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Maintenance of sweat glands by stem cells located in the acral epithelium

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ABSTRACT

The skin is responsible for a variety of physiological functions and is critical for wound healing and repair. Therefore, the regenerative capacity of the skin is important. However, stem cells responsible for maintaining the acral epithelium had not previously been identified. In this study, we identified the specific stem cells in the acral epithelium that participate in the long-term maintenance of sweat glands, ducts, and interadnexal epidermis and that facilitate the regeneration of these structures following injury. Lgr6-positive cells and Bmi1-positive cells were found to function as long-term multipotent stem cells that maintained the entire eccrine unit and the interadnexal epidermis. However, while Lgr6-positive cells were rapidly cycled and constantly supplied differentiated cells, Bmi1-positive cells were slow to cycle and occasionally entered the cell cycle under physiological conditions. Upon irradiation-induced injury, Bmi1-positive cells rapidly proliferated and regenerated injured epithelial tissue. Therefore, Bmi1-positive stem cells served as reservoir stem cells. Lgr5-positive cells were rapidly cycled and maintained only sweat glands; therefore, we concluded that these cells functioned as lineage-restricted progenitors. Taken together, our data demonstrated the identification of stem cells that maintained the entire acral epithelium and supported the different roles of three cellular classes.

1. Introduction

The skin is the largest organ in the body and has many critical functions, including prevention of moisture loss, regulation of body temperature, protection from external material, and role as a sensory organ, among others. Therefore, the skin must maintain homeostasis; when it is damaged, repair and regeneration occur promptly. This mechanism is particularly important in patients with burns and epidermolysis bullosa hereditaria because of the dramatic loss of skin, which can result in leakage of water and various nutrients. In cancer patients, antitumor drugs can lead to injury of the tongue and oral mucosa, seriously impairing patient quality of life. Furthermore, necrosis of the skin, including the acral part of the limbs, is becoming increasingly more common due to the rising prevalence of peripheral arterial disease and diabetes mellitus [1]. Therefore, further research on wound repair in both hairy and nonhairy skin regions is necessary.

Skin has two distinct population of stem cells, keratinocyte stem cells (KSCs) and melanocyte stem cells (MSCs). KSCs are located at the bulge lesion, a structure between the sebaceous gland and arrector pili muscle, and function to supply differentiated cells to the cycling portions of the hair follicle, sebaceous gland, and

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interfollicular epidermis in hairy skin [2]. However, nonhairy skin contains abundant eccrine units (sweat glands and ducts), but no hair follicles as the main skin appendage, and KSCs have not been well studied in acral skin.

Stem cells responsible for maintaining the acral epithelium have not yet been identified. The cells generated from sweat glands and ducts differentiate and migrate towards the acral epithelium. Cells that are positive for keratin 14 (Krt14), sex determining region Y (SRY)-box 9 (Sox9), and alpha6-beta1 integrin have self-renewing capacity and multipotency. In studies using the lineage tracing method, sweat glands and ducts have been shown to develop from Krt14-positive cells and Sox9-positive cells [3]. However, a considerably large fraction of epithelial cells in the interadnexal epidermis express Krt14, and other tissues, such as hematopoietic cells in the bone marrow, have very small populations of long-term stem cells [4,5], suggesting that the identities of true stem cells responsible for the maintenance of the acral epithelium are still not known.

Recently, we identified the specific stem cells in the lingual epithelium that provide long-term maintenance of keratinized epithelial cells and that can regenerate this tissue upon injury [6]. Here, we sought to identify KSCs expressing hair follicle stem cell markers, such as leucine-rich repeat-containing G protein-coupled receptor (Lgr) 5, Lgr6, and B lymphoma Mo-MLV insertion region 1 homolog (Bmi1), in mouse acral skin. Moreover, we investigated the distinctions among cells with different markers.

