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Origin of Vocal Fold Stellate Cells in the Human Macula Flava

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Abstract

Objectives: There is growing evidence that vocal fold stellate cells (VFSCs) in the human maculae flavae are tissue stem cells of the human vocal fold and that the maculae flavae are a stem cell niche. The origin of the cells in the human maculae flavae (CHMF) and the relationship with bone marrow–derived cells were investigated.

Methods: Five human adult vocal fold mucosae were investigated. The CHMF were subcultured and morphological features were assessed. Immunoreactivity to antibodies directed to cytokeratin, desmin, GFAP, vimentin, CD34, CD45, and collagen type I was investigated.

Results: Cultured CHMF formed a colony-forming unit, indicating they are mesenchymal stem cells or stromal stem cells in the bone marrow. The CHMF expressed hematopoietic markers (CD34, CD45) and collagen type I, which are the major makers for bone marrow–derived circulating fibrocytes. The cultured CHMF expressed epithelium-associated, muscle-associated, neural-associated, and mesenchymal cell–associated proteins, indicating the CHMF are undifferentiated and express proteins of all 3 germ layers.

Conclusions: The CHMF are undifferentiated cells derived from the differentiation of bone marrow cells. The results of this study are consistent with the hypothesis that the VFSCs are tissue stem cells or progenitor cells of the human vocal fold mucosa.

Keywords

vocal fold stellate cell, tissue stem cell, progenitor cell, human vocal fold, larynx

Introduction

Bone marrow–derived cells (BMDCs) have received a great deal of attention with regard to tissue development and regeneration. BMDCs are considered to contain bone marrow–derived mesenchymal stem cells, which are multipotent cells capable of self-renewal,[1,2] and to be the origin of circulating fibrocytes, which are associated with wound healing and tissue fibrosis.[3] They circulate in the peripheral blood and are distributed to organs under normal conditions. When tissue is injured, they contribute to tissue repair by cell differentiation and migrate into injured tissue as needed.[4,5]

In the vocal fold mucosa, vocal fold stellate cells (VFSCs) in the anterior and posterior maculae flavae (MFe) have been considered to be important interstitial cells in the growth, development, and aging of the human vocal fold mucosa.[6-10] The VFSCs, which have a starlike appearance in the human MFe, were discovered in our previous study.[11] These cells possess lipid droplets and store vitamin A.[12] They have many morphological differences from the conventional fibroblasts in the human vocal fold. There was considerable evidence in past studies that the VFSCs in the human MFe were involved in the metabolism of extracellular matrices that are essential for the viscoelasticity of the human vocal fold mucosa.[9,11]

The VFSCs in the human MFe are a member of the diffuse stellate cell system.[13] The concept of diffuse stellate cell system has been proposed as a result of the discovery of cells that are morphologically similar to hepatic stellate cells at extrahepatic sites,[14-16] including the human MFe.[13] All members of this system are desmin-positive cells with

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perinuclear vitamin A droplets. In addition, hepatic stellate cells are considered to be bone marrow–derived mesenchymal cells.[17]

Consequently, we hypothesize that the VFSCs, which are a member of the diffuse stellate cell system, are derived from bone marrow. The purpose of this study is to investigate the relationship between VFSCs and BMDCs.

Materials and Methods

This study was approved by the Ethical Committee of Kurume University.

Culturing of the Human Vocal Fold Stellate Cells

Two normal human vocal folds were obtained from patients who had undergone total pharyngo-laryngo-esophagectomy as initial treatment for primary hypopharyngeal cancers. The vocal folds were unaffected by the cancer. After enucleating the anterior MFe from the surgical specimens under microscope, they were minced to 1 mm^3 and cultured in MF-start medium (Toyobo, Osaka, Japan). After the primary culture, MF-medium (Mesenchymal Stem Cell Growth Medium; Toyobo) was used to subculture the cells. The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. For immunohistochemistry, the cells were plated onto Lab-tek chamber slides (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The cells were observed with an inverted-type phase-contrast microscope (Olympus, Tokyo, Japan).

