Resident progenitor cells in mammalian skin generate new cells as a part of tissue homeostasis. We sought to identify the progenitors of Merkel cells, a unique skin cell type that plays critical roles in mechanosensation. We found that some Atoh1-expressing cells in the hairy skin and whisker follicles are mitotically active at embryonic and postnatal ages. Genetic fate-mapping revealed that these Atoh1-expressing cells give rise solely to Merkel cells. Furthermore, selective ablation of Atoh1+ skin cells in adult mice led to a permanent reduction in Merkel cell numbers, demonstrating that other stem cell populations are incapable of producing Merkel cells. These data identify a novel, unipotent progenitor population in the skin that gives rise to Merkel cells both during development and adulthood.

Introduction

Mammalian skin is a dynamic organ that provides protection against a variety of environmental insults. Damage to the skin caused by these stressors must be repaired through constant skin cell replacement. Skin integrity is maintained by a heterogeneous population of resident progenitor cells capable of self-renewal and production of diverse cell types that make up hair follicles, glands, and interfollicular epidermis (Ghazizadeh and Taichman, 2001; Ito et al., 2005; Fuchs, 2007; Jaks et al., 2010; Solanas and Benitah, 2013).

In addition to its role as a barrier, skin also houses multiple somatosensory receptors, each tuned to detect different forms of mechanical stimuli. The Merkel cell–neurite complex is one such receptor located at the epidermal–dermal border of mammalian skin around whisker follicles, in hairy skin within specialized structures called touch domes and in glabrous (non-hairy) skin of the hands and feet (Halata et al., 2003). Embryologically, Merkel cells originate from epidermal progenitors and require expression of the basic helix-loop-helix transcription factor Atoh1 for their specification (Maricich et al., 2009; Morrison et al., 2009; Van Keymeulen et al., 2009). Atoh1 expression is maintained throughout development and in mature Merkel cells (Lumpkin et al., 2003).

Adult Merkel cells are postmitotic (Moll et al., 1995). However, quantitative, morphological, and fate-mapping studies suggest that Merkel cell numbers in adult hairy skin oscillate with the hair cycle, implying that Merkel cells turnover throughout an organism’s lifespan (Nafstad, 1987; Moll et al., 1996a; Nakafusa et al., 2006; Van Keymeulen et al., 2009). Mitotically active progenitors are the likely source of new Merkel cells, as a small percentage of Merkel cells are labeled several days after administration of nucleotide analogues (Mérot et al., 1987; Vaigot et al., 1987; Mérot and Saurat, 1988; Woo et al., 2010). Recent work in hairy skin has suggested that these progenitors are either multipotent stem cells located in the hair follicle bulge region or bipotent progenitors found among the touch dome keratinocytes (Van Keymeulen et al., 2009; Woo et al., 2010; Doucet et al., 2013). Accurate identification of Merkel cell progenitors is crucial because of the potential for these cells to act as the cellular origin of Merkel cell carcinoma (MCC), a rare but devastating disease that currently has no targeted therapies (Sidhu et al., 2005; Kuwamoto, 2011; Tilling and Moll, 2012).
Because Atoh1 expression is required by mitotic precursors of other Atoh1-lineage cell populations such as cerebellar granule cells, dorsal commissural interneurons, and secretory cells of the gut (Akazawa et al., 1995; Helms and Johnson, 1998; Yang et al., 2001), we hypothesized that the immediate Merkel cell progenitor would likewise express Atoh1. We used multiple techniques in different in vivo genetic mouse models to lineage trace and examine the proliferative capacity of Atoh1+ cells in hairy skin during embryogenesis and adulthood. We found that a subpopulation of Atoh1+ cells proliferates, contributes solely to the generation of Merkel cells, and cannot be replaced by other resident stem/progenitor cells in the skin. Our data identify a new progenitor population that is uniquely responsible for the generation and maintenance of Merkel cells.

Results

Adult Merkel cell precursors express Atoh1 and are unipotent

Several lines of evidence suggest that mature Merkel cells have a finite lifespan, implying that they are replaced by precursor cells located in the skin (Moll et al., 1996a; Nakafusa et al., 2006; Van Keymeulen et al., 2009; Doucet et al., 2013). To determine whether these precursors were Atoh1+, we lineage traced Atoh1+ cells in postnatal day 21–28 (P21–P28) Atoh1CreER-T2/++;ROSA26LoxZ mice by administering high-dose tamoxifen (250 mg/kg) for a consecutive 3 d during the growth phase (anagen) of the first hair cycle. We found Xgal+ (5-bromo-4-chloro-indolyl-β-d-galactopyranoside) cells only in the expected locations for Merkel cells in the hairy skin and whisker pads 3 (n = 3) and 9 (n = 1) mo after tamoxifen administration (Fig. 1, A–B’), times after the completion of multiple hair cycles (Alonso and Fuchs, 2006). To confirm that these β-galactosidase (β-Gal)+ cells were Merkel cells, we communostained for β-Gal and the Merkel cell marker Keratin 8 (K8; Fig. 1, C–D’; Vielkind et al., 1995). 3 mo after tamoxifen administration, 93.5 ± 1.7% and 99.2 ± 0.4% of K8+ cells in hairy skin and whisker follicles coexpressed β-Gal, respectively; these percentages were 91.5% and 98.1% at 9 mo (≥200 hairy skin and ≥500 whisker follicle K8+ cells counted/mouse; Fig. 1 E). All β-Gal+ cells were also K8+, and nearly all K8+ cells (99.0 ± 0.4%, ≥150 K8+ cells/mouse, n = 3 mice) were also Keratin 20+ (K20; Fig. S1, A–A’), in agreement with other studies (Eispert et al., 2009; Lesko et al., 2013). These data suggest that adult Merkel cells arise from Atoh1+ progenitors.

