© 2015. Published by The Company of Biologists Ltd | Development (2015) 142, 2533-2544 doi:10.1242/dev.123141

RESEARCH ARTICLE

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

Stephen M. Ostrowski[1,*,‡], Margaret C. Wright[2,‡], Alexa M. Bolock[3], Xuehui Geng[4] and Stephen M. Maricich[4,§]

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 (SAI) nerve fibers and act as mechanotransducers important for detecting certain forms of light touch (Ikeda 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 Shivdasani, 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 ($K14^{Cre}$) (Dassule et al., 2000), mice that express a Cre-activated reverse tetracycline transactivator ($ROSA^{rtTA}$) (Belteki et al., 2005) and mice with a tetracycline-inducible *Atoh1* transgene (Tet^{Atoh1}) (Kelly et al., 2012). These triple transgenic $K14^{Cre}$; $ROSA^{rtTA}$; Tet^{Atoh1} mice allow inducible *Atoh1* expression throughout the epidermal lineage for the duration of doxycycline administration (Fig. 1A).

Adolescent [postnatal day (P)22-P26] $K14^{Cre}$; $ROSA^{rtTA}$; Tet^{Atoh1} 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

[1]Department of Dermatology, Case Western Reserve University, Cleveland, OH 44106, USA. [2]Center for Neurosciences, University of Pittsburgh, Pittsburgh, PA 15260, USA. [3]Department of Neuroscience, University of Pittsburgh, Pittsburgh, PA 15260, USA. [4]Richard King Mellon Institute for Pediatric Research, Department of Pediatrics, University of Pittsburgh, Pittsburgh, PA 15224, USA.
*Present address: Department of Dermatology, Massachusetts General Hospital, Boston, MA 02114, USA.
‡These authors contributed equally to this work

§Author for correspondence (stephen.maricich@chp.edu)

Received 9 February 2015; Accepted 4 June 2015

Development (2015) 142, 2533-2544 doi:10.1242/dev.123141

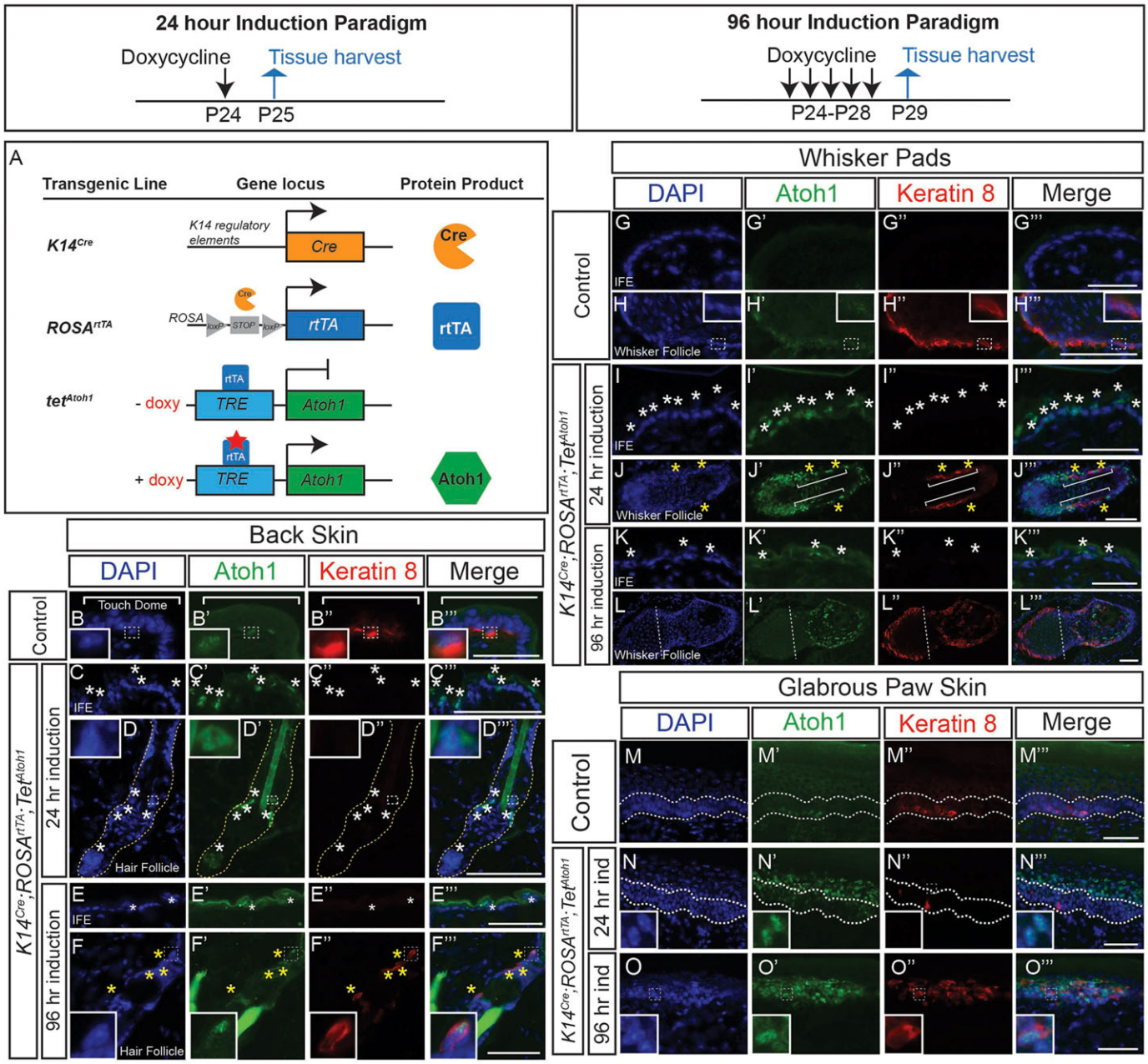

Fig. 1. Inducible *Atoh1* expression produces ectopic K8+ cells in glabrous and hairy skin of adolescent *K14*^{Cre}; *ROSA*^{rtTA}; *Tet*^{Atoh1} mice. Experimental induction paradigms are shown at the top of the figure. (A) Schematic of *K14*^{Cre}; *ROSA*^{rtTA}; *Tet*^{Atoh1} mouse alleles. Cre is produced in K14-expressing cells, which then removes the floxed stop allele upstream of rtTA at the *ROSA* locus. Upon administration of doxycycline, rtTA binds to *Tet* to drive *Atoh1* expression. (B-O‴) Sectioned back skin (B-F‴), whisker pads (G-L‴) and glabrous paw skin (M-O‴) immunostained for Atoh1 and K8 of littermate control (B-B‴,G-H‴,M-M‴) and *K14*^{Cre}; *ROSA*^{rtTA}; *Tet*^{Atoh1} mice (C-F‴,I-L‴,N-O‴) treated with doxycycline for 24 or 96 h. Asterisks denote ectopic Atoh1+ (white) and Atoh1+K8+ (yellow) cells in the interfollicular epidermis (IFE) and hair follicles of the back skin and whisker pads. Brackets (J′-J‴) mark the position of ectopic Atoh1+ cells that co-express low levels of K8. Dashed lines in D-D‴ indicate hair follicle boundaries. Dashed lines in L-L‴ separate normal Merkel cells (left) from ectopic K8+ cells (right). Dashed lines in M-N‴ mark position of normal Merkel cells; this delineation was difficult in O-O‴ owing to the large number of ectopic cells. Skin surface is at the top (B-F‴,G-G‴,I-I‴,K-K‴,M-O‴) or right (H-H‴,J-J‴,L-L‴) of panels. Hairs autofluoresce in the green channel. Boxes denote regions shown at higher magnification in insets. Scale bars: 50 µm.

