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Multipotent stem cells are effectively collected from adult human cheek skin

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ABSTRACT

Skin-derived precursor (SKP) cells are a valuable resource for tissue engineering and regenerative medicine, because they represent multipotent stem cells that differentiate into neural and mesodermal progenies. Previous studies suggest that the stem cell pool decreases with age. Here, we show that human multipotent SKP cells can be efficiently collected from adult cheek/chin skin, even in aged individuals of 70–78 years. SKP cells were isolated from 38 skin samples by serum-free sphere culture and examined for the ability to differentiate into neural and mesodermal lineages. The number of spheres obtained from adult facial skin was significantly higher than that of trunk or extremity skin. SKP cells derived from cheek/chin skin exhibited a high ability to differentiate into neural and mesodermal cells relative to those derived from eyelid, trunk, or extremity skin. Furthermore, cheek/chin skin SKP cells were shown to express markers for undifferentiated stem cells, including a high expression level of the Sox9 gene. These results indicate that cheek/chin skin is useful for the recovery of multipotent stem cells for tissue engineering and regenerative therapy.

1. Introduction

Multipotent progenitor cells derived from human adult tissues are an important resource for tissue engineering and regenerative medicine because of their accessibility and therapeutic potential [1,2]. Although pluripotent embryonic stem cells are a potential resource for regenerative medicine, use of these cells is highly restricted due to heterologous transplantation and ethical issues. Induced pluripotent stem (iPS) cells represent another potential resource; however, there are difficulties in the clinical application of iPS cells, because of potential carcinogenicity.

Skin-derived precursor (SKP) cells are multipotent stem cells isolated from the adult dermis [3] by using a method similar to that used to isolate stem cells from the central nervous system [4]. By incubating skin-derived cells in serum-free medium supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), spheres of floating cells are obtained [3]. These cells possess multipotency to differentiate into neuronal and mesoderm-derived cells [3,5,6]. SKP cells are considered to be useful for tissue regeneration therapy because they are obtained from the dermal tissues of the same patient without using expression vectors, inducers or drugs. These cells are isolated from human skin of various locations, including scalp [3,7], breast [8] and foreskin [6,9,10] tissues. The cellular potential to differentiate and the number of recovered stem cells vary significantly between different areas of the skin. However, recovery of insufficient numbers of SKP cells in the dermal tissue has presented a problem in the clinical use of these cells for tissue engineering.

In this report, we show that a large number of SKP cells are obtained from human cheek/chin skin, even in aged individuals (70–78 years of age), despite other reports suggest that the number of SKP cells decreases with age [10]. SKP cells were more effectively obtained from cheek/chin skin than from trunk or eyelid skin. The SKP cells obtained from cheek/chin skin differentiated into multiple types of neuronal and mesoderm-derived cells. These results indicate that the number of multipotent progenitor cells is significantly higher in the cheek/chin skin tissues, and that cheek/chin skin SKP cells may be useful for regenerative medicine.

2. Materials and methods

2.1. SKP sphere culture

Tissues used for this study were obtained from human skin of individuals undergoing plastic surgery. Samples were collected with patient written informed consent, with adherence to the Declaration of Helsinki protocols, and with the approval of the Ethical Committee of the Kyoto University Medical School. Culture of SKP cells was performed essentially according to the method of Toma et al. [3]. After fat tissues were removed, the dermal tissues were cut into small pieces and washed in PBS. Tissue fragments were

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digested with 1000 PU/ml dispase (Sanko Junyaku Co., Ltd., Tokyo, Japan) in D-MEM/F12 medium supplemented with 10,000 U/ml penicillin G, 10 mg/ml streptomycin sulfate, and 25 μg/ml of amphotericin B (Gibco, Grand Island, NY, USA) at 37 °C for 30 min. After the epidermis was manually removed from each tissue piece, the dermis was cut into 1 mm^3 pieces and digested with 0.5% collagenase-type I (Gibco) in D-MEM/F12 medium, with shaking at 37 °C for 1 h. Cells were recovered by centrifugation at 180*g* for 10 min, and washed twice with D-MEM/F12 medium. After passage through a 40 μm nylon mesh (BD Falcon, Bedford, MA, USA), cells were resuspended and cultured in D-MEM/F12 medium containing B-27 (Gibco), 20 ng/ml EGF (Sigma, St. Louis, MO, USA), and 40 ng/ml bFGF (Kaken Pharmaceutical Co., Ltd. Tokyo, Japan) in the presence of antibiotic and antimycotic reagents, under 5% CO_2-95% air conditions at 37 °C, using 6-well plates. Spheres were produced by plating cells with the serum-free medium in the presence or absence of 1.5% methylcellulose (Nacalai Tesque, Inc., Kyoto, Japan) at a density of 1000 cells/well, using 48-well plates, at 37 °C for 10 days.