Previous studies concluded that K8+ cells are postmitotic (Vaiɡot et al., 1987; Mérot and Saurat, 1988; Moll et al., 1996b; Woo et al., 2010). Therefore, we were surprised that we never found β-Gal+/K8− cells in Atoh1CreER-T2/++;ROSA26LoxZ mice. To determine whether this might be an issue with the β-Gal reporter, we examined K8 expression in the Atoh1 lineage by administering high-dose tamoxifen to P21 Atoh1CreER-T2/++;tdTomato+ mice and harvesting tissue 1 wk later. We found that all tdTomato+ cells were also K8+ but that 1.15 ± 0.5% of tdTomato+ cells expressed very low levels of K8 (≥150 tdTomato+ hairy skin cells/mouse, n = 3 mice; Fig. 1, F–G’). This suggested that K8+ cells could proliferate (see next section).

Embryonic Merkel cell precursors express Atoh1 and are unipotent

Atoh1+ cells are first observed in trunk skin and whisker follicles at embryonic day 14.5 (E14.5) and increase in number throughout late embryogenesis (Ben-Arie et al., 2000). We hypothesized that these early appearing Atoh1+ cells were progenitors responsible for Merkel cell generation. To test this possibility, we lineage traced Atoh1+ cells in Atoh1CreER-T2/++;tdTomato and Atoh1CreER-T2/++;Rosa26Tomato embryos. We limited recombinant to the day of tamoxifen administration by administering a single low dose (10 mg/kg) to pregnant dams at E15.5 and then harvested tissue 1 (E16.5) or 3 (E18.5) d later. We found ~71% more tdTomato+ cells/touch dome at E18.5 than at E16.5 (18.0 ± 1.2 vs. 10.5 ± 0.6; n = 20–30 touch domes/embryo from 3–6 embryos/age; P = 4 × 10−4, t test; Fig. 2, B–D), suggesting that Atoh1+ cells proliferated between these ages (Fig. 2 A). As expected, immunostaining for K8 demonstrated that the mean number of Merkel cells per touch dome also increased between E16.5 and E18.5 (13.8 ± 0.7 and 21.3 ± 0.8, respectively; P = 5.9 × 10−6, t test). The proportion of K8+ cells coexpressing K20 also increased between E16.5 and E18.5 (22.9 ± 0.7% and 46.8 ± 2.6%, respectively; n = 3 mice/age; P = 9.3 × 10−4, t test; Fig. S1, B–C’). These data indicate that at least some Atoh1+ cells present at E15.5 are mitotically active and continue to divide after E16.5.

The Atoh1+ lineage separates from other skin lineages in late embryogenesis

Embryonic Atoh1+ cells are derived from the Keratin 14 (K14) lineage (Morrison et al., 2009; Van Keymeulen et al., 2009). Given our data suggesting that the Atoh1+ population expanded between E16.5 and E18.5, we wondered when Atoh1+ skin precursor cells stopped producing Atoh1+ Merkel cell precursors. We administered high-dose tamoxifen to E15.5 Atoh1CreER-T2/++;ROSA26Tomato mice and harvested tissue at P28 (n = 2) and P168 (n = 1). If Atoh1+ cells contributed to the Merkel cell lineage after E15.5, we expected to find a large proportion of K8+/tdTomato− cells. However, we found that the vast majority of K8+ cells were tdTomato+ at P28 and P168 (94.4 ± 0.04% and 95% K8+/tdTomato+ cells in touch domes; 98.9 ± 0.5% and 93.0% K8+/tdTomato+ cells in whisker follicles, respectively; >250 hairy skin and >500 whisker follicle K8+ cells counted/mouse; Fig. 2, E–G). Conversely, no K8+/tdTomato− cells were found in E18.5 K14CreER-T2/++;Rosa26Tomato embryos that received tamoxifen at E16.5 or E17.5 (>250 hairy skin and >500 whisker follicle K8+ cells counted/mouse, n = 2 mice/age; Fig. 2, H–H’). Tamoxifen administration at E14.5, when Atoh1+ cells first arise from the K14 lineage, did yield a subset of K8+/tdTomato+ cells at E18.5 (Fig. S2). These data suggest that the full complement of Atoh1+ Merkel cell progenitors are created in a 2–3-d period beginning with the appearance of the first Atoh1+ cells in the skin at E14.5.

A subset of Atoh1+ cells in hairy skin express mitotic markers

To confirm that a population of Atoh1+ cells was mitotically active, we examined several mitotic markers in Atoh1CreER-T2/++;Rosa26Tomato and Atoh1CreER-T2/++;Rosa26Tomato mice (Lumpkin et al., 2003). We verified that the Atoh1CreER-T2/++;Rosa26Tomato alleles
369 merkel cell progenitors are Atoh1+ and unipotent. Wright et al. Gerdes, 2000). GFP+ /Ki67+ cells were present at all ages. The percentage of GFP+ cells that were also Ki67+ peaked at E14.5 in whisker follicles and at E16.5 in hairy skin and then decreased as the animals aged, reaching <1% at P21 (>500 cells/region/mouse, n = 2 mice/age; Fig. 3, B and D). GFP+/Ki67+ cells also expressed low levels of K8 (Fig. 3, E–E′), consistent with our finding in Atoh1CreER-T2+/+ ;ROSAtdTomato+ mice that all tdTomato+ cells were also K8+. A subset of GFP+ cells also expressed the M-phase marker phosphohistone H3 (PH3), suggesting that they

labeled the same cells by generating Atoh1CreER-T2+/+ ;Atoh1GFP+/+ ;ROSAtdTomato+ mice, administering high-dose tamoxifen by oral gavage at P21, and analyzing skin at P28. We found that 100% of GFP+ cells were tdTomato+ and that 98.6 ± 0.87% of tdTomato+ cells were GFP+ (>150 hairy skin K8+ cells/mouse, n = 3 mice; Fig. S3). Thus, these alleles are effectively interchangeable.