Immunocytochemical Investigations of the Cultured Human Vocal Fold Stellate Cells

Cultured VFSCs grown on the chamber slides were fixed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) for 20 minutes, after which the medium was aspirated and the cells were rinsed with PBS. The specimens were pretreated in 0.3% hydrogen peroxide–methanol for 10 minutes to block endogenous peroxidase activity. The specimens were then incubated at 4°C overnight with the following primary antibodies: 1:300 diluted anti-vimentin (ab8978, Abcam, Cambridge, England), 1:300 diluted anti-desmin (ab6322, Abcam), 1:300 diluted anti-glial fibrillary acidic protein (GFAP; ab10062, Abcam), and 1:300 diluted anti-cytokeratin (M0821, Dako, Glostrup, Denmark). After rinsing with PBS and incubating with universal secondary antibodies conjugated with horseradish peroxidase–labeled amino acid polymer (Histofine Simple Stain MAX-PO, Nichirei, Tokyo, Japan) at room temperature for 30 minutes, the immunoreactive proteins were stained with 3,3′-diaminobenzidine tetrahydrochloride for 5 to 10 minutes. The specimens were counterstained with hematoxylin, dehydrated with xylene, and mounted.

Immunohistochemical Investigations of Formalin-Fixed Human Vocal Fold Stellate Cells

Three normal human adult larynges from autopsy cases were used for immunohistochemical investigation. Any larynges that had diseases that could possibly affect the tissue of the vocal fold were excluded from the study.

CD34, CD45, and collagen type I were detected in formalin-fixed paraffin-embedded tissue by immunohistochemical analysis. All specimens were sectioned to a thickness of 4 μm and mounted on glass slides. Deparaffinized and hydrated sections were rinsed with 0.01 mol/L PBS at pH 7.4. Antigen retrieval by microwave irradiation was conducted with Target Retrieval Solution (S1699, Dako) for 10 minutes. The sections were blocked with 3% hydrogen peroxide for 10 minutes. The specimens were then incubated at 4°C overnight with the following primary antibodies: 1:100 diluted anti-CD34 (ab81289, Abcam), 1:100 diluted anti-CD45 (ab40763, Abcam), and 1:100 diluted anti-collagen type I (ab138492, Abcam). After rinsing with PBS and incubating with universal secondary antibodies conjugated with horseradish peroxidase labeled amino acid polymer (Histofine Simple Stain MAX-PO, Nichirei) at room temperature for 30 minutes, the immunoreactive proteins were stained with 3,3′-diaminobenzidine tetrahydrochloride for 5 to 10 minutes. The specimens were counterstained with hematoxylin, dehydrated with xylene, and mounted.

Results

Culturing of the Human Vocal Fold Stellate Cells

On the 13th day of the primary culture in the MF-start medium, 2 phenotypes of cells, which were fibroblast-like spindle cells and cobblestone-like squamous cells, grew out from the MFe fragments (Figure 1A). Each group of cells was individually subcultured in the MF-medium (Mesenchymal Stem Cell Growth Medium) after isolating them with a cell scraper.

Fibroblast-like spindle cells became stellate in shape and possessed slender cytoplasmic processes on the sixth day of the first passage in the MF-medium (Figure 1B). Some cells possessed small lipid droplets in the cytoplasm. The nuclei in the cells were oval-shaped, and their nucleus–cytoplasm ratios were low. These cells were morphologically similar to the VFSCs.

Cobblestone-like squamous cells were polygonal in shape and had oval-shaped nuclei. The nucleus–cytoplasm ratios were high. On the sixth day of the second passage in the MF-medium, they formed cell colonies (Figure 1C). These colonies were observed up to the fourth passage.

