Heterogeneity and Hierarchy of the Tissue Stem Cells in the Human Adult Vocal Fold Mucosa

Kiminori Sato, Shun-ichi Chitose, Fumihiko Sato, Kiminobu Sato, Takashi Kurita, Takeharu Ono and Hirohito Umeno

INTRODUCTION

Our previous research revealed the cells in the maculae flavae are tissue stem cells and the maculae flavae are a candidate for a stem cell niche of the human vocal fold mucosa. It is generally known that stem cells have heterogeneity and hierarchy in the stem cell system.

In vitro, our previous research showed that three phenotypes of cells (cobble stone-like polygonal cells, vocal fold stellate cell-like cells and fibroblast-like spindle cells) proliferated when human maculae flavae fragments were cultured. This result indicates that the cells in the maculae flavae of the human adult vocal fold have heterogeneity in vitro.

In vivo, the heterogeneity and hierarchy of the tissue stem cells in the maculae flavae of the human adult vocal fold as a vibrating tissue are of interest. In vivo, our past study revealed that the tissue stem in the maculae flavae of the human newborn vocal fold mucosa have immature heterogeneity and hierarchy in the stem cell system. However, there has been no investigation in vivo whether the cells in the maculae flavae of the human adult vocal fold have heterogeneity and hierarchy in the stem cell system.

The purpose of this study is to investigate heterogeneity and hierarchy of the cells in the maculae flavae (stem cells niche) of the human adult vocal fold mucosa in vivo.

MATERIALS AND METHODS

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guidelines on human experimentation (Kurume University) and with the Helsinki Declaration of 1975, as revised in 2008. Informed consent was obtained from the subjects after the nature of the experimental procedure was explained.

Four normal human adult vocal folds were investigated. Any diseases that could possibly affect the tissue of the
vocal fold were not observed.

The cells in the maculae flavae of the human adult vocal fold mucosa were observed using transmission electron microscopy and light microscopy including immunohistochemistry.

**Transmission electron microscopy (TEM)**

The human vocal folds were fixed in 2.5% glutaraldehyde, rinsed with cacodylate buffer solution and postfixfixed in 2% osmium tetroxide with cacodylate buffer solution. The human vocal folds were dehydrated in graded concentrations of ethanol and embedded in epoxy resin. Thin sections were made with an ultramicrotome and stained with uranyl acetate & lead citrate and tannic acid. Observation was performed using a H-7650 (HITACHI, Japan) transmission electron microscope.

**Light microscopy (immunohistochemistry)**

For light microscopy, specimens were fixed in 10% formalin, dehydrated in graded concentrations of ethanol, and embedded in paraffin. Hematoxylin-Eosin stain was used for each section, and immunohistochemical staining was carried out. Stage-specific embryonic antigen (SSEA-3) was detected histologically in formalin-fixed and paraffin-embedded tissue by immunohistochemistry, for which a universal immunoenzyme polymer method staining kit (Histofine Simple Stain MAX-PO, Nichirei, Tokyo, JAPAN) was used.

Specimens were sectioned to a thickness of 5 to 6 µm and mounted on glass slides. Deparaffinized and hydrated sections were rinsed with 0.01-mol/L phosphate buffered saline (PBS) at pH 7.4. The specimens were covered with 3% hydrogen peroxide for 10 minutes and rinsed with 0.01-mol/L PBS, followed by treatment with normal mouse serum. The specimens were then incubated with the primary antibody overnight at 4°C.

A 1:200 antibody against SSEA-3 (Abcam. Cambridge, UK, ab16286, rat monoclonal) was used.

After rinsing with PBS and labeling with the universal immuno-enzyme polymer method staining kit, a color reaction was developed with 3,3'-diaminobenzidine at room temperature. Immunoreactivity was examined by light microscopy.

**RESULTS**

**Transmission electron microscopic (TEM) findings**

Cobblestone-like polygonal cells (Figure 1), vocal fold stellate cell-like cells possessing lipid droplets in the cytoplasm (Figure 2) and fibroblast-like spindle cells (Figure 3) were intermingled in the maculae flavae of the human vocal fold mucosa in vivo under electron microscopy, indicating that the cells in the maculae flavae of the human adult vocal fold had heterogeneity in vivo.

