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ORIGINAL RESEARCH

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# Heterogeneity and hierarchy of the tissue stem cells in the human newborn vocal fold mucosa

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## Abstract

**Objectives:** There is growing evidence that the cells in the maculae flavae (MFe) are tissue stem cells and the MFe are a stem cell niche of the human vocal fold mucosa. Heterogeneity and hierarchy of tissue stem cells in the MFe of newborn vocal fold were investigated in vivo.

**Study design:** Histologic analysis of the human vocal folds.

**Methods:** Five normal human newborn vocal folds were investigated under transmission electron microscopy and light microscopy.

**Results:** Cobblestone-like polygonal cells, vocal fold stellate cell-like cells, and fibroblast-like spindle cells were intermingled in the newborn MFe in vivo, indicating that the cells in the MFe had heterogeneity. However, cobblestone-like polygonal cells were predominant. Free ribosomes were well developed in the cytoplasm. The cells in some cases formed gap junctions with each other. The cells in some cases were attached to other cells and formed cell junctions with each other. These findings indicated cells in the newborn maculae flavae possessed features of mesenchymal cells (cells in mesenchyme). Colony-forming-unit-like cell aggregate was observed, indicating the cells in the newborn MFe had stemness. The cobblestone-like polygonal cells expressed SSEA-3 (a human pluripotent stem cell marker), indicating they were at the top of a cellular hierarchy in the stem cell system.

**Conclusions:** The cells in the MFe of the human newborn vocal fold mucosa had heterogeneity and hierarchy in the stem cell system in vivo. At birth, newborn maculae flavae are ready to start the growth of the vocal fold mucosa as a vibrating tissue.

KEYWORDS

cellular heterogeneity, cellular hierarchy, maculae flavae, newborn, stem cell niche, tissue stem cell, vocal fold mucosa

## 1 | INTRODUCTION

Human adult maculae flavae, located at the anterior and posterior ends of the membranous portion of the bilateral vocal folds mucosa, are dense masses of cells and extracellular matrices.[1] At birth, newborns already have maculae flavae at the same sites as adults, however their features are different from those of adults.[2] Density of cells in the newborn maculae flavae is great, causing the maculae flavae to appear as dense masses of cells.[2] The density of cells in the newborn maculae flavae is approximately five times that of the adult maculae flavae.[2]

Maculae flavae are considered to be an important structure in the growth, development and ageing of the human vocal fold mucosa.[1-5] At birth, the maculae flavae of the newborn vocal fold are ready to start the growth and development of the human vocal fold mucosa as a vibrating tissue.[2,3]

Our previous research showed there is growing evidence that the maculae flavae are a stem cell niche containing tissue stem cells of the human vocal fold mucosa.[6-13] In vitro, Sato et al reported 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.[13] This result indicates that the cells in the maculae flavae of the human adult vocal fold have heterogeneity in vitro. Furthermore, our previous investigation showed that the cells in the maculae flavae of the human adult vocal fold have heterogeneity and hierarchy in the stem cell system in vivo (unpublished data).[14]

The heterogeneity and hierarchy of the tissue stem cells in the newborn maculae flavae before growth and development of the human vocal fold as a vibrating tissue are of interest. However, it is virtually impossible to investigate in vitro whether the cells in the maculae flavae of the human newborn vocal fold have this heterogeneity and hierarchy in the stem cell system.

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

## 2 | 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.

Five normal human newborn vocal folds obtained from autopsy cases 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 newborn vocal fold mucosa were observed using transmission electron microscopy and light microscopy including immunohistochemistry.

### 2.1 | Transmission electron microscopy (TEM)

The specimens were fixed in 2.5% glutaraldehyde at 4°C for 2 hours, rinsed with cacodylate buffer solution and postfixed in 2% osmium tetroxide with cacodylate buffer solution at 4°C for 2 hours. After rinsing with cacodylate buffer solution, the specimens were dehydrated in graded concentrations of ethanol and embedded in epoxy resin. Semithin sections were prepared with an ultramicrotome, stained with 1% toluidine blue and examined with a light microscope. Thin sections were made with an ultramicrotome. Thin sections were stained with uranyl acetate & lead citrate and tannic acid. Observation was conducted with a H-7650 (HITACHI, Japan) transmission electron microscope.