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″,D″,I″,J″,N″). Doxycycline administration for 96 h resulted in greater numbers of ectopic Atoh1+ cells in all regions (Fig. 1E-F‴,K-L‴,O-O‴). 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-B‴,G-H‴,M-M‴; Fig. 2A,D-D″,G). These data suggest that keratinocytes in different skin regions exhibit differential competence to respond to *Atoh1* expression. Unfortunately, *K14*^{Cre}; *ROSA*^{rtTA}; *Tet*^{Atoh1} 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

Development (2015) 142, 2533-2544 doi:10.1242/dev.123141

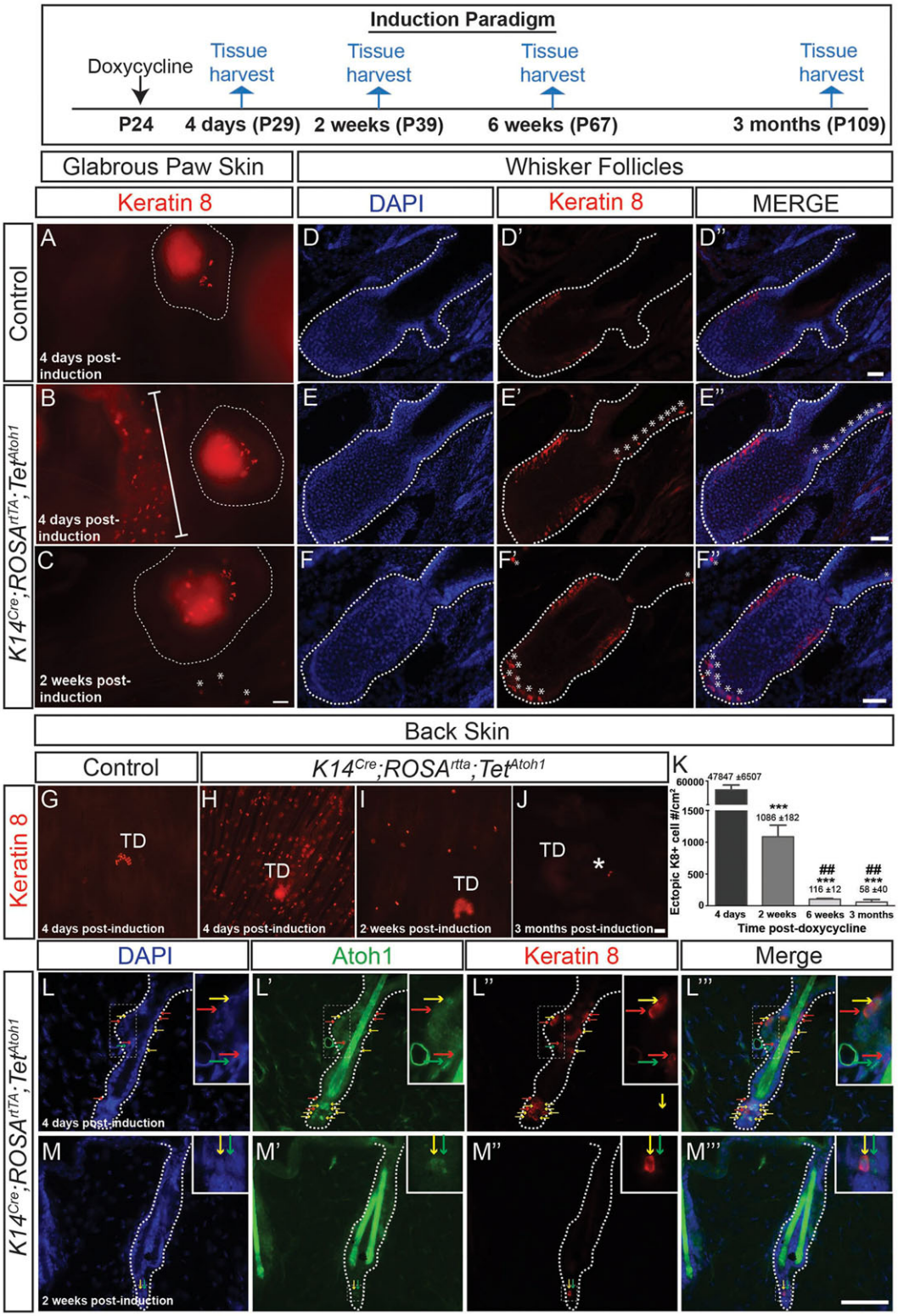

Fig. 2. Ectopic K8+ cells persist in glabrous and hairy skin of *K14*^{Cre}; *ROSA*^{rtTA}; *Tet*^{Atoh1} mice. Experimental induction paradigm is shown at the top of the figure. (A-J) Wholemount glabrous paw skin (A-C), sectioned whisker follicles (D-F″) and wholemount back skin (G-J) of control (A,D-D″,G) and *K14*^{Cre}; *ROSA*^{rtTA}; *Tet*^{Atoh1} mice four days (B,E-E″,H), two weeks (C,F-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<0.001 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 *K14*^{Cre}; *ROSA*^{rtTA}; *Tet*^{Atoh1} mouse back skin four days (L-L‴) 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.

Development (2015) 142, 2533-2544 doi:10.1242/dev.123141

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 *K14*^{Cre}; *ROSA*^{rtTA}; *Tet*^{Atoh1} 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 *K14*^{Cre}; *ROSA*^{rtTA}; *Tet*^{Atoh1} mice 4 days post-doxycycline, indicating that ectopic K8+ cells were not mitotically active (Fig. 3B-B‴). Furthermore, *K14*^{Cre}; *ROSA*^{rtTA}; *Tet*^{Atoh1} mice did not manifest skin malformations or overt changes in epidermal structure at any time point, and numbers of Ki67+K8− epidermal cells were similar in *K14*^{Cre}; *ROSA*^{rtTA}; *Tet*^{Atoh1} 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.

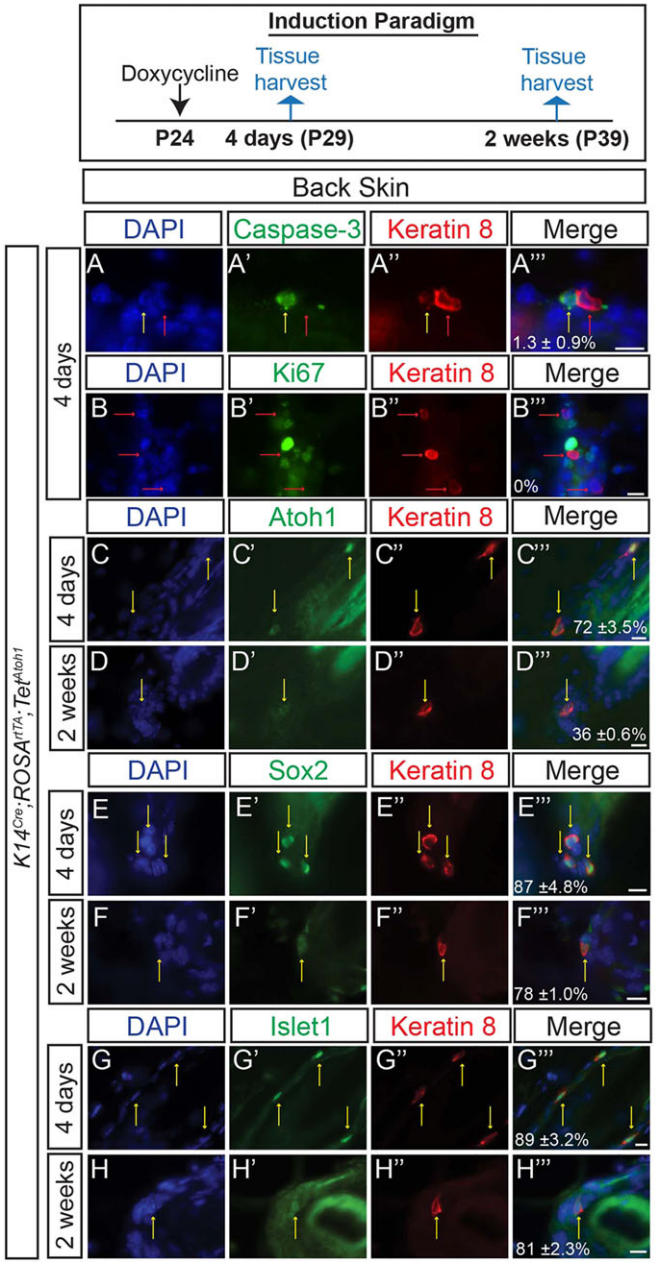

Fig. 3. Ectopic K8+ cells express Atoh1, Sox2 and Isl1, some express caspase-3, but none is mitotically active. Experimental induction paradigm is shown at the top of the figure. (A-H‴) Sectioned back skin of adolescent *K14*^{Cre}; *ROSA*^{rtTA}; *Tet*^{Atoh1} mice showing ectopic K8+ cells in hair 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.

