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Comparison of Nestin-Expressing Multipotent Stem Cells in the Tongue Fungiform Papilla and Vibrissa Hair Follicle

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ABSTRACT

We have previously reported that hair follicles contain multipotent stem cells, which express nestin and participate in follicle growth at anagen as well as in the extension of the follicle sensory nerve. The nestin-driven green fluorescent protein (ND-GFP) transgenic mouse labels all nestin-expressing cells with GFP. The hair follicle nestin-GFP cells can differentiate into neurons, Schwann cells, and other cell types. In this study, we describe nestin-expressing multipotent stem cells in the fungiform papilla in the tongue. The nestin-expressing multipotent stem cells in the fungiform papilla are located around a peripheral sensory nerve immediately below the taste bud and co-express the neural crest cell marker $p75^{NTR}$. The fungiform papilla cells formed spheres in suspension culture in DMEM-F12 medium supplemented with basic fibroblast growth factor (bFGF). The spheres consisted of nestin-expressing cells that co-expressed the neural crest marker $p75^{NTR}$ and which developed expression of the stem cell marker CD34. $P75^{NTR}$, CD34 and nestin co-expression suggested that nestin-expressing cells comprising the fungiform papilla spheres were in a relatively undifferentiated state. The nestin-expressing cells of these spheres acquired the following markers: β III tubulin typical of nerve cells; GFAP typical of glial cells; K15 typical of keratinocytes; and smooth-muscle antigen (SMA), after transfer to RPMI 1640 medium with 10% fetal bovine serum (FBS), suggesting they differentiated into multiple cell types. The results of the current study indicate nestin-expressing fungiform papilla cells and the nestin-expressing hair follicle stem cells have common features of cell morphology and ability to differentiate into multiple cell types, suggesting their remarkable similarity. J. Cell. Biochem. 115: 1070–1076, 2014.

KEY WORDS: FUNGIFORM PAPILLA; HAIR FOLLICLE; VIBRISSA; NESTIN; STEM CELLS; DIFFERENTIATION; GFP; CONFOCAL FLUORESCENCE IMAGING; PERIPHERAL NERVE; HISTOCULTURE

The fungiform papilla is a taste organ and located on the dorsal surface of the tongue, mainly at the tip margin. The fungiform papilla contains taste buds, which transduce stimuli into electro-chemical signals on gustatory sensory neurons. The fungiform papillae are innervated by the chorda tympani and lingual nerves. These sensory peripheral nerves are joined in the taste buds [Oakley and Witt, 2004; Miura and Barlow, 2010]. It is thought that the progenitor cells of the fungiform papilla are within taste buds or in its surrounding epidermis [Miura et al., 2006; Sullivan et al., 2010].

The intermediate filament protein, nestin, marks progenitor cells of the CNS [Mignone et al., 2004] and the hair follicle [Li et al., 2003], as well as mesenchymal stem cells [Pinho et al., 2013]. Nestin-expressing cells can be selectively labeled by placing green fluorescent protein (GFP) driven by the nestin promoter in transgenic mice (ND-GFP mice) [Kawaguchi et al., 2001; Li et al., 2003; Mignone et al., 2004].

We originally reported that nestin-expressing cells, brightly expressing GFP, can be visualized in the permanent upper hair follicle in the bulge area of ND-GFP mice. The nestin-expressing cells of the hair follicle have round/oval-shaped bodies with a typical diameter of 7 μm and two-three elongated processes containing club-like bodies in the bulge area surrounding the hair shaft [Li et al., 2003].

In vitro, the nestin-expressing hair follicle cells formed spheres and differentiated into neurons, glia, keratinocytes, smooth muscle cells, and melanocytes. Nestin-expressing cells in the spheres were positive for the stem cell marker CD34 [Amoh et al., 2005a].

When nestin-expressing cells from the mouse vibrissa bulge area were implanted into the gap region of the severed sciatic nerve, they effected functional nerve repair. The transplanted follicle bulge-area nestin-expressing cells transdifferentiated largely into Schwann

The authors declare that they have no competing interests.

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cells, which are known to support neuron regrowth. The transplanted mice recovered the ability to walk normally [Amoh et al., 2005b].

