Differentiation potential of the cells in the macula flava of the human vocal fold mucosa

Short title: Differentiation potential of the cells in the macula flava of the human vocal fold

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Abstract

The latest research suggests cells in the maculae flavae located at both ends of the lamina propria of the human vocal fold mucosa have stemness. This study investigated the differentiation potential of the cells in the maculae flavae of the human vocal fold mucosa.

Four normal human adult vocal folds from surgical specimens were used. After extraction of the anterior maculae flavae located at the anterior end of the lamina propria of the human vocal fold mucosa under microscope, the maculae flavae were minced, cultured and proliferated in mesenchymal stem cell growth medium and morphological features were assessed. Cell surface markers were detected using flow cytometry. Cell differentiation into adipogenic, chondrogenic and osteogenic lineages was performed. Cell's differentiation potential was assessed using a human pluripotent stem cell functional identification kit and immunohistochemistry.

Subcultured cells formed a colony-forming unit. Subcultured cells expressed CD90, CD105 and CD73 and lacked expression of CD45, CD34, CD11b, CD19 and HLA-DR. They differentiated into adipogenic, chondrogenic and osteogenic lineages. Consequently, the cell features in the maculae flavae meet the minimal criteria defining mesenchymal stromal cells. In addition, subcultured cells

differentiated into ectoderm, mesoderm and endoderm and expressed stage-specific embryonic antigen 3 (SSEA-3).

The results of this study are consistent with the hypothesis that the cells in the maculae flavae in the lamina propria of the human vocal fold mucosa are putative stem cells.

Key Words: larynx, human vocal fold mucosa, macula flava, tissue stem cells, mesenchymal stem cell, stem cell niche

Introduction

The viscoelastic properties of the lamina propria of the human vocal fold mucosa determine its vibrating behavior and depend on extracellular matrices. The fine structures of the human vocal fold mucosa influence its vibrating behavior and voice quality.

As described in detail previously (Sato K et al., 2010a), human adult maculae flavae located at both ends of the lamina propria of the vocal fold mucosa are dense masses of cells and extracellular matrices. The histological structure of the maculae flavae in the lamina propria of the human vocal fold mucosa is unique and their roles in the vocal fold as a vibrating tissue are important. However, their roles in the human vocal fold mucosa have not been clarified until recently (Sato K et al., 2010a).

Physiologically, the latest research shows maculae flavae located at both ends of the lamina propria of the human vocal fold mucosa are involved in the metabolism of extracellular matrices, which are essential for the viscoelastic properties of the human vocal fold mucosa as a vibrating tissue, and they are responsible for maintaining the characteristic layered structure of the human vocal fold mucosa (Sato K et al., 2010a). Furthermore, human maculae flavae are considered to be an

important structure in the growth, development and aging of the human vocal fold mucosa (Sato K et al., 2010b).

Clinically, the main target of regenerative medicine for the human vocal fold mucosa are restoring the viscoelastic properties of the human vocal fold mucosa. However, even now, effective treatment for the loss of the viscoelastic properties in the human vocal fold mucosa has not been established.

A clear mechanism for maintaining homeostasis of the human vocal fold mucosa as a vibrating tissue is essential to determine treatment strategies for vocal fold diseases. Recently, tissue stem cells that regenerate and maintain individual tissues have been discovered in various organs, however, the putative tissue stem cells of the human vocal fold mucosa have not been clearly established.

The latest research suggests cells in the maculae flavae located at both ends of the lamina propria of the human vocal fold mucosa have stemness (Sato K et al., 2012; Kurita T et al., 2015; Sato K et al., 2016a; Sato K et al., 2016b). However, the differentiation potential of the cells in the maculae flavae in the lamina propria of the human vocal fold mucosa has not been investigated.

The purpose of this study is to investigate the differentiation potential of the cells in the maculae flavae in the lamina propria of the human vocal fold mucosa.

Materials and methods

This study complied with the ethical standards of the relevant national ethical standards as well as the institutional guidelines on human experimentation by the Ethical Committee of Kurume University (Permission Number. 12095).

Four normal human adult vocal folds from patients ranging in age from 58 to 65 years (62.75±3.2 years, mean ± SD) who had undergone total laryngectomy with/without total glossectomy as an initial treatment for primary carcinoma of the hypopharynx or the tongue were used. All the patients were males. None of the patients underwent radiotherapy or chemotherapy. The vocal folds were unaffected by radiation or cancer invasion. And any larynges which had diseases that affect the vocal fold tissue were excluded from this study.

