

REPORT

Early-age-dependent selective decrease of differentiation potential of hair-follicle-associated pluripotent (HAP) stem cells to beating cardiac-muscle cells

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ABSTRACT

We have previously discovered nestin-expressing hair-follicle-associated pluripotent (HAP) stem cells and have shown that they can differentiate to neurons, glia, and many other cell types. HAP stem cells can be used for nerve and spinal cord repair. We have recently shown the HAP stem cells can differentiate to beating heart-muscle cells and tissue sheets of beating heart-muscle cells. In the present study, we determined the efficiency of HAP stem cells from mouse vibrissa hair follicles of various ages to differentiate to beating heart-muscle cells. We observed that the whiskers located near the ear were more efficient to differentiate to cardiac-muscle cells compared to whiskers located near the nose. Differentiation to cardiac-muscle cells from HAP stem cells in cultured whiskers in 4-week-old mice was significantly greater than in 10-, 20-, and 40-week-old mice. There was a strong decrease in differentiation potential of HAP stem cells to cardiac-muscle cells by 10 weeks of age. In contrast, the differentiation potential of HAP stem cells to other cell types did not decrease with age. The possibility of rejuvenation of HAP stem cells to differentiate at high efficiency to cardiac-muscle cells is discussed.

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Introduction

We have previously demonstrated that the neural stem-cell marker nestin is expressed in hair follicle stem cells, located in the bulge area, which are termed hair-follicle-associated pluripotent (HAP) stem cells.¹ HAP stem cells from mouse and human could differentiate to neurons, glia, keratinocytes, smooth muscle cells, and melanocytes in vitro.² HAP stem cells also could effect nerve and spinal cord regeneration in mouse models.^{3–6} We recently demonstrated that HAP stem cells differentiated to beating cardiac-muscle cells. The differentiation potential to cardiac-muscle is greatest in the upper part of the follicle. The beat rate of the cardiac-muscle cells was stimulated by isoproterenol and inhibited by propranolol.⁷

We subsequently observed that isoproterenol directs HAP stem cells to differentiate to cardiac-muscle cells in large numbers. The addition of activin A, bone-morphogenetic protein 4, and basic fibroblast growth factor, along with isoproterenol, induced the HAP-stem-cell-derived cardiac-muscle cells to form tissue sheets of beating heart-muscle cells.⁸

In the present report, we demonstrate that early aging selectively reduces the ability of HAP stem cells to differentiate to cardiac-muscle cells.

Results and discussion

Vibrissa follicles from 4-week-old mice have a high potential for differentiation to cardiac-muscle cells

We determined whether the differentiation of vibrissa (whisker) follicles to cardiac-muscle cells was age dependent. We isolated vibrissa hair follicles from 4-, 10-, 20-, and 40-week-old mice. Vibrissa follicles located near the ear side were larger than those located near the nose side (Fig. 1A). We cultured whisker follicles individually in 24-well cell-culture plates and aligned them in wells of the dish corresponding to their location in the mouse (Fig. 1B). We observed beating heart-muscle cells differentiating from the whisker follicles at 4 weeks in culture. The experiments were repeated on 4 samples. The greatest percentage of whisker follicles differentiating to beating cardiac-muscle cells was from 4-week-old mice. The percentage of whisker follicles that produced beating heart-muscle cells per one whisker pad from 4-week-old mice was: 43.01 ± 9.86 %; 10-week-old: 13.98 ± 8.12 %; 20-week-old: 21.51 ± 13.04 %; 40-week-old: 12.90 ± 3.23 % (Fig. 1C). *P*-value of 4-week-old vs. 10-week old: = 0.008; *P*-value of 4-week-old

