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A melanocyte–melanoma precursor niche in sweat glands of volar skin

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Summary
Determination of the niche for early-stage cancer remains a challenging issue. Melanoma is an aggressive cancer of the melanocyte lineage. Early melanoma cells are often found in the epidermis around sweat ducts of human volar skin, and the skin pigmentation pattern is an early diagnostic sign of acral melanoma. However, the niche for melanoma precursors has not been determined yet. Here, we report that the secretory portion (SP) of eccrine sweat glands provide an anatomical niche for melanocyte–melanoma precursor cells. Using lineage-tagged H2B-GFP reporter mice, we found that melanoblasts that colonize sweat glands during development are maintained in an immature, slow-cycling state but renew themselves in response to genomic stress and provide their differentiating progeny to the epidermis. FISH analysis of human acral melanoma expanding in the epidermis revealed that unpigmented melanoblasts with significant cyclin D1 gene amplification reside deep in the SP of particular sweat gland(s). These findings indicate that sweat glands maintain melanocyte–melanoma precursors in an immature state in the niche and explain the preferential distribution of early melanoma cells around sweat glands in human volar skin.

Introduction
Human cutaneous melanoma is a highly aggressive cancer that is resistant to traditional cancer treatments (Garbe et al., 2011; Mocellin et al., 2010; Wheatley, 2003). Acral (volar skin) melanoma is the most prevalent subtype of melanoma in the non-Caucasian population. Human volar skin of peripheral extremities is minimally exposed to UV sunlight and contains abundant eccrine sweat glands but no hair follicles as the main skin appendage (Curtin et al., 2005). The characteristic skin pigmentation pattern of acral melanoma can be recognized with a magnifying glass called a dermoscopy in a ‘parallel ridge pattern’, which reflects the preferential proliferation and differentiation of melanoma cells around sweat ducts (Oguchi et al., 1998; Saida, 2007; Saida et al., 2004, 2011). This method devised by Saida, one of the authors of this manuscript and his colleagues, has

Significance
Acral volar skin, which lacks hair follicles but contains abundant eccrine sweat glands, is highly susceptible to melanoma even without exposure to ultraviolet light. The dermoscopic pattern of parallel ridges has 99% specificity to detect acral melanomas, while the cause of the skin pigmentation pattern has been unknown. Our study is the first to identify sweat glands as the anatomical niche for melanocyte–melanoma precursor cells in mammalian volar skin.
enabled the accurate diagnosis with a 99% specificity (Saida et al., 2004) of early acral melanoma even as small as a few millimeter and has become the standard methodology for early diagnosis of melanoma in the volar skin nowadays. However, the origin of acral melanoma cells, the cause of the parallel ridge pattern, and even the existence of melanocytic cells in sweat glands have yet to be characterized. Recent reports on sweat gland-centric distribution of melanoma cells in some acral melanoma cases (Zembowicz and Kafanis, 2012) and repigmentation of the human white spot epidermis around sweat glands (Makino et al., 2013) have suggested the possibility that unknown melanocyte precursors (melanoblasts) exist in sweat glands. Thus, the skin appendage may provide a niche or special microenvironment not only for melanoblasts but also for earliest melanoma cells.

In most somatic stem cell systems, stem cells are maintained in an immature and quiescent state but become activated to undergo self-renewal to provide amplifying and differentiating progeny for tissue homeostasis (Fuchs et al., 2004; Li and Clevers, 2010). We previously identified melanocyte stem cells (McSCs) in the bulge/sub-bulge area of murine and human hair follicles and reported that the niche plays a dominant role in stem cell fate determination (Nishimura et al., 2002, 2005). The McSCs self-renew and provide their progeny not only to the hair bulb for hair pigmentation in physiological condition but also to the surrounding epidermis for skin pigmentation (Nishimura et al., 2002). During the process of epidermal repigmentation, the repigmented spots often start from the orifice of hair follicles with contiguous distribution of melanoblasts/melanocytes in the upper outer root sheath starting from the hair follicle bulge to the epidermal basal layer (Nishimura, 2011; Nishimura et al., 2002). It is thus possible that sweat glands also provide similar niche microenvironment not only for normal melanoblasts but also for early melanoma cells.

In this paper, we hypothesized that dormant immature melanocyte–melanoma precursors exist in mammalian eccrine sweat glands, which are abundantly distributed on the volar skin. We searched for the population by taking advantage of stable lineage tagging system, and we report here that such melanocyte–melanoma precursors exist in sweat glands of mammalian volar skin.

