Ectopic Atoh1 expression drives Merkel cell production in embryonic, postnatal and adult mouse epidermis

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ABSTRACT

Merkel cells are mechanosensitive skin cells whose production requires the basic helix-loop-helix transcription factor Atoh1. We induced ectopic Atoh1 expression in the skin of transgenic mice to determine whether Atoh1 was sufficient to create additional Merkel cells. In embryos, ectopic Atoh1 expression drove ectopic expression of the Merkel cell marker keratin 8 (K8) throughout the epidermis. Epidermal Atoh1 induction in adolescent mice similarly drove widespread K8 expression in glabrous skin of the paws, but in the whisker pads and body skin ectopic K8+ cells were confined to hair follicles and absent from interfollicular regions. Ectopic K8+ cells acquired several characteristics of mature Merkel cells in a time frame similar to that seen during postnatal development of normal Merkel cells. Although ectopic K8+ cell numbers decreased over time, small numbers of these cells remained in deep regions of body skin hair follicles at 3 months post-induction. In adult mice, greater numbers of ectopic K8+ cells were created by Atoh1 induction during anagen versus telogen and following disruption of Notch signaling by conditional deletion of Rbpj in the epidermis. Our data demonstrate that Atoh1 expression is sufficient to produce new Merkel cells in the epidermis, that epidermal cell competency to respond to Atoh1 varies by skin location, developmental age and hair cycle stage, and that the Notch pathway plays a key role in limiting epidermal cell competency to respond to Atoh1 expression.

KEY WORDS: Sensation, Stem cell, Touch, Mouse

INTRODUCTION

Merkel cells are specialized skin cells found at the dermal/epidermal border in mammalian hairy and glabrous skin. Mature Merkel cells contact slowly adapting type I (SA1) nerve fibers and act as mechanotransducers important for detecting certain forms of light touch (Ikedo et al., 2014; Maksimovic et al., 2014; Maricich et al., 2012, 2009; Woo et al., 2014). Merkel cells express the primitive epithelial intermediate filament proteins cytokeratin 8, 18 and 20 (K8, K18 and K20, respectively; also known as Krt8, Krt18 and Krt20) (Moll et al., 1995, 1984) as well as mechanosensitive ion channels, a variety of neuropeptides and presynaptic machinery components, such as the synaptic vesicle protein Rab3c and the vesicular glutamate transporter 2 (VGLUT2; also known as SLC17A6) (Alvarez et al., 1988; Cheng Chew and Leung, 1991; English et al., 1992; Fantini and Johansson, 1995; García-Caballero et al., 1989; Haeberle et al., 2004; Hartschuh and Weihe, 1989; Hartschuh et al., 1989, 1979, 1983). These markers have been used to reliably identify Merkel cells in a variety of species ranging from fish to humans (Moll et al., 1984; Saxod, 1996; Whitear, 1989).

Merkel cells arise from the epidermal lineage, and Merkel cell production requires the basic helix-loop-helix transcription factor Atoh1 (Maricich et al., 2009; Morrison et al., 2009; Van Keymeulen et al., 2009). Atoh1 is also important for cell fate determination of brainstem neurons, hair cells of the inner ear and neurosecretory cells of the intestinal epithelium (Ben-Arie et al., 1997; Bermingham et al., 1999; Wang et al., 2005; Yang et al., 2001). Moreover, ectopic overexpression of Atoh1 is sufficient to convert inner ear supporting cells into hair cells and intestinal enterocytes to neurosecretory cells (Kelly et al., 2012; VanDussen and Samuelson, 2010; Zheng and Gao, 2000). Whether Atoh1 expression is sufficient to direct Merkel cell specification within the epidermal lineage is unknown.

Using transgenic mice that allow inducible epidermal overexpression of Atoh1, we show that Atoh1 expression alone is sufficient to convert epidermal cells into ectopic Merkel cells as identified by expression of numerous Merkel cell markers. We show that epidermal competency to respond to Atoh1 varies by age, skin region and hair cycle stage. Furthermore, epidermal competency was limited by Notch signaling, which has been shown in other systems to antagonize endogenous and exogenous Atoh1 function (Golub et al., 2012; Kim and Shvidasani, 2011; Yamamoto et al., 2006; Zheng et al., 2000; Zine et al., 2001). These data establish the sufficiency of Atoh1 to control Merkel cell lineage specification in the skin.

RESULTS

Inducible Atoh1 expression produces ectopic K8+ cells in glabrous and hairy skin

In mouse skin, Atoh1 is normally expressed exclusively by Merkel cells located in foot pads, touch domes of hairy skin and whisker follicles (Fig. 1B-B‴, G-H‴, M-M‴). To induce Atoh1 expression in other skin regions, we crossed mice that express Cre recombinase in the epidermal lineage (K14Cre) (Dassule et al., 2000), mice that express a Cre-activated reverse tetracycline transactivator (ROSArtTA) (Belteki et al., 2005) and mice with a tetracycline-inducible Atoh1 transgene (TetAtoh1) (Kelly et al., 2012). These triple transgenic K14Cre, ROSAartTA, TetAtoh1 mice allow inducible Atoh1 expression throughout the epidermal lineage for the duration of doxycycline administration (Fig. 1A).