Isolation and Culturing of the Cells from the Macula Flava of the Human Vocal Fold

After extraction of the anterior maculae flavae in the lamina propria of the human vocal fold from surgical specimens under microscope, they were minced to 0.5 mm^3 and cultured in MF-start (primary culture medium; Toyobo, Osaka, Japan, TMMFS-001) and MF-medium (Mesenchymal Stem Cell Growth Medium; Toyobo, TMMFM-001). After using MF-

start, MF-medium was used to proliferate and subculture the cells. Cells were cultured at 37℃ in a humidified atmosphere of 5% carbon dioxide. Morphological features of the cells were observed with a phase-contrast microscope (Olympus, Tokyo, Japan).

Fluorescence activated cell-sorting analysis

Cell surface markers of the cells from the human maculae flavae cultured in the second passage were examined using direct flow cytometry. The cells were harvested and washed and then the cell suspension was adjusted to a concentration of 1.0×10^5-10^6 cells/ ml in ice-cold PBS. The primary labeled antibody was added in 1.0×10^4-10^5 cells/ μL, and 7-AAD (BD Pharmingen, Franklin, New Jersey, USA, 559925) were added for dead cell exclusion. The cells were incubated in the dark for at least 30 minutes at 4℃.

Antibodies were human-specific fluorescently labeled mouse monoclonal antibodies as follows: anti-CD45, CD34, CD11b, CD19 and HLA-DR PE (BD Stemflow™ PE Human Mesenchymal Stem Cell Lineage Antibody Cocktail, with Isotype Control, BD Pharmingen, Mouse Anti-Human, 562530), anti-CD90 Fluorescein Isothiocyanate (FITC) (BD Pharmingen, Mouse Anti-Human, 561969), anti-CD105 allophyocyanin (APC) (BD Pharmingen, Mouse Anti-Human, 562408), and anti-CD73 PE-Cy7 (BD Pharmingen, Mouse Anti-

Human, 561258). After incubation, the cells were washed three times by centrifugation at 400 gravity for 5 minutes and resuspended in 1ml of ice-cold PBS. As a measuring instrument, BD FACSVerse™ Flow Cytometer (BD Pharmingen, USA) was used.

Analysis of Differentiation of Cells from the Human Maculae Flavae Toward Adipogenic, Chondrogenic and Osteogenic Lineages

A human Mesenchymal Stem Cell Functional Identification Kit (R&D Systems, Minneapolis, Minnesota, USA, SC027B) was used to assess whether the cells in the human maculae flavae are capable of differentiating into multiple cell types including adipocytes, chondrocytes and osteocytes.

The subcultured cells in the second passage were respectively cultured in the medium with adipogenic, chondrogenic and osteogenic differentiation media to induce adipogenesis, chondrogenesis and osteogenesis. The analyzed cells were the same subcultured cells in the second passage which had been used for flow cytometry.

After 10~21 days of culturing, the cells were fixed in 4% paraformaldehyde, after which the reacted with primary antibodies against anti-mouse fatty acid binding protein 4 (FABP-4) (R&D Systems, #967799, goat polyclonal)

(1:200 dilution), aggrecan (R&D Systems, #967800, goat polyclonal) (1:200 dilution) and osteocalcin (R&D Systems, #967801, mouse monoclonal) (1:200 dilution). The negative controls were incubated without primary antibodies. For secondary antibodies, donkey anti-goat IgG conjugated with Alexa Flour 594 (ThermoFisher scientific, Waltham, Massachusetts, USA, A11058) (1:200 dilution) or donkey anti-mouse IgG conjugated with Alexa Flour 594 (ThermoFisher scientific, A21203) (1:200 dilution) were used. The nuclei were stained with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, California, USA, H-1200). Fluorescent images were observed using fluorescent microscopy (Nikon, Tokyo, Japan), and were recorded with a cooled CCD camera and the IP Lab Spectrum software (Nikon, Japan).

Analysis of Differentiation of Cells from the Human Maculae Flavae Toward Ectodermal, Mesodermal and Endodermal Lineages

Human Pluripotent Stem Cell Functional Identification Kit (R&D Systems, SC027B) was used to assess whether the cells in the human maculae flavae are capable of differentiating into ectodermal, mesodermal and endodermal lineages.

The subcultured cells of the second passage were respectively cultured in the medium with ectoderm, mesoderm and endoderm differentiation media. The analyzed cells were the same subcultured cells in the second passage which had been used for flow cytometry.

