differentiation [28]. This phenomenon might be related to the neuronal differentiation in response to the intracellular delivery of TAT-VHL peptide. The epidermis is an easily accessible source. Recently, truly pluripotent SSEA-3-positive MUSE cells were isolated from a skin fibroblast population by use of a cell sorter [36]. Although the population of somatic stem cells derived from the epidermis might contain MUSE cells, it may not be necessary to re-isolate MUSE cells from among such somatic stem cells since those stem cells themselves can differentiate into cells of the epithelial lineage in various organs. The neuronal differentiation method using TAT-VHL peptide is very simple because only the TAT-VHL peptide needs to be added to basic medium lacking serum, and it is also rapid compared with previously reported methods using various neurotrophic factors and other agents. Thus, we recommend the use of TAT-VHL for the neuronal differentiation of multipotent somatic stem cells.

4. Experimental Section

4.1. Cell Culture

Samples of human skin were sterilized with 70% ethanol for 1 min. Then, the skin was treated with dispase (concentration 1000 PU/mL) for 24 h at room temperature. Next, the epidermis was peeled off from the dermis, cut into small fragments with scissors, and digested with 0.1% trypsin for 30 min at 37 °C. Thereafter, the cells were filtered through a cell strainer (40-μm-diameter holes) and subsequently cultured in medium containing epidermal growth factor (20 ng/mL), basic fibroblast growth factor (40 ng/mL), and 2% B27 supplement (Gbico-BRL, Grand Island, NY, USA) in DMEM/F12 (1:1; Gibco-BRL) in a 5% CO_2 incubator. The cells formed numerous spheres at 2–3 weeks after the start of the primary culture. Once the primary culture was 3–4 weeks old, the spheres were dissociated by pipetting; and the cells were subcultured every two weeks. For experiments, cells at the first or second subculture were used.

4.2. Identification of Niche of Multipotent Nestin-Expressing Stem Cells Derived from Human Epidermis

For identification of the niche of pluripotent sphere-forming stem cells derived from the epidermis, a part of the cut skin, which had been fixed with Mildform (Wako, Tokyo, Japan), was embedded in paraffin, sectioned at a 10-μm thickness, and immunostained with anti-nestin antibody (BD Bioscience PharMingen, San Diego, CA, USA), anti-fibronectin antibody (Sigma, St. Louis, MO, USA), anti-CD34 antibody (Sigma) and anti-keratin 15 antibody (Acris Antibodies GmbH, Herford, Germany). As secondary antibodies, FITC-conjugated anti-mouse IgG monoclonal antibody (Sigma) and TRIC-conjugated anti-rabbit IgG polyclonal antibody were used. Finally, the nuclei were stained with DAPI (Molecular Probes, Eugene, OR, USA). Observation was performed by using a confocal fluorescence microscope (FV300, Olympus, Tokyo, Japan).

4.3. Immunocytochemical Characterization of Multipotent Stem Cells

After dissociation of the sphere-forming cells, characterization of the stem cells was performed immunocytochemically by using anti-nestin antibody, rabbit anti-fibronectin antibody (1:200; Sigma), anti-CD34 antibody, anti-NGFR p75 (Sigma), and anti-kerain 15 antibody (Acris Antibodies GmbH).

For examination of stem cell differentiation into certain cell types, ant-glial fibrillary acidic protein (GFAP) antibody (1:300; Dako, Glostrup, Denmark), anti-smooth muscle actin (SMA) antibody (1:200; Sigma), and anti-microtubule-associated protein (MAP)-2 (1:300; Sigma) were used. Immunoreactive cells were visualized by using either FITC-conjugated goat anti-mouse IgG (1:200; Sigma) or rhodamine-conjugated goat anti-rabbit IgG (1:150; Sigma) as secondary antibodies. To assess the frequency of different cell types in a given culture, we counted the number of cells immunopositive with a given antibody in 10 to 15 random non-overlapping visual fields (50–200 cells per field) in each experiment. At least three experiments were performed per condition. The degree of positivity was expressed as the ratio of immuno-positive cells to the total number of nuclei stained with DAP1.

4.4. Sphere-Forming Assay

The sphere formation assay was performed as follows [28]: Sphere-forming stem cells were dissociated into single cells by pipetting them continuously for 10 min. Then, after confirmation of their single-cell status and dilution up to 5 cells/mL, 200 μL of the cell suspension was placed into each well of a 96-well plate (mean of 1 cell per well). Then, spheres ≥50 μm in diameter in 1 plate were counted under observation by phase-contrast microscopy at 3 weeks after placement of the cells.