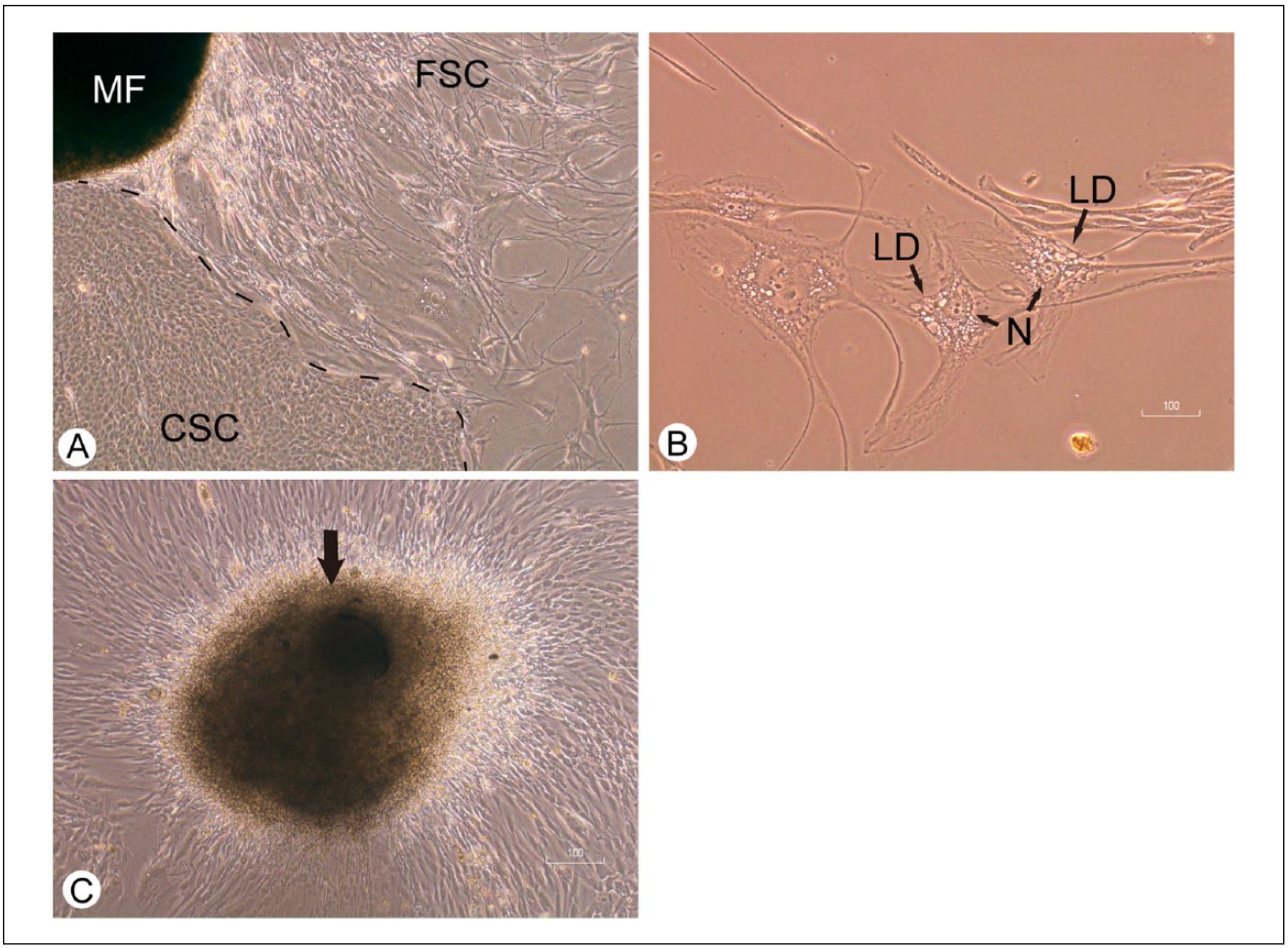

Figure 1. Phase-contrast microscopy of the cultured vocal fold stellate cells.
(A) Fibroblast-like spindle cells (FSC) and cobblestone-like squamous cells (CSC) grew out from the macula flava fragment (MF). (B) Primary cultured fibroblast-like spindle cells after 6 days. Fibroblast-like spindle cells became stellate in shape and possessed slender cytoplasmic processes (N, nucleus; LD, lipid droplets). (C) Subcultured cobblestone-like squamous cells 6 days after the second passage (Arrow: colony-forming unit).