**Results**

To compare the architecture and pigmentation pattern of human and mouse volar skin, we first performed histological analysis. As shown in Figure 1A–C, both human and mouse volar skin were characterized by a similar thickened epidermis with rete ridges extending into the underlying dermis and by the abundant presence of sweat glands (Figure 1A–C). No pigment-containing cells or melanocytic marker-expressing cells were detectable with conventional immunohistochemistry in the sweat glands found in the footpads of non-aged adult C57/BL6Cr mice (Figure 1D). In contrast, some pigment-containing cells were found in the sweat glands and the underlying epidermis from 2-year-old mice (Figure 1E and data not shown), which suggested that unpigmented melanoblasts that correspond to melanocyte stem cells or progenitors exist in non-hair-bearing volar skin. Dct-lacZ transgene (Figure 1F) and endogenous Dct protein expression, which has been used for melanocyte lineage tagging including melanocyte stem cells (McSCs) in hair follicles (Mackenzie et al., 1997; Nishimura et al., 2002), failed to tag that cell population in adult footpad skin (Figure 1H and data not shown). Thus, we examined the developmental process of melanocytes in the distal hindlimb using Dct-lacZ and expression of endogenous Dct as markers for melanoblasts. As shown in Figure 1G and Figure S1–S3, we succeeded in chronologically analyzing the distribution of migrating melanoblasts, which first appear in the developing dermis, then in the epidermis, and finally in the sweat buds, and of their eventual colonization in mature sweat glands in volar skin. This suggested that melanoblasts that colonize the sweat glands during development are maintained in a dormant and immature state with profound downregulation of lineage markers after development until they are reactivated during the physiological aging process.

Follicular McSCs, which reside in the bulge/sub-bulge area of hair follicles, are maintained in a quiescent, inactivated state during the hair cycle except for their activation for their self-renewal at the anagen growth phase (Nishimura et al., 2002, 2005, 2010). Upon genotoxic stress or aging, the follicular McSCs ectopically differentiate within the stem cell niche without renewing themselves (Inomata et al., 2009). We hypothesized that dormant immature melanoblasts that respond to aging and/or genotoxic stress exist in the sweat glands and generate mature melanocytes. To test this, we irradiated young Dct-lacZ transgenic (tg) mice and searched for melanocytic cells in mouse volar skin. Melanin-containing mature melanocytes appeared in sweat glands of the mouse volar skin within a week after 5 Gy ionizing irradiation (IR) (Figure 1K) and persisted for more than a month (Figure 1L, M), while those cells were only occasionally found in non-irradiated control skin (data not shown). As DNA damage accumulates in long-lived stem cells during aging (Nijnik et al., 2007; Rossi et al., 2007; Ruzankina et al., 2007), the above phenomenon induced by IR and the similar phenomenon associated with aging (shown in Figure 1E, I) suggest that both aging and genotoxic stress stimulate a putative stem/precursor cell population to supply pigment-producing melanocytes to the epidermis through the dermal-epidermal ducts in acral volar skin.

To identify putative stem/precursor cells which would explain the above phenomena, we developed Dct-H2B-GFP transgenic mice, in which non-dividing/slow-cycling melanocytic cells stably retain the expression of histone-GFP
fusion protein under control of the Dct promoter with the LCR element once the promoter is activated (Figure 1N and Figure S4A) (Ganss et al., 1994; Mackenzie et al., 1997; Kanda et al., 1998). As shown in Figure S4B–Y, all known melanocyte lineage cells can be efficiently tagged using GFP in these transgenic mice. The distribution pattern of GFP expressing cells was identical with that of lacZ expressing cells in Dct-lacZ transgenic mice during development (Figure S4B–J). In mouse volar skin, GFP + melanoblasts appear in the epidermis by embryonic day (E) 17.5 (Figure 1O, U), then migrate down into the epidermal buds, and finally colonize the tip of immature sweat glands (Figure 1P–R, U). This area then develops into the secretory portion (SP) of the sweat gland (Figure 1S, T). Similar migration processes were observed with Dct-lacZ transgenic mice (data not shown).

To identify the population which just colonizes the SP of sweat glands, we analyzed footpads of neonatal mice. The GFP + melanoblasts in sweat glands are located well inside the basement membrane and are surrounded by K8+ luminal keratinocytes and underlying SMA+K5+ myoepithelial cells (Figure 2A–C). These GFP + cells in the neonatal skin express melanocyte lineage marker proteins such as Mitf and Pax3 (Figure 2I, K) and downstream target genes of Mitf including Dct, tyrosinase-related protein 1 (Tyrp1), tyrosinase (Tyr), and Silv/Pmel17 (Figure 2D–F and data not shown), but none of the following non-melanogenic cell markers tested including Nestin (a neural