4.5. Induction of Neuronal Differentiation with TAT-VHL Peptide

By using the Fmoc (9-fluorenylmethyloxycarbonyl)-solid-phase method described previously [24], we chemically synthesized a peptide corresponding to the 157–171 amino-acid sequence of pVHL. The synthesized peptide was linked with the protein transduction domain (PTD) of the HIV-TAT protein (TAT-VHL peptide), thereby facilitating peptide entry into the cells. A control peptide composed of only the PTD of the TAT protein (TAT-peptide) was also synthesized. The sequences of these synthesized peptides were NH_2-YGRKKRRQRRRDTLKERCLQVVRSLVK-COOH for the TAT-VHL peptide and NH_2-YGRKKRRQRRRD-COOH for the TAT one. The isolated stem cells were incubated in DMEM/F12, and 1 μM TAT-VHL peptide or TAT peptide was delivered into the dissociated stem cells for neuronal differentiation. At first, an *in vitro* immunocytochemical study was performed. Three days after intracellular delivery of the TAT-VHL peptide in DMEM/F12 medium without growth or neurotrophic factors, the cells were observed under the phase-contrast microscope, and an immunocytochemical study was performed by using the following antibodies: anti-MAP2 antibody (1:200; Sigma), anti-tyrosine hydroxylase antibody (TH; 1:200; Merk Millipore, Billerica, MA, USA), and anti-neurofilament 200 antibody (1:200; Sigma). Immunoreactive cells were visualized by using either FITC-conjugated goat anti-mouse IgG (1:200; Sigma) or rhodamine-conjugated goat anti-rabbit IgG (1:150; Sigma) as secondary antibodies. In addition, DAPI (Molecular Probes) was used for counterstaining nuclei.

4.6. Implantation of Multipotent Stem Cells Derived from Epidermis

One hour after the TAT-VHL peptide had been transferred into the stem cells, the cells were stained with red-fluorescent PKH26PCL (Sigma), and implanted into the brains of eight-week-old Wistar rats (Charles River, Yokohama, Japan). Three weeks after the implantation, the rats were anesthetized with

Nembutal (200 mg/kg body weight) and perfused with periodate-lysine-paraformaldehyde solution. Their brains were subsequently dissected and postfixed in the same fixative for 2 h, cryopreserved in 30% sucrose for 12 h, and then embedded in Tissue Tek OCT compound (Sakura, Tokyo, Japan). Cryostat coronal sections of 14-μm thickness were prepared and used for immunohistochemistry. For immunostaining, sections were incubated with primary antibody, *i.e.*, anti-NeuN antibody (1:200; Merk Millipore, Billerica, MA, USA) or anti-Tuj-1 antibody (1:200; R&D Systems, Minneapolis, MN, USA) for 1 h at room temperature. Immunoreactive cells were visualized by using either FITC-conjugated goat anti-mouse IgG (1:200; Sigma) or rhodamine-conjugated goat anti-rabbit IgG (1:150; Sigma) as secondary antibodies. In addition, DAPI was used for counterstaining nuclei.

4.7. Statistics

Results were expressed as the mean ± standard deviation. For comparisons between values for groups, the Scheff test after the ANOVA test was used, with probabilities of less than 0.05 being considered significant (Statcel version 5.0/7.0, California, CA, USA).

4.8. Ethical Approval

This study was approved by the Ethics Committee of Yokohama City University in 2009, and the informed consent for this study was obtained from all of the patients.

5. Conclusions

In conclusion, we showed the isolation of multipotent nestin-expressing stem cells from the epidermis of facial skin of elderly humans and characterized these cells as being capable of sphere formation and strong expression of nestin, fibronectin, and CD34 but not of keratin 15. In addition, we identified the niche of these stem cells as being the outer root sheath of hair follicles and showed neuronal differentiation of these cells both *in vitro* and *in vivo* when VHL-peptide had been delivered into them. These multipotent stem cells derived from human epidermis are easily accessible and should be useful as donor cells for neuronal regenerative cell therapy.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

References

1. Abbas, O.; Mahalingam, M. Epidermal stem cells: Practical perspectives and potential uses. *Br. J. Dermatol.* **2009**, *161*, 228–236.