Nestin-expressing mouse vibrissa cells from the bulge area were also transplanted into the injured thoracic spinal cord. Most of the transplanted cells also differentiated into Schwann cells that effected repair of the spinal cord. The spinal cord recovered and extensive hind-limb locomotor performance was re-established [Amoh et al., 2008; Liu et al., 2011].

We demonstrated in three-dimensional Gelfoam® histoculture that the nestin-expressing cells in the whisker follicle bulge traffic to the truncated whisker sensory nerve and effect nerve extension and joining with other nerves in vitro [Duong et al., 2012; Mii et al., 2013]. Thus, nestin-expressing hair follicle cells have a critical role in nerve regeneration.

In the present study, we identified and characterized nestin-expressing cells in the fungiform papilla of the tongue, which is a sensory organ, as is the whisker follicle. We compared the characteristics of nestin-expressing fungiform papilla cells to the nestin-expressing cells in the hair follicle, as described in our previous studies [Li et al., 2003; Amoh et al., 2005a,b, 2008; Liu et al., 2011; Uchugonova et al., 2011a; Duong et al., 2012; Mii et al., 2013] and found a remarkable similarity of nestin-expressing cells in the two organs.

MATERIALS AND METHODS

ANIMALS

Transgenic mice with nestin-driven GFP (ND-GFP) [Kawaguchi et al., 2001; Li et al., 2003; Mignone et al., 2004] (AntiCancer, Inc., San Diego, CA), as well as red fluorescent protein (RFP) transgenic mice [Vintersten et al., 2004], at different ages (4 weeks up to 5 months) (AntiCancer, Inc.), were used for this study. All animal studies were conducted with an AntiCancer Institutional Animal Care and Use Committee (IACUC) protocol approved for this study and in accordance with the principals and procedures outlined in the National Institute of Health Guide for the Care and Use of Animals under Assurance Number A3873-1.

ISOLATION OF FUNGIFORM PAPILLA FROM ND-GFP TRANSGENIC MICE

The mice were anesthetized with a 30–50 μl ketamine solution (25 mg/ml). The tongue was removed from ND-GFP transgenic mice and sterilized with 70% isopropyl alcohol and washed in PBS three times. Using an MZ6 binocular microscope (Leica, Wetzlar, Germany), the tongue was dissected into single fungiform papillae with forceps and fine needles. The isolated fungiform papillae were used for imaging and culture.

ISOLATION OF VIBRISSA HAIR FOLLICLE FROM ND-GFP TRANSGENIC MICE

The mice were anesthetized with the 30–50 μl ketamine solution (25 mg/ml). Whisker pads from ND-GFP transgenic mice were sterilized with 70% isopropyl alcohol and washed in PBS three times. Using the MZ6 binocular microscope, the whisker pad was dissected to obtain single vibrissae follicles with forceps and fine needles [Duong et al., 2012; Mii et al., 2013]. The isolated vibrissae hair follicles were used for imaging.

CULTURE OF FUNGIFORM PAPILLA

The nestin-expressing cells in the fungiform papillae were incubated in DMEM-F12 (GIBCO/BRL, Grand Island, NY), containing B-27 (GIBCO/BRL), N2 (GIBCO/BRL), 1% penicillin and streptomycin (GIBCO/BRL) and 20 ng/ml basic fibroblast growth factor (bFGF) (EMD Millipore, Billerica, MA) at 37°C, 5% CO_2 at 100% humidity in 24-well tissue-culture dishes (Sarstedt, Newton, NC). The medium was changed every other day. The nestin-expressing cells formed spherical colonies (spheres).

For differentiation, the spheres were centrifuged and the growth factor-containing DMEM-F12 medium was removed. The spheres were resuspended into fresh RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. The spheres were cultured in collagen I-(GIBCO) coated four-well chamber slides (Fisher Scientific, Pittsburgh, PA).

CONFOCAL LASER SCANNING MICROSCOPY

A confocal laser scanning microscope (Fluoview FV1000, Olympus Corp., Tokyo, Japan) was used for two- (X,Y) and three-dimensional (3D, X,Y,Z) high-resolution imaging of fungiform papillae in culture and immunofluorescence staining. Fluorescence images were obtained using the 4×/0.10 Plan N, 10×/0.30 Plan-NEOFLUAR, 20×/0.50 UPlan FLN, and 20×/1.00w XLUMplan FL objectives.