The cells were fixed by 4% paraformaldehyde, after which they reacted with primary antibodies against Otx2 (R&D Systems, #963273, goat polyclonal) (1:200 dilution), Brachyury (R&D Systems, #963427, goat polyclonal) (1:200 dilution) and SOX17 (R&D Systems, #963121, goat polyclonal) (1:200 dilution). The negative controls were incubated without primary antibodies. For secondary antibodies, donkey anti-goat IgG conjugated with Alexa Flour 594 (ThermoFisher scientific, A11058) (1:200 dilution) was used. The nuclei were stained with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, H-1200). The fluorescent images were observed using fluorescent microscopy (Nikon, Japan), and were recorded with a cooled CCD camera and the IP Lab Spectrum software (Nikon, Japan).

Immunohistochemical Analysis of the Cultured Cells from the Human Maculae Flavae

The analyzed cells were the same subcultured cells in the second passage which had been used for flow cytometry. Subcultured cells from the human maculae flavae grown on

Lab-tek chamber slides (ThermoFisher scientific, 154526) 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 with primary antibody at 4°C overnight.

Anti-stage-specific embryonic antigen 3 (SSEA-3) antibody (Abcam, Cambridge, UK, ab16286, Rat monoclonal) (1:50 dilution) was used.

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, 424151) 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 on slide. Immunoreactivity was examined by light microscopy.

Results

Morphological features of the cultured cells

On the 4th day of the primary culture in the MF-start medium (primary culture medium), cells grew from the minced macula flava. On the 10th day of the primary culture, the cells formed colony-forming units. On the 15th day, 3 phenotypes of cells, cobblestone-like polygonal cells (Fig. 1A), vocal fold stellate cell-like cells possessing lipid droplets in the cytoplasm (Fig. 1B) and fibroblast-like spindle cells (Fig. 1C), proliferated. Since the three types of cells were mixed and were difficult to isolate, these cells were subcultured together in the MF-medium (Mesenchymal Stem Cell Growth Medium).

On the 3rd day of the first passage, the cells formed a greater number of colony-forming units than in the primary culture (Fig. 1D). These colony-forming units were observed up to the third passage.

In the second passage, fibroblast-like spindle cells occupied most of the dish and vocal fold stellate cell-like cells became inconspicuous.

Fluorescence activated cell-sorting analysis

Single cells in the living cells (8842±30.5 cells) were assessed. Most of the cells from the human maculae

flavae at the second passage expressed CD90 and CD105 (Fig. 2A) and almost all of them expressed CD73 (Fig. 2B). Furthermore, almost all of the cells which had expressed CD90, CD105 and CD73 lacked expression of CD45, CD34, CD11b, CD19 and HLA-DR (Lineage) (Fig. 2C).

The ratio of CD90 and CD105 positive cells to the total number of cells were 95.8±0.39% (mean±SD). CD73 positive cells were 95.7±0.29%. And CD45, CD34, CD11b, CD19 and HLA-DR positive cells were 0.023±0.01%.

Analysis of differentiation of cells from the human maculae flavae toward adipogenic, chondrogenic and osteogenic lineages

The cells in the medium with adipogenic differentiation media possessed lipid droplets (Fig. 3A) and expressed FABP-4 in the cytoplasm (Fig. 3B) on the 10^{th} day.

The cells in the medium with chondrogenic differentiation media aggregated at the bottom of the tube (Fig. 3C) and expressed aggrecan in the intercellular matrices (Fig. 3D) on the 14^{th} day.

The cells in the medium with osteogenic differentiation media proliferated densely (Fig. 3E) and expressed osteocalcin in the cytoplasm (Fig. 3F) on the 15^{th} day.

Analysis of the differentiation of cells from the human maculae flavae toward ectodermal, mesodermal and endodermal lineages

The cells cultured with ectoderm, mesoderm and endoderm differentiation media differentiated into the cells that expressed markers of their respective germ layers, Otx2 (ectoderm marker) (Fig. 4A), Brachyury (mesoderm marker) (Figure. 4B) and SOX17 (endoderm marker) (Fig. 4C).

Immunohistochemical Analysis

Almost all cells expressed SSEA-3 (human pluripotent stem cell marker) (Fig. 5).

