

**ROLE OF THE *ATOHI* LINEAGE IN MERKEL CELL DEVELOPMENT AND
MAINTENANCE**

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Merkel cells are mechanosensory cells found in mammalian epidermis that are important for the detection of texture, size and shape. These epidermally-derived cells are present in both glabrous and hairy skin, are innervated by slowly adapting type 1 (SA1) afferents, and express the transcription factor *Atoh1* from their specification into maturity. In this dissertation I sought to identify and characterize the generation and maintenance of the *Atoh1*⁺ Merkel cell lineage using transgenic mice. I found that embryonic *Atoh1*⁺ cells are proliferatively capable and unipotent, generating only mature Merkel cells. Contrary to previous estimates, these embryonic-born Merkel cells persist into late adulthood in both hairy and glabrous skin, making them the longest-lived post-mitotic cell present in mammalian epidermis. Despite this, production of Merkel cells in adulthood can be induced by mechanical abrasion caused by repeated skin shaving. Unlike embryonic Merkel cells, these *de novo* Merkel cells do not arise from *Atoh1*⁺ or *K14*⁺ progenitors and have a decreased probability of survival compared to original Merkel cells. Furthermore, ectopic *Atoh1* expression is sufficient to induce production of ectopic Merkel cells outside the touch dome boundaries. These ectopic Merkel cells express multiple canonical Merkel cell markers and a small percentage that reside in the hair follicle bulge can persist for at least 3 months post-induction. Competency of keratinocytes to respond to *Atoh1* varies by skin location, developmental age and hair cycle stage. Also, the Notch signaling pathway plays an instructive role in epidermal cell responsiveness to *Atoh1* expression. Together, these experiments provide novel insight into Merkel cell genesis and maintenance as well as illustrate two mechanisms by which Merkel cells production can be initiated in adulthood. They also suggest that the skin cancer, Merkel cell carcinoma, is more likely to arise from epidermal keratinocytes than the Merkel cell lineage.

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PREFACE

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
<i>Atoh1</i>	Atonal homolog 1
BDNF	Brain-derived neurotrophic factor
bHLH	basic helix-loop-helix
BMP	bone morphogenic protein
BrdU	Bromodeoxyuridine
β-Gal	Beta-galactosidase
CGRP	calcitonin gene-related peptide
CKO	conditional knockout
Cre	cre recombinase enzyme
Cre-ER	cre recombinase with estrogen receptor
Cre-PR	cre recombinase with progesterone receptor
Cy3	fluorescent cyanine dye
DAPI	4',6-diamidino-2-phenylindole
DRG	dorsal root ganglia
DTA	diphtheria toxin subunit A
E	embryonic day; <i>refers to an age</i>
EdU	5-ethynyl-2'-deoxyuridine (a modified nucleoside)
eGFP	enhanced green fluorescent protein
Ezh1/2	Enhancer Of Zeste Homolog 1 and Enhancer Of Zeste Homolog 2
flox	loxP-flanked
FM1-43	<i>N</i> -(3-Triethylammoniumpropyl)-4-(4-(Dibutylamino) Styryl) Pyridinium Dibromide
Gfi1	Growth Factor Independent Protein 1

Gli1	Glioma-Associated Oncogene Family Zinc Finger 1
GFP	Green fluorescent protein
H&E	hematoxylin and eosin stain
H3K27me3	tri-methylation of lysine 27 on histone H3
Hath1	human atonal homolog 1
Hh	hedgehog
hrs	hour(s); <i>refers to a period of time</i>
HTMR	High threshold mechanoreceptor
iDTR	inducible diphtheria toxin receptor
IFE	interfollicular epidermis
Isl1	Islet 1
K	Keratin
kg	kilogram
LacZ	gene coding for Beta-galactosidase
LTMR	Low threshold mechanoreceptor
loxP	locus of X-over P1 site recognized by Cre recombinase
MCC	Merkel cell carcinoma
MCPyV	Merkel cell polyomavirus
mg	milligram
mL	milliliter
min	minute
mm	millimeter
mo	month(s); <i>refers to an age</i>
NF200	heavy chain neurofilament
NGF	nerve growth factor
NT3	neurotrophin-3
NT4	neurotrophin-4
OE	overexpressor
P	post-natal day; <i>refers to an age</i>
Pax6	paired box 6
PBS	phosphate-buffered saline