Immunocytochemical Investigations of the Cultured Human Vocal Fold Stellate Cells

Both non-colony-forming cells (fibroblast-like spindle cells) and colony-forming cells (cobblestone-like squamous cells) expressed cytoplasmic vimentin (mesenchymal cell–associated protein; Figures 2A and 3A), desmin (muscle-associated protein; Figure 2B and 3B), GFAP (neural-associated protein; Figures 2C and 3C), and cytokeratin (epithelium-associated protein; Figures 2D and 3D).

Immunohistochemical Investigations of the Formalin-Fixed Human Vocal Fold Stellate Cells

The VFSCs in the human MFe expressed CD34 (hematopoietic stem cell marker; Figure 4A). They also expressed CD45 (leukocyte common antigen; Figure 4B) and collagen type I (Figure 4C). These proteins (CD34, CD45, and collagen type I), which are major makers of bone marrow–derived circulating fibrocytes, were present in the VFSCs.

Discussion

It is generally recognized that BMDCs are distributed in a wide variety of organs. Several reports have revealed that BMDCs in the organs and tissue stem cells in their niche, which are the local cell source, are distributed in the same location.[5,18]

The results of this study are consistent with the hypothesis that the cells, including the VFSCs, in the human MFe are tissue stem cells or transit-amplifying cells (progenitor cells) of the human vocal fold derived from the bone marrow.

Cell Division in the Human Maculae Flavae

The present investigation revealed that 2 types of cells grew out of the primary culture of the human MFe and that cobblestone-like squamous cells in the MF-medium formed cell