We immunostained hairy skin and whisker follicles from E14.5, E15.5, E16.5, E17.5, P0, and P21 Atoh1GFP+ mice for Ki67+, a marker of dividing cells (Fig. 3, A–D; Scholzen and Gerdes, 2000). GFP+/Ki67+ cells were present at all ages. The percentage of GFP+ cells that were also Ki67+ peaked at E14.5 in whisker follicles and at E16.5 in hairy skin and then decreased as the animals aged, reaching ~1% at P21 (>500 cells/region/mouse, n = 2 mice/age; Fig. 3, B and D). GFP+/Ki67+ cells also expressed low levels of K8 (Fig. 3, E–E′), consistent with our finding in Atoh1CreER-T2+/+ ;ROSAtdTomato+ mice that all tdTomato+ cells were also K8+. A subset of GFP+ cells also expressed the M-phase marker phosphohistone H3 (PH3), suggesting that they

Figure 1. Adult Merkel cell precursors express Atoh1 and are unipotent. In this and all figures, dosing and harvest paradigms are shown above the pertinent panels. [A–B′] Xgal staining of hairy skin [A and B] and whisker follicles [A′ and B′] shows the presence of labeled cells 3 (A and A′; n = 3 mice) and 9 (B and B′; n = 1 mouse) after tamoxifen. Insets in A and B are individual touch domes. [A′ and B′] Counterstain is Nuclear Fast red. [C–D] Touch domes [C–C′] and whisker follicles [D–D′] immunostained for K8 and β-Gal. (E) Percentages of K8+ cells that coexpress β-Gal at 3 (n = 3) and 9 (n = 1) after tamoxifen (TMX). Error bars show SEM. [F–G] Hairy skin from a tamoxifen-treated P28 Atoh1CreER-T2+/+ ;ROSAtdTomato+ mouse immunostained for K8 (n = 3 mice). tdTomato+ cell (arrows) that appears to be K8− at exposure times that identify other K8+ cells (F–F′) in fact expresses low levels of K8 (G–G′). Bars: (A and B, main images) 1 mm; (A and B, insets) 100 µm; (A′ and B′) 100 µm; (C–G′) 50 µm.
were actively dividing (Fig. S4, A–A”). To confirm this finding, we administered tamoxifen to Atoh1CreER-T2/+;ROSAtdTomato embryos at E15.5 (250 mg/kg) to label all Atoh1 lineage cells with tdTomato, then administered the nucleoside analogue 5-ethynyl-2’-deoxyuridine (EdU; 50 mg/kg) at E16.5 and harvested skin 4 h later to identify actively dividing cells. We found that 0.9 ± 0.1% of tdTomato/K8+ cells in the whisker follicles and 1.1 ± 1.1% in the hairy skin incorporated EdU (>200 tdTomato+ cells/region/mouse, ~1,500 tdTomato+ cells total; n = 2 mice; Fig. 3, G–G”). These data indicate that embryonic Atoh1+ cells are mitotically active.

We next examined 3 (n = 4)- and 6 (n = 2)-mo-old adult Atoh1GFP mice to determine whether GFP+/Ki67+ cells were present and whether the numbers of these cells changed during the natural hair cycle. Three of these mice were in the growth phase of the hair cycle (anagen), and three were in the resting phase (telogen). GFP+ cells in the body skin (250–500/mouse, ~1,700 GFP+ cells total) and whisker follicles (1,500–5,000/...
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Figure 3. A subset of Atoh1+ cells in hairy skin express cell proliferation markers. [A–C] Confocal images of an E14.5 whisker follicle (A–A’) and E16.5 touch dome (C–C’) from Atoh1GFP mice immunostained for GFP (A’ and C’) and Ki67 (A’‘ and C’‘), counterstained with DAPI (A and C). Whisker (A–A’) and guard hair (C–C’) follicles are outlined with dashed lines. Crosshairs are over double-labeled cells, which are also indicated by arrows. Percentages ± SEM of GFP+/Ki67+ cells are shown in A’ and C’. [B and D] The percentages of GFP+/Ki67+ cells within the GFP+ population changed from E14.5 to P21. Error bars show SEM. (n = 2 mice/age.) [E–F] E16.5 Atoh1GFP touch dome immunostained for GFP, Ki67, and K8. Insets show a GFP+/Ki67+/K8+ cell. (E–F’) P90 Atoh1CreER;ROSAtdTomato touch dome immunostained for GFP (F’) and K8 (F). Insets show a GFP+/K8+ cell. [G–G’] Single confocal z slice of touch dome from E16.5 Atoh1CreER/tdTomato mouse given tamoxifen at E15.5, EdU at 16.5, and tissue retrieved 4 h after EdU administration. EdU (G’), tdTomato (G’), K8 (G’), and merge (G’) are shown. Insets show a K8+/tdTomato+/EdU+ cell. [H] Cross section of E14.5 Atoh1GFP whisker follicle illustrating how follicles were divided into quadrants. The dotted yellow line outlines a single whisker follicle. (I) GFP+/Ki67+ cells are clustered at the top of whisker follicles (n = 2 mice/age). Bars: [all main images] 50 µm; [E–F’, insets] 10 µm; [G–G’’, insets] 5 µm.
Multiple Merkel cell progenitors are present in each touch dome and whisker follicle.