2.2. Adherent culture of SKPs

Adherent culture of SKP cells was performed essentially by following the method of Joannides et al. [2]. After primary spheres were centrifuged at 180*g* for 5 min, the pellet was resuspended in D-MEM/F12 supplemented with 10% fetal bovine serum, B-27, 20 ng/ml EGF, and 40 ng/ml bFGF, and 30–50 spheres were cultured in 10 ml medium using 10-cm tissue culture dishes. After treatment with 0.05% trypsin for 5 min, cells were collected and seeded at a density of 1000 cells/cm^2. After propagation of SKP cells, spheres were produced again by transferring SKP cells into serum-free medium.

2.3. Neurogenic differentiation

For neurogenic differentiation of SKP cells, spheres were seeded on round glass coverslips coated with poly-L-lysine, in a 24-well culture plate, at a density of 4–6 spheres/well, and cultured for 1 week in neurogenic differentiation medium (NeuroCult NS-A Differentiation Medium, Stem Cell Technologies, Vancouver, BC, Canada).

2.4. Adipogenic differentiation

Adipogenic differentiation of SKP cells was stimulated by hMSC Differentiation BulletKit, Adipogenic (Lonza, VS, Switzerland) and hMSC Differentiation BulletKit, Adipogenic (Lonza). Cells were fixed with 4% paraformaldehyde in phosphate buffer, and stained with 0.3% oil red O solution (Eastman Kodak, Rochester, NY, USA) for 10 min at 37 °C.

2.5. Osteogenic differentiation

For osteogenic differentiation of SKP cells, spheres were cultured in osteogenic induction medium (hMSC Differentiation BulletKit, Osteogenic, Lonza) for 3 weeks. Alkaline phosphatase activity was detected in cells by staining with an Alkaline Phosphatase Staining kit (Muto Pure Chemicals, Tokyo, Japan).

2.6. Quantitative analysis of differentiation

Spheres (10 spheres/6 cm dish) were cultured in D-MEM/F12 medium containing 10% fetal bovine serum (Cambrex, Walkersville, MD, USA), and then differentiation of cells was stimulated as described above. To analyze adipogenic differentiation quantitatively, the amount of triglyceride in cell lysate was determined after cells were lysed in 0.1% polidocanol (Thesit) by using a serum triglyceride determination kit (Sigma–Aldrich, St. Louis, MO, USA). Protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). To analyze osteogenic differentiation quantitatively, alkaline phosphatase activity of cell lysate was determined by using a SensoLyte pNPP Alkaline Phosphatase Assay Kit (Ana Spec, San Jose, CA, USA).

2.7. Immunofluorescence staining of neural cells and spheres

Cells after neurogenic differentiation, or spheres seeded on glass coverslips coated with poly-L-lysine were washed, and fixed with 4% paraformaldehyde in phosphate buffer at 4 °C overnight or for 1 h at room temperature, respectively. Cells were permeabilized with 0.3% (0.5% for spheres) Triton X-100 in PBS for 5 min (10 min for sperehes), washed in PBS, and blocked with BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan) at 37 °C for 30 min. Cells were incubated with rabbit anti-human nestin antibody (1:400, Santa Cruz Biotechonology, California, USA) and mouse anti-neuronal class III β-tubulin antibody clone TUJ1 (1:1000, Stem Cell Technologies) at 4 °C overnight, and Spheres were incubated with NGF receptor p75 (1:500, Millipore, Billerica, MA, USA), vimentin (1:1000, Santa Cruz, Santa Cruz, CA, USA), versican (1:500, Seikagaku Corporation, Tokyo, Japan), or fibronectin (Ab-1, 1:200, Thermo, Fremont, CA, USA). Alternatively, cells were incubated without primary antibodies as a control. After PBS washes, cells were incubated with AlexaFluor 488-conjugated goat anti-mouse IgG or AlexaFluor 546-conjugated goat anti-rabbit IgG (Invitrogen). Nuclei were stained with Hoechst 33258.