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**Figure 1.** The existence of melanocytic sources in human and mouse sweat glands of acral skin. (A, B) Organization of eccrine sweat glands in mouse and human acral skin. The hematoxylin-eosin-stained sections are from human finger skin (A) and mouse footpad skin (B). (C) Schematic diagram of a sweat gland. The structure is similar both in mice and in humans. (D, E) Hematoxylin-eosin-stained sections of footpad skin of 7-week-old (7wo) (young) (D) and 2-year-old (2 yr) (old) (E) mice. Melanin-containing cells are observed in the sweat duct, but not in the secretory portion (SP), of 2-year-old mice (arrowheads). (F) Structure of the LacZ reporter under control of the melanocyte-specific Dct promoter (Dct-LacZ). (G–I) LacZ-stained skin of the footpad of Dct-LacZ tg mice [G; 5 day old (P5), H; 7 week old (7wo) and I; 2 year old (2yo)]. LacZ+ melanocytic cells are found in the sweat duct and SP at P5 mice (arrows) and old mice, while no LacZ+ cells are detectable at 7wo (H). (J–L) LacZ-stained skin of the footpad of 5 Gy-irradiated Dct-LacZ tg mice. Dct-LacZ+ cells (arrow) appear in the sweat duct at 1 week (1 wk) after IR (K), and melanin-containing cells (arrowheads) appear in the sweat duct at 4 weeks (4 wk) after IR (L). (M) Stained with hematoxylin-eosin, (M) Fontana-Masson staining for the detection of melanin. (N) Structure of the H2B-GFP reporter under control of the melanocyte-specific Dct promoter and the LCR element (Dct-H2B-GFP). (O–T), Distribution of H2B-GFP expressing cells in the developing footpad skin of Dct-H2B-GFP tg mice at different stages. GFP+ cells migrating from the epidermis (green; arrows) toward the SP (the area encircled by the dotted line) of glands. Keratin 5+ myoepithelial/basal keratinocyte immunostaining (red) defines the gland structure. GFP+ cells are found in the epidermis at E17.5 (O) and subsequently in the sweat gland primordial buds at E18.5 (P). At postnatal day (P) 1 (Q), GFP+ cells are found in the developing ducts. The majority of GFP+ cells are settled in the SP of glands between P5 (R), 2 weeks old (2wo) (S) and 7 weeks old (7wo) (T) after birth. Nuclei are counterstained with DAPI (blue). The insets show magnified images of GFP expressing cells. (U) The distribution and number of GFP+TuJ1+ cells in the acral skin of each developmental stage. n = 3 mice each genotypes. Error bars represent SD of three independent experiments. Statistical significance at the level of P < 0.01.
progenitor marker), p75 (a neural crest progenitor marker), TuJ1 and Pgp9.5 (a peripheral nerve marker), GFAP and MPZ (Schwann cell markers), and keratin 20 (a Merkel cell marker) (Figure 2G, H, Figure S2 and data not shown). Furthermore, no significantly close association between these GFP+ melanoblasts that express melanosome markers and p75 or TuJ1 was observed.

Figure 2. Identification of slow-cycling melanoblasts located in the SP of sweat glands in acral skin using Dct-H2B-GFP transgenic (tg) mice. (A) GFP+ cells (green) in the SP (the area encircled by the dotted line) are melanin-17 rounded cells as shown in DIC image. (B, C) GFP+ cells (green) are located between keratin 8+ glandular cells (red) (B) and α-SMA+ myoepithelial cells (red)(C). GFP+ cells in the developing SP (the area encircled by the dotted line) co-express melanosomal proteins, TRP1 (D), TYR (E) and Pmel17/Silv (F) but not TuJ1 (G) or GFAP (H). TYR-immature GFP+ cells were also occasionally found in the SP (E, dotted arrow). Nuclei are counterstained with DAPI (blue). (I–P) Down-regulation of the lineage markers Pax3, Mitf, or Dct was observed in GFP+ melanoblasts in the SP of adult mice (I–N). GFP+ melanoblasts were kept in an MCM2-1 quiescent state in the adult stage (J, L, P). (Q, R) The number of GFP+TuJ1+ cells (arrowhead) in the dermis was profoundly reduced after sciatic denervation, while GFP+TuJ1+ cells (arrows) were maintained in the SP. (S, X) Immunostaining of the footpad sections of Mitf+/–;Dct-H2B-GFPtg/+ (S–U), and Mitf+/–;Dct-H2B-GFPtg/+ (V–X) mice for GFP and TuJ1 expression. GFP+TuJ1+ melanoblasts (arrows) were found in the SP of Mitf+/– mice, but not in Mitf+/– mice. GFP+TuJ1+ neurons exist outside of the glands in both types of mice. (Y) The average number of GFP+TuJ1+ melanoblasts per gland in a single footpad and per single hair follicle and of the dermal GFP+TuJ1+ melanocytes (arrows) were found in the SP of Mitf+/– mice, but not in Mitf+/– mice. GFP+TuJ1+ melanocytes (arrows) were found in the SP of Mitf+/– mice, but not in Mitf+/– mice.
ergic genes including \textit{Dct} protein during development with \textit{TuJ1}+ non-melanocytic cells in the dermis has been found during the earlier developmental processes (Figure S1–S3 and data not shown). These data indicate that GFP+ cells that migrated from the epidermis into the developing sweat glands are melanocyte lineage cells.