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 *K14*^{Cre}; *ROSA*^{rtTA}; *Tet*^{Atoh1} 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‴),

Development (2015) 142, 2533-2544 doi:10.1242/dev.123141

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 styryl 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).

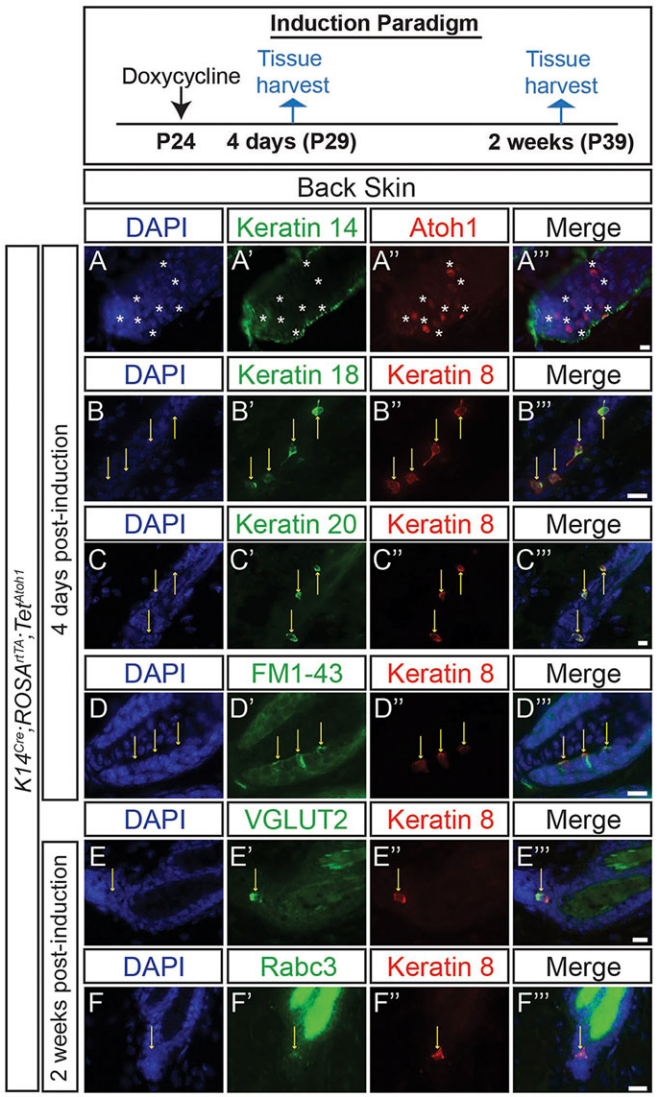

Fig. 4. Ectopic K8+ cells express mature Merkel cell markers but not keratin 14. Experimental induction paradigm is shown at the top of the figure. (A-F‴) Sectioned back skin of adolescent *K14*^{Cre}; *ROSA*^{rtTA}; *Tet*^{Atoh1} mice showing ectopic K8+ cells in hair follicles 4 days (A-D‴) and 2 weeks (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.

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 (*K14*^{Cre} and *Tet*^{Atoh1}; *ROSA*^{rtTA}) 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 *K14*^{Cre}; *ROSA*^{rtTA}; *Tet*^{Atoh1} 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 *K14*^{Cre}; *ROSA*^{rtTA}; *Tet*^{Atoh1} 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 *K14*^{Cre}; *ROSA*^{rtTA}; *Tet*^{Atoh1} 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 *K14*^{Cre}; *ROSA*^{rtTA}; *Tet*^{Atoh1} embryos (supplementary material Fig. S1D,E). Induction of *Atoh1 in utero*, even for periods as short as 24 h, resulted in

Development (2015) 142, 2533-2544 doi:10.1242/dev.123141

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

	Ectopic K8+ cells in *K14*^{Cre}; *ROSA*^{rtTA}; *Tet*^{Atoh1} mice		Touch dome K8+ cells in C57BL/6J mice	
	4 days post-doxycycline	2 weeks post-doxycycline	P0	P7
K8+K18+/K8+	100±0%	100±0%	97±0.9%	100±0%
K8+K20+/K8+	94±0.8%	95±3.9%	87±3.2%	99±0.3%
K8+FM1-43/K8+	91±5.3%	100±0%	98±0.6%	94±5.9%
K8+VGLUT2+/K8+	10±2.6%	94±2.0%	13±3.2%	95±1.0%
K8+Rab3c+/K8+	5±1.5%	69±4.3%	3±1.8%	83±4.1%

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.

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 *K14*^{Cre}; *ROSA*^{rtTA}; *Tet*^{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 *K14*^{Cre}; *ROSA*^{rtTA}; *Tet*^{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.

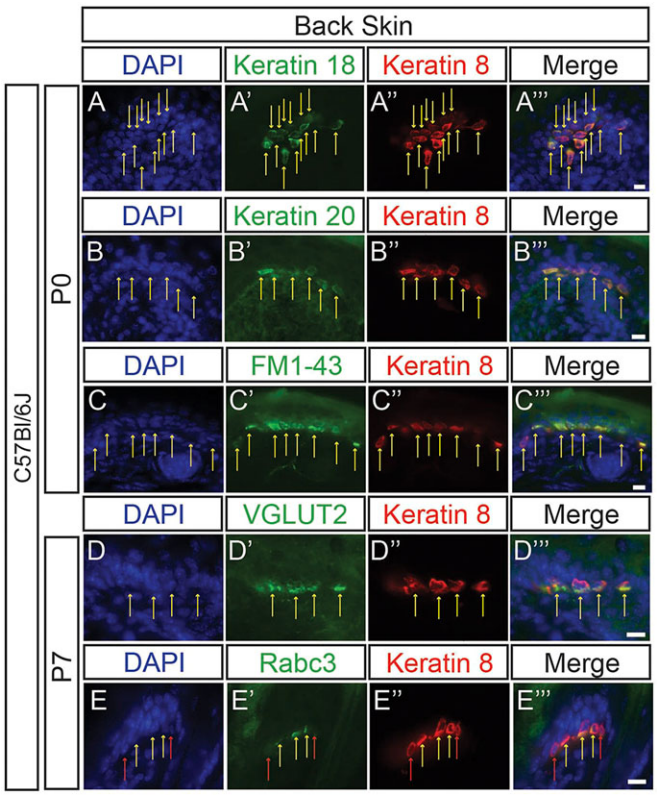

Fig. 5. Developmental time course of touch dome Merkel cell marker expression. (A-E‴) Sectioned back skin showing touch domes of wild-type C57Bl/6J P0 (A-C‴) and P7 (D-E‴) mice immunostained for or labeled with K18 (A′), K20 (B′), FM1-43 (C′), Vglut2 (D′), Rab3c (E′) and K8 (A″,B″,C″,D″,E″). Yellow arrows indicate double-positive cells; red arrows indicate K8+ cells that do not express the marker of interest. Co-expression data is quantified in Table 1. Scale bars: 10 µm.