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cultured SKP cells by using an RNeasy Mini Kit (Qiagen, Hilden, Germany). After digestion of genomic DNA with DNase I (Takara Bio, Otsu, Japan), RNA was recovered by ethanol precipitation. cDNA was synthesized by using an Advantage RT-for-PCR Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. Specific cDNAs were amplified by PCR using Blend Taq Plus DNA polymerase (Toyobo, Osaka, Japan). Primers used for amplification were as follows: Wnt-5a sense 5′-GGATGGCTGGAAGTGCAATG-3′ and antisense 5′-ACACAAACTGGTCCACGATC-3′; Sca-1 sense 5′-TGAAATCCAACCAAGAGCGG-3′ and antisense 5′-TGGCCAGCAGAGTGGAATAG-3′; Dermo-1 sense 5′-GCAAGAAGTCGAGCGAAGATG-3′ and antisense 5′-GGCAATGGCAGCATCATTCAG-3′; TWIST sense 5′-GATGATGCAGG ACGTGTCCAG-3′ and antisense 5′-TGCCATCTTGGAGTCCAGCTC-3′; Sox9 sense 5′-ATCTGAAGAAGGAGAGCGAG-3′ and antisense 5′-TCAGAAGTCTCCAGAGCTTG-3′; GAPDH sense 5′- TGGTATCGTGGAA GGACTCATGAC-3′ and antisense 5′- ATGCCAGTGAGCTTCCCGTTCAGC-3′. RT-PCR products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining.

2.9. Statistical analysis

Significance of difference between two groups was analyzed by using the Mann–Whitney U test.

3. Results

3.1. Spheres derived from adult cheek skin are multipotent precursor cells

SKP cells are a useful resource for regenerative medicine, and using a sphere culture system is an effective method for obtaining SKP cells. To analyze whether the potential to produce cultured

SKP cells differs between different areas of skin on the adult human body, spheres were produced from collected skin samples of the cheek/chin, eyelid, trunk and extremities by using serum-free methylcellulose culture (Fig. 1A and B, Supplementary Fig. S1A and B, Tables 1 and 2). Spheres derived by guided differentiation culture of adult cheek cells were found to stably maintain neurogenic, adipogenic and osteogenic differentiation potential, even those spheres obtained from a 77-year-old individual (Fig. 1C–D). Spheres cultured in neurogenic differentiation medium were clearly stained with antibodies against neuronal precursor cell makers: nestin and β III tubulin (Fig. 1C). Adipogenic or osteogenic differentiation was confirmed by oil red O (Fig. 1G) or alkaline phosphatase (Fig. 1F) staining, respectively. The multipotent characteristics of adult cheek skin-derived spheres were very similar to those of infant philtrum skin-derived spheres (Supplementary Fig. S2). Although spheres derived from adult trunk-derived skin were also able to differentiate into osteogenic and adipogenic lineages, the number of differentiated cells obtained from trunk skin-derived spheres were apparently smaller than those obtained from cheek skin-derived spheres (Supplementary Fig. S1C and D). In addition, fibroblasts obtained from adult cheek skin by using an ordinary fibroblast culture system were shown to differentiate poorly into osteogenic and adipogenic lineages, relative to that of SKP cells obtained by using a sphere