IMMUNOFLUORESCENCE STAINING

Tissues were fixed in pre-cooled 4% paraformaldehyde at room temperature (RT) for 2 h and embedded in tissue freezing medium (Triangle Biomedical Science, Durham, NC) and frozen in nitrogen for 10 min and at −80°C. Frozen sections of 7–10 μm thickness were prepared with a CM1850 cryostat (Leica). The frozen sections were washed with phosphate buffered saline (PBS) three times. Immunofluoresence staining procedures were as follows: 1) Five percent normal goat serum was applied at RT for 1 h. 2) Primary antibodies were applied at RT for 2 h. The primary antibodies used were as follows: anti-β III tubulin mAb (mouse, 1:100, Santa Cruz Biotech, Dallas, TX); anti-glial fibrillary acidic protein (GFAP) mAb (mouse, 1:250, BD Pharmingen, San Jose, CA); anti-S100 mAb (mouse, 1:200, Millipore, Billerica, MA); anti-CD34 mAb (rat, 1:100, BD Pharmingen); anti-$p75^{NTR}$ antibody (rabbit, 1:3200, Cell Signaling, Danvers, MA). 3) Secondary antibodies used were as follows: goat anti-mouse IgG Alexa Fluor® 555 (1:1,000, Cell Signaling); goat anti-rat IgG (H + L) Alexa Fluor® 555 (1:1,000, Cell Signaling); goat anti-rabbit IgG (H + L) Alexa Fluor® 555 (1:1,000, Cell Signaling), RT, dark, 1 h. 4) DAPI staining (1:48,000, Invitrogen, Carlsbad, CA) was carried out at RT in the dark for 3 min. 5) Slides were mounted with Fluoremount (Sigma, St. Louis, MO) and observed under confocal laser microscopy with the FV1000. Immunofluorescence staining was compared with positive and negative controls.

RESULTS

NESTIN-EXPRESSING CELLS IN THE FUNGIFORM PAPILLA

The anterior and dorsal surface of the tongue of ND-GFP transgenic mouse contain fungiform papillae. Each fungiform papilla expressed nestin-driven GFP (ND-GFP; Fig. 1B). A cross section of the tip of the tongue showed that the fungiform papilla had a taste bud in the