### 2.2 | 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) (a human pluripotent stem cell marker) was detected histologically in formalin-fixed and paraffin-embedded tissue by immunohistochemistry, for which a universal immuno-enzyme polymer method staining kit (Histofine Simple Stain MAX-PO, Nichirei, Tokyo, Japan) was used. An antibody against SSEA-3 (Abcam. Cambridge, UK, ab16286, rat monoclonal) 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.

After rinsing with PBS and labeling with the universal immuno-enzyme polymer method staining kit, a color reaction was developed with 3,3′-diaminobenzidine for about 5 minutes at room temperature. Immunoreactivity was examined by light microscopy.

## 3 | RESULTS

The following transmission electron microscopic and light microscopic (immunohistochemistry) findings were observed in all subjects.

### 3.1 | Transmission electron microscopy (TEM)

In vivo, 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 newborn vocal fold under electron microscopy.

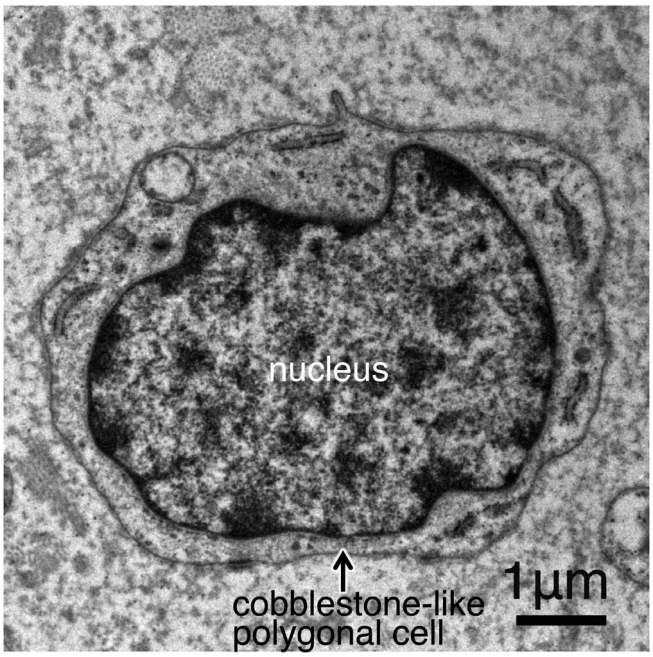


**FIGURE 1** Transmission electron microscopy of the cells in the maculae flavae of the human newborn vocal fold (uranyl acetate and lead citrate stain). Cobblestone-like polygonal cells were round and polygonal in shape with no cytoplasmic processes or no lipid droplets

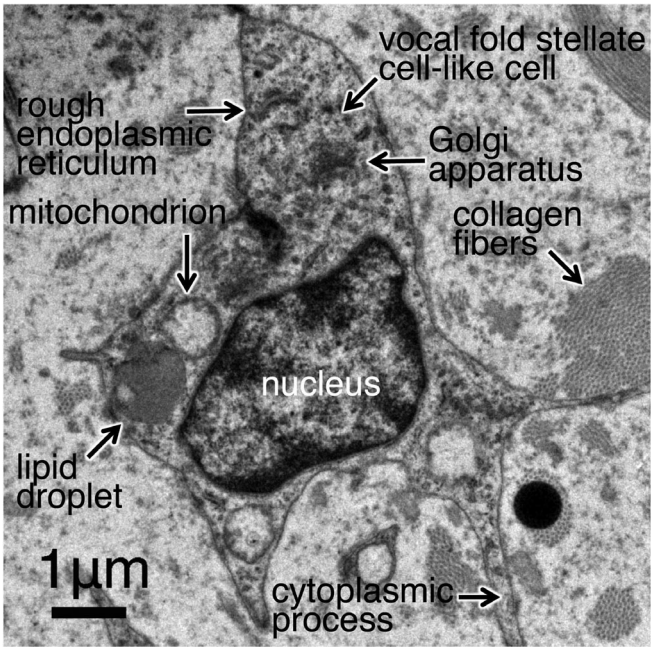


**FIGURE 2** Transmission electron microscopy of the cells in the maculae flavae of the human newborn vocal fold (uranyl acetate and lead citrate stain). Vocal fold stellate cell-like cell were irregular and stellate in shape possessing lipid droplets in the cytoplasm and cytoplasmic processes

The cells in the maculae flavae of the human newborn vocal fold had heterogeneity in vivo, however the predominant cells in the newborn maculae flavae were cobblestone-like polygonal cells.