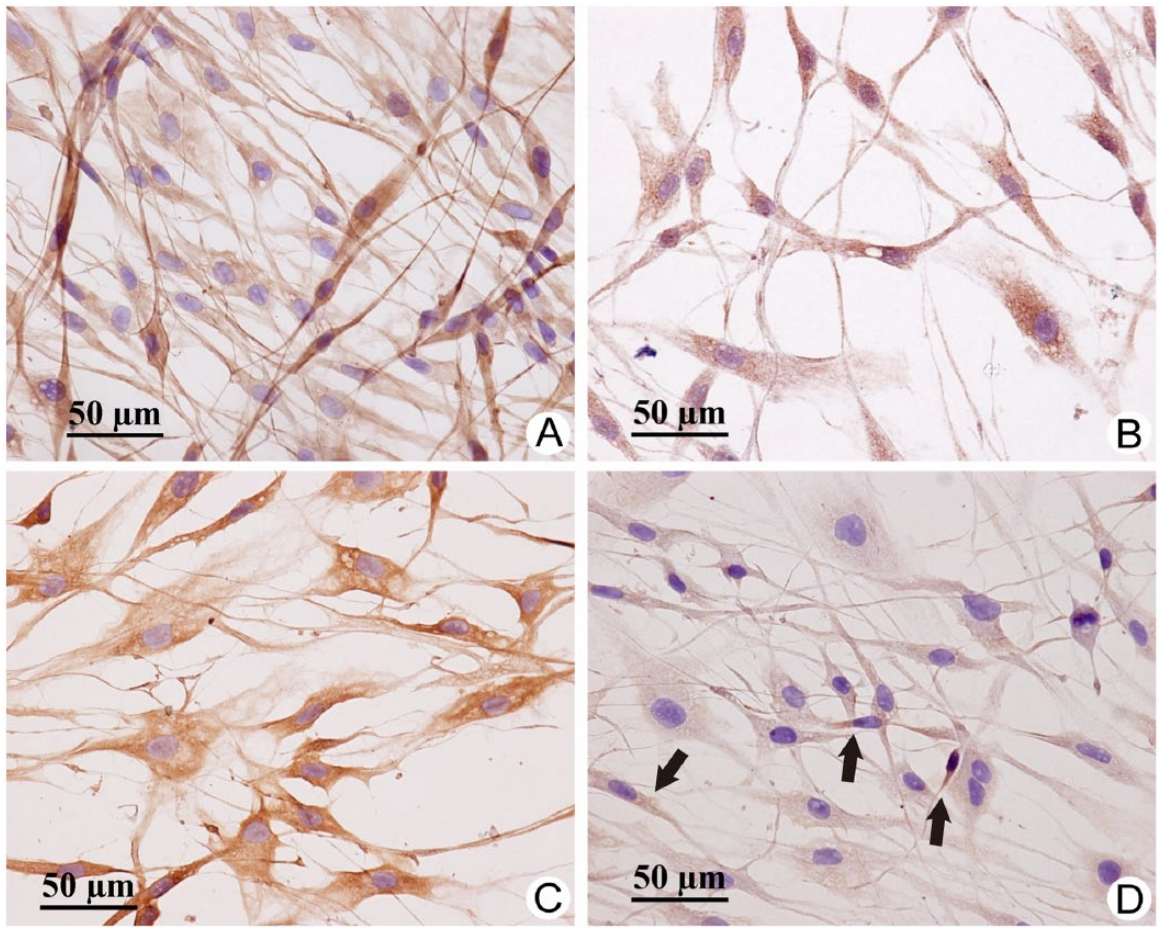

Figure 2. Immunohistochemistry of the non-colony-forming cells (fibroblast-like spindle cells): (A) vimentin; (B) desmin; (C) GFAP; (D) cytokeratin (arrows).

colonies. Colony formation is one of the characteristics of stem cells. The colony-forming unit in vitro was first described by Friedenstein et al.[19] They established that adherent fibroblastic cells that form cell colonies in in vitro culture can be isolated from the bone marrow stroma. This colony-forming unit can differentiate into cartilage, bone, and adipose tissue.[1] Such a colony is also observed in embryonic stem cells,[20] induced pluripotent stem cells,[21] and tissue stem/progenitor cells such as hepatic stem cells[22] and renal progenitor cells.[23] Therefore, the colony-forming phenomenon gives rise to the possibility that the cells, including the VFSCs, in the human MFe are tissue stem cells.

The human MFe have a high concentration of hyaluronan and the VFSCs in them express CD44, which is a hyaluronan receptor indicating that the human MFe is a hyaluronan-rich matrix.[24] A stem cell niche is composed of a group of cells in a special tissue location for the maintenance of stem cells.[25] Hyaluronan serves as an important niche component for numerous stem cell populations.[26,27] A hyaluronan-rich matrix, which is composed of the glycosaminoglycan hyaluronan and its transmembrane receptors, is able to directly affect the cellular functions of stem cells in a stem cell niche. Consequently, the human MFe should be considered a stem cell niche.[24]

The VFSCs are a member of the diffuse stellate cell system.[13] The concept of the diffuse stellate system is that stellate-shaped cells, which are morphologically similar to hepatic stellate cells that have perinuclear vitamin A droplets and express desmin,[28] are distributed in many organs. Such cells have been found at many extrahepatic sites such as the pancreas, kidneys, and intestines.[16] The VFSCs are star-like in appearance and possess slender cytoplasmic processes and lipid droplets storing vitamin A.[12] They express desmin and GFAP, which are a muscle-associated protein and a neural-associated protein, respectively.[13] These morphological features are similar to hepatic stellate cells.