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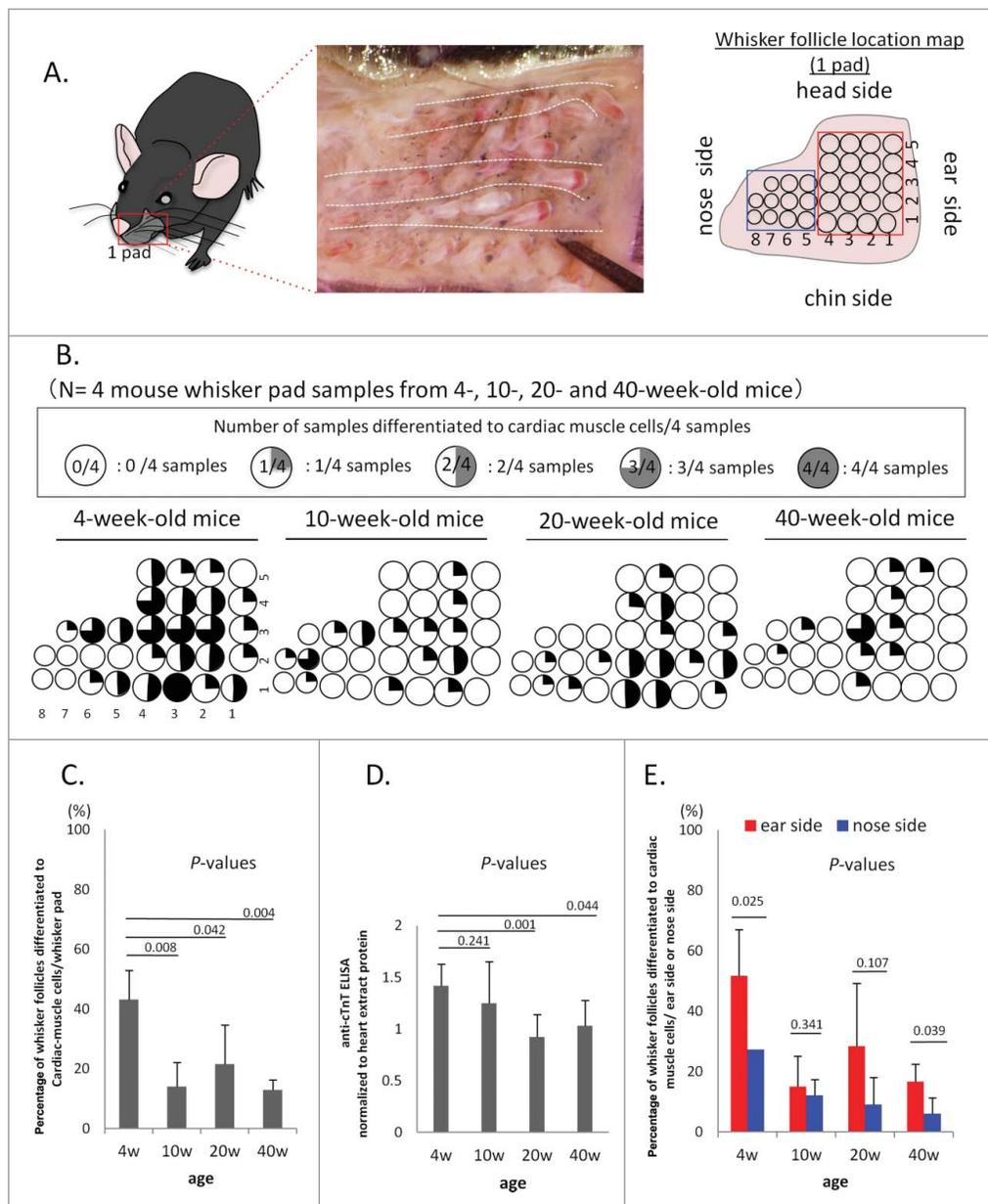


Figure 1. Whisker follicles from 4-week-old mice have greater potential for differentiation to cardiac-muscle cells than older mice. (A) Location map of mouse whisker follicles from one pad in one cheek. Blue box = nose side of the mouse whisker pad, Red box = ear side of the mouse whisker pad. (B) Whisker follicles from 4-week-old mice have a greater potential for differentiation to cardiac-muscle cells compared to 10-, 20-, and 40-week-old mice ($n = 4$ or each time point). (C) Comparison of the differentiation efficiency to cardiac-muscle cells from whisker follicles with mice of different age. (D) Anti-cTnT levels (ELISA analysis) in 4-, 10-, 20- and 40-week-old mouse cardiac-muscle cells differentiated from cultured whiskers. (E) Comparisons of whisker follicles from the ear side and nose side for differentiation potential to cardiac-muscle cells.

vs. 20-week old: = 0.042; P -value of 4-week-old vs. 40-week old: = 0.004.

