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ORIGINAL RESEARCH

Role of colony-forming tissue stem cells in the macula flava of the human vocal fold in vivo

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Abstract

Objectives: Our previous investigations showed that tissue stem cells in the maculae flavae (a stem cell niche) form colonies in vivo like stem cells in vitro. However, the roles of colony-forming cells in the maculae flavae in vivo have not yet been determined.

This study investigated the metabolism of the colony-forming tissue stem cells in the maculae flavae of the human adult vocal fold.

Study design: Histologic analysis of the human vocal folds.

Methods: Three normal human adult vocal folds were investigated under transmission electron microscopy and light microscopy including immunohistochemistry.

Results: Mitochondrial cristae of the colony-forming cells in the maculae flavae were sparse. Hence, the microstructural features of the mitochondria suggested that their metabolic activity and oxidative phosphorylation were low. Colony-forming cells strongly expressed glucose transporter-1 and glycolytic enzymes (hexokinase II, glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase A). The colony-forming cells did not express phosphofructokinase-1 but did express glucose-6-phosphate dehydrogenase indicating the cells relied more on the pentose phosphate pathway. Since the colony-forming cells expressed lactate dehydrogenase A, cells seemed to rely more on anaerobic glycolysis in an anaerobic microenvironment.

Conclusions: The present study is consistent with the hypothesis that the colony-forming tissue stem cells in the maculae flavae of the human adult vocal fold seemed to rely more on anaerobic glycolysis using the pentose phosphate pathway for energy supply in vivo. Microstructural features of the mitochondria and expressed glycolytic enzymes of the colony-forming cells in the maculae flavae suggested that the oxidative phosphorylation activity was low.

In an anaerobic microenvironment in vivo, there is likely a complex cross-talk regarding the metabolism between the colony-forming aggregated cells along the adhesion machinery and chemical signaling pathways, which reduces toxic oxygen species and is favorable to maintaining the stemness and undifferentiated states of the tissue stem cells.

Level of Evidence: NA.

KEYWORDS

colony-forming, glycolysis, human vocal fold, larynx, macula flava, metabolic activity, mitochondria, oxidative phosphorylation, tissue stem cells

1 | INTRODUCTION

The latest research shows there is growing evidence that the cells in the maculae flavae located at both ends of the lamina propria of the vocal fold mucosa are tissue stem cells of the human vocal fold and the macula flavae are a stem cell niche which is a microenvironment nurturing the tissue stem cells.[1-12]

It is a characteristic phenomenon that cultured stem cells form colonies in vitro.[13,14] This phenomenon was also observed in our previous studies, that is, the cultured cells harvested from the maculae flavae of the human adult vocal fold formed colonies in vitro.[2,4,8] Consequently, this phenomenon is consistent with the hypothesis that the cells possessing stemness reside in the maculae flavae (stem cell niche) of the human vocal fold.

Our previous study revealed, likely for the first time, tissue stem cells in the maculae flavae of the human adult vocal fold mucosa form colonies in vivo the same as in vitro.[10] Furthermore, their fine structures in vivo were investigated using electron microscopy.[10]

Generally, the making and breaking of attachments are important events in the lives of cells and provoke large changes in their internal affairs.[15] Conversely, changes in the internal state of a cell must be able to trigger the making or breaking of attachments.[15] Thus, there is a complex cross-talk between cells along the adhesion machinery and chemical signaling pathways.[15] However, the roles and physiology of colony-forming by tissue stem cells in the maculae flavae of the human adult vocal fold in vivo have not yet been determined.

The purpose of this study is to investigate the metabolism, especially glycolysis, of the colony-forming tissue stem cells in the maculae flavae of the human adult vocal fold 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.

Three normal human adult vocal folds obtained from autopsy cases were investigated. Any diseases that could possibly affect the tissue of the vocal fold were not observed.

The colony-forming tissue stem cells in the maculae flavae of the human adult vocal fold were observed using light microscopy including immunohistochemistry and transmission electron microscopy.

2.1 | 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.

Glucose transporter-1 (GLUT-1), and glycolytic enzymes (hexokinase II [HK II], phosphofructokinase-1 [PFK-1], glucose-6-phosphate dehydrogenase [G6PD], glyceraldehyde-3-phosphate dehydrogenase [GAPDH] and lactate dehydrogenase A [LDHA]) were 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.