We wondered whether each Merkel cell niche (touch dome or whisker follicle) had a single designated progenitor or multiple progenitors. To investigate this, we randomly recombined only a fraction of the \( Atoh1^{+/+} \) population by administering a single very low dose of tamoxifen (0.1 mg/mouse, the lowest tested dose to activate recombination) to \( P28 Atoh1^{CreER-T2/+};ROSA\text{lacZ} \) mice. We then harvested tissue 7 d, 3 mo, or 9 mo later and counted the number of \( K8^{+} \) cells colabeled with Xgal. We reasoned that if only one progenitor was present per niche (Fig. 4A), over time, all cells in an individual touch dome or whisker follicle would be \( K8^{+}/Xgal^{+} \) if recombination occurred in the progenitor or \( K8^{+}/Xgal^{-} \) if recombination did not occur. Conversely, if multiple progenitors were present in each niche there would continue to be a heterogeneous population of \( K8^{+}/Xgal^{+} \) and \( K8^{+}/Xgal^{-} \) cells (Fig. 4A'). We found that \( K8^{+}/Xgal^{+} \) cells were randomly distributed in whisker follicles and touch domes at all time points (Fig. 4B–D, F, and F'). The percentage of \( K8^{+}/Xgal^{+} \) cells in whisker follicles increased over time (17.4 ± 5.0% at 7 d, 32.4 ± 2.1% at 3 mo, and 48.3 ± 2.6% at 9 mo; mouse, \( \sim 11,000 \) GFP\(^{+} \) cells total) were analyzed. We found one GFP\(^{+}/Ki67^{+} \) cell in the body skin of a 3-mo-old mouse whose skin was in telogen (Fig. 3, F–F'). We then harvested tissue 7 d, 3 mo, or 9 mo later and counted the number of \( K8^{+}/Xgal^{+} \) cells per whisker follicle and back skin of \( P19–P24 \) mice (\( n = 3; >400 \) \( K8^{+} \) cells/mouse/region) after administration of EdU (50 mg/kg) and tissue harvest 4 h later. Collectively with our fate mapping data suggesting that a subpopulation of \( Atoh1^{+} \) cells continues to proliferate throughout the lifetime of the mouse, the low numbers of GFP\(^{+}/Ki67^{+} \) cells and absence of \( K8^{+}/EdU^{+} \) cells suggest that this proliferation occurs very slowly.

To determine where proliferative \( Atoh1^{+} \) cells were located, we divided whisker follicles into four equal segments and counted the number of GFP\(^{+}/Ki67^{+} \) cells in each quadrant (Fig. 3H). The vast majority of GFP\(^{+}/Ki67^{+} \) cells were found in the most superficial 25% of the whisker follicle and never in the bottom 50% (Fig. 3I). Similarly, GFP\(^{+}/Ki67^{+} \) cells in guard hair follicles of the body skin were found mostly in the infundibulum (Fig. 3C' and C'). These data suggest that proliferative \( Atoh1^{+} \) cells are located in specific hair follicle regions.
>500 K8* cells/mouse from n = 2–3 mice/time point; F(2,5) = 23.4, P < 0.01, one-way analysis of variance [ANOVA]) but remained constant in touch domes (48.3 ± 13.3% at 7 d, 42.2 ± 2.8% at 3 mo, and 47.6 ± 4.3% at 9 mo; >250 K8* cells/mouse from n = 2–3 mice/time point; F(2,5) = 0.21, P = 0.82, one-way ANOVA; Fig. 4, E and G). These results suggest that multiple progenitors reside in each Merkel cell niche but that homeostasis might be achieved differently in different body regions.

Atoh1* cells do not express K14, but a subset of Atoh1* cells does express K17.

Though embryonically derived from the K14 lineage, mature Merkel cells do not express K14 (Moll et al., 1993). To determine whether Atoh1* Merkel cell progenitors retained K14 expression, we performed K14 immunostaining in hairy skin and whizzer follicles from E16.5 Atoh1CreER/ROSA26Sortomato mice. All Atoh1* cells were K14 negative (>250 GFP* or tdTomato* hairy skin and whisker follicle cells/mouse, n = 2 mice/genotype; Fig. 5, A–B*). We postulated that K14 could be expressed in the Atoh1 lineage, but at low protein levels undetectable by immunostaining. To examine this possibility, we conditionally fate mapped the K14 lineage in adulthood by administering high-dose tamoxifen for a consecutive 3 d to P28 K14CreER/ROSA26Sortomato mice and then harvesting tissue 1 and 4 wk later. Immunostaining for K8 revealed that all K8* cells were tdTomato negative (>250 hairy skin and >500 whisker follicle K8* cells counted/mouse, n = 2 mice/age; Fig. 5, C–C*). Collectively with our data from embryonic K14CreER/ROSA26Sortomato mice (Fig. 2, H–H*), these data indicate that Atoh1* cells do not express K14 at embryonic or postnatal ages.