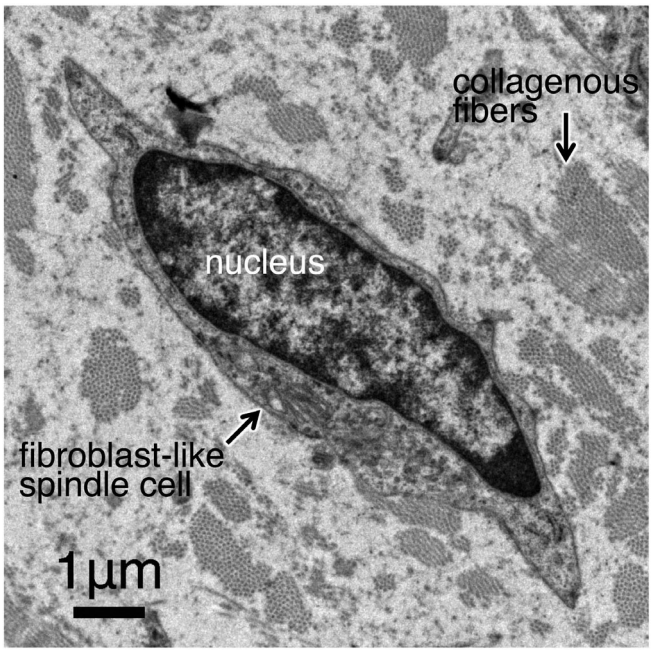


**FIGURE 3** Transmission electron microscopy of the cells in the maculae flavae of the human newborn vocal fold (uranyl acetate and lead citrate stain). Fibroblast-like spindle cells were spindle shaped or elliptic with no cytoplasmic processes

### 3.1.1 | Cobblestone-like polygonal cells

Cobblestone-like polygonal cells (Figure 1) were round and polygonal in shape with no cytoplasmic processes or no lipid droplets. The nucleus was round or oval. The nucleus-cytoplasm ratio was large and cytoplasm occupied a small area around the nucleus.

Intracellular organelles were not developed (Figure 1). Free ribosomes were well developed in the cytoplasm (Figure 4). The cells in some cases formed gap junctions with each other (Figure 4). The cells in some cases were attached to other cells and formed cell junctions with each other (Figure 5). These findings indicated cells in the newborn maculae flavae possessed features of mesenchymal cells (cells in the mesenchyme). Consequently, cobblestone-like polygonal cells are likely to be at the top of a cellular hierarchy in the stem cell system in the maculae flavae of the human newborn vocal fold.

Some basal bodies were observed in the cytoplasm (Figure 6), consequently cells in the newborn maculae flavae possess features and characteristics of epithelial cells.

### 3.1.2 | Vocal fold stellate cell-like cells

Vocal fold stellate cell-like cells (Figure 2) were irregular and stellate in shape and possessed the cytoplasmic processes. Lipid droplets approximately 1 μm in diameter were present in the cytoplasm. The nucleus was oval. The nucleus-cytoplasm ratio was relatively small and cytoplasm occupied a large area around the nucleus. Intracellular organelles such as rough endoplasmic reticulum and Golgi apparatus consisting of