A 1:250 antibody against GLUT1 (ab115730, rabbit monoclonal, Abcam, Cambridge, UK), a 1:200 antibody against HK II (ab104836, mouse monoclonal, Abcam, Cambridge, UK), a 1:50 antibody against PFKFB3 (ab181861, rabbit monoclonal, Abcam, Cambridge, UK), a 1:50 antibody against G6PD (ab106810, goat polyclonal, Abcam, Cambridge, UK), 1:50 antibody against GAPDH (ab9485, rabbit polyclonal, Abcam, Cambridge, UK) and 1:250 antibody against LDHA (ab101562, rabbit monoclonal, Abcam, Cambridge, UK) were 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 for 60 minutes 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 at room temperature. Immunoreactivity was examined by light microscopy.

2.2 | Transmission electron microscopy

For transmission electron microscopy, 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 and lead citrate. Observation was conducted with a H-7650 (HITACHI, Japan) transmission electron microscope.

To evaluate the concentration of cristae in each mitochondrion, the ratio of cristal space to intercristal and cristal space was measured with computer software (ImageJ, NIH) in 50 random mitochondria of colony-forming cells in the electron micrographs of the specimens.

3 | RESULTS

Colony-forming aggregated cells were observed in the maculae flavae of the human adult vocal fold (Figures 1 and 2). However, colony-