Next, we examined the transition of the GFP+ cell state after the neonatal stage. GFP+ melanoblasts are maintained in an immature unpigmented state in the SP with drastic downregulated expression of global melanogenic genes including Pax3 and Mitf and its downstream genes such as Dct, Dct-lacZ, and Tyr by 7 weeks after development without differentiating into mature melanocytes (Figure 2I–N). At the same time, they lose expression of Mcm2, a marker for the non-Go cell state (Kingsbury et al., 2005) after the neonatal stage, indicating that these cells go into the quiescent, inactivated state after development (Figure 2Q, P). It is notable that McSCs that reside in the hair follicle bulge also downregulate expression of Dct-lacZ and melanogenic genes when they enter the quiescent state (Nishimura et al., 2010) while maintaining high levels of GFP expression throughout the hair cycle (Figure S4 and data not shown). Thus, we concluded that melanoblasts are maintained in an immature inactivated state in the lower sweat glands after development similar to McSCs in the hair follicle bulge area.

The other GFP+ population distinguished from the melanocyte lineage in acral volar skin is the \textit{TuJ1}-expressing GFP+ cell population in the dermis outside of sweat glands (Figure 2G, arrowhead). Most of these cells appear at around prenatal stage in the dermis (data not shown) and co-express the neuronal markers \textit{TuJ1} and less frequently Pgp9.5, but not Nestin, mature Schwann cell markers such as GFAP and MPZ/P0 nor the neural crest progenitor marker, p75 (Figure 2G, H, Figure S5 and data not shown). To confirm the identity of these dermal GFP+ cells, we performed sciatic denervation. As shown in Figure 2Q, R, the GFP+ \textit{TuJ1}+ cells disappeared from the dermis, suggesting that the dermal GFP+ cells are peripheral neurons or associated neuron-dependent precursor cells (Figure 2Q, R and Figure S6). Then, we analyzed \textit{Mitfe/ce} mutant mice which specifically lack melanocyte lineage cells (Steingrimsson et al., 1994; Zimring et al., 1996). We found that \textit{Mitfe/ce} mice with the GFP reporter transgene lack GFP+ cells in the sweat glands (Figure 2S–Y). On the other hand, GFP+ \textit{TuJ1}+ cells are maintained in the dermis with neuronal morphology in \textit{Mitfe/ce} mice as well, demonstrating that the GFP+ cells in the dermis are non-melanocytic cells. To further confirm the lineage identity of GFP+ cells in the sweat glands, we took advantage of ACK2, a neutralizing antibody against Kit (c-Kit), which has been used to deplete amplifying melanoblasts in developing skin (Nishimura et al., 2002). ACK2 treatment of the neonatal skin depleted almost all GFP+ cells in sweat glands (Figure 3A, B and Figure S7), while ACK2 treatment after the neonatal stage, such as 7 weeks after birth, did not deplete GFP+ cells in the sweat glands (Figure 3B). This demonstrates that the glandular GFP+ cells are \textit{bona fide} melanoblasts which develop in Kit-dependent manner but become inactivated to go into the quiescent and immature state after the neonatal stage and survive in a Kit-independent manner similar to slow-cycling McSCs in hair follicles (Nishimura et al., 2002).

Self-renewal and quiescence are important features of somatic stem cells including hair follicle McSCs (Cotsar-
elis et al., 1990; Fuchs et al., 2004; Nishimura et al., 2002). To examine whether the GFP* cells identified here are slow-cycling cells or quiescent cells, we performed pulse-chase experiments with BrdU using our transgenic mice. As shown in Figure 3C, D, we found that GFP* cells in sweat glands are able to retain the BrdU label for more than a month, while most other cells in volar skin lost their BrdU label earlier. The BrdU retention level was stably high in the majority of GFP* melanoblasts at P25 but became steadily diluted in a subset of them at P50 and was further diluted to barely detectable levels at P100 (Figure 3C, D and Figure S8). Adjacent GFP* cells, which show an almost identical dilution level, were also found at P50 (7wo) and show unpigmented immature cell bodies.

