pro version 11.1 (SAS, Cary, NC).

Results

Retaining of BrdU Label

At day 1 post-injection, BrdU-positive cells were identified in various part of the VF, including the epithelium, the LP, and the MF. However, the numbers of the BrdU-positive cells in the LP and the MF were significantly decreased by day 10 post-injection. At day 15, only a few BrdU-positive cells remained in the epithelium (Fig. 1). Based on this result, we used 10 days as the optimal chasing period for detecting slow-cycling cells in the VF in the following experiments.

Localizations and Characteristics of Slow-Cycling Cells

At day 10 post-injection, BrdU (+) Ki-67 (+) slow-cycling cells were detected in all five subdivided regions, and among these regions, slow-cycling cells were found to be significantly abundant in the basal layer of the epithelium ($p<0.01$) (Fig. 2). To characterize the slow-cycling cells, we performed immunohistochemistry to detect E-cadherin, CK5, vimentin, CD31, S100A4, FGFR1, HAS-1, HAS2, and HAS3 (Table 2,

Fig. 3).

E-cadherin, CK5, vimentin, and CD31 are markers for epithelial, basal layer, mesenchymal, and endothelial cells, respectively. We compared the positive ratio of these surface markers between slow-cycling cells and non-slow-cycling cells (BrdU (-) or Ki-67 (-)). Non slow-cycling cells in the basal layer of the epithelium were positive for E-cadherin (100%; 219 positive cells of 219 cells) and CK5 (99.6%; 231 positive cells of 232 cells), and mostly negative for vimentin (0.70%; 2 positive cells of 286 cells) and CD31 (1.83%; 4 positive cells of 218 cells). These numbers were obtained from 10 randomly chosen high-power fields [HPFs] of epithelial basal layer. However, some slow-cycling cells in the basal layer of the epithelium were negative for E-cadherin (5% negative) and CK5 (3.2% negative), and positive for vimentin (7.3% positive) and CD31 (3.8% positive).

S100A4 has been widely accepted as a fibroblast-specific marker, and 69.0% of the present study's normal LP and MF cells expressed S100A4 (156 positive cells detected among 226 LP and MF cells from 10 randomly chosen HPFs). Only 20% of the slow-cycling cells in the LP or the MF were positive for S100A4.

FGFR1 is also known as 'basic fibroblast growth factor receptor 1,' which is expressed occasionally in the epithelial cells of normal VFs. We did not detect any FGFR1

in the slow-cycling cells. Additionally, none of the slow-cycling cells were positive for hyaluronan synthase 1 (HAS-1), HAS-2, or HAS-3, although some cells in the LP and the MF were positive for them.

Slow-Cycling Cells in Injured Vocal Folds

To examine the response of slow-cycling cells in the VFs after injury, we performed a VF incision with BrdU-labeled rats, and they were sacrificed 1 day or 3 days post-surgery. In the injured VFs, increased numbers of Ki-67-positive cells due to the infiltration of inflammatory cells and decreased numbers of BrdU-positive cells were observed (data not shown). Compared to the naive VFs, the number of slow-cycling cells in the injured VFs was significantly decreased ($p<0.01$) (Fig. 4). This reduction of slow-cycling cells was observed on both the injured and intact sides of the VFs.

The distribution of slow-cycling cells in the 1-day post-injury VFs tended to be abundant in the epithelium, but this tendency was not significant, unlike that in the naive VFs (Table 3). In the 3-days post injury VFs, the number of slow-cycling cells in the injured-side LP surpassed that in the injured-side epithelium.

Discussion

We applied a double-labeling technique that allows us to seek a cell population that has both the proliferative and slow-cycling characters, which are thought to be inherent in tissue stem cells. With this method, we can discriminate cell populations that have differentiated to the quiescent state from BrdU-retaining cells.

The dose of the BrdU and injection frequency were determined in accordance with the previous work of Leydon et al.[13] Onetime 50 mg/kg BrdU injection was applied by Tateya et al. to investigate the cell kinetics of injured VFs.[15] However, they revealed very small number of cells soaked up BrdU in intact VF. This report indicates the number of cells in S phase is so few and thus multiple-injections of BrdU is required to label more cells which have the proliferative character.

In this approach, the chasing period is important to detect true slow-cycling cells. Another research group hypothesized that the optimal chasing period in murine VFs is 150–200 h[13] based on two findings: epithelial cells divide every 30 h,[16] and BrdU labeling is detectable for up to five or six cell divisions.[17] Another study revealed the doubling time of human VF fibroblasts to be 30 h.[18] According to these reports and our own finding of a radical loss of BrdU in the MF on day 10, we used 10 days as an optimal chasing period in this study.