Adolescent [postnatal day (P)22-P26] K14Cre, ROSAartTA, TetAtoh1 mice that received doxycycline for 24 h prior to sacrifice produced Atoh1 protein throughout the foot pad epidermis, hairy skin follicular and interfollicular epidermis, and in epidermal cells within whisker follicles (Fig. 1C–D‴, I‴, J‴, N‴). However, only a fraction of
the ectopic Atoh1+ cells located in whisker follicles but not body skin or glabrous paw skin co-expressed low levels of the early Merkel cell marker K8 (Vielkind et al., 1995) (Fig. 1C″,I″,J″,N″).

Doxycycline administration for 96 h resulted in greater numbers of ectopic Atoh1+ cells in all regions (Fig. 1E‴,F‴,K‴,L‴,N‴). This longer induction paradigm also led to K8 expression throughout the paw epidermis, but in hairy skin and whisker pads K8 expression was limited to ectopic Atoh1+ cells confined to hair follicles (Fig. 1E‴,F‴,K‴,L‴,N‴). We never found ectopic Atoh1+ or K8+ cells in any skin region in control littermates (Fig. 1B‴,G‴,H‴,M‴,M‴; Fig. 2A,D–D″). These data suggest that keratinocytes in different skin regions exhibit differential competence to respond to Atoh1 expression. Unfortunately, K14Cre; ROSArtTA; TetAtoh1 mice undergoing induction for more than 24 h experienced severe weight loss, probably secondary to degeneration of the tongue epithelium causing decreased oral intake (supplementary material...
Fig. 2. Ectopic K8+ cells persist in glabrous and hairy skin of K14Cre; ROSA26mTetAtoh1 mice. Experimental induction paradigm is shown at the top of the figure. (A–J) Wholemount glabrous paw skin (A–C), sectioned whisker follicles (D–F) and wholemount back skin (G–J) of control (A,G) and K14Cre; ROSA26mTetAtoh1 mice four days (B,E,H), two weeks (C,F,I) and three months (J) post-doxycycline immunostained for K8. Dotted lines outline paw skin touch pads (A–C) and whisker follicles (D–F). Bracket (B) and asterisks (C,E,F,J) indicate ectopic K8+ cells. TD indicates normal Merkel cells within touch domes, which are sometimes out of focus because they are in a different focal plane than ectopic cells. (K) Ectopic Merkel cell density in back skin (n=2–3 mice/time point). Numbers above bars are mean±s.e.m. **P<0.01, ***P<0.001 versus 4 day time point; ##P<0.01 versus 2 week time point (ANOVA with Tukey’s pair-wise post-hoc testing). (L–M″) Sectioned K14Cre; ROSA26mTetAtoh1 mouse back skin four days (L–M″) and 2 weeks (M–M″) post-induction immunostained for Atoh1 and K8 shows ectopic Atoh1+/K8− (green arrows), Atoh1−/K8+ (red arrows) and Atoh1+/K8+ (yellow arrows) cells in hair follicles. Dotted lines outline hair follicle, and boxes denote regions shown at higher magnification in insets. Scale bars: 50 μm.
Fig. S1A-C). Therefore, we used the 24 h doxycycline administration paradigm for the rest of our experiments.

To determine how long ectopic K8+ cells survived, we induced Atoh1 expression by administering doxycycline for 24 h to adolescent K14Cre; ROSA4rtTA; TetAtoh1 mice and harvesting skin 4 days, 2 weeks, 6 weeks and 3 months after doxycycline was withdrawn (Fig. 2). In glabrous paw skin and whisker follicles, many ectopic K8+ cells were present 4 days after doxycycline administration, but very few remained 2 weeks after doxycycline administration (Fig. 2A-F″). These cells were not studied further. By contrast, ectopic K8+ cells were found in body skin hair follicle epidermis at all time points examined, but their numbers decreased between 4 days and 6 weeks post-doxycycline, then remained constant up to 3 months post-doxycycline (Fig. 2G-K). Co-immunostaining for K8 and cleaved caspase-3 4 days post-doxycycline revealed that 1.3±0.8% of ectopic K8+ cells were caspase-3+, suggesting that the decline in ectopic K8+ cell number occurred secondarily to apoptosis (Fig. 3A-A″). Rare Atoh1+K8− cells were found at 4 days and 2 weeks post-doxycycline, but the vast majority of Atoh1+ cells co-expressed K8 (Fig. 2L-M″). At 4 days post-doxycycline, ectopic K8+ cells were found throughout the hair follicle epidermis (Fig. 2L-L″) but were restricted to hair follicle bulb and bulge regions from 2 weeks post-doxycycline onwards (Fig. 2M-M″; data not shown). These data indicate that the majority of ectopic K8+ cells die over time, but that a small subset survives for at least 3 months post-Atoh1 induction.

Embryonic Atoh1+ cells undergo mitosis (Wright et al., 2015), so we wondered whether ectopic Atoh1-expressing cells were proliferative. No ectopic K8+ cells expressed the proliferation marker Ki67 (Mki67 – Mouse Genome Informatics) in K14Cre; ROSA4rtTA; TetAtoh1 mice 4 days post-doxycycline, indicating that ectopic K8+ cells were not mitotically active (Fig. 3B-B″). Furthermore, K14Cre; ROSA4rtTA; TetAtoh1 mice did not manifest skin malformations or overt changes in epidermal structure at any time point, and numbers of Ki67+K8− epithelial cells were similar in K14Cre; ROSA4rtTA; TetAtoh1 mice and control littermates 4 days post-doxycycline (interfollicular: 28.5±2.4 versus 25.8±7.6 Ki67+ cells/20× field of view, P=0.7; follicular: 34.3±4.4 versus 25.9±0.4 Ki67+ cells/20× field of view, P=0.42; t-test).