2. Materials and methods

2.1. Mice

Mice were bred and maintained at the Kansai Medical University Research Animal Facility in accordance with the Kansai Medical University guidelines. C57BL/6J, Bmi1$^{CreER/+}$ [7], Lgr5$^{EGFP-IRES-CreERT2/+}$ [8], Lgr6$^{EGFP-IRES-CreERT2/+}$ [9], Rosa26$^{CreERT2/+}$, and Rosa26$^{rbw/+}$ mice were purchased from Jackson Laboratories or generated as described previously [10,11]. The experiments were approved in advance by the Kansai Medical University Animal Welfare Committee. Tamoxifen (Sigma, St. Louis, MO, USA) was dissolved in corn oil (Sigma) and injected intraperitoneally into adult mice between postnatal weeks 4 and 6 at a concentration of 5–9 mg per 40 g body weight.

2.2. Irradiation

Mice were administered 5 Gy of whole-body irradiation using a cesium irradiator, and the tissue was collected and analyzed at the indicated time points. To observe the irradiated mice over a long-term study period, total bone marrow nucleated cells (7.5×10^6/mouse) were injected intravenously following irradiation.

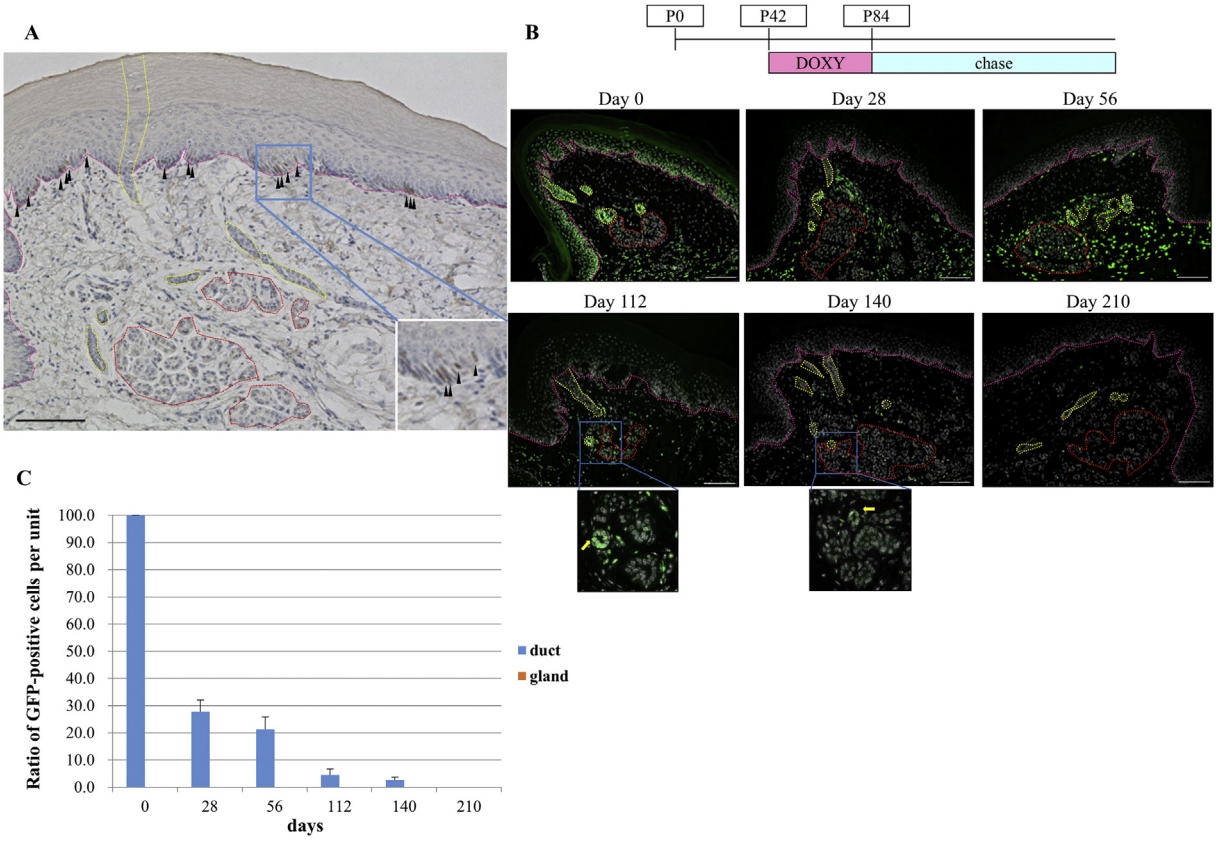

Fig. 1. Identification of proliferating cells in the acral epithelium. (A) Immunostaining of the acral epithelium with anti-Ki67 antibodies. Ki67-positive proliferating cells (black arrowheads) were expressed in the basal layer of the interadnexal epidermis. The inset is a magnified image in a part of the epidermis. (B) H2BGFP was expressed following treatment with doxycycline in Tet On H2BGFP/Rosa26-rtTA mice, as shown. H2BGFP retention occurred only in sweat ducts (yellow arrows). Magnified images of regions indicated by white rectangles in the left panels are shown in the right panels. (C) The ratio of H2BGFP-positive cells per eccrine unit at the indicated time points on sections was judged by the number of Hoechst-33342-stained nuclei. Standard error bars represent SDs. The specimens were gathered from four H2BGFP/Rosa26-rtTA (Tet On) mice (two males and two females) for each time point. The sweat ducts are indicated by a yellow dashed line, and the sweat glands are indicated by a red dashed line. The areas above the pink dashed line indicate interadnexal epithelium. Scale bars, 100 μm.

2.3. Histological analysis