PBS-T	phosphate-buffered saline with Triton-X
PFA	paraformaldehyde
PH3	phosphohistone 3
PRC	polycomb repressive complex
Rbpj	recombining binding protein suppressor of hairless
RU486	mifeprisone, progesterone blocker
rtTA	reverse tetracycline transactivator
SA1	slowly adapting type 1
SCLC	small cell lung cancer
SEM	standard error of the mean
SHH	sonic hedgehog
Sox2	SRY (sex determining region Y)-box 2
sT	small T antigen
Tet	tetracycline
TD	touch dome
TDKC	touch dome keratinocyte
tdTomato	tandem dimeric red fluorescent protein
Thy1	thy-1 cell surface antigen
TMX	tamoxifen
TrkB	tyrosine receptor kinase B
TTF-1	thyroid transcription factor-1
UV	ultraviolet
VGLUT2	vesicular glutamate transporter-2
VIP	vasoactive intestinal peptide
Wnt1	wingless-type MMTV integration site family, member 1
XGal	(5-bromo-4-chloro-indolyl- β -d- galactopyranoside)
YFP	yellow fluorescent protein
μg	micrograms
μL	microliters
μm	micrometers

1.0 INTRODUCTION

1.1 MERKEL CELL DISCOVERY AND EARLY CHARACTERIZATION

A renowned anatomist of his time, Friedrich Sigmund Merkel was the first to observe and describe the cells that were to be given his name (Kopera & Holubar, 1994; Merkel, 1875; Weissmann & Camisa, 1982). Their presence in highly sensitive skin areas and close proximity to nerves led Merkel to infer that they were likely important for the sense of touch and thus termed these cells *tastzellen*, or touch cells. He noted that only some *tastzellen* appeared to make close contact with nerves and specifically named those *Tastkörperchen*, or “little touch bodies”. Decades later, the ultrastructure within which these touch cells were found in hairy skin was described by Felix Pinkus who named them *haarscheibe*, or hair discs (Pinkus, 1902). Many factors contributed to the elusiveness of this cell population, not the least of which is their difficult identification with routine hematoxylin and eosin staining. Work by many scientists in years since has improved our ability to identify and characterize these cells with alternative fixation, sectioning, and staining techniques. The names by which these cells have been referred has also evolved throughout this time, culminating today in our study of Merkel cells within touch domes of hairy skin (Camisa & Weissmann, 1982). While putative Merkel cells (cells that share ‘Merkel cell’ characteristics) are also found in many other anatomical locations including the oral mucosa, whisker follicles, and glabrous skin of the hands, feet, and lips, their niche within touch domes is most frequently studied as it is a well-characterized model system common in multiple species (Boulais & Misery, 2007).

Merkel’s captivation with microscopy no doubt played an instrumental role in his discovery of these enigmatic cells, which he first observed in the epithelia of ducks, geese, and moles using a long osmic acid fixation followed by a methylene blue stain (Camisa & Weissmann, 1982; Merkel, 1875; 1880; Weissmann & Camisa, 1982). This type of fixation was