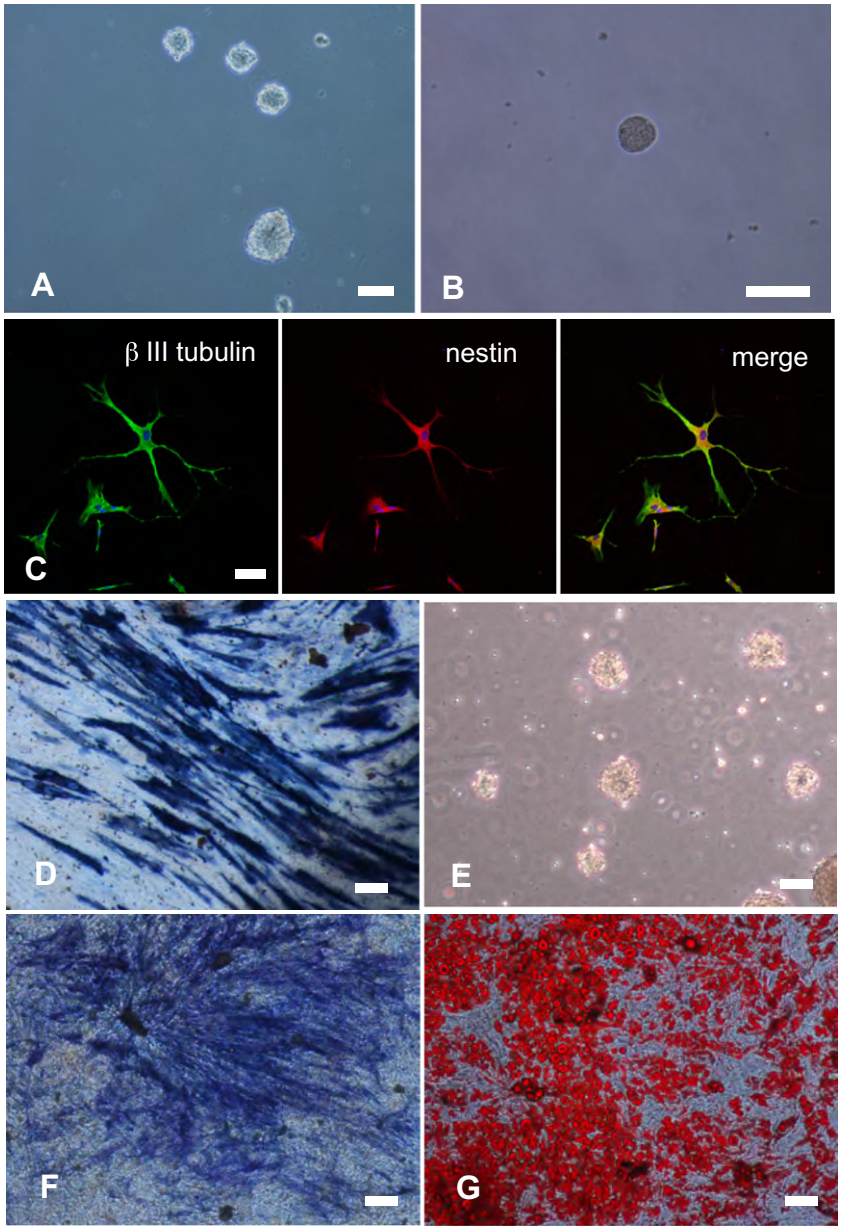

Fig. 1. Spheres derived from adult human cheek skin are composed of multipotent precursor cells. (A–D) Primary spheres were produced from cheek skin of a human adult individual (age of 77) by (A) serum-free or (B) methylcellulose culture. Bar = 100 μm. (C) Spheres shown in panel A were further cultured in neurogenic differentiation medium, and differentiated cells were analyzed by immunofluorescence staining for β III tubulin and nestin. Bar = 20 μm. (D) The same primary spheres were cultured in osteogenic induction medium, and differentiated cells were analyzed by alkaline phosphatase staining. (E–G) Secondary spheres were produced from cheek skin of a human adult individual (age of 70) after expansion of primary spheres by adherent culture method. (E) Secondary spheres were cultured in osteogenic induction medium and (F) differentiated cells were analyzed by alkaline phosphatase staining. Signals from alkaline phosphatase activity are shown in blue. (G) The same secondary spheres were cultured in adipogenic induction medium and differentiated cells were stained with oil red O. Bar = 100 μm for panels (D–G). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Number of spheres produced from various human skin samples after methylcellulose culture.