The mice were euthanized, and the collected tissues were fixed, frozen, cut, and analyzed as reported previously [12,13]. Immunostaining was performed using primary antibodies targeting Ki67 (cat. no. M7249; monoclonal rat anti-mouse Ki67, clone TEC-3; 1:50 dilution; Dako, Glostrup, Denmark) and peroxidase-labeled secondary antibodies (1:200 dilution; Invitrogen, Carlsbad, CA, USA). *In situ* hybridization was performed by standard protocols using an ISH kit (Nippon Gene, Japan). Single-molecule mRNA *in situ* hybridization was performed as described previously [14]. Fluorescent images were acquired using OLYMPUS BX63 (Olympus Corporation, Japan) and BZ-9000 (Keyence Corporation, Japan) microscopes. Hematoxylin and eosin staining was performed following a general protocol.

2.4. Three-dimensional images

Frozen tissues were cut into 80- to 120-μm sections, and images were taken with a Nikon C2 confocal microscope (Nikon Instech). The three-dimensional images were reconstructed using Nikon or Volocity software (Perkin–Elmer).

2.5. Statistical analysis

Student's *t*-test was used for statistical comparisons. Differences with *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. Slow-cycling stem cells were present in sweat ducts

To investigate proliferating cells in the acral epithelium, we first examined the expression of Ki67. Proliferating cells were expressed in the basal layer of the interadnexal epidermis, but not in the sweat ducts and glands (Fig. 1A), indicating that sweat duct and gland cells were not actually cycling.

Next, we attempted to identify and locate label-retaining cells (LRCs) in the acral epithelium. LRCs comprise only a minor subset of stem cells that cycle very slowly or enter dormancy [15]. We performed a series of pulse-chase experiments using H2BGFP/Rosa26-rtTA (Tet On) mice. During and immediately after administration of doxycycline, almost all of the cells within the tissue, except for sweat glands, exhibited H2BGFP expression. However, after 16–20 weeks (112–140 days), fluorescence was only retained within the sweat ducts (Fig. 1B–C). In the gland area, GFP expression was detected only in myoepithelial cells, but not in sweat gland cells. These pulse-chase experiments suggested that slow-cycling stem cells responsible for maintenance of the acral epithelium existed only in sweat ducts and that sweat gland cells cycled the slowest of all cell types.