the best of its time to identify and study Merkel cells, but precluded the analysis of potential innervation, as staining for neurites required formalin fixation (Munger, 1977). It is now much easier to find these cells in hairy skin by first locating touch domes, which appear as epidermal thickenings both in sectioned tissue and in whole mount human skin (Smith, 1970). Merkel cells can be distinguished using light microscopy by their positioning below the touch dome columnar epithelia, large lobulated nuclei, orientation parallel to the skin surface, and very light staining properties relative to other surrounding skin cells (Halata et al., 2003). However, these qualities can be similar to those used to identify melanocytes and Langerhans cells. Electron microscopy has surfaced as the classical method to confirm Merkel cell identity. This technique allows for the visualization of Merkel cell-specific characteristics including small villi on their apical side that penetrate between overlying keratinocytes, the desmosomes between touch domes keratinocytes and Merkel cells, the abundance of intermediate cytokeratin filaments, and large lobulated nuclei (Halata et al., 2003). Perhaps most importantly for Merkel cell identification are the electron-dense core granules concentrated near their basal side. An axon terminal with synaptic membrane specialization is closely apposed Merkel cells on this side, together comprising the Merkel disc or Merkel cell-neurite complex (Iggo & Muir, 1969; Smith, 1970). The neurite not only makes contact with Merkel cells, but actually has an elaborate terminal ending that conforms to the shape of their basal side (Munger, 1965).

The cytokeratin profile of Merkel cells is distinct from that of the surrounding epithelia. The development of cytokeratin-specific antibodies combined with immuno-electron microscopy determined that Merkel cells are unique in their mature expression of the simple and low molecular weight cytokeratins (K) 8, 18, 19, and 20 (Moll et al., 1993; Saurat, 1984). This is distinct from the overlying touch dome keratinocytes that express K17 and the interfollicular epidermal keratinocytes that express K5 and K14 (Moll et al., 1993). This cytokeratin profile is consistent between Merkel cells of multiple species, though K20 is believed to be the most specific marker (Moll et al., 1995;1996a). Today the TROMA-1 antibody specific for K8 is most routinely used to identify and characterize Merkel cells, as this cytokeratin has robust expression in maturity and is one of the first to be expressed during Merkel cell development (Perdigoto et al., 2014a; Saurat, 1984).

1.2 DEBATE OF MERKEL CELL ORIGIN

In addition to a unique cytokeratin profile, Merkel cells also express many other distinct proteins including the transcription factors *Atoh1*, *Sox2*, *Gfi1*, and *Pax6* (Ben-Arie et al., 2000; Lesko et al., 2013; Parisi & Collinson, 2012; Wallis et al., 2003), various synaptic proteins including *Rab3c*, *VGLUT2*, and *piccolo* (Haerberle et al., 2004; Hitchcock et al., 2004), and neuroactive peptides such as vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), and serotonin (Cheng Chew & Leung, 1993; Nordlind, et al., 2008; Tachibana & Nawa, 2005). This complex and distinct expression profile incited an exhaustive debate surrounding the embryonic origin of Merkel cells. The two prevailing opinions concerning Merkel cell development were that Merkel cells either derived from migrating neural crest progenitors, or from resident epidermal progenitors in the skin. The techniques available to investigate this question early on were limited, and scientists depended on gleaning information from snapshots of Merkel cells at varying stages of their development. These scientists studied a wide array of tissue types in many different model systems and mounted a wealth of indirect supporting evidence for both sides of the argument.

The location of Merkel cells during animal development and maturity was often cited as evidence for either side of this debate. While mature Merkel cells are found at the epidermal-dermal border in touch domes or at rete ridges of palmar and plantar skin, reports of their existence within the dermis led scientists to infer that they could be migrating from a non-keratinocyte origin (Breathnach, 1978; Saxod, 1978). This idea was reinforced by the similarities that Merkel cells share with neurosecretory cells, supporting the hypothesis that Merkel cells were derived from the neural crest (Taira, et al., 2002; Winkelmann, 1977). However, as pointed out by Dr. Kathleen English, these cells were never exclusively found in the dermis at an age prior to their being present in the epidermis, and one cannot infer in which direction the cells were traveling based on their location (English, 1977).