2. Yu, H.; Fang, D. Isolation of a novel population of multipotent adult stem cells from human follicles. *Am. J. Pathol.* **2006**, *168*, 1879–1888.
3. Amoh, Y.; Mii, S.; Aki, R.; Hamada, Y.; Kawahara, K.; Hoffman, R.M.; Katsuoka, K. Multipotent nestin-expressing stem cells capable of forming neurons are located in the upper, middle and lower part of the vibrissa hair follicle. *Cell Cycle* **2012**, *11*, 3513–3517.
4. Amoh, Y.; Li, L.; Katsuoka, K.; Hoffman, R.M. Multipotent nestin-expressing hair follicle stem cells. *J. Dermatol.* **2009**, *36*, 1–9.
5. Sieber-Blum, M.; Grim, M.; Hu, Y.F.; Szeder, V. Pluripotent neural crest stem cells in the adult hair follicle. *Dev. Dyn.* **2004**, *231*, 258–269.
6. Hill, R.P.; Gledhill, K.; Gardner, A.; Higgins, C.A.; Crawford, H.; Lawrence, C.; Hutchison, C.J.; Owens, W.A.; Kara, B.; James, S.E.; *et al.* Generation and characterization of multipotent stem cells from established dermal cultures. *PLoS One* **2012**, *7*, e50742.
7. Toma, J.G.; McKenzie, I.A.; Bagli, D.; Miller, F.D. Isolation and characterization of multipotent skin-derived precursors from human skin. *Stem Cells* **2005**, *23*, 727–737.
8. Liu, F.; Uchugonova, A.; Kimura, H.; Zhang, C.; Zhao, M.; Zhang, L.; Koenig, K.; Duong, J.; Aki, R.; Saito, N.; *et al.* The bulge area is the major hair follicle source of nestin-expressing pluripotent stem cells which can repair the spinal cord compared to the dermal papilla. *Cell Cycle* **2011**, *10*, 830–839.
9. Uchugonova, A.; Duong, J.; Zhang, N.; König, K.; Hoffman, R.M. The bulge area is the origin of nestin-expressing pluripotent stem cells of the hair follicle. *J. Cell. Biochem.* **2011**, *112*, 2046–2050.
10. Amoh, Y.; Li, L.; Yang, M.; Moossa, A.R.; Katsuoka, K.; Penman, S.; Hoffman, R.M. Nascent blood vessels in the skin arise from nestin-expressing hair-follicle cells. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 13291–13295.
11. Uchugonova, A.; Hoffman, R.M.; Weinige, M.; Koenig, K. Watching stem cells in the skin of living mice noninvasively. *Cell Cycle* **2011**, *10*, 2017–2020.
12. Duong, J.; Mii, S.; Uchugonova, A.; Liu, F.; Moossa, A.R.; Hoffman, R.M. Real-time confocal imaging of trafficking of nestin-expressing multipotent stem cells in mouse whiskers in long-term 3-D histoculture. *In Vitro Cell. Dev. Biol. Anim.* **2012**, *48*, 301–305.
13. Amoh, Y.; Kanoh, M.; Niiyama, S.; Kawahara, K.; Sato, Y.; Katsuoka, K.; Hoffman, R.M. Human and mouse hair follicles contain both multipotent and monopotent stem cells. *Cell Cycle* **2009**, *8*, 176–177.
14. Brownell, I.; Guevara, E.; Bai, C.B.; Loomis, C.A.; Joyner, A.L. Nerve-derived sonic hedgehog defines a niche for hair follicle stem cells capable of becoming epidermal stem cells. *Cell Stem Cell* **2011**, *8*, 552–565.
15. Biernaskie, J.; Paris, M.; Morozova, O.; Fagan, B.M.; Marra, M.; Pevny, L.; Miller, F.D. SKPs derive from hair follicle precursors and exhibit properties of adult dermal stem cells. *Cell Stem Cell* **2009**, *5*, 610–623.
16. Amoh, Y.; Li, L.; Katsuoka, K.; Penman, S.P.; Hoffman, R.M. Multipotent nestin-positive, keratin-negative hair-follicle bulge stem cells can form neurons. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 5530–5534.