3.2. Actual long-term stem cells were rarely observed in sweat glands

To identify the actual stem cells that were responsible for the long-term maintenance of the acral epithelium, we labeled all of

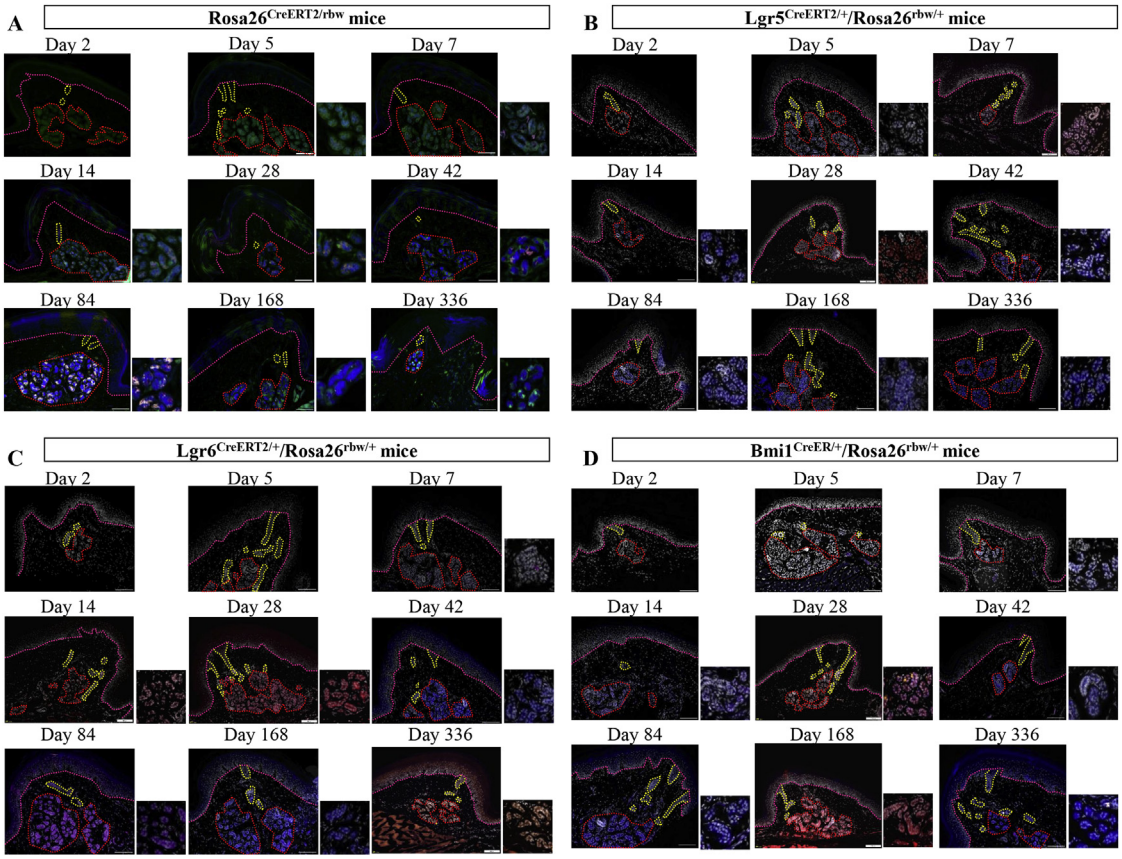

Fig. 2. Multicolor lineage tracing method. (A) Rosa26$^{CreERT2/rbw}$ mice, (B) Lgr5$^{EGFP\text{-}IRES\text{-}CreERT2/+}$/Rosa26$^{rbw/+}$ mice, (C) Lgr6$^{EGFP\text{-}IRES\text{-}CreERT2/+}$/Rosa26$^{rbw/+}$ mice, and (D) Bmi1$^{CreER/+}$/Rosa26$^{rbw/+}$ mice were labeled with tamoxifen and analyzed at the indicated time points. Labeled cells (blue, orange, or red) in the acral epithelium are presented. The sweat ducts are indicated by a yellow dashed line, and the sweat glands are indicated by a red dashed line. The areas above the pink dashed line indicate interadnexal epithelium. Scale bars, 100 μm.