Several studies have revealed that the members of the diffuse stellate system have characteristics of stem or

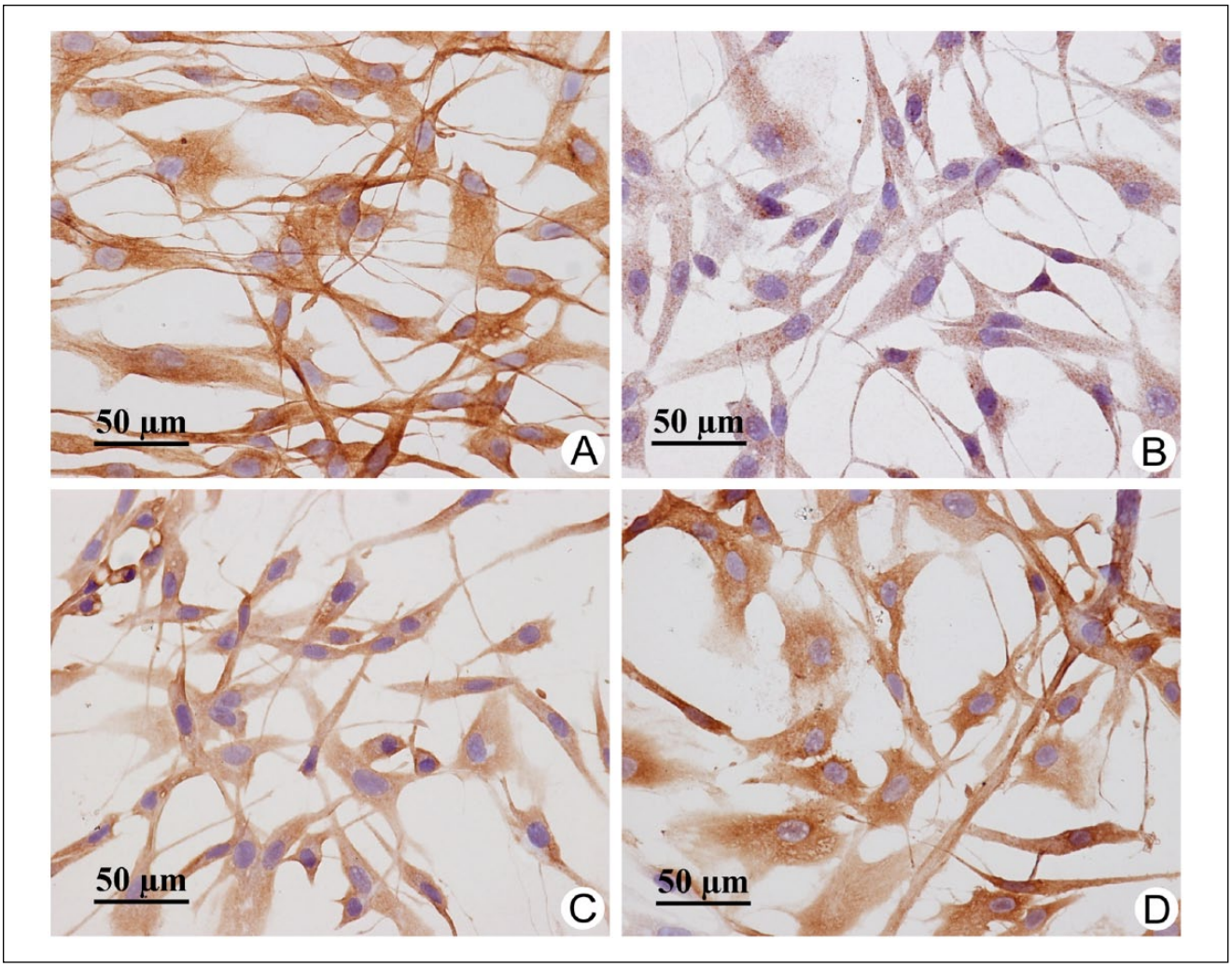

Figure 3. Immunohistochemistry of the colony-forming cells (cobblestone-like squamous cells): (A) vimentin; (B) desmin; (C) GFAP; (D) cytokeratin.

progenitor cells. Castilho-Fernandes et al[17] have shown that hepatic stellate cell lines exhibit characteristics of bone marrow–derived mesenchymal stem cells. Kordes et al[29] have revealed that hepatic stellate cells have the potential to differentiate into an endothelial or hepatocyte lineage. Kordes et al[30] have also revealed that pancreatic stellate cells have stem cell characteristics and contribute to the regeneration of injured pancreatic tissue. Hence, our results indicate that the VFSCs, which are also the member of this system, may possess characteristics of tissue stem cells or progenitor cells (transit-amplifying cells).