Discussion

Recently, tissue stem cells that regenerate and maintain individual tissues have been discovered in various organs. According to the latest research, there is growing evidence to suggest that the cells in the maculae flavae in the lamina propria of the human vocal fold mucosa are putative stem cells of the vocal fold mucosa and that the maculae flavae are a candidate for a stem cell niche (Sato K et al., 2012; Kurita T et al., 2015; Sato K et al., 2016a; Sato K et al., 2016b).

The characteristics of the cultured cells from the human maculae flavae

In this study, the cultured cells grown from the maculae flavae in the lamina propria of the human vocal fold mucosa formed colony-forming units.

Colony-forming units are one of the features of stem cells. Friedenstein et al established for the first time that adherent fibroblastic cells that form cell colonies in vitro culture can be isolated from the bone marrow stroma (Friedenstein AJ et al., 1974). Cells that form colony-forming units have multipotency (Pittenger MF et al., 1999). Such colony-forming units are also observed in embryonic stem cells (ES cells) (Thomson JA et al., 1998), induced pluripotent stem cells (iPS cells) (Takahashi K et

al., 2007), and tissue stem or progenitor cells in various organs (Suzuki A et al., 2000; Osafune K et al., 2006). Therefore, the colony-forming phenomenon gives rise to the possibility that the cells in the maculae flavae in the lamina propria of the human vocal fold mucosa have stemness.

Additionally, 3 phenotypes of cells, cobblestone-like polygonal cells, vocal fold stellate cell-like cells and fibroblast-like spindle cells proliferated from the human maculae flavae in this study. Sato et al reported there are vocal fold stellate cells possessing cytoplasmic processes and vitamin A storing lipid droplets in the cytoplasm in the maculae flavae in the lamina propria of the human vocal fold mucosa (Sato K et al., 2001; Sato K et al., 2003). Sato et al also reported that the vocal fold stellate cells are possibly transit-amplifying (progenitor) cells (Sato K et al., 2016b). However, at the present stage of our investigations, it is difficult to determine the stem cell system and hierarchy of stem cells in the maculae flavae in the lamina propria of the human vocal fold mucosa.

Minimal criteria defining multipotent mesenchymal stromal cells

The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposes

minimal criteria to define human multipotent mesenchymal stromal cells (Dominici M et al., 2006). They propose: First, multipotent mesenchymal stromal cells must be plastic-adherent when maintained in standard culture conditions. Second, multipotent mesenchymal stromal cells must express CD90, CD105 and CD73 (≧95% positive) and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules (≦ 2% positive). Third, multipotent mesenchymal stromal cells must differentiate to adipocytes, chondroblasts and osteoblasts in vitro.

In this study, subcultured cells from the maculae flavae in the lamina propria of the human vocal fold mucosa were plastic-adherent when maintained in standard culture conditions. They expressed CD90, CD105 and CD73, and lacked expression of CD45, CD34, CD11b, CD19 and HLA-DR. They differentiated into adipogenic, chondrogenic and osteogenic lineages. Consequently, the cell features in the maculae flavae in the lamina propria of the human vocal fold mucosa meet the minimal criteria defining multipotent mesenchymal stromal cells.

Stemness of the cells in the maculae flavae in the lamina propria of the human vocal fold mucosa

There is a report that vocal fold fibroblasts in the lamina propria of the vocal fold mucosa include mesenchymal stem (stromal) cells (Hanson SE et al., 2010). As described above, our investigations revealed that the cell features in the maculae flavae in the lamina propria of the human vocal fold mucosa meet the minimal criteria of mulitipotent mesenchymal stromal cells. Furthermore, our investigations showed that the cells in the maculae flavae had a different and higher differentiation potential when compared with the fibroblasts in the lamina propria of the vocal fold mucosa.

The latest research shows that intermediate filaments are structural proteins of the cytoplasm and specific to cell type and differentiation (Becker W et al., 2006). And because of the tissue specificity of intermediate filaments, cells from different tissues can be distinguished on the basis of the intermediate filament protein present (Becker W et al., 2006).

Kurita et al reported that cultured cells from the maculae flavae in the lamina propria of the human vocal fold mucosa expressed members of each of the intermediate filament protein families, and suggested that the cultured cells from the maculae flavae in the lamina propria of the human vocal fold mucosa expressed the proteins of all three germ layers and that they are undifferentiated cells (Kurita T et al., 2015).

It is of interest that previous research shows that mesenchymal stem (stromal) cells do differentiate into the cells of all three germ layers (Pittenger MF et al., 1999; Dezawa M et al., 2004; Terai S et al., 2003; Karnieli O et al., 2007). Another previous study reported the existence of a specific type of human mesenchymal stem (stromal) cell that is capable of differentiating into cells of all three germ layers and can be efficiently isolated from naive tissues as cells positive for both CD105 and SSEA-3 (Kuroda Y et al., 2010).