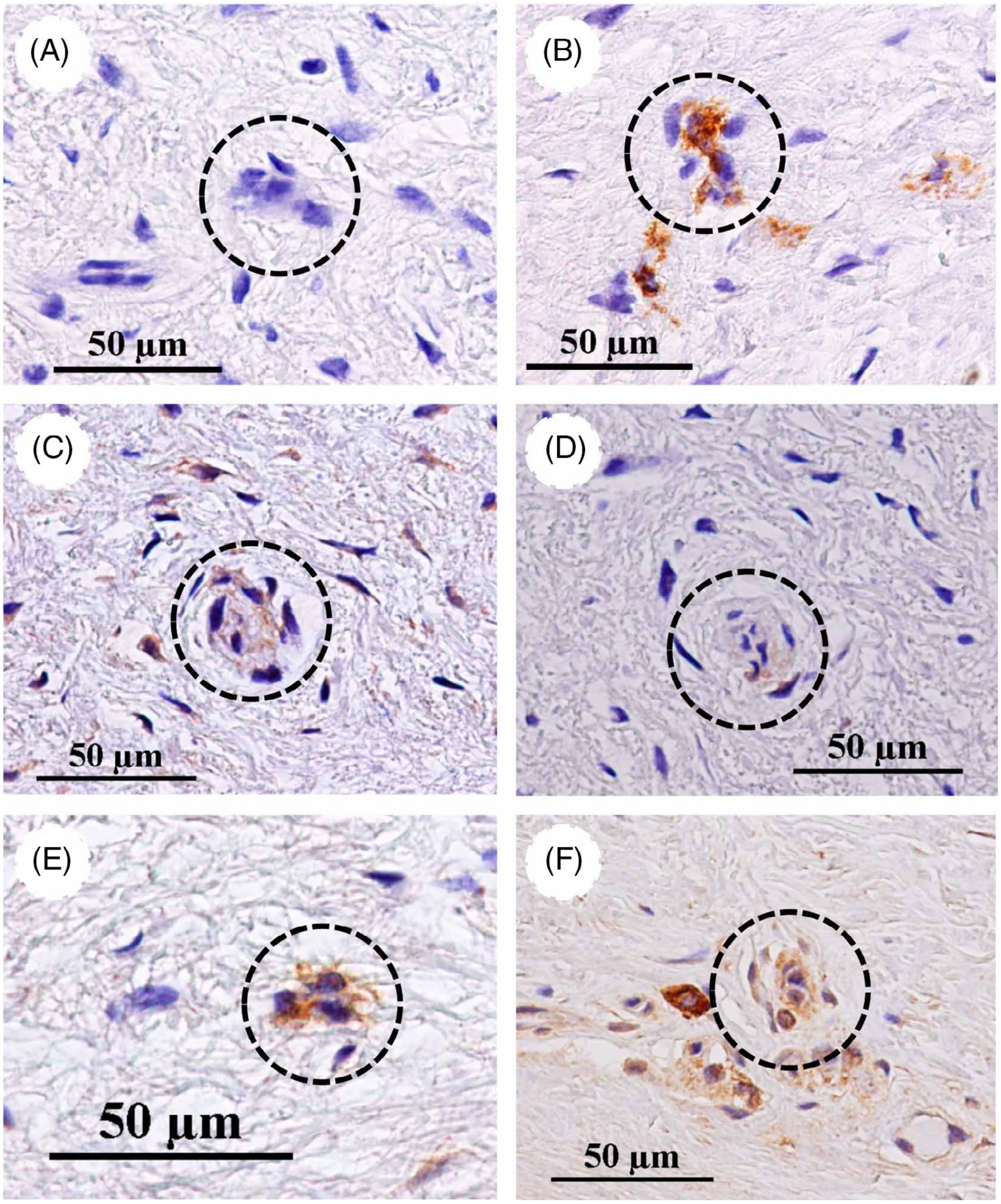

FIGURE 1 Colony-forming aggregated cells in the maculae flavae of the human adult vocal fold. A, Hematoxylin stain; B, glucose transporter-1 (GLUT-1) immunohistochemical staining; C, Hexokinase II (HK II) immunohistochemical staining; D, Glucose-6-phosphate dehydrogenase (G6PD) immunohistochemical staining; E, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) immunohistochemical staining; F, Lactate dehydrogenase A (LDHA) immunohistochemical staining. Colony-forming aggregated cells (dotted line circle) in the human adult maculae flavae strongly expressed GLUT-1 and glycolytic enzymes

forming aggregated cells were not detected in the lamina propria of the human adult vocal fold.

3.1 | Glucose transporter of the colony-forming tissue stem cells in the maculae flavae of the human vocal fold

Colony-forming aggregated cells in the human adult maculae flavae strongly expressed GLUT-1 (Figure 1B). Consequently, they had glucose transporter on the cell plasma membrane.

3.2 | Glycolytic enzymes of the colony-forming tissue stem cells in the maculae flavae of the human vocal fold

Colony-forming aggregated cells in the human adult maculae flavae expressed HK II (catalyzes glucose into glucose-6-phosphate) (Figure 1C). The first step in glucose metabolism pathways occurs when glucose enters glycolysis by phosphorylation into glucose-6-phosphate. Consequently, glucose was likely catalyzed into glucose-6-phosphate by hexokinase.

Colony-forming aggregated cells in the human adult maculae flavae did not express PFKFB3.

Colony-forming aggregated cells in the human adult maculae flavae expressed G6PD (Figure 1D), the rate-limiting enzyme of the pentose phosphate pathway.

Colony-forming aggregated cells in the human adult maculae flavae expressed GAPDH (Figure 1E). Consequently, glyceraldehyde-3-phosphate is likely catalyzed into 1,3-bisphosphoglycerate by GAPDH.

Colony-forming aggregated cells in the human adult maculae flavae expressed LDHA (Figure 1F). Consequently, pyruvate is likely catalyzed into lactate by LDHA in the anaerobic microenvironment.

3.3 | Mitochondrial morphology of the colony-forming tissue stem cells in the maculae flavae of the human vocal fold

Mitochondria were observed in the cytoplasm of the colony-forming aggregated cells in the maculae flavae of the human adult vocal fold (Figure 2). Their shape was oval.

The mitochondria consisted of a double-membrane-bounded body (limited by smooth-countered outer and inner membranes) containing matrices and cristae. Both membranes of some mitochondria were ambiguous (Figure 3).

The intercristal space was occupied by mitochondrial matrices which contained some intramitochondrial granules (dense granules), mitochondrial DNA and ribonucleoprotein granules (Figure 3). Mitochondrial inclusions were observed in the mitochondrial matrix (Figure 4).