Figure 4. Melanoblasts in the SP of sweat glands maintain themselves and provide proliferating and differentiating melanocytes to the epidermis in response to IR or oncogenic stress. (A, B) Transient cell cycle entry and commitment of GFP* cells in the SP. At around 48 hr after IR, expression of MCM2 and TRP1 was found transiently in some GFP* cells located within the SP (arrow). (C–V) Behavior of GFP* cells in sweat glands after 5 Gy IR. Immunostaining for keratin 5 (K5) was performed on footpad skin sections in Dct-H2B-GFP tg mice to chase GFP* cells (green) located within the glands after IR. While no GFP* cells exist in the duct or the epidermis in control mice (C–F), GFP* cells (arrows) exist in the dermal-epidermal duct and the epidermis after IR at 1 week (1 wk) (G–J). Bright view images merged with GFP images are shown for the epidermis (solid-line square) (D, H), the sweat duct (marked by the long-dashed line square) (E, I), and SP (the short-dashed line square) (F, J) on the right side of the large panel. Melanogenesis can be observed only in the duct or in the epidermis (H, I). K–P and Q–V, MCM2, and TYR immunostaining images are shown, respectively, on the right side of the figure. Most GFP* cells (arrows) at the SP are MCM2- (M, P), while IR-induced ductal or epidermal GFP* melanocytes (arrows) are MCM2+ (red) (N, O) and TYR* (red) (T, U, IW). The number of GFP* cells located in the SP remained stable after IR (graph in the upper column), while the number of GFP* cells located within the duct of glands after IR was significantly increased (graph in the lower column). * statistical significance at the level of P < 0.05. (X) IR-induced pigmentation of the upper portions of glands after IR was dramatically impaired when mice were pretreated with ACK2 at the neonatal stage. Error bars represent SD of three independent experiments. * statistical significance at the level of P < 0.01. Nuclei are counterstained with DAPI (blue). (Y) Pigmented GFP* cells (red arrow) were found in the duct at 4 weeks after DMBA, while GFP* cells in the SP were kept in an unpigmented state.
(Figure S8). Only a few unpigmented GFP+ cells reside in the SP in each footpad (Figure 3D) and maintained well from the neonatal to the adult stage (Figure 3C). Most of these unpigmented GFP+ melanoblasts are usually negative for the non-Go cell state marker Mcm2 and Ki67 after development (Figure 2O, P and data not shown). Therefore, these data collectively indicate that the GFP+ immature melanoblasts are an extremely rare population in tissues and are normally kept in a quiescent state after development but occasionally undergo self-renewal in the SP of the sweat glands even under normal physiological conditions. As mice that congenitally lack melanocyte lineage cells (Cable et al., 1995) are able to sweat from the footpads (Figure S9) and maintain the integrity of sweat glands (Figure 2S–X and data not shown), we assumed that the glandular melanoblasts in the SP may not be essential for the homeostasis and sweating function of sweat glands but could become pathogenic when abnormally activated.

We then determined whether the quiescent GFP+ cells were able to generate pigmented melanocytes upon activation. As aged sweat glands occasionally contain pigmented melanocytes with γ-H2AX foci formation outside of the niche (data not shown), we hypothesized that melanocyte precursors are transiently activated to respond to endogenous or exogenous genomic stress during aging. To test this, we performed chronological analysis for the fate of GFP+ melanoblasts after 5 Gy IR. GFP+ cells in the SP showed transient expression of Mcm2, a marker for the non-Go state (Kingsbury et al., 2005) (13.3 ± 4.7% in irradiated mice versus 0% of control mice [n = 3]) (Figure 4A) and melanogenic genes such as Trp1 (6.3 ± 4.5% in irradiated mice versus 0% of control mice [n = 3]) at 48 h after IR (Figure 4B). Subse-

![Figure 5](image-url)

**Figure 5.** Identification of melanoblasts in the SP of human volor sweat glands as an expandable source of early acral melanoma. (A–L) Localization of melanocyte/melanoma cells and their cell cycle status in human volor sweat glands. Human normal volor skin (A–C) and early melanoma skin (D–L) were immunostained for the lineage markers, MART1 (shown in green) and MCM2 (in red). A small number of MART1+MCM2− cells were found in the epidermis close to the sweat duct within the ridge area of the skin (A) and the SP (C), but not within the duct (B). (D) A nest of MART1+MCM2− unpigmented melanoma cells (white arrows) and MART1+MCM2low pigmented melanoma cells (red arrows) was found in the epidermis of the ridge area of the lesional skin of melanoma in situ. MART1+ cells are aligned contiguously throughout the duct (arrow; E) and SP (arrow; F) area of a particular sweat gland within the center of the lesion. At the periphery of the lesion, MART1+MCM2− cells were found in the epidermis (arrow; G), but not in sweat glands (arrow; I). At the intact margin, there are MART1+MCM2− melanoblasts, but no MART1+MCM2− cells in the epidermis (J). The SP of an identical gland contains a MART1+MCM2− melanoblast (L). Note that MART1+ cells were found in the duct of particular glands at the lesion, but not in the surrounding glands at the margin of the lesion (H and K). Magnified images of MART1+ cells are shown in the insets. Nuclei are counterstained with DAPI. (M) Table showing the distribution and proliferative status of MART1+ cells in human acral skin samples. In normal control skin samples, a small number of MART1+ cells were found in the SP and most of them are MCM2−, while those in early melanoma lesions are MCM2+ and MART1+MCM2+ proliferative cells were found in the sweat duct and SP of particular glands.