Merkel cells are derived from the K14 lineage, so our transgenic Atoh1 overexpression paradigm must have driven Atoh1 overexpression in normal Merkel cells. However, Atoh1 induction did not affect touch dome Merkel cell morphology or numbers (supplementary material Fig. S2A,B). This suggests that Atoh1 overexpression was not toxic to Merkel cells and that it did not drive excess production of K8+ cells in touch domes.

We also studied whether ectopic K8+ cells had detectable levels of Atoh1 protein. About twice as many ectopic K8+ cells co-expressed Atoh1 at 4 days compared with 2 weeks post-doxycycline, and these cells had qualitatively stronger Atoh1 immunofluorescence at the 4 day time point (Fig. 3C-D″). These data suggest that transient transgenic Atoh1 expression induced Atoh1 expression from the endogenous locus that was maintained for at least 2 weeks, and that endogenous expression levels subsequently decreased over time.

**Ectopic K8+ cells acquire Merkel cell marker expression over a time course similar to that seen during normal Merkel cell development**

Ectopic K8+ cells in hair follicles of K14Cre; ROSA4rtTA; TetAtoh1 mice looked morphologically similar to Merkel cells found in touch domes of control mice (supplementary material Fig. S2C). We wondered whether ectopic Atoh1 expression drove expression of Merkel cell markers other than K8. We looked first at expression of Sox2 and Isl1, two transcription factors expressed early in Merkel cell development (Bardot et al., 2013; Lesko et al., 2013; Perdigoto et al., 2014). The vast majority of ectopic K8+ cells co-expressed Sox2 and Isl1 at 4 days and 2 weeks post-doxycycline (Fig. 3E-H″),

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**Fig. 3. Ectopic K8+ cells express Atoh1, Sox2 and Isl1, some express caspase-3, but none is mitotically active.** Experimental induction paradigm is shown at the top of the figure. (A-H″) Sectioned back skin of adolescent K14Cre; ROSA4rtTA; TetAtoh1 mice showing ectopic K8+ cells in hair follicles four days (A-C″,E-E″,G-G″) and 2 weeks (D-D″,F-F″,H-H″) post-induction. Sections were co-immunostained for cleaved caspase-3 (A), Ki67 (B) Atoh1 (C-D″), Sox2 (E, F), Isl1 (G-H″) and K8 (A″, B″, C″, D″, E″, F″, G″, H″). Yellow arrows indicate double-positive cells. Red arrows indicate K8+ cells not expressing the marker of interest. All images were taken using the same exposure settings, demonstrating greater fluorescence intensity of each transcription factor at 4 days versus 2 weeks post-doxycycline. The average percentage of ectopic K8+ cells (±s.e.m.) co-expressing each marker is shown (n=100-200 K8+ cells from each of three mice). Scale bars: 10 μm.
demonstrating that Atoh1 expression was sufficient to induce long-lasting expression of these transcription factors. As with Atoh1 itself, the immunofluorescence levels of these proteins appeared to decrease over time.

Four days after a 24 h doxycycline pulse nearly all ectopic K8+ cells co-expressed the Merkel cell markers K18 and K20 (Fig. 4B–C‴; Table 1), but very few expressed the synaptic vesicle protein Rab3c or the vesicular glutamate transporter Vglut2 (Table 1). By contrast, two weeks post-induction ectopic K8+ cells co-expressed all four markers (Fig. 4E–F‴; Table 1). Co-expression of these markers was maintained at the 6 week and 3 month survival times (data not shown). In addition, the majority of ectopic K8+ cells were labeled by systemic administration of the steryl dye FM1-43 at 4 days and 2 weeks post-induction (Fig. 4D–D‴; Table 1), suggesting that they possessed mechanosensitive ion channels (Meyers et al., 2003).

However, NF-200 immunostaining demonstrated that ectopic K8+ cells were not innervated at any time point (data not shown). These data demonstrate that Atoh1 is sufficient to direct a subset of follicular keratinocytes to adopt several key features of Merkel cells.

Mature Merkel cells do not express keratinocyte markers (Haeberle et al., 2004; Moll et al., 1995; Wright et al., 2015), and we wondered whether ectopic Atoh1 expression in keratinocytes altered normal marker expression in these cells. Four days post-induction, we found no cells that co-expressed Atoh1 and the general keratinocyte marker K14 (Krt14 – Mouse Genome Informatics; Fig. 4A–A‴). This suggests that Atoh1 expression downregulates K14 expression as it drives these cells to switch fate away from the keratinocyte lineage.