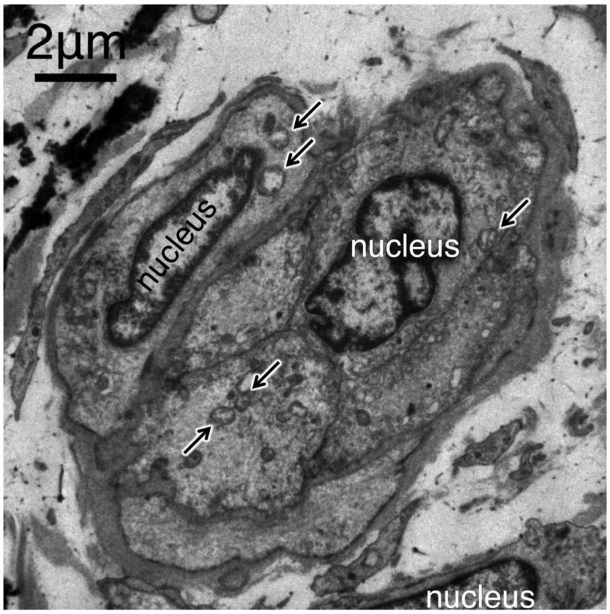

FIGURE 2 Colony-forming aggregated cells in the maculae flavae of the human adult vocal fold (TEM, tannic acid stain). Arrows: mitochondria in the cytoplasm

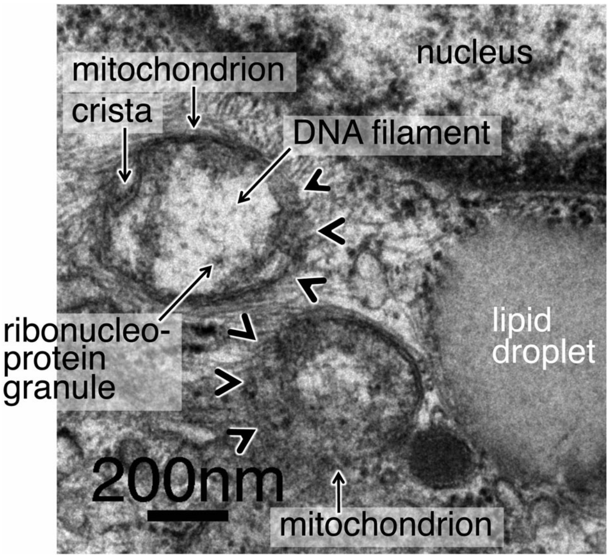

FIGURE 3 Mitochondria in the cytoplasm of the colony-forming aggregated cells in the maculae flavae of the human adult vocal fold. (TEM, tannic acid stain). The mitochondrial cristae of the colony-forming aggregated cells were sparse. In some portions smooth-countered outer and inner membranes containing matrices and cristae were ambiguous (arrowhead)

3.4 | Concentration of cristae in the mitochondrion of the colony-forming tissue stem cells in the maculae flavae of the human vocal fold

Cristae, the inner membrane forming thin folds (lamellar cristae), were observed. The ratio of cristal space to intercristal and cristal space of

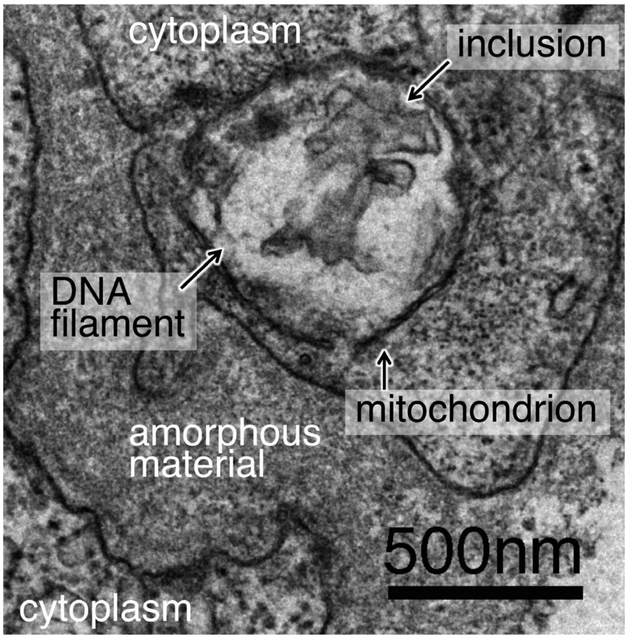

FIGURE 4 Mitochondria in the cytoplasm of the colony-forming aggregated cells in the maculae flavae of the human adult vocal fold. (TEM, tannic acid stain)