17. Kloepper, J.E.; Tiede, S.; Brinckmann, J.; Reinhardt, D.P.; Meyer, W.; Faessler, R.; Paus, R. Immunophenotyping of the human bulge region: The quest to define useful *in situ* markers for human epithelial hair follicle stem cells and their niche. *Exp. Dermatol.* **2008**, *17*, 592–609.
18. Tiede, S.; Koop, N.; Kloepper, J.E.; Fässler, R.; Paus, R. Nonviral *in situ* green fluorescent protein labeling and culture of primary, adult human hair follicle epithelial progenitor cells. *Stem Cells* **2009**, *27*, 2793–2803.
19. Garza, L.A.; Yang, C.C.; Zhao, T.; Blatt, H.B.; Lee, M.; He, H.; Stanton, D.C.; Carrasco, L.; Spiegel, J.H.; Tobias, J.W.; *et al.* Bald scalp in men with androgenetic alopecia retains hair follicle stem cells but lacks CD200-rich and CD34-positive hair follicle progenitor cells. *J. Clin. Invest.* **2011**, *121*, 613–622.
20. Amoh, Y.; Li, L.; Campillo, R.; Kawahara, K.; Katsuoka, K.; Penman, S.; Hoffman, R.M. Implanted hair follicle stem cells form Schwann cells that support repair of severed peripheral nerves. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 17734–17738.
21. Amoh, Y.; Li, L.; Katsuoka, K.; Hoffman, R.M. Multipotent hair follicle stem cells promote repair of spinal cord injury and recovery of walking function. *Cell Cycle* **2008**, *7*, 1865–1869.
22. Amoh, Y.; Kanoh, M.; Niiyama, S.; Hamada, Y.; Kawahara, K.; Sato, Y.; Hoffman, R.M.; Katsuoka, K. Human hair follicle pluripotent stem (hfPS) cells promote regeneration of peripheral-nerve injury: An advantageous alternative to ES and iPS cells. *J. Cell. Biochem.* **2009**, *107*, 1016–1020.
23. Kanno, H.; Saljooque, F.; Yamamoto, I.; Hattori, S.; Yao, M.; Shuin, T.; U, H.-S. Role of the von Hippel-Lindau tumor suppressor protein during neuronal differentiation. *Cancer Res.* **2000**, *60*, 2820–2824.
24. Kanno, H.; Nakano, S.; Kubo, A.; Mimura, T.; Tajima, N.; Sugimoto, N. Neuronal differentiation of neural progenitor cells by intracellular delivery of synthetic oligopeptide derived from von Hippel-Lindau protein. *Protein Pept. Lett.* **2009**, *16*, 1291–1296.
25. Kubo, A.; Yoshida, T.; Kobayashi, N.; Yokoyama, T.; Mimura, T.; Nishiguchi, T.; Higashida, T.; Yamamoto, I.; Kanno, H. Efficient generation of dopamine neuron-like cells from skin-derived precursors with a synthetic peptide derived from von Hippel-Lindau protein. *Stem Cells Dev.* **2009**, *18*, 1523–1532.
26. Maeda, K.; Kanno, H.; Yamazaki, Y.; Kubo, A.; Sato, F.; Yamaguchi, Y.; Saito, T. Transplantation of von Hippel-Lindau peptide delivered neural stem cells promotes recovery in the injured rat spinal cord. *Neuroreport* **2009**, *20*, 1559–1563.
27. Yamazaki, Y.; Kanno, H.; Maeda, K.; Yoshida, T.; Kobayashi, N.; Kubo, A.; Yamaguchi, Y.; Saito, T. Engrafted VHL peptide-delivered bone marrow stromal cells promote spinal cord repair in rats. *Neuroreport* **2010**, *21*, 287–292.
28. Kanno, H.; Sato, H.; Yokoyama, T.; Yoshizumi, T.; Yamada, S. The VHL tumor suppressor protein regulates tumorigenicity of U87-derived glioma stem-like cells by inhibiting the JAK/STAT signaling pathway. *Int. J. Oncol.* **2013**, *42*, 881–886.
29. Wong, C.E.; Paratore, C.; Dours-Zimmermann, M.T.; Rochat, A.; Pietri, T.; Suter, U.; Zimmermann, D.R.; Dufour, S.; Thiery, J.P.; Meijer, D.; *et al.* Neural crest-derived cells with stem cell features can be traced back to multiple lineages in the adult skin. *J. Cell. Biol.* **2006**, *175*, 1005–1015.