We next sought to determine whether acquisition of Merkel cell-specific marker expression in ectopic Atoh1+ cells occurred in a time course similar to that seen during normal Merkel cell development. Merkel cells first appear in hairy skin at embryonic day (E) 14.5 (Pasche et al., 1990), so we examined P0 and P7 control (K14Cre and TetAtoh1, ROSArtTA) mice to approximate the 4 day and 2 week time points following Atoh1 induction. At P0, the majority of K8+ cells co-expressed K18 and K20, and also took up FM1-43 (Fig. 5A–C‴; Table 1). However, very few Merkel cells in P0 mice co-expressed Rab3c or Vglut2 (Table 1). By contrast, most K8+ cells co-expressed all of these markers at P7 (Fig. 5D–E‴; Table 1). These data indicate that the time course of marker expression in ectopic Merkel cells in K14Cre, ROSArtTA, TetAtoh1 mice closely approximates that of normal Merkel cells during postnatal development, suggesting that the maturational programs controlled by Atoh1 are similar in the two populations.

Keratinocyte competency to respond to ectopic Atoh1 is linked to hair cycle stage in adolescent mice

Previous reports suggested that Merkel cell numbers in rodent hairy skin change during the hair cycle (Moll et al., 1996, Nakafusa et al., 2006), so we wondered whether hair cycle stage might affect competency of keratinocytes to respond to Atoh1 induction. Atoh1 induction for 24 h at P24–25 during anagen I followed by tissue harvest 4 days or 2 weeks later resulted in production of ~55× (P=0.011, t-test) and ~100× (P=0.004, t-test) more ectopic K8+ cells, respectively, than induction at P19–20 during telogen I (Fig. 6). We verified hair cycle stage at time of induction and time of collection on Hematoxylin and Eosin-stained tissue sections (supplementary material Fig. S3). These data suggest that factors that vary during the hair cycle control responsiveness of keratinocytes to ectopic Atoh1 expression.

Keratinocyte competency to respond to ectopic Atoh1 decreases over developmental time

To determine whether keratinocyte competency to respond to Atoh1 expression was related to developmental age, we administered doxycycline to pregnant dams for 5 days from E14.5 to E18.5, then harvested K14Cre, ROSAartTA, TetAtoh1 embryos and littermate controls at E18.5 (n=5 and n=4 from two litters, respectively). Atoh1+ and K8+ cells were present throughout hairy skin follicular and interfollicular epidermis of K14Cre, ROSAartTA, TetAtoh1 embryos, whereas Atoh1 and K8 were detected only in touch domes of littermate controls (Fig. 7A,B,E–F‴). Some ectopic K8+ cells expressed K18 and K20 (Fig. 7H–I‴). This widespread ectopic Atoh1 expression caused acantholysis of the epidermis, which sloughed from the dermis in treated E18.5 K14Cre, ROSAartTA, TetAtoh1 embryos (supplementary material Fig. S1D,E). Induction of Atoh1 in utero, even for periods as short as 24 h, resulted in

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**Figure 4. Ectopic K8+ cells express mature Merkel cell markers but not keratin 14.** Experimental induction paradigm is shown at the top of the figure. (A–F‴) Sectioned back skin of adolescent K14Cre, ROSAartTA, TetAtoh1 mice showing ectopic K8+ cells in hair follicles 4 days (A–D‴) and 2 weeks (E–F‴) post-induction immunostained for or labeled with K14 (A), Atoh1 (A‴), K18 (B), K20 (C), FM1-43 (D), Vglut2 (E), Rab3c (F) and K8 (B‴, C‴, D‴, E‴, F‴). Asterisks indicate Atoh1+ K14– cells; yellow arrows indicate double-positive cells. Co-expression data is quantified in Table 1. Scale bars: 10 μm.
embryonic or early postnatal lethality of transgenic pups, preventing us from studying postnatal ages with this paradigm.

We next evaluated the effects of Atoh1 induction during early postnatal development by treating K14Cre; ROSA\textsuperscript{rTA}; Tet\textsuperscript{Atoh1} mice with doxycycline from P2 to P4 and examining the skin at P4 (n=6 mice/genotype). As in adolescent mice, Atoh1+ cells were detected throughout the follicular and interfollicular epidermis of K14Cre; ROSA\textsuperscript{rTA}; Tet\textsuperscript{Atoh1} mice, but K8+ cells were seen almost exclusively within the follicular epidermis and the associated infundibulum (Fig. 7D,E,G-G‴). Atoh1+ and K8+ cells were confined to touch domes of P4 control littermates (Fig. 7C). Ectopic K8+ cell densities were significantly different from one another compared with those observed at E18.5 and when different locations were compared (two-way ANOVA F=29.18, P=0.0006; post-hoc pairwise Scheffé test: E18.5 interfollicular versus P4 interfollicular, P=0.002; E18.5 follicular versus P4 interfollicular, P=0.001; P4 interfollicular versus P4 follicular, P=0.006). These data indicate that epidermal competency to respond to Atoh1 expression is widespread during embryogenesis, but becomes restricted to hair follicle epidermis shortly after birth.