Cardiac troponin (cTnT) also had higher levels in cardiac-muscle cells differentiating from cultured whisker follicles from 4-week-old mice than from whisker follicles from 10-, 20-, and 40-week-old mice (4-weeks-old; vs. 10-week-old, $P = 0.241$; 4-week-old vs. 20-week-old, $P = 0.001$; 4-week-old vs. 40-week-old, $P = 0.044$) (Fig. 1D).

We compared whisker follicles located in the ear side with whisker follicles located in the nose side for differentiation to beating cardiac-muscle cells. Whisker follicles located in the ear side differentiated to beating cardiac-muscle cells in greater amounts than those located in the nose side. A higher percentage of the whisker follicles produced beating cardiac-muscle

cells in the ear side in mice of all ages tested: (4-week-old: ear side $51.7 \pm 15.3\%$, nose side $27.3 \pm 0\%$; 10-week-old: ear side $15.0 \pm 10\%$, nose side $12.1 \pm 5.2\%$; 20-week-old: ear side $28.3 \pm 20.8\%$, nose side $9.0 \pm 9.0\%$; 40-week-old: ear side $16.7 \pm 5.8\%$, nose side $6.0 \pm 5.2\%$) (Fig. 1E). Ear side vs. nose side, 4-week-old, $P = 0.025$; ear side vs. nose side, 10-week-old, $P = 0.341$; ear side vs. nose side, 20-week-old, $P = 0.107$; ear side vs. nose side, 40-week-old, $P = 0.039$.

The greatest number of cells that differentiated to beating cardiac-muscle cells was from whisker follicles from 4-week-old mice: 4-week-old: $18 \pm 0.58 \times 10^5$; 10-week-old: $6 \pm 1.00 \times 10^5$; 20-week-old: $11 \pm 2.50 \times 10^5$; 40-week-old: $10 \pm 1.50 \times 10^5$ per whisker pad (Fig. 2A). P -value of 4-week-old vs. 10-week-old, = 0.00003; P -value of 4-week-old vs.