Generally, the role of intact VF epithelium has been regarded as a barrier to various

injuries, and as a contributor to the maintenance of optimal VF hydration by ion and water transport.[19,20] Our study revealed that a significant number of slow-cycling cells are located in the rat VF epithelium, which implies that the VF epithelium could be a storage site of putative stem cells. Leydon et al. also demonstrated the existence of label-retaining cells in the VF epithelium,[13] and our results are consistent with this.

We found that some of the slow-cycling cells in the epithelium were negative for epithelial markers and positive for mesenchymal and endothelial markers. These results suggest that the character of slow-cycling cells is not defined only by their locations, and they imply a possible phenotypical transition between epithelial, mesenchymal and endothelial cells. Further, the relatively low expression ratio of S100A4 and the lack of FGFR1, HAS1, HAS2, and HAS3 expression in slow-cycling cells might reflect the immature nature of slow-cycling cells.

We used S100A4 as a surface marker of the fibroblasts. It is known that macrophages are also stained with S100A4. However, Jecker et al. proposed only 10.9 macrophages were identified in 1 mm^2 of rat VF,[21] and macrophages are mainly distributed in sub-epithelial compartment of the VF.[22] Considering rat VFs have 1mm length and 0.2mm thickness,[23] macrophages account for such a small part in S100A4 (+) cells. And their distribution are different from that of slow-cycling cells. So we considered

S100A4 (+) cells are highly specific to fibroblasts.

In the injured rat VF, it is reported that the wounds are completely covered with hypertrophic epithelium within 3 days, and then the epithelial cells start to differentiate to squamous-looking cells in 7 days.[24] Quick reconstruction of the epithelial barrier would contribute to the protection of the healing site from additional insult. A 2014 study revealed that epithelial cells play an active role during the early phase of wound healing by secreting EGF and TGF-β1.[25]

Our present data demonstrated the time-dependent reduction of slow-cycling cells in the injured rat VFs. One possible reason is that with VF injury, slow-cycling cells are damaged and lost by necrosis or phagocytosis. However, we also observed this reduction of slow-cycling cells in the contralateral, uninjured VF. These results infer the effects of inflammatory cytokines, which accelerate the cell cycle during the wound healing process, and the results indicate that the slow-cycling cells might be diffused to the adjacent tissues or to the other side of the VF. A similar result was reported, as the wounded cornea1 epithelium stimulates cell proliferation in the distant epithelium.[26]

We found that 3 days after injury, slow-cycling cells were rarely observed in the epithelium and distributed mainly to the LP. Previous work described the post-injury migration of slow-cycling cells from the hair follicles to the epidermis,[27] and from renal

tubules to the interstitium,[28] and a similar response of the slow-cycling cells in the VF are expected. Further studies are warranted to clarify the roles and the sources of slow-cycling cells.

The limitation of this study is that an underestimation of the number of slow-cycling cells is technically inevitable for several reasons. First, as BrdU is soaked up by nuclei only in the S phase, a certain number of slow-cycling cells would be overlooked if they are temporarily quiescent during BrdU administration.[13] Second, some of the Ki-67 negative cells were just quiescent at evaluation and did not lose their self-renewal feature. However, the double-labeling technique enabled us to evaluate a cell population that possessed self-renewal capacities and slow-cycling features with high specificity. Given the purpose of this preliminary experiment to find a potential candidate for tissue-specific stem cells of the VF, our results are meaningful.

We demonstrated the locations and characteristics of slow-cycling cells as a step to detect tissue-specific stem cells in the VF. Immunohistochemistry revealed that most of them are located in the epithelium and that they are kept in a relatively undifferentiated state. The surface markers of slow-cycling cells implied the possibility of stem cell migration between the epithelium and stroma, and the injury model demonstrated their recruitment during the wound healing process. Further studies including another tracing

technique and in vitro investigations are needed to examine these cells' migration, pluripotency and roles.

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Fig. 1. Distribution of BrdU-positive cells in axial sections of rat vocal folds (VFs). Representative examples of immunohistochemistry for BrdU on day 1 (A), day 5 (B), day 10 (C), and day 15 (D) post-BrdU injection. BrdU-positive cells were located in the epithelium, lamina propria (LP), and macula flava (MF) at day 1 and day 5, and their number was significantly reduced in the LP and MF on days 10 and 15. Scale bar: 300 μm.