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 *K14*^{CreER}; *ROSA*^{rtTA}; *Tet*^{Atoh1}; *RBPj*^{flox} mice. We used the tamoxifen-inducible *K14*^{CreER} allele (Vasioukhin et al., 1999) rather than the constitutively expressed *K14*^{Cre} allele because constitutive ablation of *Rbpj* in the

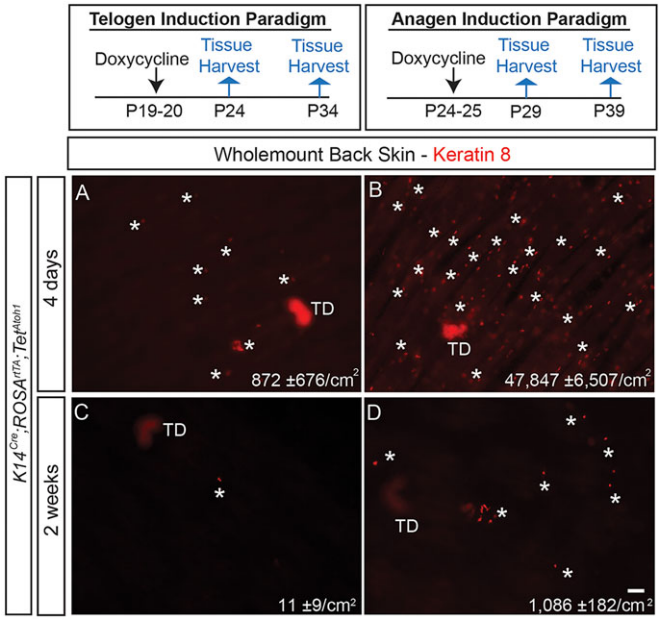

Fig. 6. Keratinocyte competency to respond to ectopic *Atoh1* varies by hair cycle stage. Experimental induction paradigms are shown at the top of the figure. (A-D) Wholemount back skin from *K14*^{Cre}; *ROSA*^{rtTA}; *Tet*^{Atoh1} mice induced during telogen (P19; A,C) or anagen (P24; B,D), taken 4 days (A,B) or 2 weeks (C,D) post-induction and immunostained for K8. Asterisks denote individual or groups of ectopic K8+ cells. Numbers indicate average densities of ectopic K8+ cells (±s.e.m.; *n*=3 mice/hair cycle stage). TD, touch dome. Scale bar: 50 µm.

Development (2015) 142, 2533-2544 doi:10.1242/dev.123141

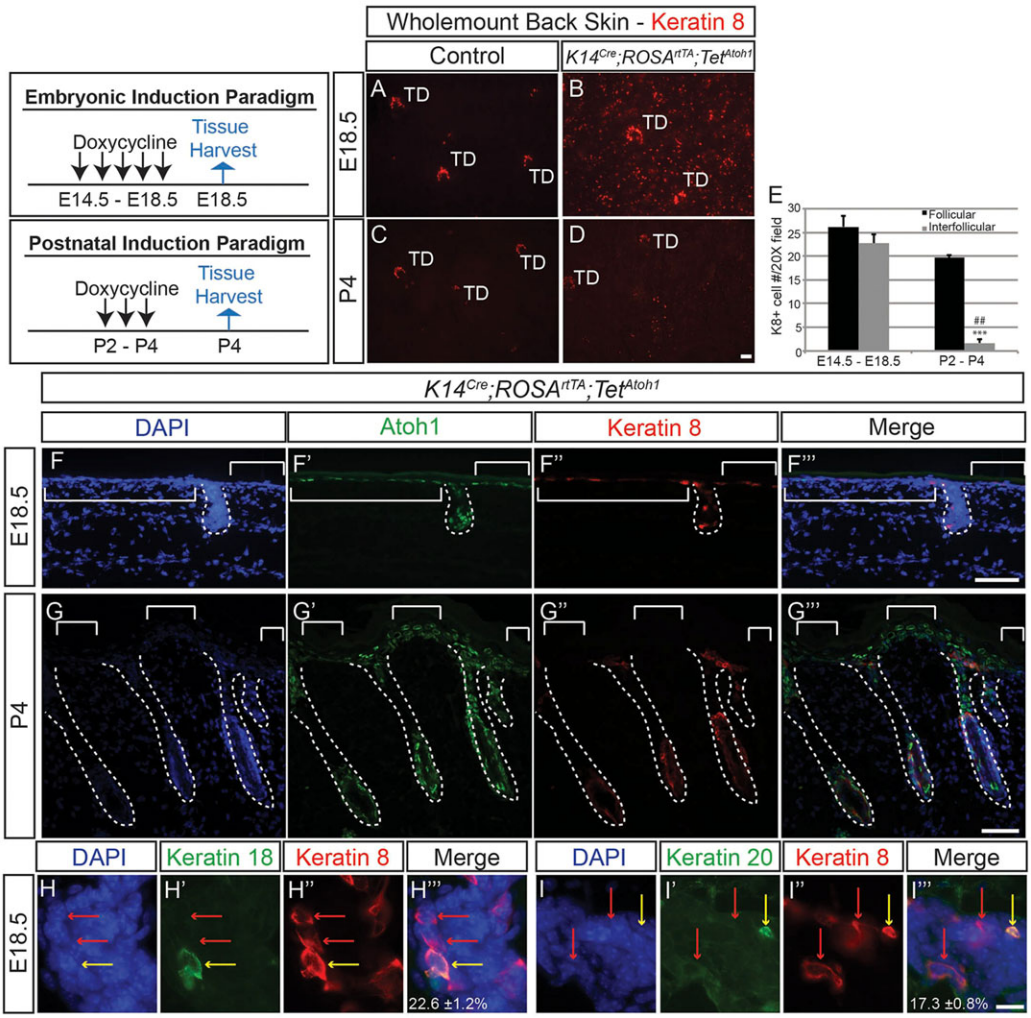

Fig. 7. Keratinocyte competency to respond to ectopic *Atoh1* decreases as animals age. Experimental induction paradigms are shown at the top of the figure. (A-I‴) Wholemount (A-D) and sectioned (F-I‴) back skin immunostained for K8 (A-D,F″,G″,H″,I″) and Atoh1 (F′,G′), K18 (H′) or K20 (I′) from E18.5 (A,B, F-F‴,H-I‴) and P4 (C,D,G-G‴) $K14^{Cre}$; $ROSA^{rtTA}$; Tet^{Atoh1} mice (B,D,F-I‴) and control littermates (A,C) given doxycycline for the indicated times. (E) Ectopic Merkel cell number in follicular and interfollicular skin (K8+ cells per 20× field ±s.e.m.; *n*=2-3 mice/age). ***P<0.001 versus E14.5-E18.5 follicular; $^{##}P$<0.01 versus E14.5-E18.5 interfollicular (two-way ANOVA with post-hoc pairwise Scheffé test). Hair follicles are outlined with dashed lines and interfollicular skin indicated with brackets. Red arrows indicate K8+ cells and yellow arrows K8+K18+ or K8+K20+ cells; percentages (±s.e.m.) of double-labeled cells are shown (*n*=100-200 K8+ cells from each of three mice). TD, touch dome. Scale bars: (A-G‴) 50 µm; (H-I‴) 10 µm.

epidermis causes perinatal lethality (Blanpain et al., 2006). The $K14^{CreER}$ 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.