A recent study suggested that bipotential K17* progenitor cells located in touch domes give rise to touch dome keratinocytes and Merkel cells (Doucet et al., 2013). To determine whether K17* cells are also Atoh1* cells we coimmunostained adult C57BL/6J mice and then harvested tissue 1 and 4 wk later. Immunostaining for K8 revealed that all K8* cells were tdTomato negative (>250 hairy skin and >500 whisker follicle K8* cells counted/mouse, n = 2 mice/age; Fig. 5, C–C*). Collectively with our data from embryonic K14CreER/ROSA26Sortomato mice (Fig. 2, H–H*), these data indicate that Atoh1* cells do not express K14 at embryonic or postnatal ages.

Atoh1* progenitors are the only source of Merkel cells in adult mice.

To determine whether other skin progenitor populations could produce Merkel cells in the absence of Atoh1* progenitors, we genetically ablated Atoh1* cells by administering high-dose tamoxifen for a consecutive 3 d to P28 Atoh1CreER/ROSA26Sortomato mice and Atoh1CreER/ROSA26Sortomato littermate controls. We used only Atoh1CreER/ROSA26Sortomato mice as controls because they have slightly fewer K8* cells per touch dome than their Atoh1+/+ siblings (18.1 ± 1.0 and 25.3 ± 1.8, respectively; >600 K8* cells/mouse; n = 5 mice/genotype; P = 0.007, t test). Back and belly skin (1 cm2) and whisker pads were harvested 1, 3, and 6 mo after tamoxifen administration (n = 2 mice/genotype/time point).

Discussion

We have identified a novel, Atoh1* progenitor population located in the infundibulum of guard hairs and whisker follicles that arises at embryonic ages, is maintained through adulthood, and produces only Merkel cells. The percentage of Atoh1* cells that express mitotic markers is highest during embryonic development when Merkel cells are first produced and then steadily decreases with age, falling to scarcely detectable levels in adulthood. The low percentages of Atoh1*/Ki67* cells in touch domes (0.06%) and whisker follicles (0.009%) and absence of K8*/EdU* cells in adult mice are most likely secondary to a slow rate of precursor division, shortened cell cycle/S-phase duration, and/or a shortened period of Ki67 expression after re-entry from G0 (Gerdes et al., 1984). Atoh1* progenitors must continue to produce new Merkel cells in the whisker follicles of adult animals given our fate-mapping data in Atoh1CreER/ROSA26Sortomato mice given very low-dose tamoxifen (Fig. 4 E). Data from these experiments favor the interpretation that Merkel cell precursor division rates decrease as mice age, because the number of labeled cells doubled (17–34%) between 7 d and 3 mo after tamoxifen administration but then increased only ~40% from 3 to 9 mo after tamoxifen (34–48%). These data suggest that mature Merkel cells in adult animals might have much longer lifespans than previously thought and that they are only rarely replaced. Our findings are consistent with previous studies that used morphology, marker expression, and incorporation of labeled nucleotide analogues to identify rare dividing Merkel cells in developing and adult animals (Mérot et al., 1987; Vaigot et al.,...
Figure 5. The Merkel cell lineage is K14−, but a subset of cells is K17+. (A–A’). Single confocal z-slice image of a touch dome immunostained for GFP and K14 in the body skin of an E16.5 Atoh1GFP mouse. (B–B’). All tdTomato+ cells in adult Atoh1CreER-T2/+;ROSAtdTomato mouse touch domes are K14−. (C–C’). Single confocal z-slice image of whole-mount touch dome preparation from a P60 K14CreER;ROSAtdTomato mouse given tamoxifen (TMX) at P28 and immunostained for K8 (C). There is no signal colocalization. (D) Whisker follicles from an adult C57BL/6J mouse immunostained for K17 (D’) and K8 (D”). Insets show one K8+K17+ cell (as indicated by asterisks) and one K8+K17− cell. (E) Single confocal z-slice image of touch dome from back skin of an adult C57BL/6J mouse immunostained for K8 and K17 showing signal colocalization. Bars: (main images) 50 µm; (D–D”, insets) 10 µm.
375Merkel cell progenitors are Atoh1+ and unipotent. Wright et al. (2010) found that this lone cell was found in telogen stage skin. This finding, coupled with our data from adolescent mice showing that 1% of GFP+ cells are Ki67+ at P21 during the first telogen (Fig. 3D), suggests that Merkel cell precursors, though rarely mitotically active, can divide during the resting stage of the hair cycle.

Our data demonstrate that unipotent Atoh1+ Merkel cell progenitors are the only source of adult Merkel cells, because no new Merkel cells are formed after ablation of Atoh1+ cells in Atoh1CreER-T2/++;ROSAΔTA mice. This finding contrasts with a study that touch dome keratinocytes and Merkel cells share a 1987; Mérot and Saurat, 1988; Woo et al., 2010). One limitation of our study is that the use of the Atoh1GFP and Atoh1CreER-T2 alleles prevented us from separating progenitors and mature Merkel cells because both populations express Atoh1.

Previous work suggested that Merkel cell number is highest during anagen and lower during other stages of the hair cycle (Moll et al., 1996a; Nakafusa et al., 2006). We were unable to establish a connection between hair cycle stage and Atoh1+ cell proliferation, primarily because of the low number of GFP+/Ki67+ cells (one) that we found in adult hairy skin. It is notable, however, that this lone cell was found in telogen stage skin. This finding, coupled with our data from adolescent mice showing that ~1% of GFP+ cells are Ki67+ at P21 during the first telogen (Fig. 3D), suggests that Merkel cell precursors, though rarely mitotically active, can divide during the resting stage of the hair cycle.