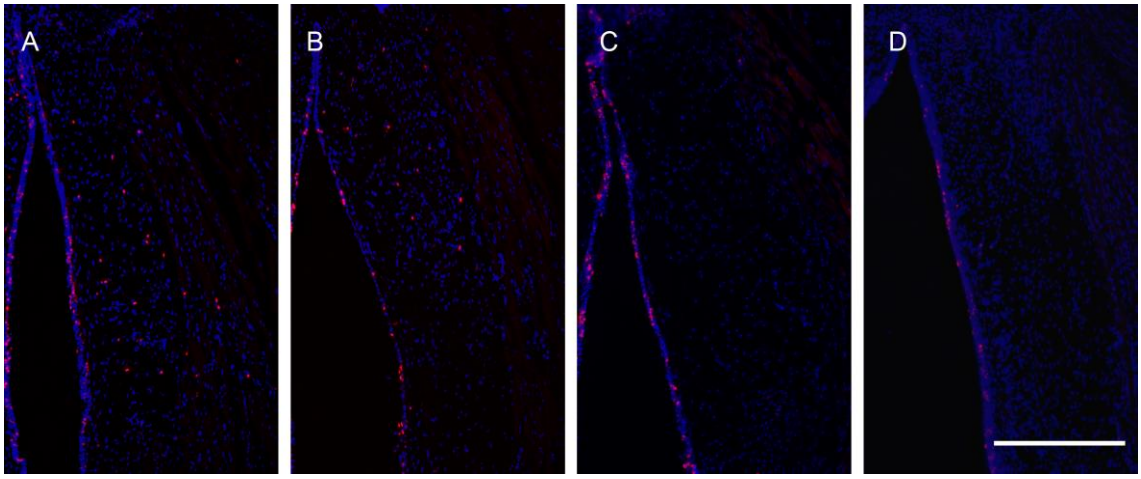

Fig. 2. Localization of slow-cycling cells 10 days after BrdU injection.

(A) An example VF simultaneously immunostained with BrdU and Ki-67. Scale bar: 300 μm. (B, B', B'') These three panels were obtained from one section and represent one of the slow-cycling cells at high magnification. Arrowheads indicate a slow-cycling cell, double-positive for BrdU and Ki-67. Scale bar: 10 μm. (C) The average numbers of slow-cycling cells per one section of lateral VF were counted by their region. Significantly more slow-cycling cells were located in the basal layer of the epithelium. Error bars: the standard deviation. *P<0.01.

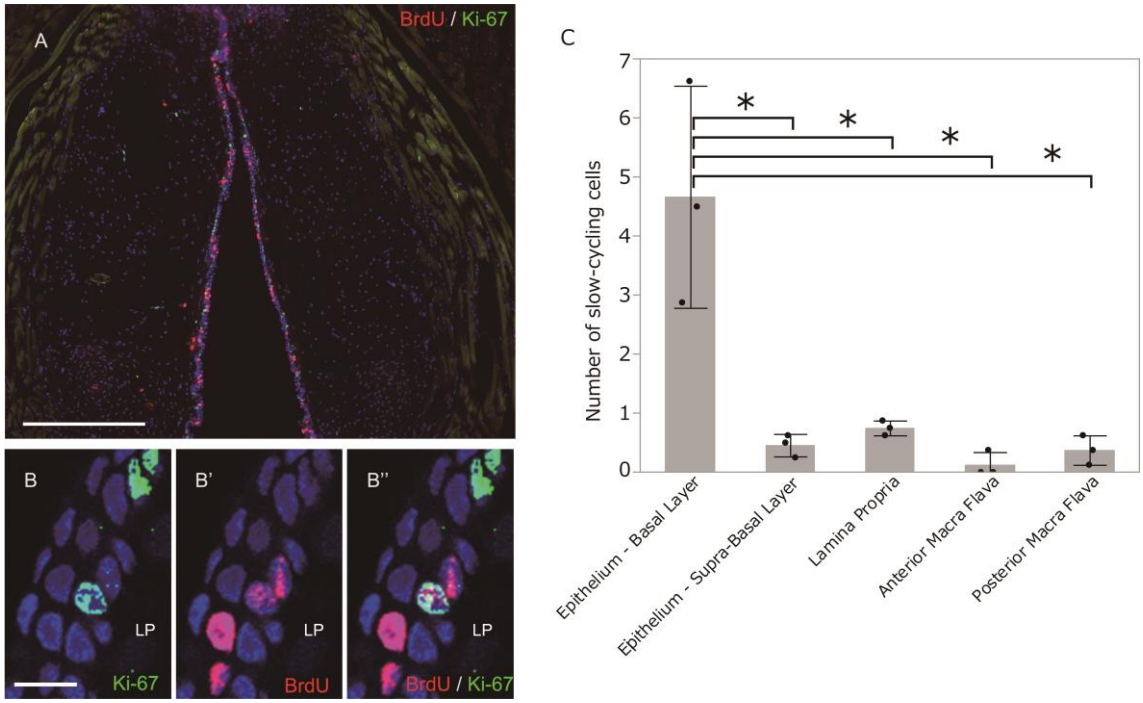

Fig. 3. Characterization of slow-cycling cells.