the epithelial cells with random colors and followed their fate using Rosa26$^{CreERT2/rbw}$ mice [10,16,17]. In this system, CreERT2, an inducible mutant of Cre, is expressed in all of the cells in the body. The administration of tamoxifen, which activates CreERT2, changes the color of every cell independently and randomly from green (GFP) to blue (mCerulean) [18], orange (mOrange), or red (mCherry), as illustrated in Supplemental Fig. 1 [17]. Compared with conventional lineage tracing methods [11], this multicolor lineage tracing method enables the number of stem cell clones in the region to be visualized and allows for cell clusters derived from different stem cell clones to be distinguished easily [10,12,13,19]. In this experiment, labeled sweat gland cells supplied differentiated cells to the cornified layer, as demonstrated by the finding that epithelial cells with four colors existed as a mixture (Fig. 2A). This finding indicated that multiple clones proliferated independently. However, after 4 weeks, areas from the sweat gland to the cornified layer were occupied by single-color cells that seemed to be the progeny of a single stem cell. This result suggested that very few cells were actual long-term stem cells in the sweat glands and that a single stem cell could survive in a single eccrine unit after 4 weeks, whereas most of the sweat glands and duct cells comprised transit-amplifying cells.

3.3. *Lgr6-positive cells were short-term stem cells, and Bmi1-positive cells were long-term stem cells in the eccrine unit*

Next, to identify specific markers for acral KSCs, we crossed various CreERT2 knock-in mice with Rosa26$^{rbw/+}$ mice, in which CreERT2 was expressed under different stem cell marker promoters. We then examined whether acral epithelial cells were labeled. We examined Lgr5 [20], Lgr6 [9,21], and Bmi1 [22,23], previously reported as hair follicle stem cell markers. In Lgr5$^{EGFP\text{-}IRES\text{-}CreERT2/+}$/Rosa26$^{rbw/+}$ mice, tamoxifen-induced cells remained as single cells for approximately 5 days and then started dividing. However, these Lgr5-positive cell clusters were located only in sweat glands (Fig. 2B), indicating that Lgr5-positive cells acted as specific sweat gland stem cells for an extended period. In contrast, tamoxifen-induced cell clusters derived from Lgr6-positive cells reached the surface of the cornified layers by 2 weeks in Lgr6$^{EGFP\text{-}IRES\text{-}CreERT2/+}$/Rosa26$^{rbw/+}$ mice (Fig. 2C). After this period, the labeled cells were maintained for more than 48 weeks. Compared with Lgr6$^{EGFP\text{-}IRES\text{-}CreERT2/+}$/Rosa26$^{rbw/+}$ mice, Bmi1-positive cells remained as single cells for an extended duration (approximately 7 days) in Bmi1$^{CreER/+}$/Rosa26$^{rbw/+}$ mice (Fig. 2D). Furthermore, the cell clusters derived from Bmi1-positive cells reached the surface of the cornified layers slower than those from Lgr6-positive cells by 4 weeks. To investigate stereoscopic structures, we captured three-dimensional images taken 12 weeks after tamoxifen administration using confocal microscopy (Supplemental Video 1A–D). These three-dimensional images supported the above findings, indicating that Lgr6-positive cells were rapid-cycling, short-term stem cells, whereas Bmi1-positive cells were slow-cycling, long-term stem cells in the eccrine unit (Supplemental Fig. 2).

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.09.022.