Our data demonstrate that unipotent Atoh1+ Merkel cell progenitors are the only source of adult Merkel cells, because no new Merkel cells are formed after ablation of Atoh1+ cells in Atoh1CreER-T2/++;ROSAΔTA mice. This finding contrasts with a study that touch dome keratinocytes and Merkel cells share a
common K17+ progenitor (Doucet et al., 2013). We found that ~28% of K8+ touch dome cells coexpress K17, which coincides well with that study’s finding that ~11% of K8+ Merkel cells were YFP+ just 24 h after tamoxifen administration to K17CreER-T2+/ROSA26Sortm1. Because we show that all K8+ cells are Atoh1+, and that the Atoh1+ lineage gives rise only to Merkel cells, the most parsimonious explanation is that touch domes contain two separate populations of K17+ precursors: one is Atoh1+/K8+/K17+ and gives rise only to Merkel cells, whereas the other is Atoh1+/K8−/K17+ and gives rise only to keratinocytes. Interestingly, we found that K8−/K17− cells are not limited to touch domes, as ~9% of K8− cells in whisker follicles are also K17+. Therefore, we hypothesize that either all or a subset of the Atoh1+/K8+/K17+ cells are the Merkel cell progenitors. Further studies are needed to determine whether K17 is expressed only by Merkel cell progenitors or also by mature Merkel cells.

In contrast, we were unable to find evidence supporting the assertion that adult Merkel cell precursors express K14 (Van Keymeulen et al., 2009), as we never observed GFP+K14+ cells in Atoh1+;ROSA26Sortm1 mice, K14+tdTomato+ cells in Atoh1CreER-T2+/ROSA26Sortm1 mice, or K8+tdTomato+ cells in K14CreER-T2+/ROSA26Sortm1 mice at adult ages. As predicted, we did find K8+/tdTomato+ cells in K14CreER-T2+/ROSA26Sortm1 mice treated with tamoxifen at E14.5 when Atoh1+ cells first arise from the K14 lineage (Vielkind et al., 1995; Morrison et al., 2009), suggesting that our findings were not secondary to a technical issue of some sort. Notably, a microarray experiment conducted on early postnatal skin also failed to detect K14 expression in purified Merkel cells (Haebeler et al., 2004). One difference between our study and the previous study is that our analyses were restricted to hairy body skin and whiskers, whereas Van Keymeulen et al. (2009) analyzed the glabrous skin of the feet. So, it is possible that Atoh1+ precursors in different body regions express different markers. Regardless, if other Atoh1+ precursor or stem cell populations (such as the K15+ bulge stem cell population also proposed by Van Keymeulen et al., 2009) were responsible for maintaining the adult Merkel cell population in whisker follicles and body skin, we would expect to find production of new Merkel cells after tamoxifen administration to Atoh1CreER-T2+/ROSA26Sortm1 mice. The absence of this compensation even with survival times of 6 mo after tamoxifen suggests that other skin precursor and stem cell linesages are either incapable of generating Merkel cells or can do so only under special conditions. Many skin stem cells have restricted lineages in adult animals under normal conditions but can give rise to additional lineages after wounding (GhaziZadeh and Taichman, 2001; Ito et al., 2005; Levy et al., 2005; Jaks et al., 2010). Whether skin stem cells can produce Merkel cells or their precursors after wounding is unknown, as is the ability of Merkel cell progenitors to give rise to other skin lineages; we are currently testing both of these possibilities.

Two lines of evidence illuminate potential differences in precursor allocation and/or Atoh1 expression levels in precursors and mature Merkel cells in touch domes versus whisker follicles. Very low-dose tamoxifen administration to adult Atoh1CreER-T2+/ROSA26Sortm1 mice led to increasing percentages of K8+/Xgal+ cells in the whiskers at 1 wk (17%), 3 mo (34%), and 9 mo (48%) survival times (Fig. 4 E). Conversely, more K8+ cells were present in the whiskers of Atoh1CreER-T2+/ROSA26Sortm1 mice 28 d (127/follicle) than 3 mo (1.6/follicle) after treatment (Fig. 6 I). These data suggest that recombination occurred preferentially in Atoh1+ Merkel cell progenitors and that this population expanded in the Atoh1CreER-T2+/ROSA26Sortm1 mice and was deleted in the Atoh1CreER-T2+/ROSA26Sortm1 mice. In contrast, ~50% of touch dome cells were K8+/Xgal+ 1 wk, 3 mo, and 9 mo after treatment in Atoh1CreER-T2+/ROSA26Sortm1 mice (Fig. 4 G), and very few K8+ cells remained in the touch domes of Atoh1CreER-T2+/ROSA26Sortm1 mice 1 mo after treatment (Fig. 6 D). This suggests that progenitors and mature Merkel cells in the touch domes underwent similar levels of recombination in both cases. One explanation for these results is that Atoh1+ Merkel cell progenitors in the whiskers express higher levels of Atoh1 than mature Merkel cells, making them more likely to undergo recombination at limiting doses of tamoxifen, whereas progenitors and mature Merkel cells in the touch domes express similar levels of Atoh1. Another possibility is that the percentage of Atoh1+ progenitor cells is higher in touch domes than in whisker follicles. Interestingly, the percentage of K8+/K17+ cells in touch domes (28%) is higher than that found in whiskers (9%), which would support our hypothesis that the Atoh1+/K8+/K17+ population is the progenitor population. A third explanation is that designated progenitors are present in the whisker follicles but that any Atoh1+ cell in the touch dome is capable of division. This explanation seems unlikely given that the number of YFP+ Merkel cells decreases over time after tamoxifen administration (Vielkind et al., 1995; Ben-Arie et al., 2000; Morrison et al., 2009; Richardson et al., 2009). Our present results demonstrate that these early born Atoh1+ cells are mitotically active and that they give rise only to Merkel cells, suggesting that they form a self-renewing population of progenitors that is maintained through adulthood. The first appearance of these cells at E14.5–E15.5 in hairy skin makes the Merkel cell lineage one of the first committed lineages within the hair follicle, with specification taking place at the same time or before that of multipotent stem cells that inhabit the bulge region (Vidal et al., 2005; Nowak et al., 2008). Merkel cell progenitor commitment also occurs several days before specification of other unipotent progenitors such as those of the sebaceous gland lineage (Horsley et al., 2006). Thus, from the earliest times, the Atoh1+ lineage is a separate skin lineage.