Examples of slow-cycling cells negative for E-cadherin (A, A'), negative for CK5 (B, B'), positive for vimentin (C, C'), and positive for CD31 (D, D'). Typical expression pattern of surface markers in vocal folds (E–M): E-cadherin in the whole epithelium (E), CK-5 in the basal layer of the epithelium (F), vimentin in several cells in the LP and MF (G), CD31 in several cells in the LP (H), S100A4 in over half of the cells in the LP and MF (I), FGFR1 in a few cells in the epithelium (J), HAS1 in several cells in the LP and MF (K), HAS2 nowhere in the VF (L), and HAS3 in several cells in the LP and MF (M). Arrowheads indicate slow-cycling cells. Scale bar: 10 μm.

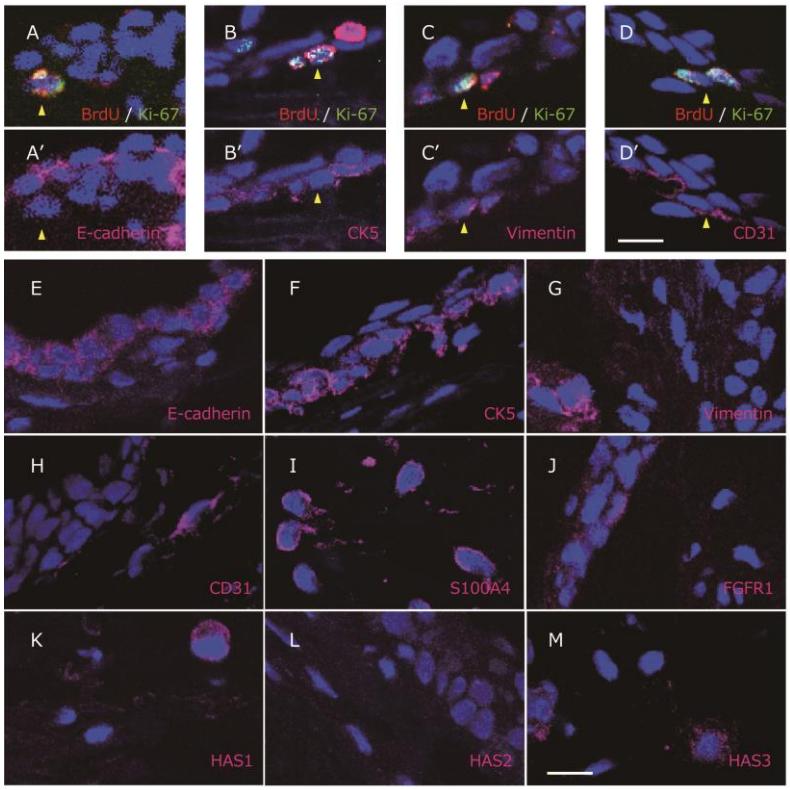

Fig. 4. Slow-cycling cell post injury.

(A) Example of post-day 1 right-side VF injury. (B) The numbers of slow-cycling cells at post-day 1 injury, post-day 3 injury, and an untouched naive control VF are shown, divided into the injured side and intact side. Scale bar: 300 μm. Error bars: std. dev. *P<0.01.