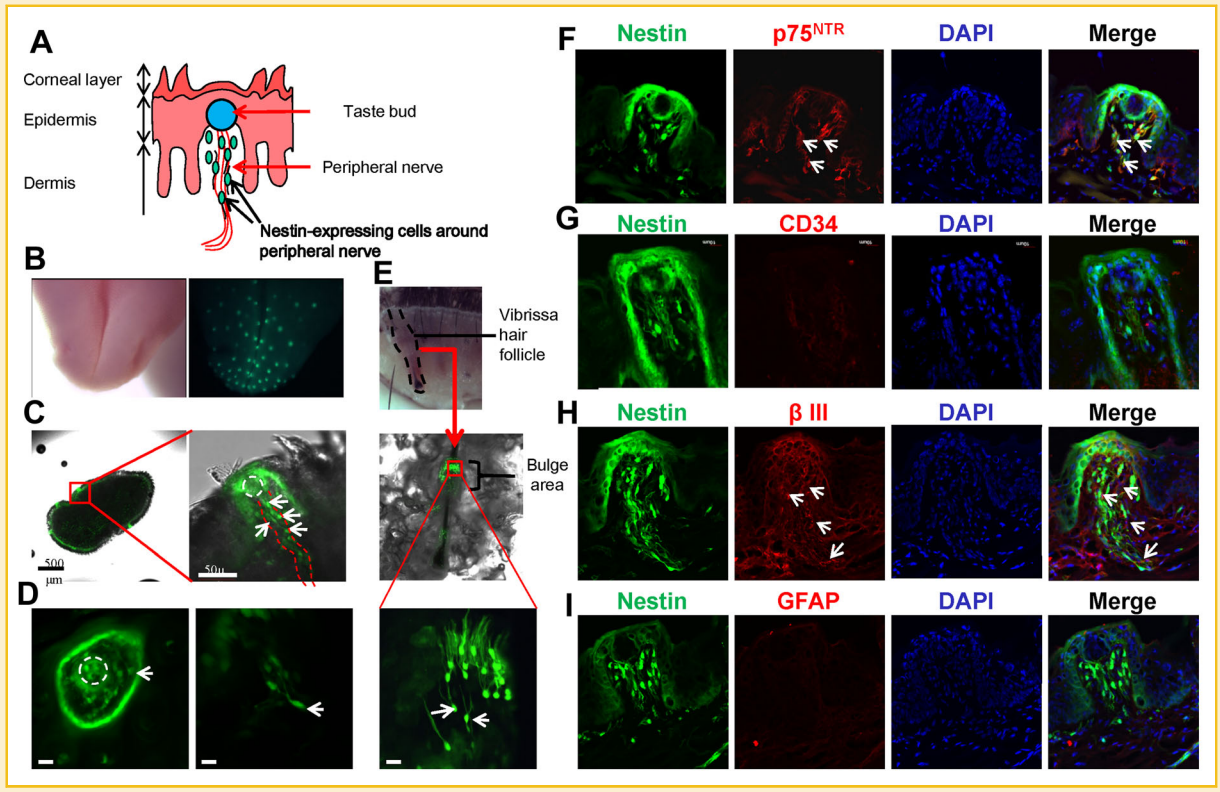

Fig. 1. Nestin GFP-expressing cells in the fungiform papilla are similar to nestin-expressing cells in the whisker hair follicle bulge. A: A schematic figure of the fungiform papilla. The fungiform papilla comprises a taste bud in the epidermis and a peripheral nerve from the dermis joined to the taste bud. We identified nestin-expressing cells around the peripheral nerve. B: The anterior and dorsal surface of the tongue of nestin-driven (ND)-GFP transgenic mice contain fungiform papillae. Each fungiform papilla expresses GFP. C: A cross section of the tip of the tongue shows that the fungiform papilla contains a taste bud (white dashed circle) in the epidermis and a sensory peripheral nerve (red dashed line) in the dermis joined to the taste bud. ND-GFP-expressing cells (white allows) were seen around the peripheral sensory nerve. D: Left: cross section of a taste bud (white dashed circle); right: longitudinal section of a fungiform papilla. The ND-GFP-expressing fungiform papilla cells (white arrows) have round/oval-shaped bodies, with a typical diameter of 7–10 μm and two-three elongated processes. White bar: 10 μm. E: Vibrissa hair follicles removed from ND-GFP transgenic mice have nestin-expressing cells in its bulge area. The nestin-expressing hair follicle cells (white arrows) have round/oval-shaped bodies with a typical diameter of 7 μm and two-three long elongated processes. White bar: 10 μm. F: The ND-GFP-expressing fungiform papilla cells co-express $p75^{NTR}$ (white arrows). White bar: 10 μm. G: CD34 staining was negative in the ND-GFP-expressing fungiform papilla cells. H: The ND-GFP-expressing fungiform papilla cells are located around β III tubulin-positive axons (white arrows). White bar: 10 μm. I: GFAP staining was negative in the nestin GFP-expressing fungiform papilla cells. White bar: 10 μm.

epidermis and a sensory peripheral nerve in the dermis, joined to the taste bud. ND-GFP-expressing cells were seen around the peripheral sensory nerve (Fig. 1A,C). The ND-GFP-expressing fungiform papilla cells had round/oval-shaped bodies with a typical diameter of 7 μm and two-three elongated processes (Fig. 1D). The morphology of ND-GFP-expressing fungiform papilla cells was very similar to nestin-expressing hair follicle cells [Li et al., 2003; Liu et al., 2011; Uchugonova et al., 2011a,b] (Fig. 1E).

The ND-GFP-expressing fungiform papilla cells co-expressed the neural crest cell marker $p75^{NTR}$ (Fig. 1F) but were negative for the stem cell marker CD34 (Fig. 1G). ND-GFP-expressing fungiform papilla cells did not express β III tubulin. However, the fungiform papilla nestin-expressing cells were located around β III tubulin-expressing axons of the peripheral nerve (Fig. 1H). The glial cell markers GFAP and S100 were negative in the ND-GFP-expressing fungiform papilla cells (Fig. 1I). The ND-GFP-expressing hair follicle cells have a similar pattern of marker expression as the fungiform papilla ND-GFP cells [Amoh et al., 2005a] (Table I).