The epidermal placodes that give rise to whisker follicles in the snout and tylotrich (guard hair) follicles in the hairy skin develop at E12.5 and E14.5, respectively, whereas Atoh1 expression is first seen in these regions at E14.5 (Vielkind et al., 1995; Ben-Arie et al., 2000; Morrison et al., 2009; Richardson et al., 2009). Our present results demonstrate that these early born Atoh1+ cells are mitotically active and that they give rise only to Merkel cells, suggesting that they form a self-renewing population of progenitors that is maintained through adulthood. The first appearance of these cells at E14.5–E15.5 in hairy skin makes the Merkel cell lineage one of the first committed lineages within the hair follicle, with specification taking place at the same time or before that of multipotent stem cells that inhabit the bulge region (Vidal et al., 2005; Nowak et al., 2008). Merkel cell progenitor commitment also occurs several days before specification of other unipotent progenitors such as those of the sebaceous gland lineage (Horsley et al., 2006). Thus, from the earliest times, the Atoh1+ lineage is a separate skin lineage.

Our study does not identify the factors that control the initial specification of Atoh1+ cells from the K14 lineage. Recent work suggests that the transcription factor Sox2 is a direct, positive regulator of Atoh1, whereas the Polycomb repressive complex
negatively regulates Merkel cell specification through repression of Sox2 (Bardot et al., 2013; Lesko et al., 2013). Deletion of Sox2 in the developing skin reduces the number of Merkel cells in embryonic mice but does not preclude their production nor their expression of multiple canonical markers (Bardot et al., 2013; Lesko et al., 2013). Sox2 and Atoh1 are expressed concomitantly in the epidermis starting at E15.5; however, the genetic cascades and signaling molecules necessary for the initiation of Atoh1 and Sox2 expression are unknown. Future experiments are required to determine which Atoh1+ cells in the developing skin ultimately give rise to the Merkel cell lineage.

Our findings have potential implications for understanding the genesis of MCC, a rare and devastating skin cancer for which there are no truly effective treatments aside from surgical excision. Although the cell type of origin of MCC tumors is unknown (Tilling and Moll, 2012), evidence that MCC arises from Merkel cells or their precursors comes from expression of Hath1, the human Atoh1 homologue, in MCC tumor lines and primary tumor cells, along with other Merkel cell markers such as K20, chromogranin A, synaptophysin, and neuron-specific enolase (Leonard et al., 2002; Heiskala et al., 2010). Assuming MCC arises from the Merkel cell lineage, it is plausible that Atoh1+ Merkel cell precursors might be the cell type of origin given their unpigmented and mitotic activity. Further experiments are necessary to test this hypothesis.

**Materials and methods**

**Mice**

Atoh1<sup>CreER</sup> (JAX 013593; The Jackson Laboratory; Lumpsik et al., 2003), ROSA<sup>Atoh1</sup> (JAX 003474; Soriano, 1999), ROSA<sup>CRE</sup> (JAX 007914; Madieu et al., 2010), ROSA<sup>SfGFP</sup> (JAX 006699; Voehringer et al., 2008), K1<sup>Cre</sup> (JAX 005107; Vasioukhin et al., 1999), and Atoh1<sup>CreER-T2</sup> (open reading frame of the Atoh1 locus is replaced with CreER-T2; Fujiyama et al., 2009) mice were maintained in accordance with International Animal Care and Use Committee guidelines at the Case Western Reserve University and the Children’s Hospital of Pittsburgh of the University of Pittsburgh Medical Center. For embryonic ages, the plug date was designated as E0.5.

**Tamoxifen and EdU administration**

Tamoxifen (Sigma-Aldrich) was dissolved in a 9:1 corn oil/ethanol solution at a 1% or 5% concentration. Mice were briefly anesthetized with isoflurane, and tamoxifen was administered by oral gavage. For lineage tracing, tamoxifen was administered as a single dose of 0.1 mg (5 mg/kg; low-dose adult), 0.4 mg (~10 mg/kg; low-dose embryonic), or 250 mg/kg (high dose) on either a consecutive 1 or 3 d as indicated in the text. EdU (Invitrogen) was dissolved in sterile PBS at a 10-mM concentration and was injected intraperitoneally into adult mice or pregnant females.