Sex	Age	Region	Number of spheres
M	0	Cheek	71
M	0	Philtrum	74
M	0	Philtrum	74
M	0	Philtrum	43
M	0	Philtrum	41
F	0	Philtrum	70
M	54	Chin	62
F	77	Cheek	56
F	33	Cheek	44
M	34	Cheek	71
M	70	Cheek	91
M	78	Cheek	162
F	70	Cheek	55
M	80	Upper eyelid	53
F	71	Upper eyelid	59
F	60	Upper eyelid	49
M	64	Chest	12
F	58	Chest	8
M	36	Abdomen	8
M	33	Abdomen	18
F	71	Buttock	6
F	25	Shoulder	19
M	33	Arm	16
M	83	Thigh	44
M	54	Thigh	34
F	29	Thigh	12

Table 2
List of skin samples used in osteogenic and adipogenic differentiation and quantitative analysis.

Sex	Age	Region
M	35	Chin
M	70	Cheek
M	78	Cheek
F	50	Cheek
M	36	Abdomen
M	35	Abdomen
M	34	Abdomen
F	74	Abdomen
F	71	Upper eyelid
M	74	Upper eyelid
F	60	Upper eyelid
M	60	Upper eyelid

culture system (Supplementary Fig. S3). These observations suggest that multipotent SKP cells are effectively collected by sphere culture of adult cheek skin.

3.2. SKP cells are more effectively collected from adult facial skin than other adult skin

To determine whether cheek/chin skin offers an advantage in producing multipotent SKP cells, we compared the number of primary spheres obtained after culturing the same number of skin-derived cells collected from various areas: cheek/chin, eyelid, trunk, and extremities (Fig. 2). The number of spheres produced was significantly higher for cheek/chin skin as compared to that of extremity or trunk skin (Fig. 2A). Eyelid skin also exhibited a high potential to produce primary spheres relative to that of extremity or trunk skin. Comparison between adult and infant facial skin indicated that adult cheek/chin skin has an ability to produce primary spheres comparable to that of infant facial (philtrum and cheek) skin (Fig. 2B). These observations indicated that facial (cheek/chin and eyelid) skin has a high potential to produce SKP cells at a statistically significant level when compared to that of other (extremity and trunk) skin.

3.3. Adult cheek/chin skin-derived spheres maintain significantly high potency to differentiate into adipogenic and osteogenic lineages

To examine whether cheek/chin and eyelid skin-derived spheres maintain high potency to differentiate, as compared to trunk-skin derived spheres, secondary spheres produced after expansion of primary spheres were further cultured in media specialized for osteogenic or adipogenic differentiation (Fig. 2C and D). Differentiation culture in osteogenic induction medium indicated that cells differentiated from cheek/chin and trunk skin-derived spheres contain a higher amount of triglyceride, a marker for adipogenic differentiation, than that of eyelid skin-derived spheres (Fig. 2C). Analysis of the potential to differentiate into an osteogenic lineage was determined by using alkaline phosphatase activity, which indicated that cheek/chin skin-derived spheres maintain a higher potential to differentiate into osteogenic cells than that of spheres derived from eyelid or trunk skin (Fig. 2D). These results clearly indicate that cheek/chin-derived spheres maintain multipotency to differentiate, whereas eyelid skin-derived or trunk skin-derived spheres are less efficient to differentiate into multiple types of cells.

3.4. Adult cheek/chin skin-derived spheres maintain expression of undifferentiated cell makers

Of the skin locations examined, cheek/chin skin was the most effective in producing multipotent SKP cells. To examine whether cheek/chin skin-derived SKP cells differ from other SKP cells in protein expression patterns, five SKP-specific marker proteins including nestin, fibronectin, NGF receptor p75, vimentin, and versican [9–11] were examined by using immunofluorescence staining of spheres (Fig. 3). SKP cells from cheek/chin, eyelid and trunk skin were all stained with antibodies against the five SKP markers, and no significant difference in staining patterns was observed. These results suggest that SKP cells obtained from different skin locations maintain the basic expression of SKP markers, even though they differ in the potency to differentiate.