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We detected MART1+ unpigmented melanoblasts with increased sensitivity (Figure S10). As shown in human acral volar skin using immunohistochemical staining, the upper sweat glands in response to oncogenic or other genotoxic stimuli. These data indicate that the GFP+ cells in the SP maintain and renew themselves and are also able to generate differentiated progeny even in the absence of neuronal cells in the dermis. Furthermore, when GFP+ cells in the SP are depleted by administration of ACK2 at the neonatal stage, mature pigmented melanocytes did not appear in the sweat glands or in the epidermis of adult acral skin even after 5 Gy IR (Figure 4I, and data not shown), underlining the regenerating capability of the McSCs and their contribution to skin pigmentation.

Oncogenic stimuli such as exposure to carcinogens/mutagens and genetic alterations involved in human melanoma induce melanoma formation in mice (Larue and Beermann, 2007; Walker et al., 2011). We tested whether carcinogens also activate McSCs. As shown in Figure 4Y, DMBA application on mouse footpads also induced the appearance of pigmented GFP+ cells in sweat glands. These data clearly demonstrate that McSCs provide their pigmented progeny with the upper sweat glands in response to oncogenic or other genotoxic stimuli.

We searched for a similar McSC-like population in human acral volar skin using immunohistochemical staining with increased sensitivity (Figure S10). As shown in Figure 5, we detected MART1+ unpigmented melanoblasts, which are located specifically in the SP of human sweat glands (Figure 5C and Figure S11C, F). Expression of other melanogenic genes (Figure S12 and S13) and MCM2 were not detectable in those cells in the adult skin (Figure 5C and data not shown). We also searched for a similar population in the sweat glands of non-volar skin areas such as the face and abdomen, but such melanoblasts were undetectable in those areas (data not shown). These data indicate that the population resides preferentially in the sweat glands of volar skin, at least in physiological conditions, and these immature and quiescent melanoblasts are most likely to be human McSC-like precursor cells.

Human acral skin forms a ridge and furrow pattern on the surface. Early acral melanoma shows preferential proliferation/pigmentation in the ‘ridge epidermis’, which corresponds to crista profunda intermedia (CPI) situated under the surface ridge and contains sweat ducts in the middle of the CPI in human acral epidermis. To address the possibility that human melanoma precursors reside in the sweat glands of acral skin, we selected cases in which the skin sections cover the whole sweat gland structure and analyzed the distribution of melanocyte lineage cells in human acral melanoma. Notably, these lower-positioned MART1+MCM2+ cells in the SP and the lower duct are distributed contiguously or sparsely from the SP to the ridge epidermis only in particular sweat gland(s) but not in other surrounding glands in the lesion (Figure 5D–L). We examined 10 serial sections for each early melanoma originally evaluated as melanoma in situ (Figure 5M, samples D–F) and more advanced melanoma (samples G and H). Each case of early acral melanoma possesses at least one sweat gland, which contains proliferating MART1+ cells distributed in the sweat gland starting from the SP to the intra-epidermal duct within the lesion (Figure 5D–F and M). Although it is believed that the distribution of early acral melanoma cells is limited within the epidermis (‘in situ’), we found their scattered or contiguous distribution in some sweat glands. Importantly, the SP and the duct of other surrounding sweat
glands are often not colonized by MART1+MCM2+ cells. Considering the features of mouse sweat gland McSCs, these findings suggest that human sweat gland melanocyte precursors are also only transiently activated for their renewal upon a stress but are able to generate abnormal melanocyte–melanoma precursors which keep proliferating in the niche in particular sweat glands during aging. As the structure of these sweat glands is well maintained, the cycling precursors in the SP are most likely to keep generating their amplifying progeny which migrate toward the epidermis where they mature into histologically recognizable melanoma cells and spread horizontally within the epidermis with some colonization preference to the ridge epidermis in early acral melanoma (Figure S14).