Differentiation of Cells in the Human Maculae Flavae

The component proteins of intermediate filaments are specific to cell types and their differentiation. The present study revealed that the cultured cells, including VFSCs, express vimentin, desmin, GFAP, and cytokeratin, which are the members of the intermediate filament protein family. These findings indicate that the cultured cells, including VFSCs, have the component proteins of intermediate filaments for all 3 germ layers.

Vimentin is the major subunit protein of intermediate filaments of mesenchymal cells such as mesenchymal stem cells, fibroblasts, and myofibroblasts. GFAP is also a member of the intermediate filament protein family and is characteristically expressed in neural crest–derived cells, including glial cells. These cells are derived from ectoderm. Desmin belongs to the intermediate filament protein family as well. Members of this family are found in muscle cells derived from mesoderm. Cytokeratin is also an intermediate filament. It is expressed in epidermal and epithelial cells, which originate from ectoderm and endoderm. Therefore, the cultured cells, including VFSCs,

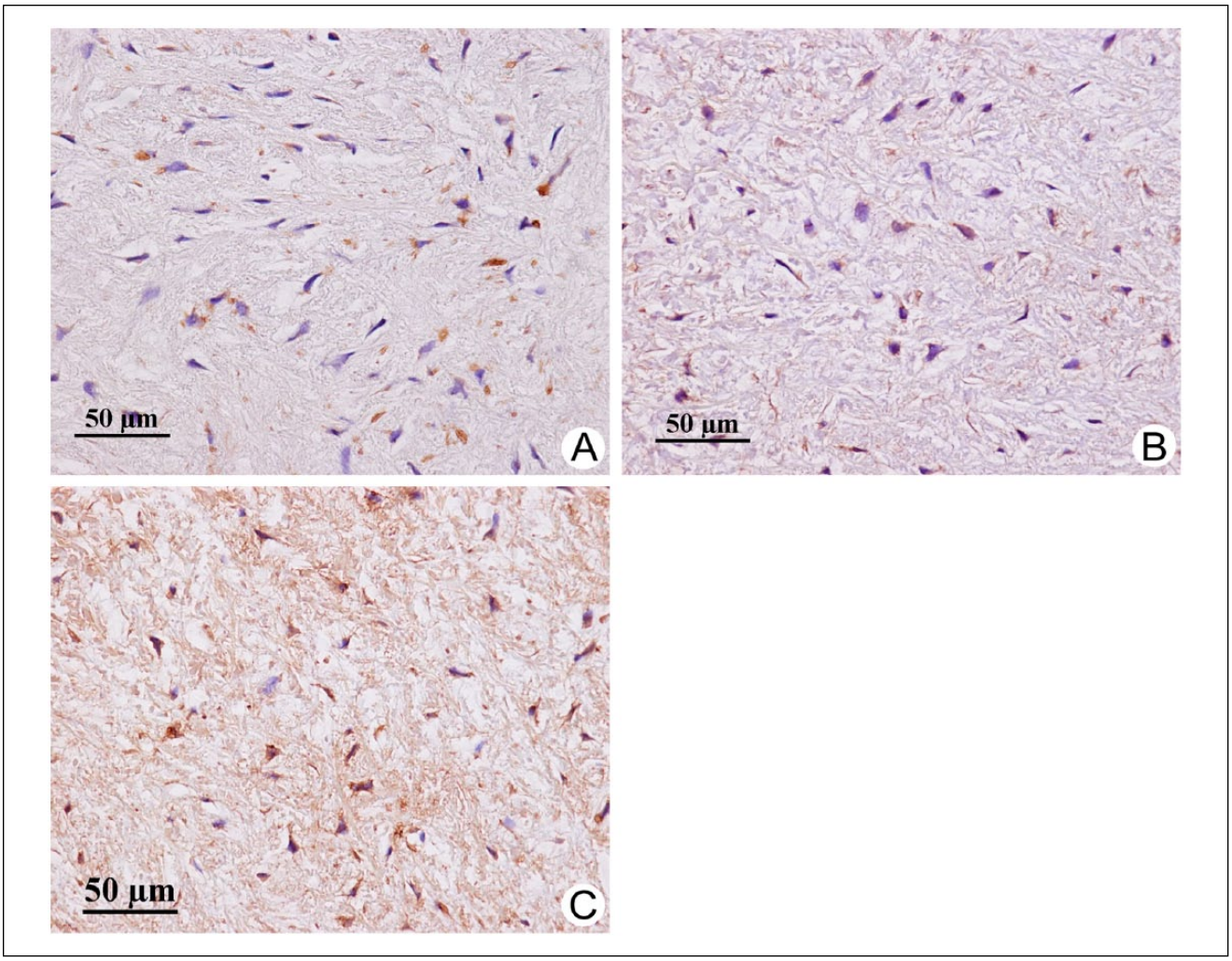

Figure 4. Immunohistochemistry of the vocal fold stellate cells in the human macula flava: (A) CD34; (B) CD45; (C) collagen type I.

are undifferentiated cells and express the intermediate proteins of all 3 germ layers.

The Relationship Between Bone Marrow–Derived Cells and Cells in the Human Maculae Flavae

Circulating fibrocytes were first described as blood-borne fibroblast-like cells by Bucala et al.[3] They were found to be unique cells because they co-expressed hematopoietic markers as well as collagen type I and other mesenchymal markers. CD34 (hematopoietic stem cell marker), CD45 (leukocyte common antigen), and collagen type I are major markers for circulating fibrocytes derived from bone marrow.

The present study revealed that CD34, CD45, and collagen type I were expressed in the cultured cells in the human MFe. Consequently, cells including VFSCs in the human MFe arise not from resident interstitial cells of the vocal fold mucosa but from the differentiation of bone marrow cells via peripheral circulation.