### Table 1. Ectopic K8+ cells in K14\textsuperscript{Cre}; ROSA\textsuperscript{rTA}; Tet\textsuperscript{Atoh1} mice express Merkel cell markers in a time course similar to that seen during normal Merkel cell development

<table>
<thead>
<tr>
<th>Ectopic K8+ cells in K14\textsuperscript{Cre}; ROSA\textsuperscript{rTA}; Tet\textsuperscript{Atoh1} mice</th>
<th>Touch dome K8+ cells in C57BL/6J mice</th>
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<td>4 days post-doxycycline</td>
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<td>K8+K18+/K8+</td>
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<td>K8+K20+/K8+</td>
<td>94±0.8%</td>
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<td>K8+FMI43/K8+</td>
<td>91±5.3%</td>
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<td>K8+VGLUT2+/K8+</td>
<td>10±2.6%</td>
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<tr>
<td>K8+Rab3c+/K8+</td>
<td>5±1.5%</td>
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<td>100±0%</td>
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<td>95±3.9%</td>
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<tr>
<td>100±0%</td>
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<td>94±2.0%</td>
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<td>69±4.3%</td>
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At least 100 K8+ cells/mouse, n=3 mice/time point or age were analyzed. No marker+ K8− cells were found. Data is shown as average ±s.e.m.

Notch signaling regulates keratinocyte competency to respond to Atoh1 induction

In the cochlea, deletion of the Notch effector proteins Hes1 and Hes5 increases hair cell production, and inhibition of Notch signaling expands support cell competency to respond to ectopic Atoh1 expression (Kelly et al., 2009; Zheng et al., 2000; Zine et al., 2001). To test whether Notch signaling similarly affected competency of the epidermal lineage to respond to Atoh1, we conditionally deleted the universal Notch effector gene Rbpj in K14Cre\textsubscript{ER}; ROSA\textsuperscript{rTA}; Tet\textsuperscript{Atoh1}; RBPjflox mice. We used the tamoxifen-inducible K14Cre\textsubscript{ER} allele (Vasioukhin et al., 1999) rather than the constitutively expressed K14Cre allele because constitutive ablation of Rbpj in the...
epidermis causes perinatal lethality (Blanpain et al., 2006). The K14CreER allele directs efficient recombination within the interfollicular epidermis while causing only limited recombination within follicular epidermis (Peterson et al., 2015; Wong and Reiter, 2011; Zhang et al., 2009) (Fig. 8A–A″), which limited the amount of ectopic Atoh1 expression that was driven in hair follicles. K14CreER; ROSA26R; RBPjflx/flx, K14CreER; ROSA26R; TetAtoh1; RBPj+/+ and K14CreER; ROSA26R; TetAtoh1; RBPjflx/flx mice were given three doses (250 mg/kg) of tamoxifen via oral gavage between P19 and P24 to activate K14CreER, followed by Atoh1 induction by doxycycline for 4 days from P24 to P28 and tissue was harvested on P28 or 3 months later (n=2−3 mice/genotype/time point). A few ectopic K8+ cells were present in K14CreER; ROSA26R, TetAtoh1; RBPjflx/flx mice, demonstrating that Rbpj ablation alone was sufficient to allow their production (Fig. 8B). Because mosaic recombination occurred in hair follicles, at P28 only relatively small numbers of K8+ cells (albeit more than obtained through Rbpj deletion alone) were present in the hairy skin of K14CreER; ROSA26R, TetAtoh1; RBPjflx/flx mice (Fig. 8C). By contrast, K14CreER; ROSA26R, TetAtoh1; RBPjflx/flx and K14CreER; ROSA26R, TetAtoh1; RBPjflx/flx had large numbers of ectopic K8+ cells (Fig. 8D,E), suggesting that acute ablation of Notch signaling greatly enhanced epidermal competency to respond to Atoh1 overexpression. These differences were statistically significant across genotypes (one-way ANOVA F=8.27, P=0.022; post-hoc pairwise Scheffé tests: K14CreER; ROSA26R, TetAtoh1; RBPjflx/flx versus K14CreER; ROSA26R, TetAtoh1; RBPjflx/flx P=0.05 and K14CreER; ROSA26R, TetAtoh1; RBPjflx/flx versus K14CreER; ROSA26R, TetAtoh1; RBPjflx/flx P=0.042). As in K14CreER; ROSA26R, TetAtoh1 mice, all ectopic K8+ cells were present in follicular epidermis and were not innervated in any of the four genotypes (data not shown). We found no difference in the number of touch dome K8+ cells between K14CreER; ROSA26R, TetAtoh1; RBPjflx/flx and K14CreER; ROSA26R, TetAtoh1; RBPjflx/flx mice (19±2.0, 17±0.4, 17±3.4, and 17±0.2 K8+ cells/touch dome, respectively; one-way ANOVA F=0.21, P=0.8873). Three months post-induction (P140), K14CreER; ROSA26R, TetAtoh1; RBPjflx/flx
mice had K8+ cells, but whether these were ectopic or part of touch domes was impossible to determine because of hair loss with near-complete destruction of hair follicles, skin thickening and cutaneous cyst formation similar to that reported following Rbpj deletion using a K15CrePR1 allele (Demehri and Kopan, 2009). K14CreER; ROSAartTA; TetAtoh1; RBPj+/+ and K14CreER; ROSAartTA; TetAtoh1; RBPjlox/lox and K14CreER; ROSAartTA; TetAtoh1; RBPjlox/lox mice were dosed with tamoxifen and doxycycline as indicated, then back skin harvested and immunostained for K8 immediately (B-E) or 3 months later (F-I). Boxes indicate areas shown at higher magnification in insets. TD, touch dome. Scale bars: 50 μm.