Around the same time, other scientists were beginning to describe a population of cells present in the epidermis termed ‘transitional cells’, which shared both Merkel cell and keratinocyte characteristics (English, 1974; 1977; Saxod, 1978; Tachibana & Nawa, 1980). It was thought that these cells were putative Merkel cell progenitors undergoing early stages of differentiation and maturation. Transitional and mature Merkel cells were identifiable before the

presence of other migrating neural-crest derivatives, and before the afferent terminals of their innervating neurons (Boulais & Misery, 2007). Combined with the presence of desmosomes and light keratin filaments in mature Merkel cells, many scientists believed that resident epidermal progenitors within the touch dome compartment were responsible for generating Merkel cells.

A handful of transplant and surgical studies in different model systems sought to directly test Merkel cell origin. In an early study, all presumptive neural crest cells and their derivatives were removed from the larvae of mole salamanders (Tweedle, 1978). Merkel cells formed even in these aneurogenic salamanders, indicating that Merkel cells of amphibian species arose from non-neural crest progenitors. A couple decades later, Moll et al. performed a transplant study with human fetal palmar and plantar skin xenografted into the subcutaneous tissue of adult nude mice and analyzed 4-8 weeks later (Moll et al., 1990). Human fetal tissue was taken and transplanted between 8 and 11 weeks of gestational age, before and during the time at which Merkel cells are first observed in the skin. Regardless of the age or tissue source, large numbers of Merkel cells of human origin were seen in the xenografts at the time of tissue analysis, indicating an epidermal origin of mammalian Merkel cells or Merkel cell progenitors derived from another cell population were brought along. Consistent with that finding, cultured keratinocyte sheets from humans lack Merkel cells in culture, but Merkel cells can be found after grafting, indicating their derivation from epithelial progenitors (Compton et al., 1990).

In contrast to that work, Halata and Grim grafted the leg bud of quail embryos into the leg bud space of chick embryos (and vice versa) and analyzed the Merkel cell and keratinocyte populations in the grafted areas after animal development (Grim & Halata, 2000; Halata et al., 1990). Cells were characterized as being of quail or chick origin on the basis of variable heterochromatin structure between the species (Le Douarin, 1969). They found that Merkel cells in the grafted areas had heterochromatin structures most like that of the host embryo, while all epidermal cells had heterochromatin structures like that of the grafted species (Grim & Halata, 2000; Halata et al., 1990). The authors infer that avian Merkel cells must have migrated from a non-keratinocyte source, likely the neural crest.

Many differences exist between these studies, most notable of which is the analyzed species. Merkel cell ultrastructure as judged by electron microscopy is seemingly similar between avian and mammalian systems and the cells also have similar protein expression profiles (Grim & Halata, 2000). However, their location differs, as mammalian Merkel cells are

found in the basal layer of the epidermis while avian Merkel cells are found in the dermis (Munger, 1977; Saxod, 1978). It is unclear whether these experiments demonstrate a true species divergence in Merkel cell origin, or if these discrepancies are due to differing technical approaches.

With the advent of sophisticated genetic techniques offered by transgenic mice, fate-mapping and conditional gene deletion approaches have greatly advanced our understanding of mammalian Merkel cell development. Using Cre-loxP technology, Szeder et al. fate mapped the neural crest lineage with *Wnt1^{Cre}* mice crossed to a *ROSA^{LacZ}* reporter (Szeder et al., 2003). In this system, the *Wnt1+* lineage irreversibly expresses the reporter gene *LacZ*, which codes for the enzyme β -galactosidase (β -gal). The authors analyzed whisker follicles of embryonic day (E)16.5 *Wnt1^{Cre};ROSA^{LacZ}* mice and found β -gal positivity in the areas of K8+ Merkel cells (Szeder et al., 2003). A complicating factor to this analysis is the very close proximity of Merkel cells to their innervating afferents, which are known to derive from the *Wnt1+* neural crest lineage (Le Douarin & Kalcheim, 1999). Presented images were insufficient to determine the precise localization of β -gal as either in the Merkel cell or the innervating afferent, and therefore these experiments could not provide incontrovertible support for a neural crest origin of Merkel cells.