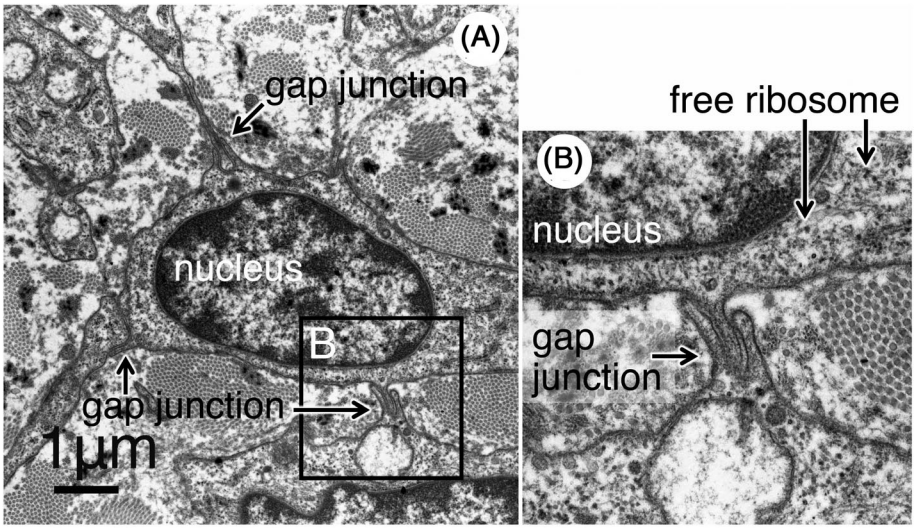


**FIGURE 4** Transmission electron microscopy of the cells in the maculae flavae of the human newborn vocal fold (tannic acid stain). Cobblestone-like polygonal cells form gap junctions, A, B, with each other. Free ribosomes were well developed in the cytoplasm, B. B, region B in panel A

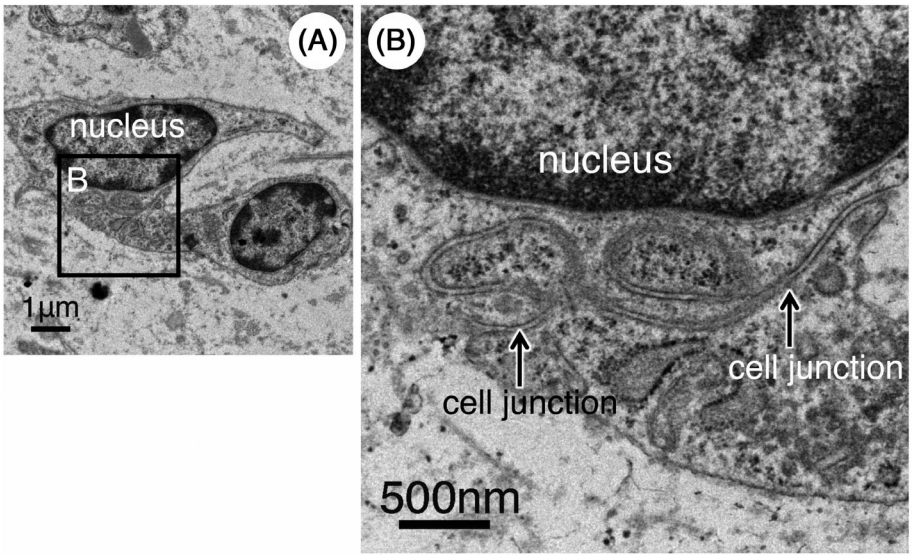


**FIGURE 5** Transmission electron microscopy of the cells in the maculae flavae of the human newborn vocal fold (uranyl acetate and lead citrate stain). Cells were attached to other cells and formed cell junctions with each other. B, region B in panel A

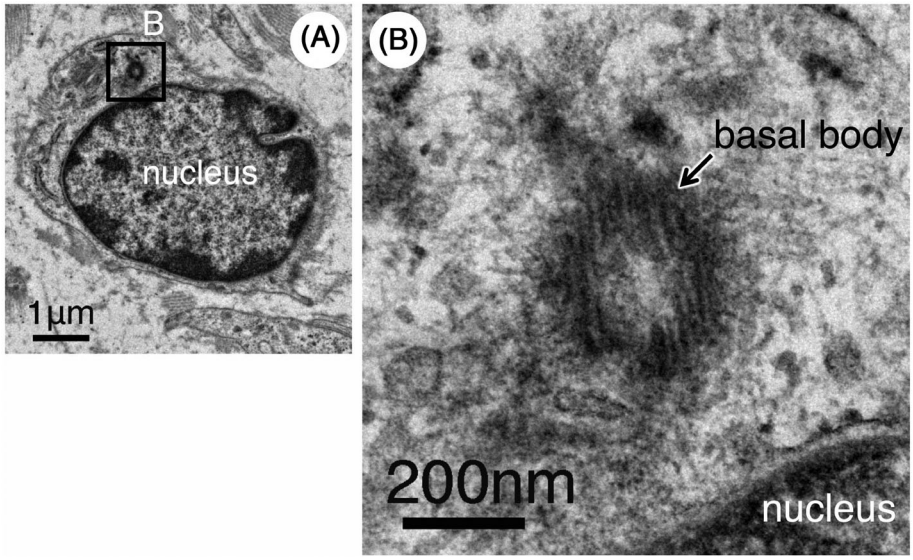


**FIGURE 6** Transmission electron microscopy of the cells in the maculae flavae of the human newborn vocal fold (uranyl acetate and lead citrate stain). A basal body was observed in the cytoplasm of the cobblestone-like polygonal cells. B, region B in panel A