ND-GFP-EXPRESSING FUNGIFORM PAPILLA CELLS FORMED SPHERES IN CULTURE

The fungiform papillae containing ND-GFP-expressing cells were cultured in DMEM/F12 medium with bFGF. The ND-GFP-expressing cells gradually formed spheres. After 4–8 weeks, the ND-GFP-expressing spheres migrated (Fig. 2A). ND-GFP-expressing spheres co-expressed $p75^{NTR}$ and CD34, although they did not express

TABLE I. Markers Expressed by Nestin-Expressing Cells in the Fungiform Papilla and Hair Follicle

	Fungiform papilla	Hair follicle[a]
CD34	−	−
p75NTR	+	+
β III tubulin	−	−
GFAP	−	−
S100	−	+

[a]These data are from Amoh et al., 2005a.

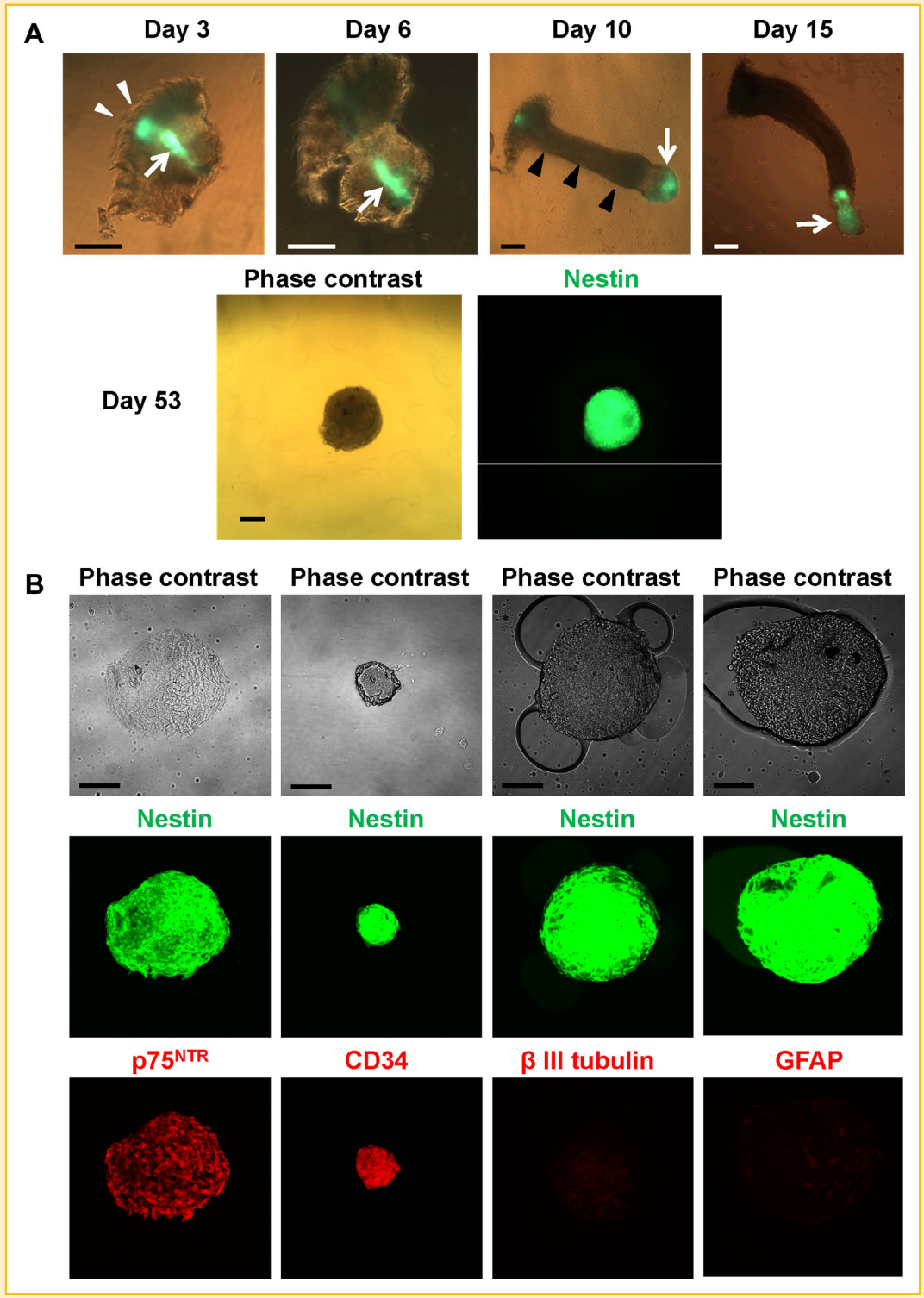

Fig. 2. ND-GFP-expressing fungiform papilla cells form spheres in culture. A: The ND-GFP-expressing cells were cultured from the fungiform papilla in DMEM/F12 medium containing bFGF. On day 3, ND-GFP-expressing cells (white arrow) were located below the taste bud (white arrowheads). By day 6, the ND-GFP-expressing cells proliferated. By day 10, the ND-GFP-expressing cells formed spheres (black arrowheads). After 4–8 weeks, more ND-GFP-expressing fungiform papilla cells formed spheres. Bars: 100 μm. B: The spheres expressing nestin co-express $p75^{NTR}$ and CD34 but did not express β III tubulin and GFAP. Bar: 100 μm.

TABLE II. Markers Expressed by Spheres of Nestin-Expressing Cells Derived from the Fungiform Papilla and Hair Follicle

	Fungiform papilla	Hair follicle[a]
CD34	+	+
p75NTR	+	+
β III tubulin	−	−
GFAP	−	−
S100	−	−

[a]These data are from Amoh et al., 2005a.

CD34 before culture. Nestin, $p75^{NTR}$, and CD34 expression indicates the cells within the spheres were in a relatively undifferentiated state. ND-GFP-expressing spheres did not express β III tubulin and GFAP (Fig. 2B). Thus, spheres formed from nestin-expressing fungiform papilla cells were similar to spheres formed from nestin-expressing hair follicle cells [Amoh et al., 2005a] (Table II).

MARKERS EXPRESSED BY NESTIN-EXPRESSING FUNGIFORM PAPILLA CELLS CULTURED IN FETAL BOVINE SERUM

The ND-GFP-expressing fungiform papilla spheres were switched to RPMI 1640 medium containing 10% FBS from DMEM/F12