In this study, the cells from the maculae flavae in the lamina propria of the human vocal fold mucosa differentiated into cells that express markers of all three germ layers, and expressed SSEA-3. This suggests that the cells in the maculae flavae in the lamina propria of the human vocal fold mucosa have the capacities of not only the mesenchymal stem (stromal) cells but also other kinds of stem cells.

Slow-dividing cell populations in the vocal fold mucosa

Adult tissue stem cells are maintained in a quiescent state and proliferate extremely slowly with stem cell properties. Adult tissue stem cells consistently retain labeled DNA since stem cells divide more infrequently than other cells.

Sato et al reported that the very small number of cells in the basal layer of the stratified squamous epithelium and the lamina propria of the rat vocal fold mucosa retained BrdU labeling. On the other hand, the cells in the maculae flavae of the rat vocal fold mucosa retained BrdU labeling, that is, label-retaining cells reside in the maculae flavae (Sato K et al, 2018). This phenomenon indicates the division cycles of the cells in the maculae flavae are slow and different from other cells in the vocal fold mucosa (Sato K et al, 2018). Consequently, the results are consistent with the hypothesis that the cells in the maculae flavae are putative stem cells of the vocal fold mucosa.

Side population cells in the vocal fold mucosa

Side population cells are regarded as a cell population enriched with stem cells or progenitor cells. Yamashita et al reported side population cells were identified in the epithelium and subepithelial tissue including the human maculae flavae of the human vocal fold mucosa (Yamashita M et al., 2007). Gugatschka et al reported side population cells in the anterior and posterior maculae flavae of the human vocal fold mucosa increased significantly in the lamina propria of an injured vocal fold mucosa and participated in the early

stage of wound healing of rat vocal fold mucosa (Gugatschka M et al., 2011).

The two reports suggest that the maculae flavae in the lamina propria of the vocal fold mucosa contain stem cells or progenitor cells which regenerate the vocal fold tissue.

Stem cell niche in the human vocal fold mucosa

The structural and biochemical microenvironment that confers stemness upon cells in multicellular organisms is referred to as the stem cell niche (Li L and Xie T., 2005). The stem cell niche regulates or supports the proliferation, maintenance of the stemness, and differentiation of stem cells.

Hyaluronan serves as an important niche component for numerous stem cell populations (Haylock DN and Nilsson SK., 2006; Preston M and Sherman LS., 2011). Sato et al reported, since the cells in the human maculae flavae express CD44 (a cell surface hyaluronan receptor) and are surrounded by a high concentration of hyaluronan, the maculae flavae in the lamina propria of the human vocal fold mucosa are a hyaluronan-rich pericellular matrix and are a candidate for a stem cell niche, which is a microenvironment nurturing a pool of stem cells (Sato K et al., 2012).

Sato et al also reported, by comparing proliferation patterns of cocultured cells from the human maculae flavae with DMEM (Dulbecco's modified Eagle's medium) and with mesenchymal stem cell growth medium, a proper microenvironment is necessary to be effective as a stem cell niche maintaining the stemness of the cells from the maculae flavae in the lamina propria of the human vocal fold mucosa (Sato K et al., 2016b).

The results of these studies are consistent with the hypothesis that the maculae flavae are a candidate for a stem cell niche of the human vocal fold mucosa.

Regeneration of the Human Vocal Fold Mucosa

Regeneration of the vocal fold requires three important elements: cell therapy, development and implementation of a scaffold, and the use of growth factors. The cells in the maculae flavae are a potential endogenous cell source for vocal fold regeneration and will provide the tools for future therapeutic approaches. Further investigations are needed regarding the stem cell system of the human vocal fold mucosa, that is, how the cells in the maculae flavae, located at both ends of the vocal fold mucosa, differentiate into surrounding interstitial cells including fibroblasts in the lamina propria of the human vocal fold mucosa.

Conclusions

In this study of the differential potential of the cells in the maculae flavae in the lamina propria of the human vocal fold mucosa, the cells in the maculae flavae not only had the features of multipotent mesenchymal stromal cells but also had the capacity to differentiate into cells of all three germ layers. Consequently, the cells in the human maculae flavae have the capacities of not only the mesenchymal stem (stromal) cells but also other kinds of stem cells.