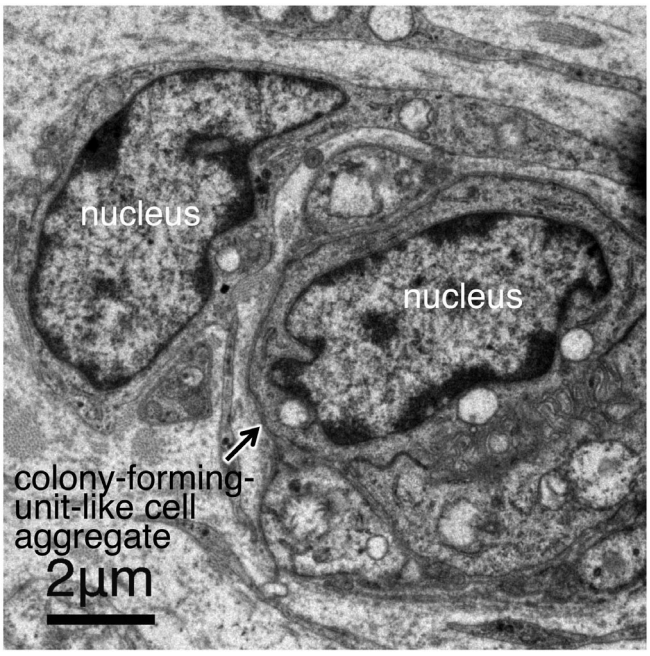


**FIGURE 7** Transmission electron microscopy of the cells in the maculae flavae of the human newborn vocal fold (uranyl acetate and lead citrate stain). Colony-forming-unit-like cell aggregate was observed in the human newborn maculae flavae in vivo

lamellae were present. A few small mitochondria were present. Along the surface of the cells, a number of vesicles were present.

### 3.1.3 | Fibroblast-like spindle cells

Fibroblast-like spindle cells (Figure 3) were spindle-shaped or elliptic, with no cytoplasmic processes and no lipid droplets. The nuclei were elliptic. The nucleus-cytoplasm ratio was large. Poorly developed intracellular organelles were apparent. Along the surface of the cells, few vesicles could be seen.

### 3.1.4 | Colony-forming-unit-like cell aggregate

Colony-forming-unit-like cell aggregate was present in the maculae flavae of the human newborn vocal fold in vivo (Figure 7).

## 3.2 | Light microscopy (immunohistochemistry)

The cobblestone-like polygonal cells in the human newborn maculae flavae expressed SSEA-3 (a human pluripotent stem cell marker) (Figure 8). The vocal fold stellate cell-like cells and fibroblast-like spindle cells poorly expressed SSEA-3 (Figure 8).

This finding suggested cobblestone-like polygonal cells are at the top of a cellular hierarchy in the stem cell system in the maculae flavae of the human newborn vocal fold (Figure 9).

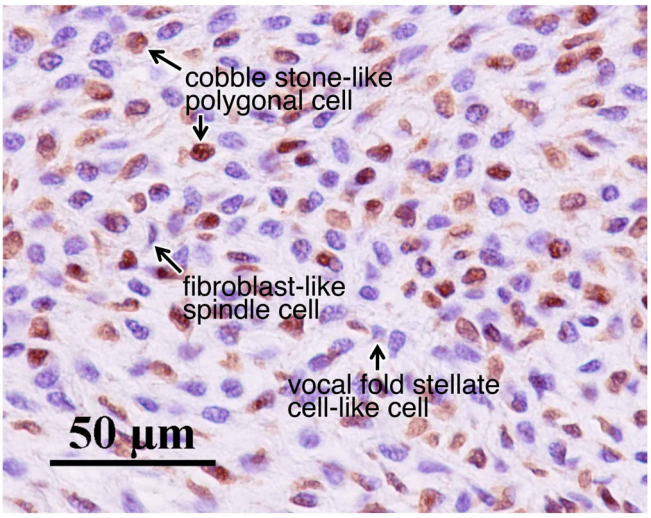


**FIGURE 8** SSEA-3 immunohistochemical staining of the cells in the maculae flavae of the human newborn vocal fold. Predominant cells in the newborn maculae flavae were cobblestone-like polygonal cells. SSEA-3 was detected in the cobblestone-like polygonal cells. The vocal fold stellate cell-like cells and fibroblast-like spindle cells poorly expressed SSEA-3