To analyze whether SKP cells obtained from cheek/chin skin differ from other undifferentiated SKP cells in gene expression patterns, semiquantitative PCR was used to compare mRNA expression levels of five undifferentiated SKP markers: Wnt-5a, Twist, Sox9, Sca-1, and Dermo-1 [3,10,11]. All five markers were expressed in each of the spheres examined, including those obtained from adult cheek/chin, trunk, and eyelids (Fig. 4). Intriguingly, significantly higher expression level of Sox9 was detected in cheek/chin-derived spheres than in trunk-derived or eyelid-derived spheres. Sox9 is known as an undifferentiated stem cell marker for neural crest progenitor cells [12]. These observations suggest that SKP cells derived from cheek/chin skin are undifferentiated to a greater degree than SKP cells obtained from trunk and eyelid skin.

4. Discussion

Human SKP cells are multipotent precursor cells, and are a useful resource for regenerative therapy and transplantation medicine. Execution of these therapies requires a substantial amount of multipotent SKP cells. Previous studies demonstrate that multipotent stem cells can be isolated from human scalp [3,7] and foreskin [6,9,10]. In the study presented here, SKP cells were isolated more efficiently from adult cheek/chin and eyelid skin than from trunk or extremity skin (Figs. 1 and 2A, B). SKP cells from cheek/chin skin demonstrated a greater ability to differentiate into neural and mesodermal lineages than SKP cells derived from eyelid, trunk or extremity skin (Figs. 1, 2C, D, and Supplementary Fig. S1).