1) **Cobblestone-like polygonal cells**

Cobblestone-like polygonal cells (Figure 1) were round and polygonal in shape with no cytoplasmic processes or lipid droplets. The nucleus-cytoplasm ratio was relatively large, and intracellular organelles were not developed. Sometimes, another cell was attached to the cobblestone-like polygonal cell.

2) **Vocal fold stellate cell-like cells**

Vocal fold stellate cell-like cells (Figure 2) were irregular and stellate in shape and possessed slender cytoplasmic processes. Lipid droplets were present in the cyto-
plasm and they were 1 to 2 µm in diameter. The nucleus
was oval. The nucleus-cytoplasm ratio was small, and
intracellular organelles such as rough endoplasmic reticu-
rum and Golgi apparatus consisting of lamellae were pre-
ent. A few small mitochondria were present. Along the
surface of the cells, a number of vesicles were present.

3) Fibroblast-like spindle cells

Fibroblast-like spindle cells (Figure 3) were spindle-
shaped or oval, with no cytoplasmic processes and few li-
pid droplets. The nuclei were elliptic. The nucleus-cyto-
plasm ratio was large. Poorly developed intracellular
organelles such as rough endoplasmic reticulum and Golgi
apparatus were apparent. Along the surface of the cells,
few vesicles could be seen.

Light microscopic (immunohistochemistry) findings

Among the three phenotypes of cells which have multi-
potency in the human adult maculae flavae, the cobble-
stone-like polygonal cells expressed SSEA-3 (Figure 4), a
human pluripotent stem cell marker. This result suggests
cobblestone-like polygonal cells are at the top of a cellular
hierarchy in the stem cell system in the maculae flavae of
the human adult vocal fold.

DISCUSSION

Our previous investigations showed that the tissue
stem cells reside in the maculae flavae (a stem cell niche)
of the human vocal fold mucosa. Tissue stem cells
generate different cell types for the specific tissue or or-
gan in which they live. Consequently, it is important to
distinguish from other kinds of stem cells such as mesen-
chymal stem cells. Regarding the human vocal fold mu-
cosa, mesenchymal stem cells residing in the human vo-
cal fold mucosa are not tissue stem cells. Side population cells and slow-cycling cells are categories of cells, consequently, they are not completely equivalent
to stem cells.

Heterogeneity of the Cells in the Maculae Flavae of the
Human Adult Vocal Fold Mucosa

Since vocal fold stellate cells (stellate in shape and pos-
sessing vitamin A-storing lipid droplets) contained in the
human maculae flavae were discovered in our labora-
tory, they have attracted notice as a new category of
cells in the human vocal fold. However, our recent re-
search shows that three phenotypes of cells, cobble stone-
like polygonal cells, vocal fold stellate cell-like cells, and fi-
broblast-like spindle cells, proliferated when human
macula flava fragments were cultured in vitro.

The present study revealed that cobblestone-like polyg-
onal cells, vocal fold stellate cell-like cells possessing lipid
droplets in the cytoplasm and fibroblast-like spindle cells
were intermingled in the maculae flavae (stem cells niche) of the human vocal fold mucosa in vivo under electron microscopy. Consequently, the same three phenotypes of cells, which can be observed in vitro, reside in the maculae flavae (stem cells niche) of the human vocal fold in vivo. Consequently, the cells residing in the macula-
flavae (stem cells niche) are suggested to have hetero-
genility in vivo. The vocal fold stellate cells are most
likely one of the phenotypes of cells in the maculae flavae
(stem cell niche).

Hierarchy of the Cells in the Maculae Flavae of the Human
Adult Vocal Fold Mucosa

Fig. 3. Fibroblast-like spindle cells (TEM, tannic acid stain).

Fig. 4. SSEA-3 is detected in the cobblestone-like polygonal
cells in the human maculae flavae, shown by immuno-
histochemical staining.
Stem cell system has hierarchy of cells composed of stem cells, progenitor cells (transient amplifying cells) and differentiated cells.