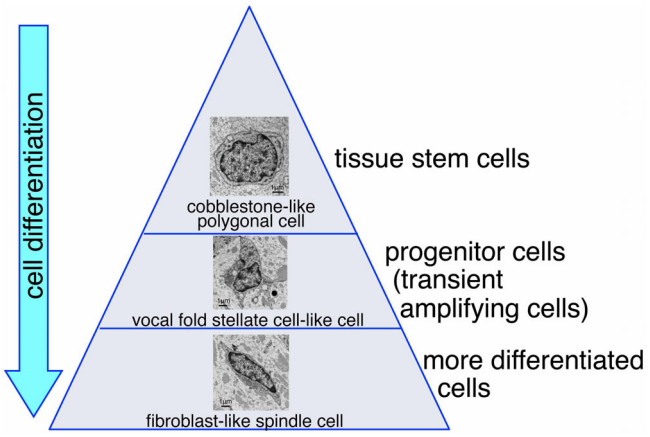


**FIGURE 9** Heterogeneity and hierarchy of the cells in the maculae flavae of the human newborn vocal fold. The cells in the maculae flavae of the human newborn vocal fold had heterogeneity (cobblestone-like polygonal cells, vocal fold stellate cell-like cells, fibroblast-like spindle cells) in vivo. Cobblestone-like polygonal cells are likely to be at the top of the cellular hierarchy in the stem cell system in the maculae flavae of the human newborn vocal fold

## 4 | DISCUSSION

Tissue stem cells (also referred to as tissue-specific stem cells, somatic stem cells or adult stem cells) have the capacity to self-renew and generate functionally differentiated cells that replenish cells lost throughout an organism's lifetime.[11] Tissue stem cells reside in a niche, whereby a complex microenvironment maintains their multipotency.[11]

In this study, cellular heterogeneity and hierarchy of the tissue stem cells in the maculae flavae (stem cells niche) of the human newborn vocal fold were investigated in vivo.

### 4.1 | Tissue stem cells and stem cell niche of the human vocal fold mucosa

Our previous research showed there is growing evidence that the maculae flavae are a stem cell niche containing tissue stem cells of the human vocal fold mucosa.[6-13] Typically, tissue stem cells generate different cell types for the specific tissue or organ (ie, human vocal fold mucosa) in which they live. Consequently, it is necessary to distinguish them from other kinds of stem cells such as mesenchymal stem cells. Regarding the human vocal fold mucosa, mesenchymal stem cells residing in the human vocal fold mucosa[15,16] are not tissue stem cells. Side population cells[17] and slow-cycling cells[18] are larger categories of cells, consequently, they are not completely equivalent to tissue stem cells.

### 4.2 | Heterogeneity of the cells in the maculae flavae of the human newborn vocal fold

Since vocal fold stellate cells (stellate in shape and possessing vitamin A-storing lipid droplets) contained in the human maculae flavae were discovered in our laboratory,[19,20] they have attracted notice as a new category of cells in the human vocal fold. However, our recent 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 adult macula flava fragments were cultured in vitro.[13] Therefore, the vocal fold stellate cells are most likely one of the phenotypes of cells in the maculae flavae of the human vocal fold.

This study revealed that the three phenotypes of cells resided and intermingled in the maculae flavae of the human newborn vocal fold in vivo under electron microscopy. Hence the cells in the newborn maculae flavae had cellular heterogeneity in vivo. However, the predominant cells in the newborn maculae flavae were cobblestone-like polygonal cells.

### 4.3 | Stemness of the cells in the maculae flavae of the human newborn vocal fold

Our previous research showed that the cells in the human newborn maculae flavae expressed proteins of all three germ layers.[8] And the cells expressed telomerase reverse transcriptase, indicating that telomerase resides in these cells.[8] Consequently, the cells in the human newborn maculae flavae are undifferentiated and multipotent.