**DISCUSSION**

Our data demonstrate that ectopic Atoh1 expression is sufficient to drive K8+ cell production in the embryonic and postnatal mammalian epidermis. These ectopic cells exhibit multiple characteristics of mature Merkel cells, including morphology, marker expression and FM1-43 dye uptake. In addition, they acquire these characteristics in a time frame comparable to that of Merkel cells during normal touch dome development, suggesting that the developmental programs controlled by Atoh1 expression are similar in both populations. These data indicate that Atoh1 expression is sufficient to direct epidermal cells to become Merkel cells. These findings are reminiscent of those seen in the ear and intestine, where ectopic Atoh1 expression drives production of supernumerary hair cells and secretory/endocrine cells, respectively (Kelly et al., 2012; Lin et al., 2011; VanDussen and Samuelson, 2010).

Cellular competency to respond to the robust doxycycline-induced ectopic Atoh1 expression seen in K14Cre; ROSAartTA; TetAtoh1 mice varied by age, location and hair cycle stage (Figs 1, 2, 6, 7). Developmental and geographical differences in competency also occur in the developing ear, where ectopic Atoh1 expression drives new hair cell production within and outside of the sensory epithelium of neonatal mice, only in the sensory epithelium of slightly older mice, and not at all in juvenile mice (Kelly et al., 2012). In both systems, variations in cellular responses are likely to be secondary to a multitude of factors that include epigenetic alterations, coexpression of transcription factors that act as activators or repressors, signaling through various genetic pathways (i.e. BMP, SHH, Wnt, etc.) and potentially even cell-cycle stage. In the ear, competency depends upon Sox2 expression in the target tissue, which occurs throughout the sensory epithelium and Kölliker’s organ at early ages but becomes restricted to supporting cells as mice age (Hume et al., 2007; Kelly et al., 2012). This is unlikely to be the case in the skin because Sox2 is expressed only by developing and mature Merkel cells and not by other epidermal cells (Bardot et al., 2013; Lesko et al., 2013). Instead, we have identified Notch signaling as one of the factors that regulates epidermal competency to respond to Atoh1. Coupling conditional deletion of the universal Notch effector Rbpj with Atoh1 transgene induction in K14CreER; ROSAartTA; TetAtoh1; RBPjlox/lox mice caused a marked increase in the number of ectopic K8+ cells (Fig. 8), a finding consistent with the antagonistic relationship between Atoh1 and Notch signaling in the ear and intestine during normal development and in the setting of Atoh1 overexpression (Golub et al., 2012; Kelly et al., 2012; Kim and Shivdasani, 2011; Lanford et al., 2000; Zheng et al., 2000; Zine et al., 2001). Interestingly, Rbpj deletion in the absence of Atoh1 transgene
expression was itself sufficient to create a few ectopic K8+ cells, suggesting that the Notch pathway normally represses Atoh1 expression in at least a subset of epidermal cells. Variations in Notch pathway signaling occur during development and during different phases of the hair cycle, which might help to explain why Atoh1 induction produces more ectopic K8+ cells in embryos than in adult mice and in anagen versus telogen (Ambler and Watt, 2010; Favier et al, 2000; Powell et al., 1998). Furthermore, Notch1 is expressed by most Merkel cell carcinomas (MCCs) (Panelos et al., 2009), but whether Atoh1/Notch antagonism participates in MCC pathogenesis has not been investigated. Future studies will address the role of Notch signaling in regulating endogenous Atoh1 expression in both normal Merkel cell development and MCC.

The density of ectopic K8+ cells in the hairy skin of K14Cre; ROSA26TA; TetAtoh1 mice decreased substantially from 4 days to 3 months post-induction, probably secondary to apoptotic cell death (Fig. 2; Fig. 3A-A‴). Previous reports suggest that mature Merkel cells live ~7-8 weeks (Doucet et al., 2013; Van Keymeulen et al., 2009), a time frame too long to explain the large decrease in cell numbers between 4 days and 2 weeks post-induction. Ectopic K8+ cell numbers also decreased dramatically between 2 and 6 weeks post-induction, at which point the density of ectopic K8+ cells remained constant up to 3 months (Fig. 2K). These long-lived ectopic K8+ cells were confined to deep regions (bulge and bulb) of hair follicles from 2 weeks post-doxycycline onwards, suggesting that these locations are permissive niches that facilitate survival. In normal touch domes, Merkel cell survival depends upon SAI innervation (Burgess et al., 2013; Moll et al., 1996; Nafstad, 1987; Nakafusa et al., 2006), and are permissive niches that facilitate survival. In normal touch domes, Merkel cell survival depends upon SAI innervation (Burgess et al., 2013; Moll et al., 1996; Nafstad, 1987; Nakafusa et al., 2006; Van Keymeulen et al., 2009), but whether Atoh1/Notch antagonism participates in MCC pathogenesis has not been investigated. Future studies will address the role of Notch signaling in regulating endogenous Atoh1 expression in both normal Merkel cell development and MCC.