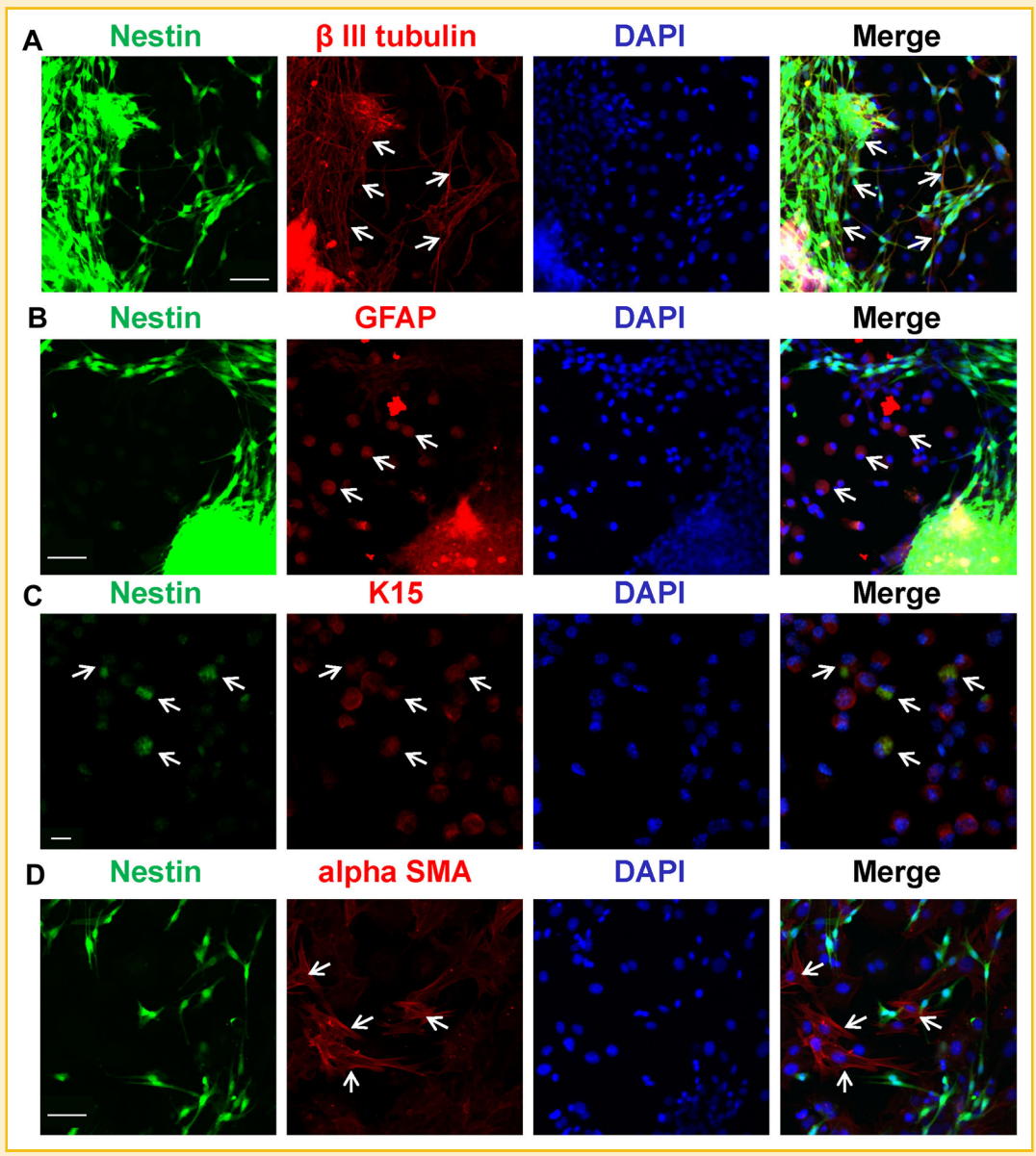

Fig. 3. Differentiation of nestin GFP-expressing fungiform papilla cells in vitro. A: The nestin GFP-expressing fungiform papilla spheres were switched to RPMI 1640 medium containing 10% FBS from DMEM/F12 containing B-27, N2, and bFGF. Seven days after medium switch, β III tubulin-positive spindle neuron cells, which maintained ND-GFP expression, were observed. White bar: 50 μm. B: At 7 days, the nestin-GFP expressing cells differentiated into GFAP-expressing glial cells. White bar: 50 μm. C: At 5 days of culture in RPMI 1640 medium with FBS, some nestin-GFP expressing cells differentiated into K15-expressing keratinocytes (white arrows). White bar: 10 μm. D: At 4 weeks after culture in medium with FBS, some nestin-GFP expressing cells also differentiated into α-SMA-expressing smooth-muscle cells. White bar: 50 μm.

TABLE III. Percent of Cells From Nestin-GFP-Expressing Fungiform Papillae (FP) Cells and Hair Follicles (HF) Expressing Various Markers

Cell type	% positive cells from FP, mean ± SEM[a]	% positive cells from HF, mean ± SEM[b]
β III tubulin-positive neurons	55.2 ± 4.3[c]	48 ± 8[c]
GFAP-positive glial cells	22.6 ± 5.1[c]	30 ± 8[c]
K15-positive keratinocytes	28.4 ± 2.7[d]	18 ± 5[f]
Alpha SMA-positive smooth-muscle cells	23.3 ± 3.1[e]	2 ± 2[e]