A clear and direct demonstration of Merkel cell origin came in 2009, 134 years after Merkel cells were first described. Using similar Cre-loxP technology, Morrison et al. conditionally deleted the transcription factor *Atoh1* from the *Wnt1+* (neural crest) or *K14+* (epidermal) lineages (Morrison et al., 2009). *Atoh1* is necessary for Merkel cell development and its absence precludes K8+ cell formation (Maricich et al., 2009). In P0 *Wnt1^{Cre};Atoh1^{CKO}* mice that lack *Atoh1* specifically in the neural crest lineage, K8+ Merkel cells were formed in touch domes and whisker follicles. However, loss of *Atoh1* from the epidermal lineage in *K14^{Cre};Atoh1^{CKO}* mice led to an absence of K8+ Merkel cells from touch domes, whisker follicles, and paws (Morrison et al., 2009). This was independently verified by Van Keymeulen et al. who also found no K8+ Merkel cells in *K14^{Cre};Atoh1^{CKO}* mice (Van Keymeulen et al., 2009). They also performed additional fate-mapping experiments with *Pax3^{Cre}* (neural crest), *Wnt1^{Cre}*, and *K14^{Cre}* mice crossed to a *ROSA^{YFP}* reporter, demonstrating that K8+ cells were all YFP+ only in *K14^{Cre};ROSA^{YFP}* mice (Van Keymeulen et al., 2009). Likewise, K18+ Merkel cells in *K14^{Cre};Ezh1/2^{CKO}* mice were found to lack H3K27me3 histone methylation, similar to other

epithelial-derived tissues, while tissues of all other origins retained this methylation signature (Bardot et al., 2013). Together, these experiments incontrovertibly demonstrate the epidermal origin of mammalian Merkel cells.

1.3 GENETIC REGULATION OF MERKEL CELL DEVELOPMENT

As mentioned above, discovery of the basic helix-loop-helix (bHLH) transcription factor *Atoh1* in Merkel cells was instrumental to our understanding of Merkel cell development. While *Atoh1* expression in the skin is limited to Merkel cells, many other cell types in the body also rely on its expression. This includes cerebellar granule cells and their progenitors (Akazawa et al., 1995; Ben-Arie et al., 1996), neurons in the retrotrapezoid nucleus (Rose et al., 2009), precursor cells of the D1 class of commissural interneurons in the spinal cord (Akazawa et al., 1995; Ben-Arie et al., 1996), inner ear hair cells (Ben-Arie et al., 2000), and secretory cells of the intestinal epithelia (Yang et al., 2001). With the exception of Merkel cells and intestinal secretory cells, all of these cell types lose *Atoh1* expression after differentiation and maturity.

Atoh1 is a single exon gene whose bHLH motif allows it to heterodimerize with other HLH proteins and bind to E-box DNA consensus sequences to regulate gene expression (Cai & Groves, 2014; Massari & Murre, 2000). The 3' coding region for *Atoh1* also contains this E-box sequence, allowing for *Atoh1* to autoregulate its own expression levels (Helms et al., 2000). *Atoh1* also has numerous serine residues available for phosphorylation and has been thought to undergo cytoplasmic shuttling during cell differentiation, indicating potential post-transcriptional regulation of its function (Akazawa et al., 1995; Cai et al., 2013; Camisa & Weissmann, 1982). This transcription factor is known to play multiple roles during lineage allocation and maturation in inner ear hair cells, illustrating a complex wealth of responsibility (Cai et al., 2013; Chonko et al., 2013).