The results of this study are consistent with the hypothesis that the cells in the maculae flavae in the lamina propria of the human vocal fold mucosa are putative stem cells.

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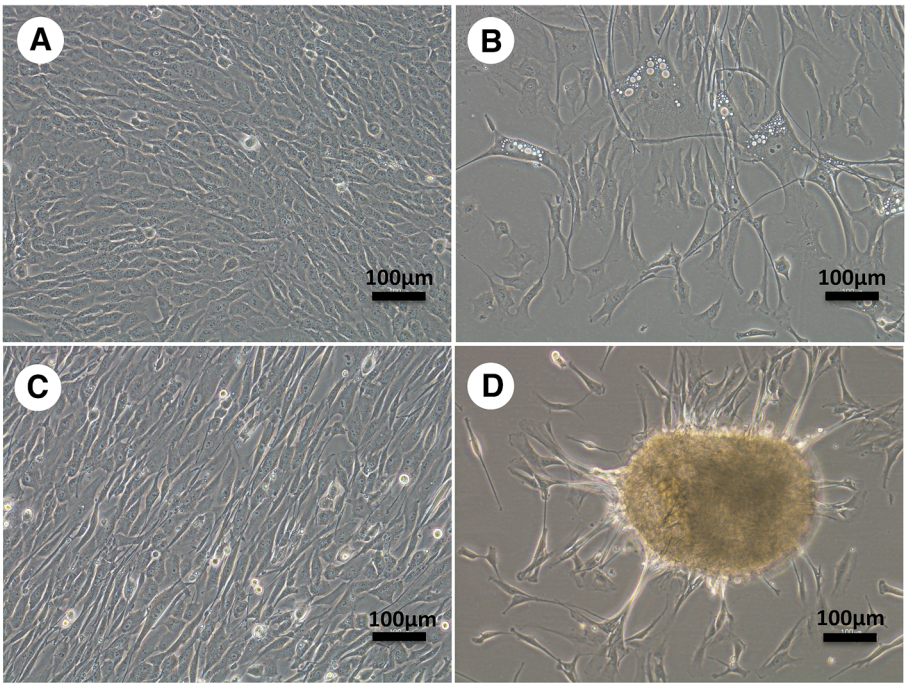
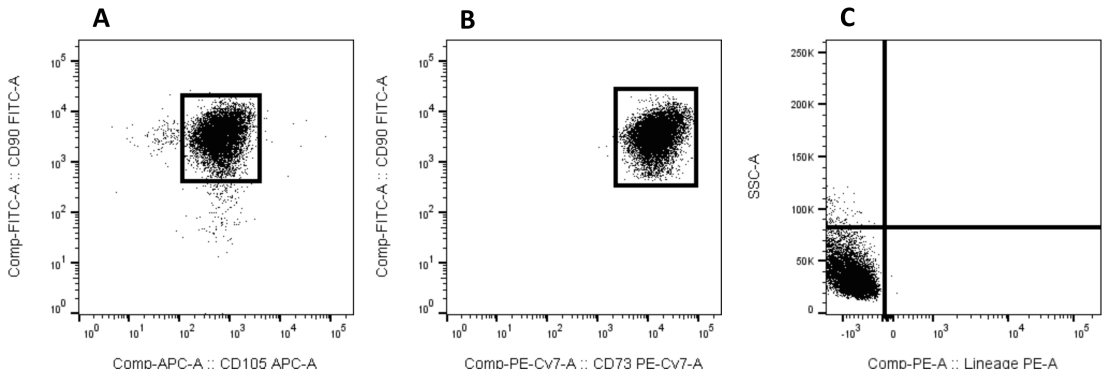