[a]In order to detect β III tubulin, GFAP, K15, and alpha SMA, we cultured a total of 16 spheres so that there were 4 spheres for each marker determination.
[b]Data in this column are from Amoh et al., 2005a.
[c]At 7 days after transfer to RPMI 1640 medium containing 10% FBS.
[d]At 5 days after transfer to RPMI 1640 medium containing 10% FBS.
[e]At 4 weeks after transfer to RPMI 1640 medium containing 10% FBS.
[f]At 2 weeks after transfer to RPMI 1640 medium containing 10% FBS.

containing B-27, N2, and bFGF. Under this condition, the cells within the spheres began to express new markers. Many cells expressed β III tubulin, a marker of neurons, while maintaining ND-GFP expression (Fig. 3A). ND-GFP-expressing fungiform papilla cells also expressed GFAP, a marker associated with glial cells (Fig. 3B). ND-GFP-expressing fungiform papilla cells also expressed K15, a marker associated with keratinocytes (Fig. 3C). ND-GFP-expressing fungiform papilla cells also expressed α-SMA, a marker associated with smooth-muscle cells (Fig. 3D). Approximately 55% of ND-GFP-expressing fungiform papilla expressed β III tubulin (Table III). These results suggest that the fungiform papilla cells of the tongue are multipotent stem cells similar to those in the hair follicle.