$K14^{CreER}$; $ROSA^{rtTA}$; $RBPj^{flox/flox}$, $K14^{CreER}$; $ROSA^{rtTA}$; Tet^{Atoh1}; $RBPj^{+/+}$, $K14^{CreER}$; $ROSA^{rtTA}$; Tet^{Atoh1}; $RBPj^{flox/+}$ and $K14^{CreER}$; $ROSA^{rtTA}$; Tet^{Atoh1}; $RBPj^{flox/flox}$ mice were given three doses (250 mg/kg) of tamoxifen via oral gavage between P19 and P24 to activate $K14^{CreER}$, 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 $K14^{CreER}$; $ROSA^{rtTA}$; $RBPj^{flox/flox}$ 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 $K14^{CreER}$; $ROSA^{rtTA}$; Tet^{Atoh1}; $RBPj^{+/+}$ mice (Fig. 8C). By contrast, $K14^{CreER}$; $ROSA^{rtTA}$; Tet^{Atoh1}; $RBPj^{flox/+}$ and $K14^{CreER}$; $ROSA^{rtTA}$; Tet^{Atoh1}; $RBPj^{flox/flox}$ 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: $K14^{Cre}$; $ROSA^{rtTA}$; $RBPj^{flox/flox}$ versus $K14^{Cre}$; $ROSA^{rtTA}$; Tet^{Atoh1}; $RBPj^{flox/flox}$ P=0.05 and $K14^{Cre}$; $ROSA^{rtTA}$; Tet^{Atoh1}; $RBPj^{+/+}$ versus $K14^{Cre}$; $ROSA^{rtTA}$; Tet^{Atoh1}; $RBPj^{flox/flox}$ P=0.042). As in $K14^{Cre}$; $ROSA^{rtTA}$; Tet^{Atoh1} 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 $K14^{CreER}$; $ROSA^{rtTA}$; $RBPj^{flox/flox}$, $K14^{CreER}$; $ROSA^{rtTA}$; Tet^{Atoh1}; $RBPj^{+/+}$, $K14^{CreER}$; $ROSA^{rtTA}$; Tet^{Atoh1}; $RBPj^{flox/+}$ and $K14^{CreER}$; $ROSA^{rtTA}$; Tet^{Atoh1}; $RBPj^{flox/flox}$ mice (19±2.0, 17±0.4, 17±4, and 17±0.2 K8+ cells/touch dome, respectively; one-way ANOVA F=0.21, P=0.8873). Three months post-induction (P140), $K14^{CreER}$; $ROSA^{rtTA}$; $RBPj^{flox/flox}$ and $K14^{CreER}$; $ROSA^{rtTA}$; Tet^{Atoh1}; $RBPj^{flox/flox}$

Development (2015) 142, 2533-2544 doi:10.1242/dev.123141

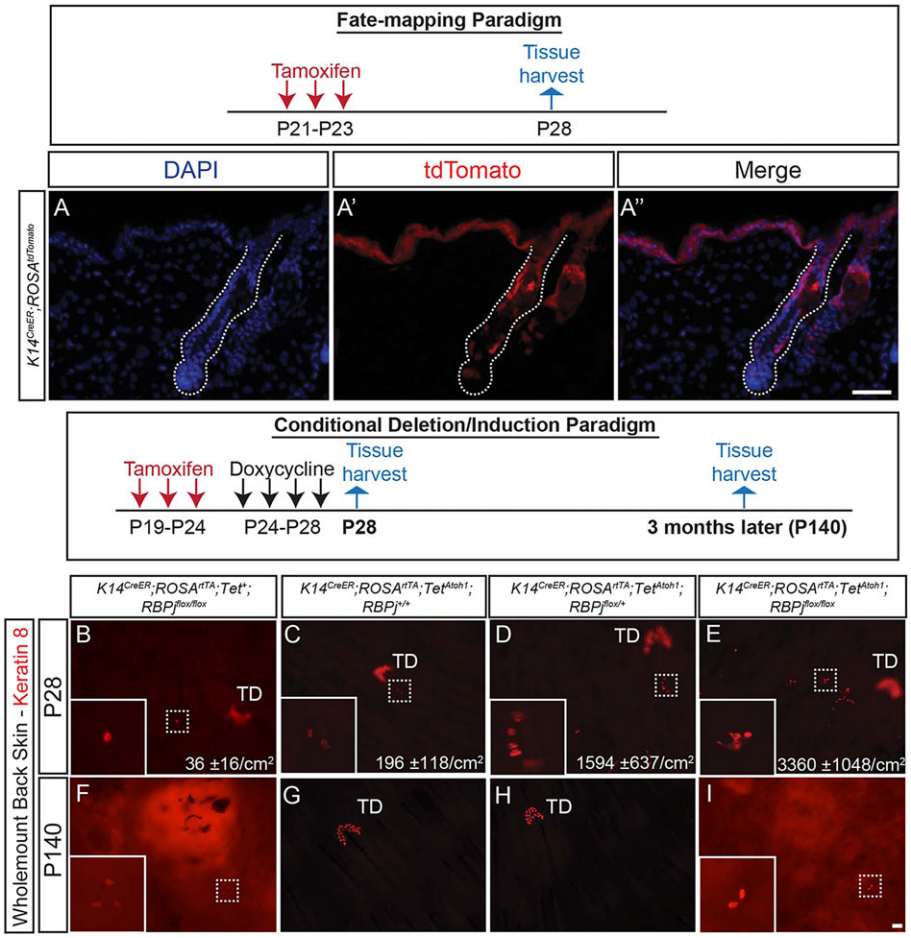

Fig. 8. Loss of Notch signaling enhances epidermal competency to respond to *Atoh1*. Experimental induction paradigms are shown at the top of each panel set. (A-A″) Sectioned back skin from an adolescent *K14*^{CreER}; *ROSA*^{tdTomato} mouse that received tamoxifen for three consecutive days shows patchy expression of tdTomato in hair follicles (outlined with dotted line). (B-I) Adolescent *K14*^{CreER}; *ROSA*^{rtTA}; *RBPj*$^{flox/flox}$, *K14*^{CreER}; *ROSA*^{rtTA}; *Tet*^{Atoh1}; *RBPj*$^{+/+}$, *K14*^{CreER}; *ROSA*^{rtTA}; *Tet*^{Atoh1}; *RBPj*$^{flox/+}$ and *K14*^{CreER}; *ROSA*^{rtTA}; *Tet*^{Atoh1}; *RBPj*$^{flox/flox}$ 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.

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 *K15*^{CrePR1} allele (Demehri and Kopan, 2009). *K14*^{CreER}; *ROSA*^{rtTA}; *Tet*^{Atoh1}; *RBPj*$^{+/+}$ and *K14*^{CreER}; *ROSA*^{rtTA}; *Tet*^{Atoh1}; *RBPj*$^{flox/+}$ mice had K8+ cells only in touch domes (Fig. 8G,H). These data demonstrate that Notch signaling regulates competency to respond to ectopic *Atoh1* expression in the skin of juvenile mice.

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 *K14*^{Cre}; *ROSA*^{rtTA}; *Tet*^{Atoh1} 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 *K14*^{CreER}; *ROSA*^{rtTA}; *Tet*^{Atoh1}; *RBPj*$^{flox/flox}$ 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

Development (2015) 142, 2533-2544 doi:10.1242/dev.123141

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 $K14^{Cre}$; $ROSA^{rtTA}$; Tet^{Atoh1} 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 seen 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., 1974; English et al., 1983) and is influenced either directly or indirectly by neurotrophins (Cronk et al., 2002; Fundin et al., 1997). Ectopic K8+ cells in our system were never innervated regardless of location or time after *Atoh1* induction, suggesting that nerve-derived factors were unlikely to maintain them. However, neurotrophins such as BDNF, NGF, NTF3 (NT-3) and NTF4 (NT-4) are all produced by the follicular epidermis and play roles in hair follicle morphogenesis and hair cycle control (Botchkarev et al., 1998a,b, 1999; Botchkareva et al., 2000). Whether these or other hair follicle-derived factors facilitate ectopic K8+ cell survival requires further study.

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 $K14^{Cre}$; $ROSA^{rtTA}$; Tet^{Atoh1} 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 (Bossuyt 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

$K14^{Cre}$ (Jax #004782) (Dassule et al., 2000), $ROSA^{rtTA,eGFP}$ (Jax #005572) (Belteki et al., 2005), $ROSA^{tdTomato}$ (Jax #007914) (Madisen et al., 2010) and Tet^{Atoh1} 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 $ROSA^{rtTA,eGFP}$ mice is undetectable without immunostaining (data not shown), and we refer to this allele as $ROSA^{rtTA}$ because it was used only for rtTA expression. $K14^{CreER}$ (Jax #005107) (Vasioukhin et al., 1999) and $RBPJ^{flox}$ mice (Han et al., 2002) were maintained on mixed genetic backgrounds. All animal work was conducted in accordance with Case Western Reserve University and University of Pittsburgh Institutional Animal Care and Use Committee guidelines. At least three mice/genotype were analyzed for each of the experiments unless otherwise stated.