30. Hoffman, R.M. The potential of nestin-expressing hair follicle stem cells in regenerative medicine. *Expert Opin. Biol. Ther.* **2007**, *7*, 289–291.
31. Nishio, Y.; Koda, M.; Kamada, T.; Someya, Y.; Yoshinaga, K.; Okada, S.; Harada, H.; Okawa, A.; Moriya, H.; Yamazaki, M. The use of hemopoietic stem cells derived from human umbilical cord blood to promote restoration of spinal cord tissue and recovery of hindlimb function in adult rats. *J. Neurosurg. Spine* **2006**, *5*, 424–433.
32. Barker, R.A. Winder H: Immune problems in central nervous system cell therapy. *J. Am. Soc. Exp. Neuro. Ther.* **2004**, *1*, 472–481.
33. Yamada, H.; Dezawa, M.; Shimazu, S.; Baba, M.; Sawada, H.; Kuroiwa, Y.; Yamamoto, I.; Kanno, H. Transfer of the von Hippel-Lindau tumor sup-pressor gene to neuronal progenitor cells in treatment for Parkinson's disease. *Ann. Neurol.* **2003**, *54*, 352–359.
34. Higashida, T.; Jitsuki, S.; Kubo, A.; Mitsushima, D.; Kamiya, Y.; Kanno, H. Skin-derived precursors differentiate into dopaminergic neuronal cells in the brains of Parkinson's disease model rats. *J. Neurosurg.* **2010**, *113*, 648–655.
35. Mahrour, N.; Redwine, W.B.; Florens, L.; Swanson, S.K.; Martin-Brown, S.; Bradford, W.D.; Staehling-Hampton, K.; Washburn, M.P.; Conaway, R.C.; Conaway, J.W. Characterization of Cullin-box sequences that direct recruitment of Cul2-Rbx1 and Cul5-Rbx2 modules to Elongin BC-based ubiquitin ligases. *J. Biol. Chem.* **2008**, *283*, 8005–8013.
36. Wakao, S.; Kitada, M.; Kuroda, Y.; Shigemoto, T.; Matsuse, D.; Akashi, H.; Tanimura, Y.; Tsuchiyama, K.; Kikuchi, T.; Goda, M.; *et al.* Multilineage-differentiating stress-enduring (Muse) cells are a primary source of induced pluripotent stem cells in human fibroblasts. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 9875–9880.

〔VI〕

平成25年度第1回班会議

プログラム

平成２５年度厚生労働科学研究費補助金（難治性疾患克服研究事業）

「フォン・ヒッペル・リンドウ病の診療指針に基づく診断治療体制確立の研究」

平成２５年度第１回班会議

日　時：　平成２５年７月２２日（月）　１３時００分～１５時００分
場　所：　キャンパス・イノベーションセンター東京　２階　「多目的室１」

1. 研究代表者　挨拶　高知大学医学部泌尿器科学教室　執印 太郎
2. 研究分担者の追加について
3. 昨年度の研究成果
4. 今後の難病研究と難病対策（制度改変について）
5. 本年の班研究の進め方
6. VHL iPS 細胞について（京都大学　中村英二郎先生）
7. 次回、症例検討会の日程
8. VHL 病症例検討（1 症例）

〔Ⅶ〕

平成 25 年度症例検討会

プログラム

平成２５年度厚生労働科学研究費補助金（難治性疾患克服研究事業）

「フォン・ヒッペル・リンドウ病の診療指針に基づく診断治療体制確立の研究」

平成２５年度第１回症例検討会

日　時：　平成２５年１１月１３日（水）　１８時００分～１９時００分

1. VHL 病症例検討（2 症例）　高知大学
   症例１　58 歳女性
   症例２　18 歳女性

2. 重症度分類　調査のお願い

3. 平成 26 年度厚生労働省科学研究費の公募について

4. 次回、症例検討会の日程調整

平成２５年度厚生労働科学研究費補助金（難治性疾患克服研究事業）「フォン・ヒッペル・リンドウ病の診療指針に基づく診断治療体制確立の研究」

平成２５年度第２回症例検討会

日　時：　平成２５年１２月１９日（木）　１７時００分～１８時００分

1. 平成 26 年度厚生科研の申請について

2. 医師主体の重症度分類調査について

3. 症例１　北海道大学　篠原先生
   症例１　27 歳女性

4. 症例２　横浜市立大学　矢尾先生
   症例２　28 歳男性

5. 次回、症例検討会の日程調整

平成２５年度厚生労働科学研究費補助金（難治性疾患克服研究事業）
「フォン・ヒッペル・リンドウ病の診療指針に基づく診断治療体制確立の研究」

平成２５年度第３回症例検討会

日　時：　平成２６年２月２７日（木）　１７時００分～１８時００分

1. **平成 25 年度報告書作成の依頼**

2. **症例１　高知大学**
　　症例１　38 歳男性

3. **連絡事項**