CCND1 gene amplification has been found in 23.8–44.4% of early acral melanoma cases (North et al., 2008; Sauter et al., 2002; Takata et al., 2005), yet the exact distribution of the CCND1-amplified melanoma cells in the volar skin has not been studied. We thus tested the distribution of CCND1-amplified cells in the lesional skin by DNA-FISH analysis. As shown in Figure 6A, CCND1-amplified cells were found in the SP in multiple cases of early acral melanoma, which were originally evaluated as melanoma in situ (n = 3/4 cases) with their contiguous distribution along the duct up to the epidermis. It is

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Melanocyte stem cells in sweat glands

Figure 6. CCND1 gene amplification in the SP within a melanoma lesion and a schematic for McSc (mice) or McSC-like cell (human) development, maintenance, and melanomagenesis. (A) Histograms showing the distribution of copy number variation of 11q13 CCND1 genomic regions in the tumor center of early acral melanomas. Colored bars (red, blue, green, and yellow) show individual samples derived from acral melanoma cases, while colored bars (black and white) show control normal samples. The Cep11 centromeric region was used to normalize the number of FISH signals between cells at different cell cycle stages. (B) Box plot showing the copy number differences between the epidermis and SP from melanoma and normal skin. Copy number amplification of the CCND1 gene is found in the SP of sweat glands within the lesion but not in the normal skin and is significantly lower than that seen in the epidermis. *, statistical significance at the level of P < 0.05. (C) The fate and behavior of melanocyte lineage cells in sweat glands are summarized and combined with a hypothetical schematic for melanoma initiation from McSCs. Proliferating melanoblasts (red) that colonized the SP of the sweat glands enter a quiescent (Go) state just after development and become McSCs maintained in a dormant state (blue). These cells are activated transiently by extrinsic or intrinsic stresses such as genotoxic stress and during the normal aging process. Conversely, the corresponding population in the SP maintains its immaturity and cycling activity to renew the population and to generate their amplifying progenies which acquire further genetic alterations during their migration and maturation into noticeable melanoma cells in the epidermis.
notable that the \textit{CCND1} amplification level in melanoma cells is significantly higher in the epidermis than in the SP (Figure 6B and data not shown). The opposite pattern (less amplification in the epidermis) has not been found in any acral melanoma cases examined. The significant increase in \textit{CCND1} gene amplification level in the epidermis indicates that melanoma cells in the SP not only maintain themselves in an immature unpigmented state in particular gland(s) but also can provide their amplifying progeny, which then undergo more amplification in the epidermis. We thus concluded that the SP of acral sweat glands serves as the reservoir of early melanoma precursors which can generate noticeably mature melanoma cells with more oncogenic alterations and expansion in the epidermis (Figure 6C).

Discussion

In this study, we identified McSCs, which possess somatic stem cell characteristics, in sweat glands in murine volar skin. We found that the SP of the sweat glands are the anatomical niche not only for those melanoblasts but also for early human melanoma precursors with \textit{CCND1} gene amplification. As far as we know, this is the first identification of the niche for oncogenically mutated early cancer cells in human solid cancer tissues, while leukemogenic mutations such as \textit{BCR-ABL} mutations have been found in hematopoietic stem cells (Rossi et al., 2008), the exact niche for early cancer cells remain unknown even in solid cancer as far as we know. Visualy detectable pigmentation of the earliest melanoma in situ lesions such as those with a few mm in diameter is the suitable system to tackle this problem and allowed us to search for the niche where melanoblasts with early genetic melanoma alteration are hiding deep in the skin.

McSCs in sweat glands are an extremely rare population in the skin compared with McSCs in hair follicles and are more resistant to X-ray, but possess many similar stem cell characteristics. They are normally immature and slow-cycling but transiently renew themselves not only physiologically but also in response to genotoxic stress and provide an amplifying and differentiating progeny into the epidermis through the ascending sweat duct which connect the SP to the epidermis. As those McSCs are maintained in an immature and quiescent state in the SP, where sweat gland keratinocytes are also maintained in a quiescent state (Lu et al., 2012; Nakamura and Tokura, 2009), both in mice and in humans, the SP seem to provide a special niche microenvironment for a fixed number of McSC-like cells in an inactivated immature state (shown schematically in Figure 6C scheme). In contrast, immature but amplifying McSC-like cells or immature melanoma precursor cells in human volar skin were found in the SP of particular sweat glands of early acral melanoma lesions. As these McSC-like cells do not form a tumor mass within the SP, but amplifying and differentiating melanoma cells are distributed in the upper area of the gland(s) and the surrounding epidermis, it is most likely that mutated McSC-like cells in the SP keep providing their amplifying and differentiating progeny toward the epidermis as tumor-initiating cells or cancer stem cells. The melanoma cell distribution pattern, the \textit{CCND1} amplification pattern in the SP versus in the epidermis of those early melanoma cases, and the immaturity of those melanoma cells in the SP collectively suggest that the McSC-like cells in the SP are the initial origin of human acral melanoma at least in some cases. As copy number amplification requires the DNA replication process (Hastings et al., 2009), it is most likely that these mutated McSC-like cells in the SP of particular sweat gland(s) generate more amplified subclones which expand for further progression outside of their niche mainly in the epidermis during early melanomagenesis. The origin of melanoma cells, thus, may explain the preferential proliferation and pigmentation of acral melanoma cells in the CPI around epidermal ducts. However, our data do not exclude the possibility that some acral melanomas also originate directly from epidermal melanocytes and migrate down to the SP of some sweat glands to colonize the niche and become unpigmented and obtain the immature property common to McSCs upon their colonization of the SP (Figure 6C and Figure S14). Recently reported melanoma variant called ‘syringotropic melanoma’, which shows the prominent involvement of sweat glands (Zembowicz and Kafanas, 2012) may represent one of the two possibilities. The radial distribution pattern of melanoma cells from the sweat glands suggests that melanoma cells can be originated from McSC-like cells in the SP of sweat glands, but further studies are necessary. In either case, our data indicated that sweat glands provide an anatomical niche not only for normal McSC-like cells but also for early melanoma cells in the volar skin and suggested that renewing melanoma cells with early genetic alteration (s) in the SP can further evolve into subclones with more genetic alterations within the epidermis.