Bone marrow cells contribute to tissue fibrosis and regeneration.[4,5] Circulating fibrocytes are also considered to contribute to normal tissue repair and pathological fibrotic responses.[3] Under pathological conditions, such as tissue injury or inflammation, they migrate into damaged tissue sites and subsequently differentiate into myofibroblasts, fibroblasts, and other differentiated cells at the sites. These cells participate in tissue repair and fibrosis through the production of extracellular matrix proteins, including collagen.

Our previous studies indicated that the VFSCs in the human MFe play an important role in the metabolism of extracellular matrices essential for the viscoelastic properties of the lamina propria of the human vocal fold mucosa.[9,11] The VFSCs constantly synthesize collagenous fibers, including

reticular fibers, as well as other extracellular matrices such as elastic fibers and hyaluronan under normal conditions in the human adult vocal fold. These functions are similar to those of circulating fibrocytes.

There are a few reports that suggest that cells in the MFe participate in regeneration in a similar manner to BMDCs, including circulating fibrocytes. Side population cells that contain a large number of stem cells have been identified in epithelium and subepithelial tissue, including the anterior and posterior MFe under normal condition.[31] The number of side population cells significantly increase in Reinke's space of an injured vocal fold starting on day 3, with a peak at day 7, followed by a decrease back to baseline value on day 14 after injury.[32] These cells in the MFe participate in the early stages of wound healing.[32] Considering both the results of the previous reports and the results of our present investigation, the cells including the VFSCs in the human MFe may be tissue stem cells or progenitor cells (transit-amplifying cells) in the human vocal fold mucosa.

Conclusion

The cells including the VFSCs in the human MFe arise not from resident interstitial cells but from the differentiation of bone marrow cells. They are undifferentiated cells derived from the bone marrow. The results of this study are consistent with the hypothesis that the cells including the VFSCs in the human maculae flavae are tissue stem cells or progenitor cells (transit-amplifying cells) of the human vocal fold mucosa.

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Declaration of Conflicting Interests

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