The three phenotypes of cells in the human maculae flavae have multipotency in vitro\(^{10}\). The present study showed that, among the three phenotypes of cells in the human adult maculae flavae, the cobblestone-like polygonal cells in the human macula flava expressed SSEA-3 (a human pluripotent stem cell marker), suggesting the cobblestone-like polygonal cells has not only multipotency but also pluripotency. Consequently, cobblestone-like polygonal cells are at the top of the cellular hierarchy in the human adult maculae flavae (Figure 5). Our previous research shows that the fibroblasts in the tissue surrounding the human maculae flava do not express CD44 (mesenchymal stem cell marker), however, CD44-positive fibroblast-like spindle cells are observed at the periphery of the human maculae flavae and the cells in the macula flava appear to differentiate into fibroblasts in the surrounding tissue\(^{2,8}\). Consequently, fibroblast-like spindle cells in the human maculae flavae are likely at the bottom of the cellular hierarchy in the human maculae flavae (Figure 5). Therefore, vocal fold stellate cell-like cells in the human maculae flavae are likely at the second level of the cellular hierarchy (Figure 5). This suggests that the vocal fold stellate cell-like cells are likely progenitor cells or transiently amplifying cells in the stem cell system of the human vocal fold mucosa.

This study suggested that hierarchy of the cells in the stem cell system was observed in the maculae flavae of the human adult vocal fold mucosa in vivo.

**Heterogeneity and Hierarchy of the Cells in the Maculae Flavae of the Human Newborn Vocal Fold Mucosa**

Our previous research showed that, in the maculae flavae of the human newborn vocal fold in vivo, the three phenotypes of cells resided and intermingled\(^{13}\). Hence, the cells in the newborn maculae flavae already had cellular heterogeneity in vivo\(^{13}\). However, the predominant cells in the newborn maculae flavae were cobblestone-like polygonal cells\(^{13}\).

As for the cellular hierarchy, cells in the newborn maculae flavae had cellular hierarchy and the cobblestone-like polygonal cells are at the top of the cellular hierarchy in the human newborn maculae flavae and the stem cell system\(^{13}\).

In spite of the cellular heterogeneity and hierarchy being immature, the tissue stem cells in the newborn maculae flavae are ready to start the growth of the human vocal fold mucosa as a vibrating tissue at birth\(^{13}\).

**Stem cell plasticity in the Maculae Flavae of the Human Adult Vocal Fold Mucosa**

Stem cell plasticity is the possibility that adult mammalian stem cells (tissue stem cells) may be capable of differentiating across tissue lineage boundaries\(^{23}\). Under certain circumstances, these cells may transdifferentiate to a much wider spectrum of differentiated progeny than previously anticipated\(^{23}\).

Our previous study revealed that cultured cells from the maculae flavae of the human vocal fold mucosa differentiated into cells that express markers of all three germ layers and expressed SSEA-3 (pluripotent stem cell marker) in vitro\(^{10}\).

In the present study, cobblestone-like polygonal cells in the human maculae flavae expressed SSEA-3 in vivo. The tissue stem cells in the maculae flavae of the human vocal fold mucosa may have stem cell plasticity.

The limitation of this study is even though the three phenotypes of cells reside in the stem cell niche (human adult maculae flavae), they are not necessarily in the same lineage. Identification and tracking cell populations using a lineage tracing method are necessary using animal models.

**CONCLUSIONS**

The results of this study are consistent with the hypothesis that the tissue stem cells in the maculae flavae (stem cell niche) of the human adult vocal fold mucosa have cellular heterogeneity and hierarchy in the stem cell system in vivo.
Acknowledgement

This investigation was supported by a Grant-in-Aid for Scientific Research (number 18 K 09362) from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

Conflict of Interest

The authors declare no potential conflict of interest.