DISCUSSION

In this study, we characterized nestin-expressing cells in the fungiform papilla located around a peripheral sensory nerve, immediately below the taste bud. These cells co-expressed the neural-crest cell marker $p75^{NTR}$. The fungiform papilla nestin-expressing cells formed spheres in suspension culture in DMEM-F12 medium with bFGF. The spheres consisted of nestin-expressing cells that still co-expressed $p75^{NTR}$ and developed the expression of the stem cell marker CD34 while in culture. Nestin, $p75^{NTR}$ and CD34 expression indicated the cells in the fungiform papilla spheres were in a relatively undifferentiated state. After transfer to fetal bovine serum-containing medium, the nestin-expressing fungiform papilla cells of the spheres could express β III tubulin, GFAP, and K15, which are associated with neurons, glial cells, and keratinocytes, respectively, and as well as SMA, associated with smooth muscles, suggesting they differentiate into multiple stem cells and are therefore multipotent.

The characteristics of nestin-expressing fungiform papilla cells described above are remarkably similar to nestin-expressing hair follicle cells which we had already reported [Amoh et al., 2005a,b, 2008; Li et al., 2003; Liu et al., 2011; Uchugonova et al., 2011a; Duong et al., 2012; Mii et al., 2013]. One difference noted was greater expression of SMA in the differntiating fungiform papilla nestin-exprssing cells than in corresponding hair follicle nestin-expressing cells [Amoh et al., 2005a]. This result may be due to the different functions and locations of the two organs. The nestin-expressing hair follicle cells are located near the terminals of sensory peripheral nerves in the follicle bulge area. They also have round/oval-shaped bodies with a typical diameter of 7 μm and two-three elongated processes as do the nestin-expressing fungiform papilla cells [Li et al., 2003; Liu et al., 2011; Uchugonova et al., 2011b; Duong et al., 2012; Mii et al., 2013]. Thus the morphology of the nestin-expressing cells of the two organs is very similar. The nestin-expressing hair follicle cells also can form spheres in suspension culture, where they co-express CD34 and $p75^{NTR}$ [Amoh et al., 2005a; Mii et al., 2013] as do the nestin-expressing fungiform papilla cells. The nestin-expressing hair follicle cells also can express markers associated with different cell lineages and thus differentiate into multiple cell types [Amoh et al., 2005a].

Olfactory ensheathing cells (OECs) express $p75^{NTR}$ and nestin [Pastrana et al., 2007]. Adult retina contains neuronal progenitors which express nestin and $p75^{NTR}$ [Ahmad et al., 2000; Walcott and Provis, 2003; Harada et al., 2006]. What the fungiform papilla, hair follicle, OECs, and retina have in common, is that they are all located at a peripheral sensory nerve terminal. The presence of nestin-expressing cells at nerve endings suggests that they may supply cells for nerve growth or regeneration after nerve damage and become an appropriate growth target of the axons extending from the proximal side of the injured nerve.

Circumvallate papilla also contain taste buds [Abdoul-Azize et al., 2013]. Future studies will determine if circumvallate papilla also contain nestin-expressing cells and what are their properties.

Taste-sensory nerve damage occurs as a result of various diseases and therapies including radiotherapy and chemotherapy. The loss of taste decreases the patient's quality of life [Miura and Barlow, 2010]. The fungiform papilla should be studied for its potential for regeneration of taste. For example, future studies may be performed to determine if cultured fungiform papilla nestin-expressing cells can be used for taste regeneration in chemotherapy patients and other patients who have lost taste sensation.

DEDICATION

This paper is dedicated to the memory of A.R. Moossa, M.D.

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