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).

Development (2015) 142, 2533-2544 doi:10.1242/dev.123141

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 Superfrost 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 [TROMA1, Developmental Studies Hybridoma Bank (DSHB); 1:20], rabbit anti-keratin 14 (Covance, PRB-155P; 1:1000), mouse anti-keratin 18 (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 Superfrost 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 mM 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 cm^2 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 μm^2 areas from each mouse and extrapolating to ectopic K8+ cells/cm^2. 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.

Acknowledgements

The authors thank members of the Maricich lab and Dr Sharyl Fyffe-Maricich for thoughtful discussions of the data; Dr Stefanie Altieri for help with statistics; Drs Timothy Sanders and David Ricks for assistance with confocal imaging; Dr Ping Chen and Michael Kelly for providing the *Tet*^{Atoh1} mice; and Drs Matthew Kelley and Thomas Coate for providing the Atoh1 antibody.

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.

Funding

This work was supported by the Dermatology Foundation (S.M.O.); the National Institutes of Health [T32-AR007569 to S.M.O., R01-AR059114 to S.M.M., P30-AR39750 to S.M.M.]; and the Richard King Mellon Institute for Pediatric Research (S.M.M.). Deposited in PMC for release after 12 months.

Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.123141/-/DC1

References

Alvarez, F. J., Cervantes, C., Villalba, R., Blasco, I., Martínez-Murillo, R., Polak, J. M. and Rodrigo, J. (1988). Immunocytochemical analysis of calcitonin gene-related peptide and vasoactive intestinal polypeptide in Merkel cells and cutaneous free nerve endings of cats. *Cell Tissue Res.* **254**, 429-437.

Ambler, C. A. and Watt, F. M. (2010). Adult epidermal Notch activity induces dermal accumulation of T cells and neural crest derivatives through upregulation of jagged 1. *Development* **137**, 3569-3579.

Bardot, E. S., Valdes, V. J., Zhang, J., Perdigoto, C. N., Nicolis, S., Hearn, S. A., Silva, J. M. and Ezhkova, E. (2013). Polycomb subunits Ezh1 and Ezh2 regulate the Merkel cell differentiation program in skin stem cells. *EMBO J.* **32**, 1990-2000.

Belteki, G., Haigh, J., Kabacs, N., Haigh, K., Sison, K., Costantini, F., Whitsett, J., Quaggin, S. E. and Nagy, A. (2005). Conditional and inducible transgene expression in mice through the combinatorial use of Cre-mediated recombination and tetracycline induction. *Nucleic Acids Res.* **33**, e51.

Ben-Arie, N., Bellen, H. J., Armstrong, D. L., McCall, A. E., Gordadze, P. R., Guo, Q., Matzuk, M. M. and Zoghbi, H. Y. (1997). Math1 is essential for genesis of cerebellar granule neurons. *Nature* **390**, 169-172.

Bermingham, N. A., Hassan, B. A., Price, S. D., Vollrath, M. A., Ben-Arie, N., Eatock, R. A., Bellen, H. J., Lysakowski, A. and Zoghbi, H. Y. (1999). Math1: an essential gene for the generation of inner ear hair cells. *Science* **284**, 1837-1841.

Blanpain, C., Lowry, W. E., Pasolli, H. A. and Fuchs, E. (2006). Canonical notch signaling functions as a commitment switch in the epidermal lineage. *Genes Dev.* **20**, 3022-3035.

Bossuyt, W., Kazanjian, A., De Geest, N., Van Kelst, S., De Hertogh, G., Geboes, K., Boivin, G. P., Luciani, J., Fuks, F., Chuah, M. et al. (2009). Atonal homolog 1 is a tumor suppressor gene. *PLoS Biol.* **7**, e39.

Botchkarev, V. A., Botchkareva, N. V., Albers, K. M., van der Veen, C., Lewin, G. R. and Paus, R. (1998a). Neurotrophin-3 involvement in the regulation of hair follicle morphogenesis. *J. Invest. Dermatol.* **111**, 279-285.

Botchkarev, V. A., Welker, P., Albers, K. M., Botchkareva, N. V., Metz, M., Lewin, G. R., Bulfone-Paus, S., Peters, E. M. J., Lindner, G. and Paus, R. (1998b). A new role for neurotrophin-3: involvement in the regulation of hair follicle regression (catagen). *Am. J. Pathol.* **153**, 785-799.

Botchkarev, V. A., Botchkareva, N. V., Welker, P., Metz, M., Lewin, G. R., Subramaniam, A., Bulfone-Paus, S., Hagen, E., Braun, A., Lommatzsch, M. et al. (1999). A new role for neurotrophins: involvement of brain-derived neurotrophic factor and neurotrophin-4 in hair cycle control. *FASEB J.* **13**, 395-410.

Botchkareva, N. V., Botchkarev, V. A., Albers, K. M., Metz, M. and Paus, R. (2000). Distinct roles for nerve growth factor and brain-derived neurotrophic factor in controlling the rate of hair follicle morphogenesis. *J. Invest. Dermatol.* **114**, 314-320.

Burgess, P. R., English, K. B., Horch, K. W. and Stensaas, L. J. (1974). Patterning in the regeneration of type I cutaneous receptors. *J. Physiol.* **236**, 57-82.

Cheng Chew, S. B. and Leung, P. Y. (1991). Immunocytochemical evidence of a met-enkephalin-like substance in the dense-core granules of mouse Merkel cells. *Cell Tissue Res.* **265**, 611-614.

Cronk, K. M., Wilkinson, G. A., Grimes, R., Wheeler, E. F., Jhaveri, S., Fundin, B. T., Silos-Santiago, I., Tessarollo, L., Reichardt, L. F. and Rice, F. L. (2002). Diverse dependencies of developing Merkel innervation on the trkA and both full-length and truncated isoforms of trkC. *Development* **129**, 3739-3750.

Dassule, H. R., Lewis, P., Bei, M., Maas, R. and McMahon, A. P. (2000). Sonic hedgehog regulates growth and morphogenesis of the tooth. *Development* **127**, 4775-4785.

Demehri, S. and Kopan, R. (2009). Notch signaling in bulge stem cells is not required for selection of hair follicle fate. *Development* **136**, 891-896.

Doucet, Y. S., Woo, S.-H., Ruiz, M. E. and Owens, D. M. (2013). The touch dome defines an epidermal niche specialized for mechanosensory signaling. *Cell Rep.* **3**, 1759-1765.

English, K. B., Kavka-Van Norman, D. and Horch, K. (1983). Effects of chronic denervation in type I cutaneous mechanoreceptors (Haarscheiben). *Anat. Rec.* **207**, 79-88.

English, K. B., Wang, Z.-Z., Stayner, N., Stensaas, L. J., Martin, H. and Tuckett, R. P. (1992). Serotonin-like immunoreactivity in Merkel cells and their afferent neurons in touch domes from the hairy skin of rats. *Anat. Rec.* **232**, 112-120.

Fantini, F. and Johansson, O. (1995). Neurochemical markers in human cutaneous Merkel cells. An immunohistochemical investigation. *Exp. Dermatol.* **4**, 365-371.

Favier, B., Fliniaux, I., Thélu, J., Viallet, J. P., Demarchez, M., Jahoda, C. A. B. and Dhouailly, D. (2000). Localisation of members of the notch system and the differentiation of vibrissa hair follicles: receptors, ligands, and fringe modulators. *Dev. Dyn.* **218**, 426-437.

Fundin, B. T., Silos-Santiago, I., Ernfors, P., Fagan, A. M., Aldskogius, H., Dechiara, T. M., Phillips, H. S., Barbacid, M., Yancopoulos, G. D. and Rice, F. L. (1997). Differential dependency of cutaneous mechanoreceptors on neurotrophins, trk receptors, and P75 LNGFR. *Dev. Biol.* **190**, 94-116.