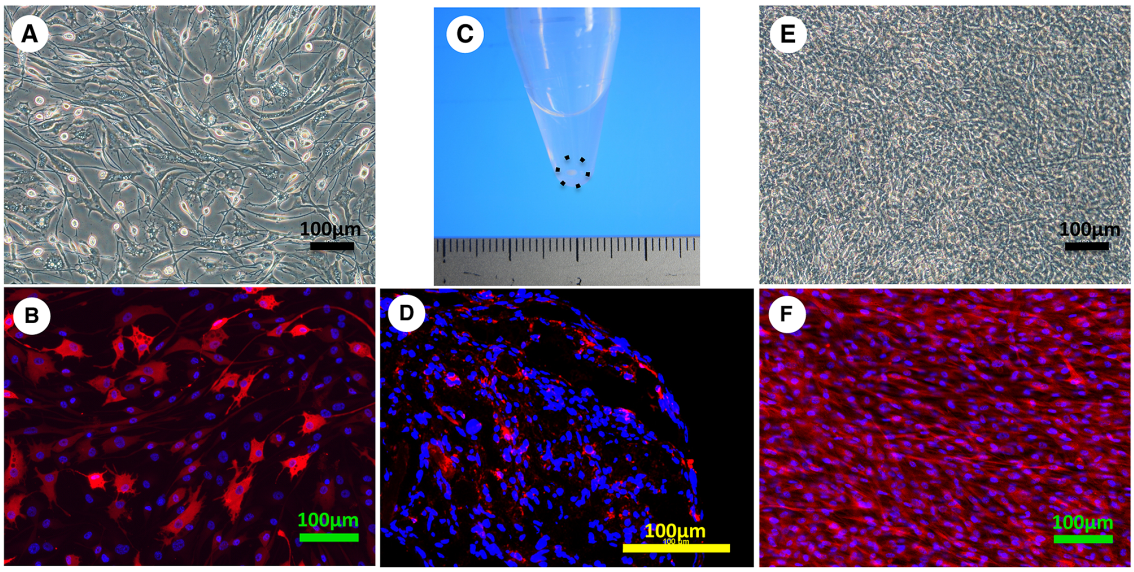
Fig. 1



	Number of positive cells	Ratio of positive cells to total cells (%)
CD90+ and CD105+ cells	8475±63.4	95.8±0.39
CD90+, CD105+ and CD73+ cells	8459±54.0	95.7±0.29
Lineage+ cells	2±0.82	0.023±0.01

*Lineage: CD45, CD34, CD11b, CD19 and HLA-DR

Fig. 2


A
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C
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100μm

Fig. 3

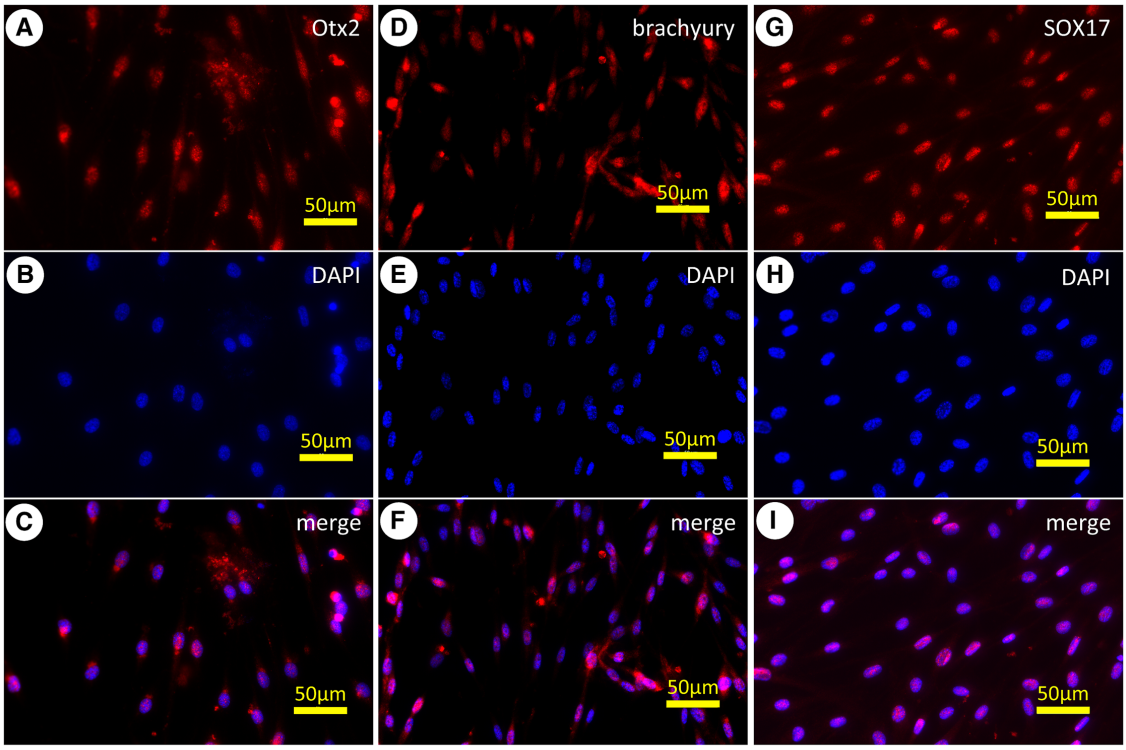
A
Otx2
50μm
D
brachyury
50μm
G
SOX17
50μm
B
DAPI
50μm
E
DAPI
50μm
H
DAPI
50μm
C
merge
50μm
F
merge
50μm
I
merge
50μm
Ectoderm
Mesoderm
Endoderm