Moreover, the fact that acral McSC-like cells are activated to renew themselves upon genomic stress such as IR may explain the IR-refractory characteristics and genomic instability frequently found in early acral melanoma (Curtin et al., 2006). As amplifying McSC-like cells or melanoma precursors with an oncogenic mutation reside in the SP located deep in the subcutaneous fat, sufficient deep surgical excision of the niche lesion and accurate staging of acral melanoma with careful evaluation of tumor depth and involvement of McSC-like melanoma precursors may improve the prognosis of melanoma patients. Further studies on the melanocyte precursors as a melanoma origin or tumor-initiating cells, their topographical dynamics for clonal evolution, and their potential for therapeutic target are necessary to combat this devastating cancer.
Methods
See Supporting Information for detailed methods.

Mice
Dct-H2B-GFP mice were newly generated as described in Supporting Information. Dct-LacZ transgenic mice were a kind gift from Dr. Ian Jackson. Mitf-/- mice were a kind gift from Dr. M. Lynn Lamoreux (Zimring et al., 1996).

BrdU injection
Mice were injected subcutaneously at 24-h intervals with 20 µl BrdU solution in PBS at 2 mg/ml.

ACK2 treatment
For the treatment of neonatal mice, 200 µg ACK2 was injected into the peritoneal cavity at days 0, 2, and 4 after birth (Nishimura et al., 2002). For the treatment of adult mice, 1 mg ACK2 was injected into the peritoneal cavity at the days 1, 3, 5, 7, and 9 after plucking at P49.

Irradiation
Mice were irradiated at a dose of 5 Gy using 1.0-mm aluminum filters.

Histology
Immunohistochemistry was performed as previously described (Inomata et al., 2009).

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Conflict of interests
The authors declare no competing financial interests.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Distribution of developing melanoblasts and peripheral neurons in the distal hindlimb of mouse embryos.

Figure S2. Migration of developing melanoblasts into the epidermis for their eventual colonization of the footpad epidermis in mice.

Figure S3. Dct-expressing melanoblasts in sole skin do not co-express a neural lineage marker TuJ1.

Figure S4. Characterization of Dct-H2B-GFP transgenic (tg) mice.

Figure S5. GFP+ cells in the sweat glands of Dct-H2B-GFP tg mice are distinct from Nestin+ neural progenitors, p75+ neural crest progenitors, TuJ1+ neurons, and GFAP+ Schwann cells.

Figure S6. GFP+ melanoblasts in the SP of sweat glands of denervated footpads provide their pigmented progenies to the epidermis after IR.

Figure S7. Depletion of developing McSCs in acral skin eradicates the IR-induced appearance of melanocytes in sweat glands and in the epidermis.

Figure S8. Slow-cycling GFP+ melanoblasts exist in the SP area of sweat glands in adult acral skin under physiological conditions.

Figure S9. Sweating response is detectable in melanocyte-deficient KitW/W− mice.

Figure S10. Low level of MART1 is expressed by quiescent McSC-like cells in human hair follicles.

Figure S11. Human MART1+ melanoblasts are located in the epidermis and SP of sweat gland in the acral skin.

Figure S12. Expression of most melanogenic proteins is undetectable in human melanoblasts in the SP of sweat glands in infant skin.

Figure S13. Human melanoblasts in the SP of sweat glands do not express HMB45, TYR, or Ki67 in adult skin.

Figure S14. Hypothetical scheme of melanomagenesis in human acral skin.