García-Caballero, T., Gallego, R., Rosón, E., Fraga, M. and Beiras, A. (1989). Calcitonin gene-related peptide (CGRP) immunoreactivity in the neuroendocrine Merkel cells and nerve fibres of pig and human skin. *Histochemistry* **92**, 127-132.

Golub, J. S., Tong, L., Ngyuen, T. B., Hume, C. R., Palmiter, R. D., Rubel, E. W. and Stone, J. S. (2012). Hair cell replacement in adult mouse utricles after targeted ablation of hair cells with diphtheria toxin. *J. Neurosci.* **32**, 15093-15105.

Gould, E., Albores-Saavedra, J., Dubner, B., Smith, W. and Payne, C. M. (1988). Eccrine and squamous differentiation in Merkel cell carcinoma: an immunohistochemical study. *Am. J. Surg. Pathol.* **12**, 768-772.

Haeberle, H., Fujiwara, M., Chuang, J., Medina, M. M., Panditrao, M. V., Bechstedt, S., Howard, J. and Lumpkin, E. A. (2004). Molecular profiling reveals synaptic release machinery in Merkel cells. *Proc. Natl. Acad. Sci. USA* **101**, 14503-14508.

Han, H., Tanigaki, K., Yamamoto, N., Kuroda, K., Yoshimoto, M., Nakahata, T., Ikuta, K. and Honjo, T. (2002). Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision. *Int. Immunol.* **14**, 637-645.

Hartschuh, W. and Weihe, E. (1989). Pancreastatin-like immunoreactivity in epidermal Merkel cells of pig and man. *Neurosci. Lett.* **98**, 258-263.

Hartschuh, W., Weihe, E., Büchler, M., Helmstaedter, V., Feurle, G. E. and Forssmann, W. G. (1979). Met-enkephalin-like immunoreactivity in Merkel cells. *Cell Tissue Res.* **201**, 343-348.

Hartschuh, W., Weihe, E., Yanaihara, N. and Reinecke, M. (1983). Immunohistochemical localization of vasoactive intestinal polypeptide (VIP) in Merkel cells of various mammals: evidence for a neuromodular function of the Merkel cell. *J. Invest. Dermatol.* **81**, 361-364.

Hartschuh, W., Weihe, E. and Yanaihara, N. (1989). Immunohistochemical analysis of chromogranin A and multiple peptides in the mammalian Merkel cell: further evidence for its paraneuronal function? *Arch. Histol. Cytol.* **52** Suppl., 423-431.

Heiskala, K., Arola, J., Heiskala, M. and Andersson, L. C. (2010). Expression of Reg IV and Hath1 in neuroendocrine neoplasms. *Histol. Histopathol.* **25**, 63-72.

Helms, A. W., Abney, A. L., Ben-Arie, N., Zoghbi, H. Y. and Johnson, J. E. (2000). Autoregulation and multiple enhancers control Math1 expression in the developing nervous system. *Development* **127**, 1185-1196.

Hewitt, J. B., Sherif, A., Kerr, K. M. and Stankler, L. (1993). Merkel cell and squamous cell carcinomas arising in erythema ab igne. *Br. J. Dermatol.* **128**, 591-592.

Hume, C. R., Bratt, D. L. and Oesterle, E. C. (2007). Expression of LHX3 and SOX2 during mouse inner ear development. *Gene Expr. Patterns* **7**, 798-807.

Iacocca, M. V., Abernethy, J. L., Stefanato, C. M., Allan, A. E. and Bhawan, J. (1998). Mixed Merkel cell carcinoma and squamous cell carcinoma of the skin. *J. Am. Acad. Dermatol.* **39**, 882-887.

Ikeda, R., Cha, M., Ling, J., Jia, Z., Coyle, D. and Gu, J. G. (2014). Merkel cells transduce and encode tactile stimuli to drive Aβ-afferent impulses. *Cell* **157**, 664-675.

Kelly, J. B., van Adel, B. A. and Ito, M. (2009). Anatomical projections of the nuclei of the lateral lemniscus in the albino rat (Rattus norvegicus). *J. Comp. Neurol.* **512**, 573-593.

Kelly, M. C., Chang, Q., Pan, A., Lin, X. and Chen, P. (2012). Atoh1 directs the formation of sensory mosaics and induces cell proliferation in the postnatal mammalian cochlea in vivo. *J. Neurosci.* **32**, 6699-6710.

Kim, T.-H. and Shivdasani, R. A. (2011). Genetic evidence that intestinal Notch functions vary regionally and operate through a common mechanism of Math1 repression. *J. Biol. Chem.* **286**, 11427-11433.

Lanford, P. J., Shailam, R., Norton, C. R., Ridley, T. and Kelley, M. W. (2000). Expression of Math1 and HES5 in the cochleae of wildtype and Jag2 mutant mice. *J. Assoc. Res. Otolaryngol.* **1**, 161-171.

Lemasson, G., Coquart, N., Lebonvallet, N., Boulais, N., Galibert, M. D., Marcorelles, P. and Misery, L. (2012). Presence of putative stem cells in Merkel cell carcinomas. *J. Eur. Acad. Dermatol. Venereol.* **26**, 789-795.

Leonard, J. H., Cook, A. L., Van Gele, M., Boyle, G. M., Inglis, K. J., Speleman, F. and Sturm, R. A. (2002). Proneural and proneuroendocrine transcription factor expression in cutaneous mechanoreceptor (Merkel) cells and Merkel cell carcinoma. *Int. J. Cancer* **101**, 103-110.

Lesko, M. H., Driskell, R. R., Kretzschmar, K., Goldie, S. J. and Watt, F. M. (2013). Sox2 modulates the function of two distinct cell lineages in mouse skin. *Dev. Biol.* **382**, 15-26.

Li, L., Rutlin, M., Abraira, V. E., Cassidy, C., Kus, L., Gong, S., Jankowski, M. P., Luo, W., Heintz, N., Koerber, H. R. et al. (2011). The functional organization of cutaneous low-threshold mechanosensory neurons. *Cell* **147**, 1615-1627.

Lin, V., Golub, J. S., Nguyen, T. B., Hume, C. R., Oesterle, E. C. and Stone, J. S. (2011). Inhibition of Notch activity promotes nonmitotic regeneration of hair cells in the adult mouse utricles. *J. Neurosci.* **31**, 15329-15339.

Madisen, L., Zwingman, T. A., Sunkin, S. M., Oh, S. W., Zariwala, H. A., Gu, H., Ng, L. L., Palmiter, R. D., Hawrylycz, M. J., Jones, A. R. et al. (2010). A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat. Neurosci.* **13**, 133-140.

Maksimovic, S., Nakatani, M., Baba, Y., Nelson, A. M., Marshall, K. L., Wellnitz, S. A., Firozi, P., Woo, S.-H., Ranade, S., Patapoutian, A. et al. (2014). Epidermal Merkel cells are mechanosensory cells that tune mammalian touch receptors. *Nature* **509**, 617-621.

Maricich, S. M., Wellnitz, S. A., Nelson, A. M., Lesniak, D. R., Gerling, G. J., Lumpkin, E. A. and Zoghbi, H. Y. (2009). Merkel cells are essential for light-touch responses. *Science* **324**, 1580-1582.

Maricich, S. M., Morrison, K. M., Mathes, E. L. and Brewer, B. M. (2012). Rodents rely on Merkel cells for texture discrimination tasks. *J. Neurosci.* **32**, 3296-3300.

Meyers, J. R., MacDonald, R. B., Duggan, A., Lenzi, D., Standaert, D. G., Corwin, J. T. and Corey, D. P. (2003). Lighting up the senses: FM1-43 loading of sensory cells through nonselective ion channels. *J. Neurosci.* **23**, 4054-4065.

Moll, R., Moll, I. and Franke, W. W. (1984). Identification of Merkel cells in human skin by specific cytokeratin antibodies: changes of cell density and distribution in fetal and adult plantar epidermis. *Differentiation* **28**, 136-154.