3.4. *Lgr5 was expressed only in sweat glands*

To examine the position of these stem cells in the acral skin, we performed *in situ* hybridization with Lgr5, Lgr6, and Bmi1 probes (Fig. 3A–C). Similar to the results of the multicolor lineage tracing experiments, Lgr5 was expressed only in sweat glands, and Lgr6 was expressed in sweat glands, ducts, and interadnexal epithelium. On the other hand, Bmi1 was expressed only in sweat glands, but

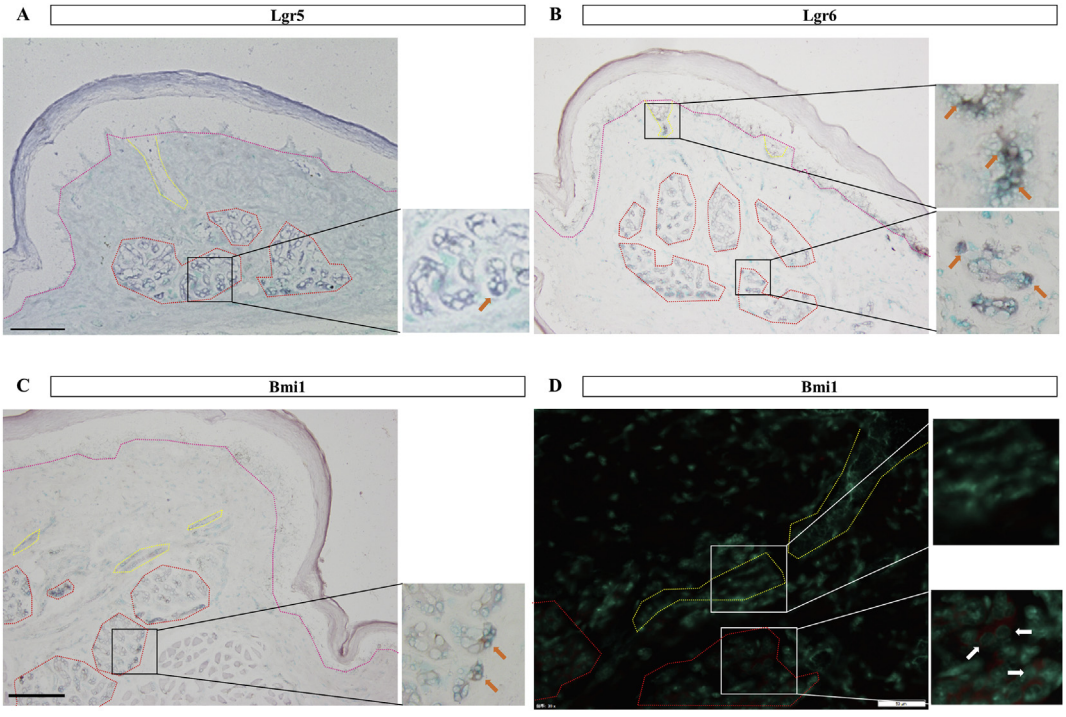

Fig. 3. Location of stem cells. *In situ* hybridization of the acral epithelium with (A) Lgr5, (B) Lgr6, and (C) Bmi1 probes. The sweat ducts are indicated by a yellow dashed line, and the sweat glands are indicated by a red dashed line. The areas above the pink dashed line indicate interadnexal epithelium. Scale bars, 100 μm. (D) Single-molecule mRNA *in situ* hybridization with Alexa594-labeled 48 Bmi1 oligonucleotide DNA probes. The red dots, indicating Bmi1-high cells, were detected (white arrow) in sweat glands (red dashed lines), but not in sweat ducts (yellow dashed lines). Scale bars, 50 μm.

not in ducts or the interadnexal epithelium. Furthermore, we next performed single-molecule mRNA *in situ* hybridization [14] to detect Bmi1-positive cells (Fig. 3D). Bmi1-positive cells were localized only in sweat glands, as was shown by *in situ* hybridization. These findings from the multicolor lineage tracing method and *in situ* hybridization indicated that Lgr6 was expressed in both stem cells and differentiated cells, but was downregulated in differentiated cells. On the other hand, Bmi1 was expressed only in stem cells localized in sweat glands.