Colony forming in vitro is one of the characteristics of stem cells.[21] Cultured cells harvested from maculae flavae of the human adult vocal fold, especially cobble stone-like polygonal cells, form a colony-forming unit in vitro.[7,9,10] The present electron microscopic study revealed that colony-forming-unit-like cell aggregate can be observed in the human newborn maculae flavae in vivo. Although it is ambiguous whether this phenomenon in vivo is the same as the one called a colony-forming unit in vitro, colony-forming-unit-like cell aggregate in vivo indicates cells in the human newborn maculae flavae have stemness.

### 4.4 | Mesenchymal cells (cells in the mesenchyme) and the cells in the newborn maculae flavae

The mesenchyme is a primordial embryonic connective tissue consisting of mesenchymal cells, usually stellate in form, supported in interlaminar jelly.[22] The creation of mesenchymal cells is a remarkable process, one where a tightly knit, impervious epithelium suddenly extends filopodia from its basal surface and gives rise to migrating cells.[23]

Intracellular organelles are not developed and free ribosomes are well developed in the cytoplasm of mesenchymal cells. Close and tight junctions are prominent between mesenchymal cells.[24,25] The present electron microscopic study showed that the cobblestone-like polygonal cells in the newborn maculae flavae possess the morphological features of mesenchymal cells (cells in the mesenchyme). Moreover, the cells in the newborn maculae flavae presented basal bodies, and thus possessed the features and characteristics of epithelial cells. Our previous research showed that the cells in the human newborn maculae flavae expressed proteins of all three germ layers including cytokeratin.[8] These phenomenon are consistent with the fact that the cells in the newborn maculae flavae possessed the characteristics of epithelial cells. Consequently, cobblestone-like polygonal cells have characteristics of mesenchymal cells (cells in the mesenchyme), indicating the cells are at the top of a cellular hierarchy in the stem cell system in the maculae flavae of the human newborn vocal fold.

### 4.5 | Hierarchy of the cells in the maculae flavae of the human newborn vocal fold mucosa

Stem cell systems have a hierarchy of cells composed of stem cells, progenitor cells (transient amplifying cells) and differentiated cells. This study showed the hierarchy of the cells in the stem cell system in the maculae flavae of the human newborn vocal fold mucosa in vivo.

Our previous research showed that the cells in the human newborn maculae flavae expressed proteins of all three germ layers and possessed telomerase.[8] These results suggest the three phenotypes of cells are undifferentiated cells and have the ability of multipotency.

This study showed that the predominant cells in the newborn maculae flavae were cobblestone-like polygonal cells and the cells in the newborn maculae flavae possessed features of mesenchymal cells (cells in the mesenchyme). In addition, this study revealed that the cobblestone-like polygonal cells in the human newborn maculae flavae expressed SSEA-3 (a human pluripotent stem cell marker), suggesting

the cobblestone-like polygonal cells are at the top of the cellular hierarchy in the human newborn maculae flavae and the stem cell system (Figure 9).

Our previous research showed that the fibroblasts in the tissue surrounding the human maculae flavae 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 maculae flavae appear to differentiate into fibroblasts in the surrounding tissue.[6,11] Consequently, fibroblast-like spindle cells in the human maculae flavae are likely at the bottom of the cellular hierarchy in the human maculae flavae and stem cells system (Figure 9). Therefore, vocal fold stellate cell-like cells in the human maculae flavae are likely at the second level of the cellular hierarchy (Figure 9). 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.

### 4.6 | Stem cell plasticity in the maculae flavae of the human newborn vocal fold mucosa

Stem cell plasticity is the possibility that tissue stem cells may be capable of differentiating across tissue lineage boundaries.[26] Under certain circumstances, these cells may transdifferentiate to a much wider spectrum of differentiated progeny than previously anticipated.[26]

Our previous study revealed that cultured cells from the adult maculae flavae expressed SSEA-3 (pluripotent stem cell maker) in vitro.[13] Furthermore, the cobblestone-like polygonal cells in the adult maculae flavae expressed SSEA-3 in vivo (unpublished data).[14]

In this study, cobblestone-like polygonal cells in the human newborn maculae flavae expressed SSEA-3 in vivo. The tissue stem cells in the human newborn maculae flavae may have stem cell plasticity.

## 5 | 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 newborn vocal fold have cellular heterogeneity and hierarchy in the stem cell system in vivo.

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.

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### CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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