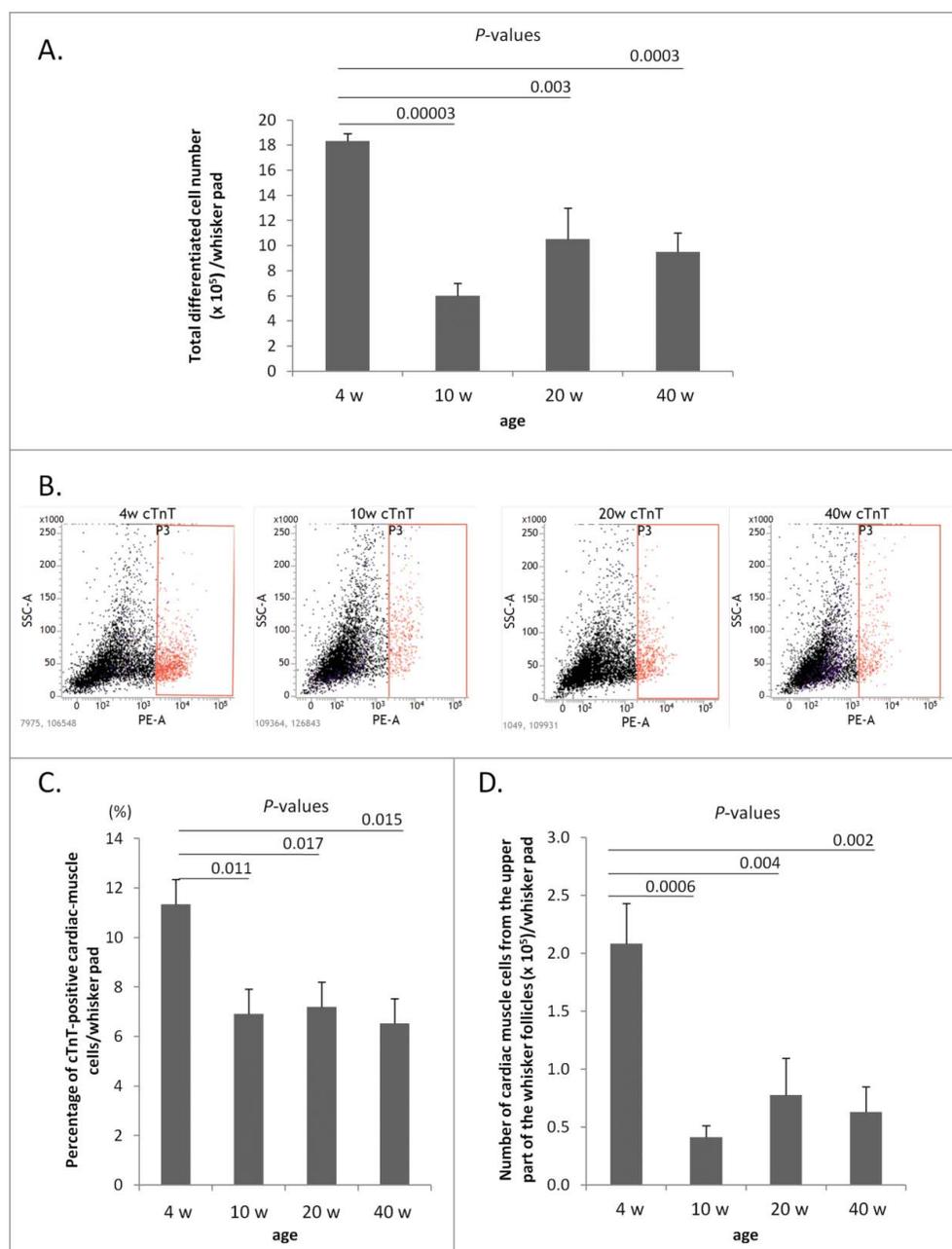


Figure 2. Differentiation to cardiac-muscle cells from whisker follicles from 4-, 10-, 20- and 40-week-old mice. (A) The total differentiated cardiac-muscle cell number from the upper part of whisker follicles from the ear side of the whisker pad, cultured in DMEM with 10% FBS. (B) Number of cTnT-positive cells differentiated from the whisker follicles from the ear side of the whisker pad, shown by flow cytometry. (C) Percentage of cTnT-positive cardiac-muscle cells differentiated from whisker follicles. (D) The number of cardiac-muscle cells differentiated from the upper part of whisker follicles.

20-week-old, = 0.003; *P*-value of 4-week-old, vs. 40-week-old, = 0.0003.

Flow cytometry analysis showed that cTnT-positive cells differentiating from whisker follicles from 4-week-old mice were greater than from 10-, 20-, and 40-week-old mice (Fig. 2B, C). *P*-value of 4-week-old vs. 10-week-old, = 0.011; *P*-value of 4-week-old vs. 20-week-old, = 0.017; *P*-value of 4-week-old vs. 40-week-old, = 0.015.

The number of cardiac-muscle cells differentiated from the upper part of hair follicles from 4-week-old mice was significantly greater from 10-, 20-, and 40-week-old mice: 4-week-old: $2.08 \pm 0.35 \times 10^5$; 10-week-old: $0.41 \pm 0.10 \times 10^5$; 20-week-old: $0.78 \pm 0.32 \times 10^5$; 40-week-old: $0.63 \pm 0.22 \times 10^5$ (Fig. 2D). *P*-value of 4-week-old vs. 10-week-old, = 0.0006;

P-value of 4-week-old vs. 20-week-old, = 0.004; *P*-value of 4-week-old vs. 40-week-old, = 0.002.