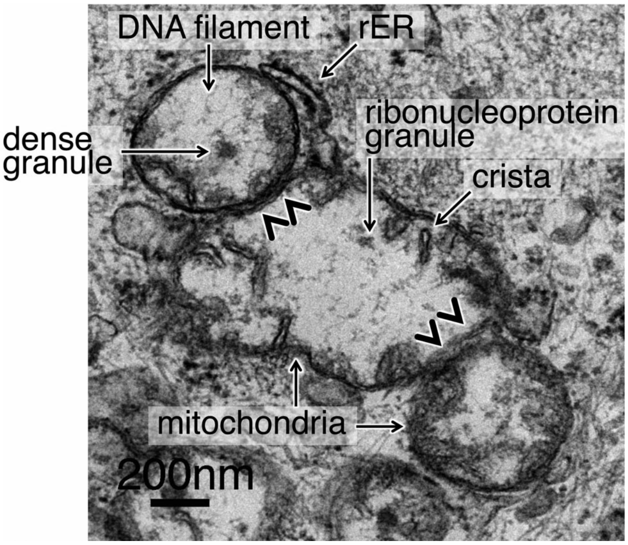

FIGURE 5 Impending division or fusion of the mitochondria in the cytoplasm of the colony-forming aggregated cells in the maculae flavae of the human adult vocal fold. (TEM, uranyl acetate and lead citrate stain). Each mitochondrial outer and inner membrane adjacent to the membrane of another mitochondrion (arrowhead) had disappeared. Mitochondria fused to rough endoplasmic reticulum (rER) in the cytoplasm

the mitochondria in the colony-forming aggregated cells was 3.8 ± 2.3% (average ± SD). Hence, the characteristic feature of the mitochondrial cristae of the colony-forming aggregated cells in the human adult maculae flavae was that they were sparse.

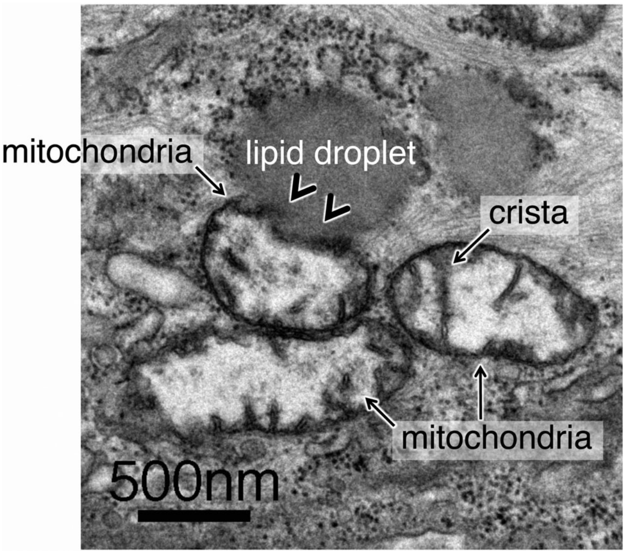

FIGURE 6 Mitochondria in the cytoplasm of the colony-forming aggregated cells in the maculae flavae of the human adult vocal fold. (TEM, uranyl acetate and lead citrate stain). A single mitochondrion fused to the surface of a lipid droplet in the cytoplasm (arrowhead)

3.5 | Mitochondrial division and fusion of the colony-forming tissue stem cells in the maculae flavae of the human vocal fold

Mitochondrial profiles suggested impending division or fusion (Figure 5). However, the static electron micrographs could not on their own indicate the direction in which the process was moving.

3.6 | Mitochondrial associations with other organelles of the colony-forming tissue stem cells in the maculae flavae of the human vocal fold

Some mitochondria spread out over or fused to the surface of a lipid droplet in the cytoplasm (Figure 6). Furthermore, both the mitochondrial outer and inner membranes adjacent to the membranes of the lipid droplets had disappeared.