REFERENCES

1) Sato K, Shirouzu H, Nakashima T : Irradiated macula flava in the human vocal fold mucosa. Am J Otolaryngol 29 : 312-318, 2008.
2) 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 121 : 51-56 , 2012.
3) Sato K, Umeno H, Nakashima T : Vocal fold stem cells and their niche in the human vocal fold. Ann Otol Rhinol Laryngol 121 : 798-803, 2012.
4) Kurita T, Sato K, Chitose S et al : Origin of vocal fold stellate cells in the human macula flava. Ann Otol Rhinol Laryngol 124 : 698-705, 2015.
5) Sato K, Chitose S, Kurita T et al : Cell origin in the macula flava of the human newborn vocal fold. J Laryngol Otol 130 : 650-655, 2016.
6) Sato K, Chitose S, Kurita T et al : Microenvironment of macula flava in the human vocal fold as a stem cell niche. J Laryngol Otol 130 : 656-661, 2016.
7) Sato K : The macula flava of the human vocal fold as a stem cell microenvironment. Stem cell microenvironment and beyond. (Birbrair A ed), 171-186, Springer, Switzerland, 2017.
8) Sato K : Tissue stem cells and the stem cell niche of the human vocal fold mucosa. Functional Histoanatomy of the Human Larynx (Sato K), 165-177, Springer, Singapore, 2018.
9) Sato K, Kurita T, Chitose S et al : Distribution of label-retaining cells and their properties in the vocal fold mucosa. Laryngoscope Invest Otolaryngol 4 : 76-82, 2019.
10) Sato F, Chitose S, Sato K et al : Differentiation potential of the cells in the macula flava of the human vocal fold mucosa. Acta Histochem 121 : 164-170, 2019.
11) Sato K, Chitose S, Sato K et al : Metabolic activity of cells in the macula flava of the human vocal fold from the aspect of mitochondrial microstructure. Laryngoscope Invest Otolaryngol 4 : 405-409, 2019.
12) Sato K : Heterogeneity of stem cells in the human vocal fold mucosa. Stem Cells Heterogeneity in Different Organs. (Birbrair A ed), 63-80, Springer, Switzerland, 2019.
13) Sato K, Chitose S, Sato F et al : Heterogeneity and hierarchy of the tissue stem cells in the human newborn vocal fold mucosa. Laryngoscope Investig Otolaryngol 5 : 903-910, 2020.
14) Sato K, Chitose S, Sato F et al : Glycolytic activity of the tissue stem cells in the macula flava of the human vocal fold. Laryngoscope Investig Otolaryngol 6 : 122-128, 2021.
15) Sato K, Chitose S, Sato F et al : Role of colony-forming tissue stem cells in the macula flava of the human vocal fold in vivo. Laryngoscope Investig Otolaryngol 6 : 283-290, 2021.
16) Sato K, Chitose S, Sato K et al : Energy metabolism of cells in the macula flava of the newborn vocal fold from the aspect of mitochondrial microstructure. J Laryngol Otol : in press, 2021.
17) Hanson SE, Kim J, Johnson BH et al : Characterization of mesenchymal stem cells from human vocal fold fibroblasts. Laryngoscope 120 : 546-551, 2010.
18) Peng H, Ming L, Yang R et al : The use of laryngeal mucosa mesenchymal stem cells for the repair the vocal fold injury. Biomaterials 34 : 9026-9035, 2013.
19) Yamashita M, Hirano S, Kanemaru S et al : Side population cells in the human vocal fold. Ann Otol Rhinol Laryngol 116 : 847-852, 2007.
20) Kawai Y, Kishimoto Y, Suzuki R et al : Distribution and characteristics of slow-cycling cells in rat vocal folds. Laryngoscope 126 : E164-170, 2016.
21) Sato K, Hirano M, Nakashima T : Stellate cells in the human vocal fold. Ann Otol Rhinol Laryngol 110 : 319-325, 2001.
22) Sato K, Hirano M, Nakashima T : Vitamin A-storing stellate cells in the human vocal fold. Acta Otolaryngol 123 : 106-110, 2003.
23) Wagers AJ, Weissman IL : Plasticity of adult stem cells. Cell 116 : 639-648, 2004.