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Mature Merkel cells are post-mitotic (Moll et al., 1995), but quantitative, morphological and fate-mapping studies suggest that Merkel cells turnover throughout an organism’s lifespan (Doucet et al., 2013; Moll et al., 1996; Nafstad, 1987; Nakafusa et al., 2006; Van Keymeulen et al., 2009). These data imply the existence of a Merkel cell precursor that gives rise to new Merkel cells. We recently showed that embryonic Merkel cell precursors express Atoh1 and are unipotent (Wright et al., 2015). However, three lines of evidence suggest that ectopic K8+ cells created by ectopic Atoh1 expression are post-mitotic. First, no ectopic K8+ cells expressed the proliferative cell marker Ki67 (Fig. 3B-B‴). Second, ectopic K8+ cell numbers remained constant between 6 weeks and 3 months post-induction, suggesting that there was neither cell death nor replication (Fig. 2K). Third, clusters of K8+ cells were never observed at these time points, suggesting that clonal expansion of individual cells did not occur (Fig. 2). Therefore, ectopic Atoh1 expression alone is insufficient to produce Merkel cell precursors from the keratinocyte lineage, demonstrating that other factors in addition to Atoh1 are required for Merkel cell precursor production. Furthermore, our data suggest that Atoh1 expression in basal epidermal keratinocytes, which are normally proliferative, removes their ability to divide without affecting proliferation of surrounding Atoh1− cells. This provides more supportive evidence that Atoh1 expression induces a cell-autonomous cellular fate switch away from the keratinocyte fate to that of a true mature Merkel cell fate.

Our data also shed light on the relationship between Atoh1 and Sox2, which is expressed from early stages of Merkel cell development (Bardot et al., 2013; Lesko et al., 2013). Ectopic Atoh1 expression in K14Cre; ROSA26TA; TetAtoh1 mice was sufficient to initiate persistent Sox2 and Isl1 expression, providing in vivo evidence that both genes are downstream of Atoh1. This is consistent with the observation that Sox2+ epidermal cells are absent from the skin of Atoh1-null mice (Perdigoto et al., 2014). Upregulation of Sox2 and Isl1 combined with Atoh1 autoregulation (Helms et al., 2000) might contribute to maintained Atoh1 expression seen following Atoh1 transgene silencing. Further studies are necessary to determine whether maintained Sox2 and Isl1 expression depends upon continuous Atoh1 expression.

Finally, our results are potentially relevant for understanding MCC ontogeny. MCC has long been thought to derive from Merkel cells or their precursors because tumor cells exhibit immunohistochemical and ultrastructural similarities to normal Merkel cells and often express HATH1, the human Atoh1 homolog (Heiskala et al., 2010). However, over 50% of MCC tumors and cell lines do not express HATH1 (Leonard et al., 2002; Van Gele et al., 2004), and other lines of evidence suggest that MCC might arise from keratinocytes, skin stem cells or even immune B cells (Zur Hausen et al., 2013; Hewitt et al., 1993; Youker, 2003). The existence of K14+ MCC tumor cells (Lemasson et al., 2012) and mixed MCC/squamous cell carcinoma with/or without eccrine differentiation (Gould et al., 1988; Iacocca et al., 1998; Szadowska et al., 1989) further suggest a non-Merkel cell origin for MCC. Ectopic expression of Atoh1 in our system failed to produce skin tumors regardless of age of induction or survival time, supporting the viewpoint that Atoh1 acts as a tumor suppressor in the skin (Bosuut et al., 2009). However, our demonstration that ectopic Atoh1 expression alone was sufficient to drive expression of multiple Merkel cell markers suggests that MCC need not arise from the Merkel cell lineage, but that marker expression in these tumors might be driven solely by Atoh1 expression. Therefore, driving ectopic HATH1 expression in human skin, coupled with dysregulated cell division, could potentially cause MCC. One potential mechanism for this could be infection by the Merkel cell polyoma virus (McPV), whose small T-antigen drives oncogenic transformation (Verhaegen et al., 2015). Further work is necessary to determine whether this mechanism operates in MCC.

MATERIALS AND METHODS

Mice

K14Cre (Jax #004782) (Dassule et al., 2000), ROSA26TA::GFP (Jax #005572) (Belteki et al., 2005), ROSA26R (Jax #007914) (Madisen et al., 2010) and TetAtoh1 mice (a generous gift of Dr Ping Chen, Emory University, Atlanta, GA, USA) (Kelly et al., 2012) were maintained on a congenic C57BL/6 genetic background. We verified a report (Kelly et al., 2012) that eGFP fluorescence in ROSA26TA::GFP mice is undetectable without immunostaining (data not shown), and we refer to this allele as ROSA26TA. Ectopic expression seen following Atoh1 expression seen following Atoh1 transgene silencing. Further studies are necessary to determine whether maintained Sox2 and Isl1 expression depends upon continuous Atoh1 expression.

Tamoxifen administration

For induction of Rbpj deletion in the keratinocyte lineage, tamoxifen (250 mg/kg; Sigma) dissolved in a 1:9 ethanol:corn oil solution was administered to P18-P24 mice by oral gavage every other day for three doses, followed by doxycycline administration for indicated durations (see below).
Doxycycline administration

Doxycycline-containing chow (200 mg/kg; BioServ) was provided ad libitum to transgenic and control pregnant dams from E14.5-E18.5 (plug date designated at E0.5) for embryonic experiments or to P22-P26 mice for 24 or 96 h for adolescent/adult experiments. For early postnatal ages, doxycycline (1 mg in 100 µl of 100% ethanol) was applied to the entire back and flank skin of pups twice per day for 3 days (P2-P4).