In order to determine whether the differentiation to neurons, glial cells, smooth muscle cells, keratinocytes, and melanocytes changed with age, the upper parts of hair follicles from 4-, 10-, 20-, and 40-week-old mice were cultured in Mattek culture plates. Immunofluorescence staining showed that hair follicle differentiation potential to other cell types was maintained with age (Fig. 3).

The mRNA level of stem-cell-marker genes does not show any significant difference with age

We also measured the expression level of stem cell markers with age including nestin, Nanog, Oct 3/4, Sox 2,

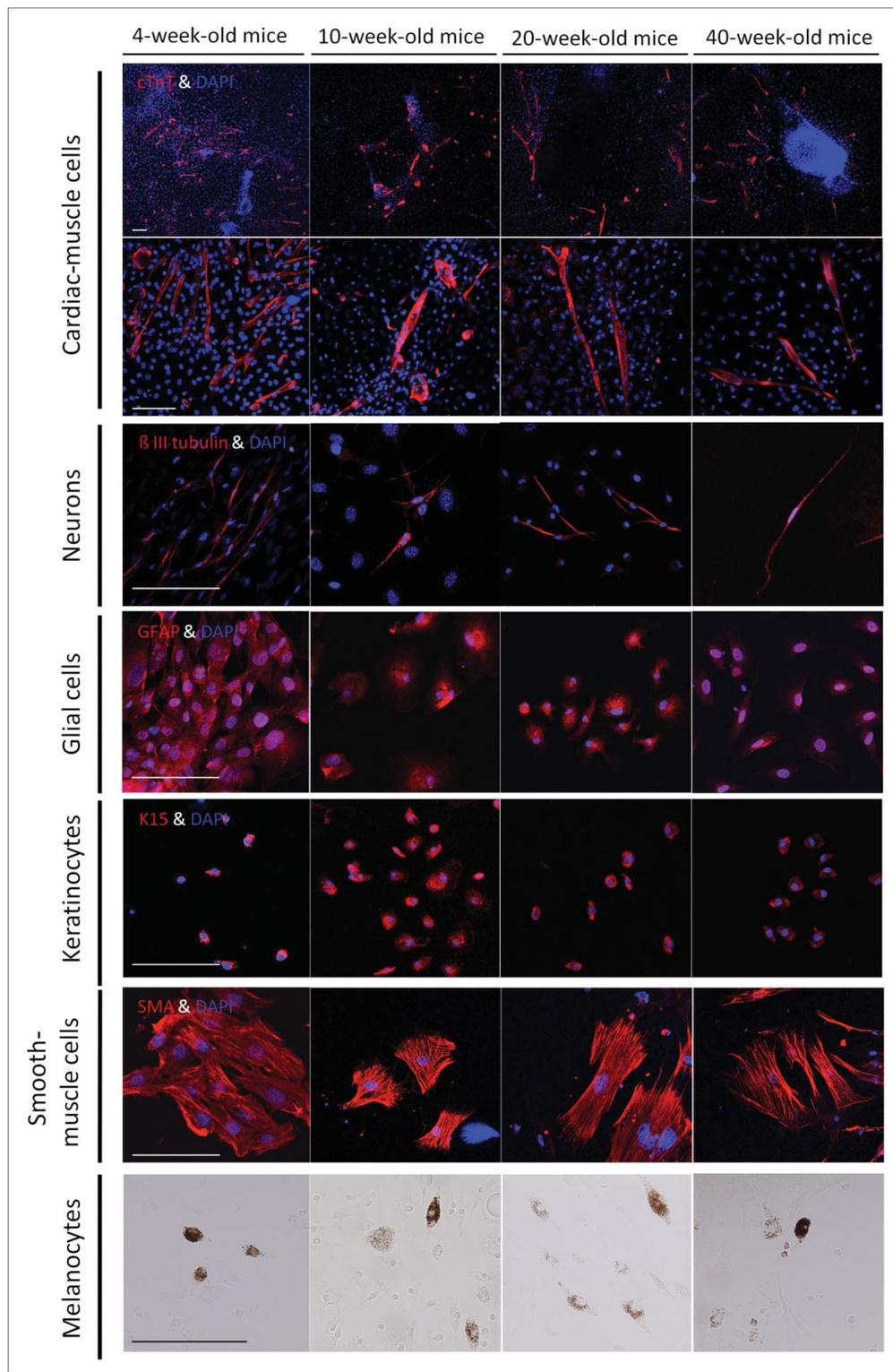


Figure 3. The differentiation potential to various cell types from whisker follicles from mice of different ages. Immunofluorescence staining showed that whisker follicle HAP stem cells from 4-, 10-, 20-, and 40-week-old mice differentiated to cardiac muscle cells, neurons, glial cells, keratinocytes and smooth muscle cells. Red = cTnT, tubulin, GFAP, Keratin 15 (K15) and smooth muscle actin (SMA). Blue = DAPI. Bar = 100 μ m. Phase-contrast images showed that HAP stem cell from 4-, 10-, 20-, and 40-week-old mice also differentiated to melanocytes. Bar = 100 μ m.

and SSEA1 in the upper parts of whisker follicles from 4-, 10-, 20-, and 40-week-old mice using real-time RT-PCR. Expression of GAPDH in whiskers of 4-week-old mice was used as a control. None of these stem-cell makers showed a change in expression in the whisker follicle with age (Fig. 4) ($P > 0.05$).

Nishimura et al. showed that hair graying is caused by defective self-maintenance of melanocyte stem cells in aging mice.⁹ Cheng et al.¹⁰ showed induced pluripotent stem (iPS) cells from bone marrow-derived myeloid cells from aged mice had lower differentiation efficiency than iPS cells from young mice. Horibe et al.¹¹ showed that human