3.5. *Bmi1-positive cells were characterized as injury-inducible reserve stem cells*

Next, we examined the role of Bmi1-positive stem cells in the regeneration of injured acral epithelium. We irradiated Bmi1$^{CreER/+}$/Rosa26$^{rbw/+}$ mice at doses of 5 Gy and administered tamoxifen just before irradiation. We then followed the fate of the labeled Bmi1-positive stem cells. Under both conditions, labeled Bmi1-positive stem cells started proliferating at around day 4, and their progeny reached the surface of the cornified layer by 7 days after irradiation (Fig. 4A). Compared with observations made under normal physiological conditions, the proliferation of Bmi1-positive cells was much faster after irradiation (Fig. 4B).

4. Discussion

In this study, we aimed to identify KSCs expressing hair follicle stem cell markers in mouse acral skin and investigate the distinctions among cells with different markers. We found that LRCs, considered slow-cycling stem cells, existed in sweat glands; Lgr5-positive cells were rapid-cycling progenitor cells that maintained only sweat glands, whereas Lgr6-positive stem cells were rapid-cycling stem cells that maintained the entire eccrine unit to the interadnexal epidermis. Moreover, Lgr6 was also expressed in differentiated cells. Bmi1-positive stem cells were slow-cycling cells that occasionally entered the cell cycle under physiological conditions. However, after irradiation-induced injury, the Bmi1-positive stem cells rapidly proliferated and could regenerate injured epithelial tissue, as shown in the schematic in Fig. 4C.

Previously, various keratin proteins, including K7, K8, K18, and K19, have been reported to represent specific sweat gland markers in humans [24]. However, in mice, no effective markers have been found that can distinguish sweat gland cells from sweat duct cells. In the current study, Lgr5-positive cells were expressed only in sweat glands, suggesting that Lgr5 could be a useful sweat gland marker in mice.

Studies in several tissues have shown that two types of stem cells exist, namely, slow-cycling, long-term stem cells and rapidly proliferating, short-term stem cells, which have distinct roles in tissue maintenance and regeneration [4,5]. In this study, we found that the acral epithelium also had two types of stem cells: Lgr6-positive rapid-cycling, short-term stem cells and Bmi1-positive slow-cycling, injury-inducible reserve stem cells. The skin protects the organism from external insults and therefore undergoes rapid and continuous regeneration supported by stem cells. Additionally, following damage to the skin, repair and regeneration