**Tissue processing**

Mice were euthanized by cervical dislocation, and skin was dissected into cold PBS. Embryos were dissected from pregnant dams and decapitated before tissue dissection. Skin processed for immunohistochemistry was fixed in 4% PFA for 30 min (adult tissue) or 24 h (embryonic skin), or overnight (whole embryos), washed in PBS, and cryopreserved in 30% sucrose/PBS. Skin for Xgal staining was fixed in cold 4% PFA for 15 min, washed in cold PBS, and stained in Xgal overnight at 37°C. Embryonic skin and whisker follicles were dissected before incubation in Xgal. Tissue was washed and passifixed for 2 h in 4% PFA before imaging. Tissue was also prepared for cryosectioning and counterstained with Nuclear Fast red (Sigma-Aldrich). Hair cycle stage was determined on hematoxylin and eosin-stained section by analyzing hair follicle dimensions, depth into the subcutaneous tissue, and the shape and size of the dermal papilla and hair bulb as previously described (Müller-Röver et al., 2001).

**Histology**

Tissue was embedded in optimum cutting temperature (O.C.T.; Thermo Fisher Scientific) and serially sectioned on a cryostat (18950M; Leica) at 25 µm. Slides were vacuum dried, rehydrated in PBS, and blocked with 5% normal donkey serum or 3% normal rabbit (in 0.1% PBS-PBS) before incubation with primary antibodies. After primary antibody incubation, sections were washed and incubated for 30 min at room temperature in blocking solution containing the appropriate secondary antibodies obtained from Jackson ImmunoResearch Laboratories, Inc.

**Embryonic ages**

E14.5, E15.5, E16.5, E17.5, P0, and P21, respectively. GFP was removed and washed for 5–8 h in 0.3% PBS-T. Tissue was incubated in 4% PFA for 30 min (adult tissue) or overnight (whole embryos), washed in PBS, and cryopreserved in 30% sucrose. Tissue was washed in cold PBS, and postfixed for 2 h in 4% PFA before imaging. Tissue for cryosectioning was fixed in 30% sucrose for 24 h at 4°C before cryopreservation in OCT and sectioning on a cryostat (1950M; Leica) at 12 µm. Sections were stained with the nuclear probe DAPI (1:1,000; Alexa Fluor 488–conjugated donkey anti–rabbit; 711-545-152), Alexa Fluor 488–conjugated donkey anti–rat (712-545-150), Alexa Fluor 568–conjugated donkey anti–mouse (713-655-150), Cy3–conjugated donkey anti–mouse secondary antibody (715-655-150), and Alexa Fluor 647–conjugated donkey anti–rabbit (711-655-152), Cy3–conjugated donkey anti–rabbit secondary antibody (711-655-152), Alexa Fluor 647–conjugated donkey anti–rabbit (712-655-150). Sections were stained with the nuclear probe DAPI (1:1,000; Roche Molecular Biochemicals) and visualized using an inverted epifluorescence microscope (Axio Observer; Carl Zeiss) with a 40× objective. Images were stored as single z-series or single z slices (as noted in the figures) consisting of 25 µm optical slices collected every 0.35 µm. Images in Fig. 6 (G–I) are stitched from z-stacked 40× images. Multiple 3D images were acquired for the desired region of interest with 10% overlap between adjacent images.

**Images**

Images were compiled and stitched using Velocity software with the standard brightness correction. Nonconfocal images were acquired with a confocal microscope (Zeiss LSM 510 META; Carl Zeiss) using a 40× C-Apochromat, NA 1.2, water immersion objective. Images presented here are maximum intensity projections of a z series consisting of 1-µm optical slices collected every 0.5 µm (optical interval setting determined by LSM 510 software, ARIA 4.2). All other confocal images were acquired with an inverted microscope (Axio Observer; Carl Zeiss) on a Zeiss Axiovert 200M with a 40× C-Apochromat, NA 1.2, water immersion objective. Images presented here are maximum intensity projections of a z series consisting of 1-µm optical slices collected every 0.35 µm. Images in Fig. 6 (G–I) are stitched from z-stacked 40× images. Multiple 3D images were acquired for the desired region of interest with 10% overlap between adjacent images.

**Cell counts**

Cell counts were performed on a microscope (DM5000 B; Leica) using an HCX Plan Apochromat 40×, 1.25 NA and an HC Plan Apochromat 10×, 0.4 NA objective, a camera (DFC420; Leica), and Leica Acquisition Software v4.2. Images were cropped, and brightness and contrast were enhanced for publication quality with Photoshop and/or Illustrator (Adobe).

**Merkel cell progenitors are Atoh1** and unpigmented. • Wright et al. 377.
Although the analysis of whisker follicles is not completed, we have determined the timing and frequency of proliferation in the whisker follicle during development. The labeling data, along with our in vivo experiments, strongly suggest that the mitotic rate in the whisker follicle is high compared to the body skin. Specifically, the labeling frequency of Ki67 in the whisker follicles is greater than that in the body skin. This finding is consistent with previous reports that the rate of cell division is higher in the whisker follicle compared to other skin areas.

In conclusion, our results provide new insights into the dynamics of cell proliferation in the whisker follicle. By combining the use of tdTomato, EdU, and histology, we have been able to trace the fate of individual cells and identify the progenitor cells that give rise to mature keratinocytes. These findings have important implications for understanding the mechanism of keratinocyte differentiation and the role of the whisker follicle in the development of the skin.

**References**


Merkel cell carcinoma.

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