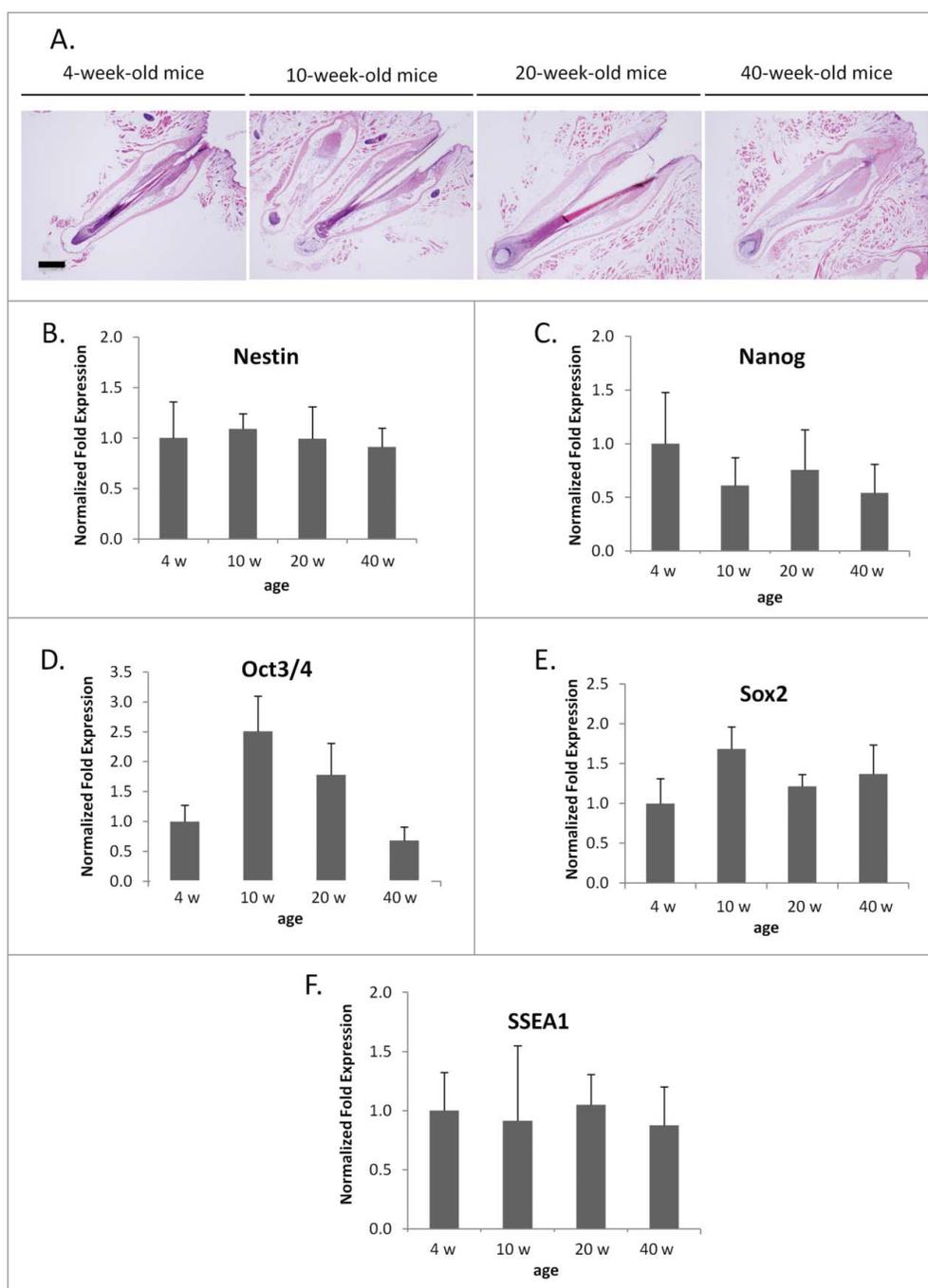


Figure 4. mRNA levels of stem cell marker genes from whisker hair follicles of various age. (A) Hematoxylin and eosin staining of whisker follicles isolated from 4-, 10-, 20-, and 40-week-old mice. Bar = 200 μ m. (B-F) Comparisons of mRNA levels of stem-cell-marker genes: nestin (B), Nanog (C), Oct 3/4 (D), Sox 2 (E), SSEA1 (F) were determined with PCR. Results are mean \pm SD for 3 samples each. The mRNA level of stem-cell-marker genes did not show any significant differences with whisker age.

mobilized dental-pulp stem cells (MDPSCs) from aged donors decreased in differentiation potential, migration, and proliferation compared to MDPSCs from young donors. We have recently shown that transplanted hair follicles decreased their hair-growth potential with age and HAP stem cells changed their distribution in the aged follicles. However, senescence of hair follicles could be reversed.¹² Perhaps the age-related reduction in HAP stem cell ability to differentiate to beating heart-muscle cells can also be rejuvenated.

We have recently shown that HAP stem cells and whole whisker follicles can be cryopreserved.^{9,10} HAP stem cells

show great promise for regenerative medicine, since they differentiate to many useful cell types, are easily accessible, non-tumorigenic, and are not immunogenic, because they are available from each patient. Since the