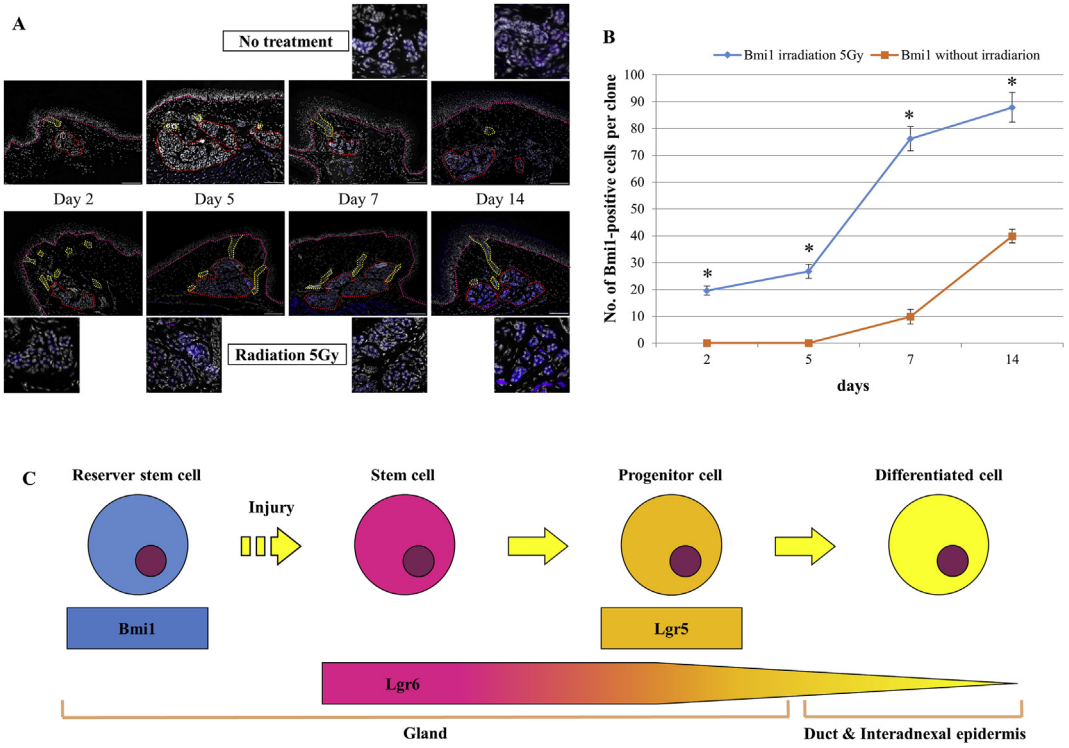

Fig. 4. Regeneration of the acral epithelium from Bmi1-positive stem cells after irradiation and schematic representation of stem cell markers in acral epithelium. (A) Bmi1-positive stem cells were labeled with tamoxifen and analyzed at the indicated time points in Bmi1$^{CreER/+}$/Rosa26$^{rbw/+}$ mice without irradiation. Rapid regeneration was observed in Bmi1$^{CreER/+}$/Rosa26$^{rbw/+}$ mice after irradiation. The mice were irradiated with 5 Gy and analyzed at the indicated time points. The sweat ducts are indicated by a yellow dashed line, and the sweat glands are indicated by a red dashed line. The areas above the pink dashed line indicate interadnexal epithelium. Scale bars, 100 μm. (B) The cell clusters derived from single Bmi1-positive cells expanded more quickly in irradiated mice than in mice without irradiation. Student's *t*-tests were used to evaluate the differences between mean values at each time point. Standard error bars represent SDs. The specimens were collected from six mice (three males and three females) for each time point. *$P < 0.01$. (C) Lgr6-positive stem cells were rapid-cycling stem cells that maintained the entire eccrine unit in the interadnexal epidermis. Lgr5-positive cells were rapid-cycling progenitor cells that maintained only sweat glands. Bmi1-positive stem cells were slow-cycling cells that occasionally entered the cell cycle under physiological conditions. However, in injured skin, Bmi1-positive stem cells rapidly proliferated and could regenerate injured epithelial tissue.

must occur promptly. Importantly, while the two types of stem cells described above have different roles in general homeostasis, both of them function cooperatively to repair damaged tissue.

In summary, we identified the specific stem cells in the acral epithelium that maintained the sweat glands, ducts, and interadnexal epidermis for an extended period and that could regenerate these structures following injury. Future studies are needed to determine whether human acral skin is similar to that observed in mice, with the two distinct subsets of stem cells, and to investigate which cells contribute to the development of carcinomas in the acral epithelium.

Author contributions

S.O. performed the majority of experiments. T.T., H.Y., Y.K., T.O., S.K., K.T., K.I., K.S., N.N., H.O., Y.T., N.A., H.H., N.Y., K.K., F.Y., and H.O. helped with the experiments and prepared samples. H.U. supervised the project. S.O. and H.U. interpreted the results and wrote the manuscript.

Disclosure of potential conflicts of interest

The authors indicate that they have no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.09.022.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.09.022.

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