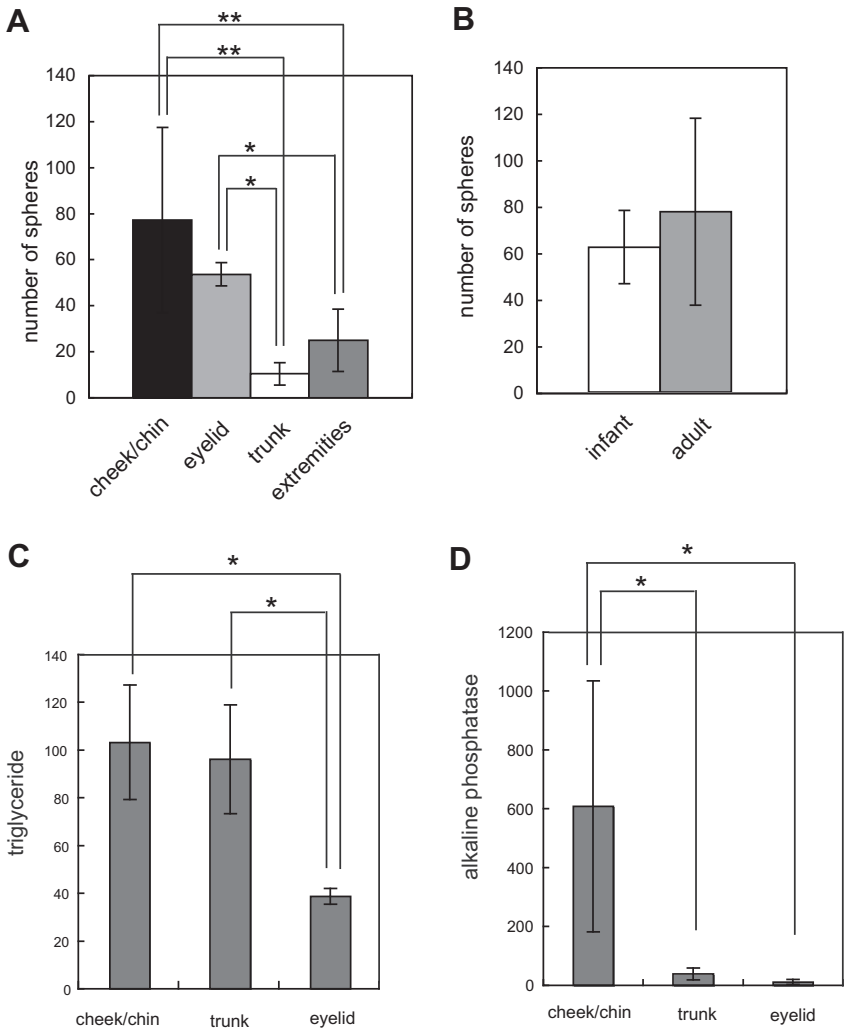

Fig. 2. (A and B) Potential to produce primary spheres is high in cheek/chin skin, regardless of age. (A) The number of spheres produced from cheek/chin or eyelid skin was significantly higher than that of spheres produced from trunk or extremities skin. Adult skin cells were obtained from cheek/chin (ages 33–78, $n = 7$), eyelid (ages 60–80, $n = 3$), trunk (ages 33–71, $n = 5$) or extremity (ages 25–83, $n = 5$) skin samples (Table 1). Skin-derived cells (48,000 cells) were seeded onto methylcellulose. After culture for 10 days, numbers of spheres were counted (mean ± SD). $^{**}p < 0.01$; $^{*}p < 0.05$. (B) Number of spheres produced from face skin shows no significant difference between adult (ages 33–78, cheek and chin, mean ± SD; $n = 7$) and infant (age 0, philtrum and cheek, mean ± SD; $n = 6$). (C, D) Spheres produced from cheek/chin skin maintain a high multipotency. Secondary spheres produced from adherent cell culture of primary spheres were differentiated by culturing in specific differentiation medium. (C) Amount of triglyceride was determined for secondary spheres derived from cheek/chin, trunk and eyelid skin after differentiation culture, to evaluate adipogenic differentiation potential of spheres (mean ± SD; $n = 4$). (D) Amount of alkaline phosphatase was determined for secondary spheres after differentiation culture, to estimate osteogenic differentiation potential of spheres (mean ± SD; $n = 4$). $^{*}p < 0.05$.

Previous studies suggest that the stem cell pool decreases with age [10,11]. Here, however, multipotent SKP cells were effectively isolated from cheek skin even in aged individuals (ages 70–78, Tables 1 and 2). These results indicate that cheek/chin skin is useful for recovering multipotent SKP cells as a potential source for regenerative medicine.

SKP cells from cheek/chin skin showed a great potential to differentiate relative to SKPs prepared from other skin. During development, the facial dermis is differentiated from neural crest cells [13], whereas the dorsal and ventral trunk dermis originate from somite and lateral plate mesoderm, respectively [14,15]. Neural crest-derived dermal papilla and whisker follicles are a niche for multipotent SKP cells [7,11,16,17]. Notably, SKP cells obtained from cheek/chin skin express a significantly higher level of sox9, an undifferentiated stem cell marker, than that of SKP cells obtained from eyelid or trunk skin (Fig. 4). Sox9 is a transcription factor categorized in the Sry-related high-mobility-group box family [18], and plays an important role in cell survival and epithelial-mesenchymal transition (EMT) in the neural crest [12,19]. Neural crest cells migrate to various parts of the body after EMT and differentiate into diverse types of tissues including neurons, bones and connective tissues [20]. SKP cell characteristics are similar to multipotent neural crest stem cells [11]. The increased expression of Sox9 in cheek/chin skin-derived SKP cells observed here may mimic the microenvironment of neural crest cells immediately before or during EMT. Consistent with the same notion, a relatively high expression level of Twist, a transcription factor that induces EMT to produce a stem cell state [21], was also detected. These observations indicate that SKP cells obtained from cheek/chin skin are similar to neural crest multipotent stem cells just prior to or during EMT.

Neural crest-like stem cells are found in hair follicles, particularly in dermal papilla [11,17]. The cheek/chin skin-derived SKP cells reported in the present study likely originated from hair follicles, because skin of the human face contains a large number of fine soft hairs, and the density of hair follicles is higher on the face