Moll, I., Kuhn, C. and Moll, R. (1995). Cytokeratin 20 is a general marker of cutaneous Merkel cells while certain neuronal proteins are absent. *J. Invest. Dermatol.* **104**, 910-915.

Moll, I., Paus, R. and Moll, R. (1996). Merkel cells in mouse skin: intermediate filament pattern, localization, and hair cycle-dependent density. *J. Invest. Dermatol.* **106**, 281-286.

Morrison, K. M., Miesegaes, G. R., Lumpkin, E. A. and Maricich, S. M. (2009). Mammalian Merkel cells are descended from the epidermal lineage. *Dev. Biol.* **336**, 76-83.

Nafstad, P. H. (1987). Evidence of turnover of mammalian Merkel cells. *J. Anat.* **151**, 57-64.

Nakafusa, J., Narisawa, Y., Shinogi, T., Taira, K., Tanaka, T., Inoue, T. and Misago, N. (2006). Changes in the number of Merkel cells with the hair cycle in hair discs on rat back skin. *Br. J. Dermatol.* **155**, 883-889.

Panelos, J., Batistatou, A., Paglierani, M., Zioga, A., Maio, V., Santi, R., Pimpinelli, N., De Giorgi, V., Santucci, M. and Massi, D. (2009). Expression of Notch-1 and alteration of the E-cadherin/beta-catenin cell adhesion complex are

observed in primary cutaneous neuroendocrine carcinoma (Merkel cell carcinoma). *Mod. Pathol.* **22**, 959-968.

Pasche, F., Mérot, Y. and Saurat, J.-H. (1990). Relationship between Merkel cells and nerve endings during embryogenesis in the mouse epidermis. *J. Invest. Dermatol.* **95**, 247-251.

Perdigoto, C. N., Bardot, E. S., Valdes, V. J., Santoriello, F. J. and Ezhkova, E. (2014). Embryonic maturation of epidermal Merkel cells is controlled by a redundant transcription factor network. *Development* **141**, 4690-4696.

Peterson, S. C., Eberl, M., Vagnozzi, A. N., Belkadi, A., Veniaminova, N. A., Verhaegen, M. E., Bichakjian, C. K., Ward, N. L., Dlugosz, A. A. and Wong, S. Y. (2015). Basal cell carcinoma preferentially arises from stem cells within hair follicle and mechanosensory niches. *Cell Stem Cell* **16**, 400-412.

Powell, B. C., Passmore, E. A., Nesci, A. and Dunn, S. M. (1998). The Notch signalling pathway in hair growth. *Mech. Dev.* **78**, 189-192.

Saxod, R. (1996). Ontogeny of the cutaneous sensory organs. *Microsc. Res. Tech.* **34**, 313-333.

Szadowska, A., Wozniak, L., Lasota, J., Giryn, I., Mirecka, B. and Wolska, H. (1989). Neuroendocrine (Merkel cell) carcinoma of the skin: a clinico-morphological study of 13 cases. *Histopathology* **15**, 483-493.

Van Gele, M., Boyle, G. M., Cook, A. L., Vandesompele, J., Boonefaes, T., Rottiers, P., Van Roy, N., De Paepe, A., Parsons, P. G., Leonard, J. H. et al. (2004). Gene-expression profiling reveals distinct expression patterns for Classic versus Variant Merkel cell phenotypes and new classifier genes to distinguish Merkel cell from small-cell lung carcinoma. *Oncogene* **23**, 2732-2742.

Van Keymeulen, A., Mascré, G., Youseff, K. K., Harel, I., Michaux, C., De Geest, N., Szpalski, C., Achouri, Y., Bloch, W., Hassan, B. A. et al. (2009). Epidermal progenitors give rise to Merkel cells during embryonic development and adult homeostasis. *J. Cell Biol.* **187**, 91-100.

VanDussen, K. L. and Samuelson, L. C. (2010). Mouse atonal homolog 1 directs intestinal progenitors to secretory cell rather than absorptive cell fate. *Dev. Biol.* **346**, 215-223.

Vasioukhin, V., Degenstein, L., Wise, B. and Fuchs, E. (1999). The magical touch: genome targeting in epidermal stem cells induced by tamoxifen application to mouse skin. *Proc. Natl. Acad. Sci. USA* **96**, 8551-8556.

Verhaegen, M. E., Mangelberger, D., Harms, P. W., Vozheiko, T. D., Weick, J. W., Wilbert, D. M., Saunders, T. L., Ermilov, A. N., Bichakjian, C. K., Johnson, T. M. et al. (2015). Merkel cell polyomavirus small T antigen is oncogenic in transgenic mice. *J. Invest. Dermatol.* **135**, 1415-1424.

Vielkind, U., Sebzda, M. K., Gibson, I. R. and Hardy, M. H. (1995). Dynamics of Merkel cell patterns in developing hair follicles in the dorsal skin of mice, demonstrated by a monoclonal antibody to mouse keratin 8. *Acta Anat. (Basel)* **152**, 93-109.

Wang, V. Y., Rose, M. F. and Zoghbi, H. Y. (2005). Math1 expression redefines the rhombic lip derivatives and reveals novel lineages within the brainstem and cerebellum. *Neuron* **48**, 31-43.

Whitear, M. (1989). Merkel cells in lower vertebrates. *Arch. Histol. Cytol.* **52** Suppl., 415-422.

Wong, S. Y. and Reiter, J. F. (2011). Wounding mobilizes hair follicle stem cells to form tumors. *Proc. Natl. Acad. Sci. USA* **108**, 4093-4098.

Woo, S.-H., Ranade, S., Weyer, A. D., Dubin, A. E., Baba, Y., Qiu, Z., Petrus, M., Miyamoto, T., Reddy, K., Lumpkin, E. A. et al. (2014). Piezo2 is required for Merkel-cell mechanotransduction. *Nature* **509**, 622-626.

Wright, M. C., Reed-Geaghan, E. G., Bolock, A. M., Fujiyama, T., Hoshino, M. and Maricich, S. M. (2015). Unipotent, Atoh1+ progenitors maintain the Merkel cell population in embryonic and adult mice. *J. Cell Biol.* **208**, 367-379.

Yamamoto, N., Tanigaki, K., Tsuji, M., Yabe, D., Ito, J. and Honjo, T. (2006). Inhibition of Notch/RBP-J signaling induces hair cell formation in neonate mouse cochleas. *J. Mol. Med.* **84**, 37-45.

Yang, Q., Bermingham, N. A., Finegold, M. J. and Zoghbi, H. Y. (2001). Requirement of Math1 for secretory cell lineage commitment in the mouse intestine. *Science* **294**, 2155-2158.

Youker, S. R. (2003). Merkel cell carcinoma. *Adv. Dermatol.* **19**, 185-205.

Zhang, Y. V., Cheong, J., Ciapurin, N., McDermitt, D. J. and Tumbar, T. (2009). Distinct self-renewal and differentiation phases in the niche of infrequently dividing hair follicle stem cells. *Cell Stem Cell* **5**, 267-278.

Zheng, J. L. and Gao, W.-Q. (2000). Overexpression of Math1 induces robust production of extra hair cells in postnatal rat inner ears. *Nat. Neurosci.* **3**, 580-586.

Zheng, J. L., Shou, J., Guillemot, F., Kageyama, R. and Gao, W. Q. (2000). Hes1 is a negative regulator of inner ear hair cell differentiation. *Development* **127**, 4551-4560.

Zine, A., Aubert, A., Qiu, J., Therianos, S., Guillemot, F., Kageyama, R. and de Ribaupierre, F. (2001). Hes1 and Hes5 activities are required for the normal development of the hair cells in the mammalian inner ear. *J. Neurosci.* **21**, 4712-4720.

Zur Hausen, A., Rennspiess, D., Winnepenninckx, V., Speel, E.-J. and Kurz, A. K. (2013). Early B-cell differentiation in Merkel cell carcinomas: clues to cellular ancestry. *Cancer Res.* **73**, 4982-4987.