HAP stem cells decrease, their potential for differentiation to beating heart-muscle cells at an early age, and hair follicles become senescent with age, banking hair follicles at an early age may be beneficial to every person for potential future use.

Previous studies indicated that stem cells may be able to be rejuvenated such as MTOR inhibitors, such as rapamycin,¹¹ or modulators of TP53 by nutlin-3a.¹²

Future experiments will determine if such treatments can rejuvenate the ability of aging hair follicles to more efficiently differentiate to cardiac-muscle cells.

Materials and methods

Mice

C57BL/6J mice; 4-, 10-, 20- and 40-week-old mice, were obtained from CLEA (Kawasaki, Japan) and used to isolate whisker hair follicles. All animal experiments were conducted according to the Guidelines for Animal Experimentation at Kitasato University.

Whisker follicles and culture

The whisker follicles from mice were isolated as described previously.³ The upper parts of whisker follicles were seeded onto BD Matrigel™ (basement-membrane matrix growth-factor reduced) (BD Biosciences, Bedford, MA), and cultured in DMEM (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS), 50 µg/ml gentamycin (GIBCO, Grand Island, NY), 2 mM L-glutamine (GIBCO) and 10 mM HEPES (MP Biomedicals, Santa Ana, CA). Beating cardiac-muscle cells were recorded by video microscopy (ScopPad-500, GelleX, Tokyo, Japan).