Some close association between mitochondria and rough endoplasmic reticulum in the cytoplasm was observed (Figure 5).

4 | DISCUSSION

Cultured stem cells form colonies in vitro.[13,14] Hence, colony-formation is one of the characteristic phenomenon of stem cells in vitro. However, the role of colony-forming stem cells has not yet been completely determined.

This phenomenon was also observed in our previous studies and the cultured cells harvested from the maculae flavae of the human adult vocal fold formed colonies in vitro.[2,4,8] Furthermore, our previous study revealed that the cells in the maculae flavae of the human adult vocal fold form colonies in vivo like stem cells in vitro.[10] However, the role of colony-forming tissue stem cells in the maculae flavae of the human adult vocal fold in vivo has been ambiguous.

4.1 | Roles of colony formation by tissue stem cells in the maculae flavae of the human adult vocal fold in vivo

Pieters and van Roy reported that E-cadherin-mediated cell-cell adhesion is a driving force in survival, self-renewal and pluripotency maintenance of naive embryonic stem cells in vitro.[16] Wong et al reported that an important role for gap junctional intercellular communication has been demonstrated in human embryonic stem cells with respect to colony growth and cell survival in vitro.[17] Thus there is a complex cross-talk between colony-forming stem cells along the adhesion machinery and chemical signaling pathways.

Our previous light and electron microscopic study revealed that, in the maculae flavae of the human adult vocal fold, colony-forming aggregated cells attached through the intermediary of E-cadherin mediated adhesive junctions in vivo.[10] Adhesive junctions link cells together into tissues, thereby enabling cells to function as a unit.[18] Colony-forming tissue stem cells in the maculae flavae (stem cell niche) of the human adult vocal fold are likely to be some sort of functional unit. Cell-cell junctions are likely to send signals into the cell interior. However, the significance and physiology of colony formation by tissue stem cells in the maculae flavae of the human adult vocal fold in vivo have not yet been completely determined.

4.2 | Glycolysis of colony-forming tissue stem cells in the maculae flavae of the human adult vocal fold in vivo

Our previous research suggested that the tissue stem cells in the maculae flavae of the human adult vocal fold seem to rely more on anaerobic glycolysis, especially by the pentose phosphate pathway, for energy supply in comparison with oxidative phosphorylation.[9,19]

The present study showed that the colony-forming tissue stem cells expressed GLUT-1, glycolytic enzymes (HK II, GAPDH, and LDHA), anaerobic glycolytic enzymes (LDHA), and G6PD (the rate-limiting enzyme of the pentose phosphate pathway) indicating the colony-forming tissue stem cells seem to rely more on anaerobic glycolysis using the pentose phosphate pathway in vivo.

The availability of oxygen determines which of the two pathways is followed.[20] Under anaerobic conditions, NADH cannot be reoxidized thorough the respiratory chain, and pyruvate is reduced to lactate catalyzed by lactate dehydrogenase.[20] Under aerobic conditions, pyruvate is transported into the mitochondria and undergoes oxidative decarboxylation to acetyl-CoA then oxidation to CO_2 in the tricarboxylic acid (TCA) cycle (citric acid cycle) (oxidative phosphorylation).[20]

The present study showed that colony-forming tissue stem cells in the human maculae flavae expressed lactate dehydrogenase (LDHA). Consequently, pyruvate is likely to be reduced to lactate catalyzed by LDHA under an anaerobic microenvironment in the colony-forming tissue stem cells in the maculae flavae of the human adult vocal fold. Furthermore, pyruvate is not likely to be transported into the mitochondria or undergo oxidative decarboxylation to acetyl-CoA then oxidation to CO_2 in the TCA cycle (citric acid cycle) (oxidative phosphorylation). Hence, the colony-forming tissue stem cells seem to rely more on anaerobic glycolysis for energy supply in comparison with oxidative phosphorylation.

From the functional morphological point of view, there is likely to be a complex cross-talk between cells along the adhesion machinery and chemical signaling pathways regarding the metabolism between the colony-forming aggregated tissue stem cells in an anaerobic microenvironment.