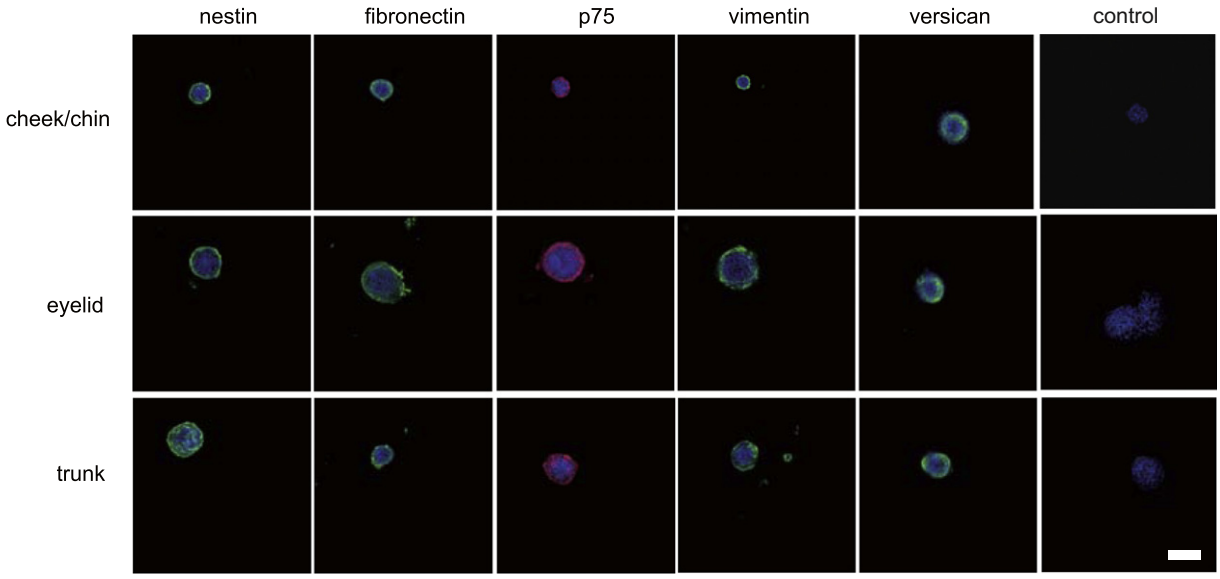

Fig. 3. Spheres obtained from human skin express SKP marker proteins. Spheres derived from cheek/chin, trunk and eyelid skin were analyzed by immunofluorescence staining. Fixed samples were incubated with antibodies against nestin, fibronectin, NGF receptor p75, vimentin, and versican, followed by incubation with AlexaFluor 488-conjugated goat anti-mouse IgG (for nestin, fibronectin, NGF, vimentin, and versican) or AlexaFluor 546-conjugated goat anti-rabbit IgG (for p75). Bar = 100 μm.

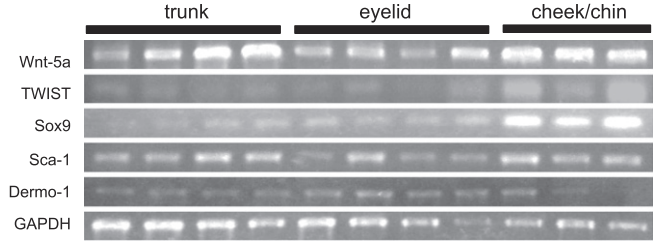

Fig. 4. Undifferentiated cell maker genes are expressed in skin-derived spheres and cheek/chin skin-derived spheres express a high level of Sox9. Expression of SKP markers, including Wnt-5a, Twist, Sox9, Sca-1, and Dermo-1 genes, in spheres derived from cheek/chin, trunk and eyelid skin was analyzed by RT-PCR, followed by agarose gel electrophoresis.

than on other parts of the body (e.g., trunk and extremities) [22,23]. SKP cells produced from eyelid had a lesser potential to differentiate than that of those produced from cheek/chin skin. Since eyelid skin is very thin and distinctive from other areas of skin [24], SKP cells obtained from eyelid and cheek/chin may originate from different cells, at least in part. Sox9 is known to be expressed in the outer root sheath or bulge of hair follicles, and is essential for the formation of the hair stem cell compartment [25]. Similarly, Sox9 is required for the maintenance of mammary stem cells [26]. In addition to dermal papilla, outer root sheath or bulge in hair follicles has been reported as a source of multipotent cells that are able to differentiate into neuronal cells [27–29]. Thus, the spheres produced from cheek/chin skin may contain cells derived from outer root sheath or bulge, in addition to cells derived from dermal papilla, although this possibility remains to be examined.

Nevertheless, these observations indicate that the number of multipotent progenitor cells is significantly higher in cheek/chin skin tissues and that these cells are undifferentiated high-quality SKP cells useful for tissue engineering and regenerative therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.069.

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