Anti-cardiac-troponin T (cTnT) ELISA analysis of differentiated cells from the upper parts of whisker follicles

The upper parts of mouse whisker follicles were cultured in 24-well cell plates (Corning, Kennebunk, ME) for 4-weeks. The cells were fixed and incubated with anti-cardiac-troponin T (cTnT) mouse monoclonal antibody (1:2000, Gene Tex, Hsinchu City, Taiwan) for 1 hour. The cells were then incubated with HRP-protein A/G Purified Recomb® (1:10000, Thermo scientific, Rockford, IL) for 1 hour. The whisker follicles were removed from the well and the remaining cells were incubated with TMB+Substrate-Chromogen (Dako, Carpinteria, CA) for 15 minutes. H₂SO₄ 1 N was then added to each well. The supernatant was transferred to 96-well EIA/RIA plates (Corning, Corning, NY) and measured with a Multi-Functional Microplate Reader SPECTRA FLUOR Plus (Wako, Tokyo, Japan).

Real-time polymerase chain reaction (RT-PCR) of stem cell marker genes

Total RNA from 20 upper parts of hair follicles was purified with RNeasy® Plus Mini Kit (QIAGEN, Hilden, Germany) and treated with the OneStep™PCR Inhibitor-Removal Kit (ZYMO RESEARCH, Irvin, CA) to remove melanin. c-DNA was synthesized with the SuperScript®VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). Real-time PCR, on CFX96 (Bio-Rad, Hercules, CA) with TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) and TaqMan Probes, was performed as follows: GAPDH: Mm99999915_g1; Nanog: Mm02019550_s1; nestin: Mm00450205_m1; Oct 3/4: Mm03053917_g1; Sox 2: Mm03053810_s1; and SSEA1: Mm00487448_s1. The mRNA levels were normalized with GAPDH. Results are mean ± SD for 3 samples each.

Immunofluorescence staining of differentiated cells from the upper part of whisker follicles

The upper part of whisker follicles was cultured in MatTek plates (MatTek, Ashland, MA) for 4-weeks. The cells were incubated with anti-cardiac-troponin T (cTnT) mouse monoclonal antibody (1:250, Gene Tex, Hsinchu City, Taiwan); anti-β III tubulin mouse monoclonal antibody (1:250, Tuj1 clone, Covance, CA); anti-glial fibrillary acidic protein (GFAP) chicken polyclonal antibody (1:250; abcam, UK); anti-smooth-muscle actin (SMA) mouse monoclonal antibody (1:250; Lab Vision, Fremont, CA); and anti-keratin 15 (K15) mouse monoclonal antibody (1:250; Lab Vision, UK) in blocking buffer (4 % BSA, 0.5 % Triton X100, 0.04 % NaN₃ in PBS) at room temperature for 3 hours. The cells were then incubated with goat anti-mouse IgG conjugated with Alexa Flour 568® (1:400, Molecular Probes, Eugene, Oregon) or goat anti-chicken IgG conjugated with Alexa Flour 568® (1:400, Molecular Probes, Eugene, Oregon) and 4', 6-diamino-2-phenylindole, dihydrochloride (DAPI) (Molecular Probes) in blocking buffer for 2h at room temperature. Fluorescence staining was visualized using a laser-scanning microscope (710) (Carl Zeiss, Oberkochen, Germany).

cTnT flow cytometry of differentiated cells from the upper part of whisker follicles

The upper part of hair follicles was cultured in 6-well flat bottom plates (Corning, Kennebunk, ME) for 4-weeks. The cells were incubated with anti-cTnT mouse monoclonal antibody (1:200) as the primary antibody. The secondary antibody was goat anti-mouse IgG H&L phycoerythrin (1:500; Abcam, Cambridge, UK). The cells were measured with a FACS Verse (BD Bioscience) and were analyzed with FACS suite™ software (BD Bioscience). Flow-cytometry results are presented as mean ± SD for 3 samples each.

Statistical analysis

The experimental data are expressed as the mean ± SD. Statistical analyses were performed with the unpaired Student's *t*-test with a *P*-value of < 0.05 considered significant.

Dedication

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

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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