4.3 | Oxidative phosphorylation of colony-forming tissue stem cells in the maculae flavae of the human adult vocal fold in vivo

Most of the TCA cycle (citric acid cycle) enzymes are located in the matrices of the mitochondria, and electron transport and oxidative phosphorylation enzymes form molecular assemblies in or on the inner mitochondrial membrane covering the wall and cristae.[21] The inner membrane and its spheres of the mitochondria are the site of oxidative phosphorylation.[21] There is a positive correlation between the metabolic activity of a tissue and the number and size of mitochondria and also the number, size, surface area and concentration of cristae.[21] The number of cristae per mitochondrion is much greater in cells with high-energy requirements than in those having a lower rate of metabolism.[22]

The present study showed that the mitochondrial cristae of the colony-forming aggregated tissue stem cells in the human adult maculae flavae were sparse. Furthermore, inner membranes of some mitochondria were ambiguous. The microstructural features of the mitochondria of the colony-forming aggregated tissue stem cells in the maculae flavae suggested that their metabolic activity and oxidative phosphorylation are low in vivo.

4.4 | Mitochondrial associations of colony-forming tissue stem cells in the maculae flavae of the human adult vocal fold in vivo

Mitochondria are often located near a supply of substrate or at sites in the cell known to require the ATP generated by the mitochondria.[21]

The human maculae flavae contain vocal fold stellate cells that are stellate in shape and possess lipid droplets in their cytoplasm.[23-25] Our recent research showed that the vocal fold stellate cells are most likely one of the phenotypes of cells in the maculae flavae of the human vocal fold.[8,11,12] However, the roles of lipid droplets in the cytoplasm of vocal fold stellate cells have been ambiguous.

In this study, some mitochondria were close to or fused to the surface of a lipid droplet in the cytoplasm of the colony-forming aggregated tissue stem cells in the maculae flavae. These microstructural features suggested that the lipid droplets in the cytoplasm supplied fatty acid degraded by beta-oxidation in the mitochondria. Since the mitochondria contain many of the enzymes (fatty acid oxidases) necessary for the metabolism of triglycerides,[21] these microstructural features also suggested that this brings the mitochondrial enzymes into close association with the lipidic substrate. The colony-forming aggregated tissue stem cells in the maculae flavae may have shifted to the utilization of lipids to some extent for their metabolic needs.

4.5 | Metabolic activity of colony-forming tissue stem cells in the maculae flavae of the human adult vocal fold in vivo

Oxidative stress shortens the life span of stem and progenitor cells, among which reactive oxygen species (ROS) accelerate aging through random and sequential damage to cell components.[26]

ROS are continuously generated by normal metabolic processes such as oxidative phosphorylation.[27] The inner mitochondrial membrane covering the wall and cristae are the site of oxidative phosphorylation.[21] The oxidative phosphorylation in the mitochondria is the major source of endogenous ROS.[27] There is usually a good correlation between the metabolic rate and the level of ROS generated by mitochondria.[26]

In this study, microstructural features of the mitochondria suggested that the metabolic activity and oxidative phosphorylation of the colony-forming aggregated tissue stem cells in the maculae flavae were low indicating the intracellular ROS production is suppressed. The colony-forming aggregated cells in the human maculae flavae seem to rely more on anaerobic glycolysis using the pentose phosphate pathway for energy supply in comparison with oxidative phosphorylation. The metabolism of the colony-forming aggregated tissue stem cells in the human maculae flavae seems to be favorable to maintaining the stemness and undifferentiated states in the stem cell system.

5 | CONCLUSIONS

The present study is consistent with the hypothesis that the colony-forming aggregated tissue stem cells in the human maculae flavae seem to rely more on anaerobic glycolysis using the pentose phosphate pathway for energy supply in an anaerobic microenvironment in vivo. On the other hand, the microstructural features of the mitochondria of the colony-forming cells in the maculae flavae suggest that the oxidative phosphorylation is low.

In an anaerobic microenvironment in vivo, there is likely a complex cross-talk regarding the metabolism between the colony-forming aggregated cells along the adhesion machinery and chemical signaling pathways, which reduces toxic oxygen species and is favorable to maintaining the stemness and undifferentiated states of the tissue stem cells.

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CONFLICT OF INTEREST

None.

FINANCIAL DISCLOSURE

None.

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