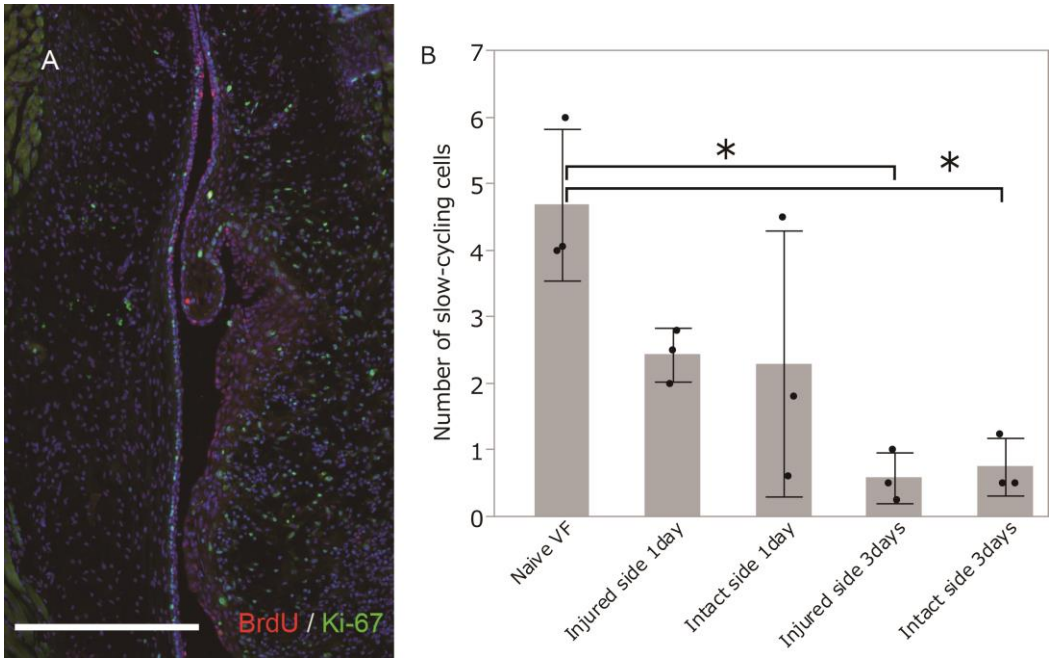

Table 1. Antibodies in immunohistochemistry

Antibody	Host animal	Dilution	Source
BrdU	Mouse	1:100	BD
Ki-67	Rabbit	1:100	Neo Markers
E-cadherin	Mouse	1:100	BD
CK5	Rabbit	1:2000	Covance
Vimentin	Mouse	1:100	BD
CD31	Rat	1:100	BD
S100A4	Rabbit	1:100	Abcam
FGFR1	Rabbit	1:100	Santa Cruz
HAS1	Goat	1:100	Santa Cruz
HAS2	Rabbit	1:100	Santa Cruz
HAS3	Goat	1:100	Santa Cruz
Mouse IgG Alexa488	Donkey	1:500	Invitrogen
Rabbit IgG Alexa555	Donkey	1:500	Invitrogen
Mouse IgG Alexa633	Donkey	1:500	Invitrogen
Rabbit IgG Alexa633	Donkey	1:500	Invitrogen
Goat IgG Alexa633	Donkey	1:500	Invitrogen

Rat	IgG Alexa633	Donkey	1:500	Invitrogen

Table 2. Positive ratio of slow-cycling cell surface markers by their region

	Epi-BL	**Epi-SBL**	**LP**	**AMF**	**PMF**
E-cadherin	38 / 40	2 / 2	0 / 9	0 / 6	0 / 2
CK5	30 / 31	0 / 3	0 / 7	0 / 7	0 / 2
Vimentin	3 / 41	0 / 6	0 / 4	2 / 10	2 / 8
CD31	2 / 52	0 / 5	0 / 13	0 / 3	0 / 2
S100A4	0 / 26	- / -	0 / 5	2 / 5	- / -
FGFR1	0 / 65	0 / 8	0 / 10	0 / 12	0 / 3
HAS1	0 / 44	0 / 5	0 / 11	0 / 11	0 / 1
HAS2	0 / 63	0 / 6	0 / 6	0 / 9	0 / 2
HAS3	0 / 35	0 / 10	0 / 10	0 / 5	0 / 1

Surface marker positive slow-cycling cells/Total slow-cycling cells. Epi-BL: epithelium-basal layer, Epi-SBL: epithelium-supra-basal layer, LP: lamina propria, AMF: anterior macula flava, PMF: posterior macula flava.

Table 3. Distribution of slow-cycling cells in injured vocal folds

	Day 1	**Day 3**
Intact side Epi-BL	1.67	0.5
Intact side Epi-SBL	0.17	0.17
Intact side LP	0	0.08
Intact side AMF	0.3	0
Intact side PMF	0.17	0
Injured side Epi	1.67	0.17
Injured side LP	0.33	0.41
Injured side AMF	0.13	0
Injured side PMF	0.3	0

The average numbers of slow-cycling cells per one section of lateral VF are shown by their region. Epi-BL: epithelium-basal layer, Epi-SBL: epithelium-supra-basal layer, LP: lamina propria, AMF: anterior macula flava, PMF: posterior macula flava, Epi: epithelium.