FM dye injections

Fixable FM1-43 dye (4 mg/kg; FM1-43FX; Life Technologies) was injected intraperitoneally and mice were sacrificed 24 h later (Meyers et al., 2003).

Tissue harvest

Embryos (E18.5), and pups were euthanized by decapitation, tails collected for genotyping, and skin dissected. Adolescent/adult mice were euthanized by cervical dislocation and skin was shaved and depilated with Surgicream. Skin was dissected and either snap frozen in Optimal Cutting Temperature medium (OCT, Thermo Fisher Scientific) or immersion fixed for 1 h in ice-cold 4% paraformaldehyde. Skin for wholemount immunostaining was washed and stored in 1× PBS, whereas skin for sectioning was cryoprotected in 30% sucrose, embedded in OCT, and cryosectioned onto Fisher Superfrst Plus slides at 10 or 20 µm using a Leica CM1950 cryostat.

Immunostaining

We used the following primary antibodies: chicken anti-Atoh1 (generous gift of Drs Tom Coates and Matthew Kelley, NIDCD/NIH; 1:10,000), rabbit anti-cleaved caspase-3 (Cell Signaling, 9661S; 1:250), rabbit anti-Islet 1 (Abcam, cat #AB109517; 1:200), rat anti-keratin 8 (Clone RGE53, Millipore; 1:200), mouse anti-keratin 20 (clone Ks20.8, Life Technologies; 1:100), rabbit anti-Ki67 (Thermo Fisher Scientific, RM9106-S1; 1:500), rabbit anti-Rab3c (Genetex, cat #GTX13047; 1:1000), rabbit anti-Sox2 (Millipore, cat #AB5603; 1:400), rabbit anti-NF200 (Sigma-Aldrich, NF142; 1:500) and rabbit anti-VGLUT2 (Synaptic Systems, cat #135402; 1:3000). Secondary antibodies (Jackson Immunoresearch) were used at a dilution of 1:250.

Wholemount immunostaining was carried out using a published protocol (Li et al., 2011) with modifications. Fat was removed and the skin cut into 2×2 mm pieces, rinsed in 1× PBS, washed with 1× PBS/0.3% Triton X-100 (0.3% PBST) every 30 min for 5-8 h, then incubated with primary antibodies in 0.3% PBST/5% goat or donkey serum/20% DMSO at room temperature for 3-5 days. Samples were washed with 0.3% PBST every 30 min for 5-8 h, transferred to secondary antibodies in 0.3% PBST/5% goat or donkey serum/20% DMSO and incubated at room temperature for 2-4 days, washed with 0.3% PBST every 30 min for 5-8 h then mounted on Fisher Superfrst Plus slides using Prolong Gold (Invitrogen).

For sectioned tissue, slides were washed in 1× PBS, blocked in 5% normal donkey serum (NDS) for 30 min, and incubated in primary antibody diluted in blocking solution overnight at 4°C. Slides were washed in 1× PBS, incubated in secondary antibody diluted in blocking solution for 30 min at room temperature, washed in 1× PBS, counterstained with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride; Fisher; 1:1000), and mounted in Prolong Gold.

Immunostaining for Islet1, Ki67 and Sox2 required antigen retrieval consisting of slide immersion in sub-boiling 10 m sodium citrate buffer for 10 min, followed by 30 min at room temperature. Slides were then stained as described above.

Image acquisition

Fluorescent images were acquired with a Leica DM 5500B epifluorescence microscope using HCX PL-APO 40×1.25 NA, HCX PL-APO 20×0.70 NA and HC PL-APO 10×0.4 NA objectives, Leica DFC420 camera and Leica Acquisition Software v4.2 or an inverted Zeiss Axio Observer on a PerkinElmer UltraVIEW VoX spinning disc confocal with C-APO 40×1.1 NA water immersion objective, Hamamatsu C9100-13 camera and Volocity software. Images were further processed using Adobe Photoshop.

Cell counts

In wholemount preparations of back skin (n=2-5 mice/time point), K8+ cells within (n=10-25 touch domes/mouse) and outside of (1 cm² area of skin/mouse) touch domes were counted except for the 4 day time point, for which the high density of ectopic K8+ cells necessitated counting two 200 µm² areas from each mouse and extrapolating to ectopic K8+ cells/cm². In sectioned tissue, at least 100 ectopic (non-touch dome-associated) K8+ cells were counted per mouse per time point/age and scored for co-labeling with the designated marker (n=3 mice/time point or age). Examining a similar number of slides from littermate controls confirmed the absence of ectopic K8+ cells.

Follicular and interfollicular epidermal Ki67+ cell number in P29 mice and K8+ cell number in E18.5 and P4 mice were determined in tissue sections. Five representative follicles were imaged at 20× and all Ki67+ or K8+ cells in each field of view were counted within the hair follicle (follicular) and in the epidermis around the follicle (interfollicular), then averaged for each mouse (n=2-3 mice/genotype/age). Cell counts were compared by one-way ANOVA followed by Scheffé pairwise comparison testing or Student’s t-test as indicated.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

S.M.O. and S.M.M. designed the study; S.M.O., A.M.B., M.C.W. and X.G. performed the experiments; S.M.O., M.C.W., A.M.B., X.G. and S.M.M. analyzed the data; S.M.O., M.C.W. and S.M.M. wrote the manuscript with input from the other authors.

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Supplementary material

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