Fig. 4

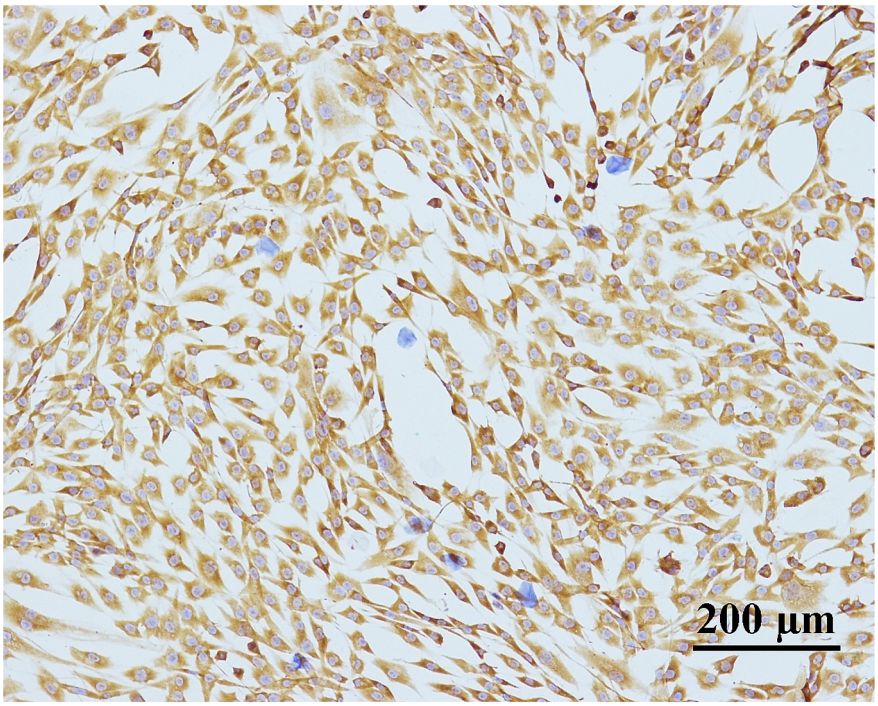

Fig. 5

Figure Legends

Fig 1. Morphological features of the cultured cells

Three phenotypes of cells proliferated (primary culture); (A) Cobble stone-like polygonal cells, (B) Vocal fold stellate cell-like cells, (C) Fibroblast-like spindle cells.

(D) The cells formed many colony-forming units (the first passage).

Fig 2. Fluorescence activated cell-sorting analysis of the cells from the human macula flava

(A) Cells expressed CD90 and CD105 (area enclosed in the box).

(B) CD90- and CD105-positive cells expressed CD73 (area enclosed in the box).

(C) Almost all cells that expressed CD90, CD105 and CD73 lacked expression of CD45, CD34, CD11b, CD19 and HLA-DR (Lineage).

Fig 3. Differentiation of the cells from the human maculae flavae toward adipogenic, chondrogenic and osteogenic lineages

Adipogenic differentiation: (A) Cells possessed lipid droplets, (B) Cells expressed FABP-4 in the cytoplasm (red) (The nuclei were counterstained blue with DAPI).

Chondrogenic differentiation: (C) Cells aggregated at the bottom of the tube, (D) Cells expressed aggrecan in the intercellular matrices (red) (The nuclei were counterstained blue with DAPI).

Osteogenic differentiation: (E) Cells proliferated densely, (F) Cells expressed osteocalcin in the cytoplasm (red) (The nuclei were counterstained blue with DAPI).

Fig 4. Differentiation of the cells from human maculae flavae toward three germ layers

Cells expressed markers of all three germ layers in the nucleus (red) (The nuclei were counterstained blue with DAPI): (A-C) Cells expressed Otx2 (ectoderm marker) in the nucleus, (D-F) Cells expressed brachyury (mesoderm marker)

in the nucleus, (G-I) Cells expressed SOX17 (endoderm marker) in the nucleus.

Fig 5. Immunohistochemistry of cultured cells from human maculae flavae

Cells expressed SSEA-3 (human pluripotent stem cell marker).