While *Atoh1* is currently the single most important transcription factor known to regulate Merkel cell development, Merkel cells also express several other developmental transcription factors whose roles have been recently studied, including *Sox2*, *Isl1*, and *Pax6*. Embryonic *Sox2* expression is initiated almost concurrently with that of *Atoh1* in the epidermal compartment where Merkel cells arise (Lesko et al., 2013). However, when conditionally ablated from the

epidermis in *K14^{Cre};Atoh1^{GFP};Sox2^{lox/lox}* mice, Merkel cells still form and express *Atoh1* (as indicated by GFP) and K8 (Bardot et al., 2013). While there is no difference in the density of guard hairs or K8+ clusters in these mice, they have 50% fewer K8+ cells per touch dome at P0 and *Atoh1*+ cells are absent by P35 (Bardot et al., 2013; Lesko et al., 2013). These Merkel cells express the synaptic functional markers VGLUT2, Rab3c, piccolo, and Cav2.1 but fail to express K18 and K20 (Bardot et al., 2013; Lesko et al., 2013). Given this data, it seems likely that *Sox2* is functioning as a Merkel cell maturation and/or survival factor, but is not necessary for their initial specification.

Epigenetic regulation of *Sox2* expression by the Polycomb repressive complex (PRC), plays a role in Merkel cell lineage allocation, as has been studied in *K14^{Cre};Ezh1/2^{CKO}* mice. The PRC is composed of multiple proteins that are recruited to chromatin where they methylate histones and subsequently alter their epigenetic structure. *Ezh1* and *Ezh2* are two histone methyltransferases responsible for catalyzing this histone mark. In their absence, the PRC is unable to repress *Sox2* expression within *K14*-lineal cells of *K14^{Cre};Ezh1/2^{CKO}* mice, resulting in increased numbers of K18+ and K20+ Merkel cells at P0 and P90 (Bardot et al., 2013). This phenotype is a direct effect of *Sox2* expression, as Merkel cell numbers are attenuated back to that of control mice when *Sox2* is removed in P0 *K14^{Cre};Ezh1/2^{CKO};Sox2^{CKO}* mice (Bardot et al., 2013). This indicates that ectopic Merkel cell formation in *K14^{Cre};Ezh1/2^{CKO}* mice was dependent on *Sox2*, and that *Sox2* can regulate *Atoh1* expression. It is postulated that removal of PRC repression directly from *Sox2* and possibly *Atoh1* permits the allocation of greater numbers of Merkel-lineal cells. It is unclear whether these events happen concomitantly, or if *Sox2* or *Atoh1* is expressed first, as they are both initially seen at the same embryonic age and are able to regulate expression of the other (Bardot et al., 2013). It is unlikely that the PRC represses *Atoh1* expression, as removal of *Sox2* in a *K14^{Cre};Ezh1/2^{CKO}* mouse was in itself sufficient to bring Merkel cell number back to control averages. If *Atoh1* was de-repressed in this model, it is likely that higher Merkel cell numbers would still be present even without *Sox2*. Also, gene expression data in *Ezh1/2*-null epidermal stem cells did not indicate a role for PRC in *Atoh1* regulation (Ezhkova et al., 2011).

There appears to be a functional redundancy in the role some of these transcription factors play during Merkel cell development. Within the skin *Isl1* is another transcription factor whose expression is limited to Merkel cells and is involved in the development and maintenance

of other cell lineages (Habener et al., 2005; Laugwitz et al., 2008; Perdigoto et al., 2014a). Loss of *Isl1* alone in *K14^{Cre};Isl1^{CKO}* mice had no effect on Merkel cell number or expression profile at P0 or in adulthood. However, when combined with loss of *Sox2* in *K14^{Cre};Isl1^{CKO}; Sox2^{CKO}* mice, the average number of Merkel cells was lower than that of *K14^{Cre};Sox2^{CKO}* mice. While this is an interesting phenomenon, likely due to gene redundancy, it is perhaps more interesting to note that many Merkel cells persist in the absence of both of these transcription factors and that these cells continue to express *Atoh1*, as indicated by GFP presence in *K14^{Cre};Atoh1^{GFP};Sox2^{CKO};Isl1^{CKO}* mice (Perdigoto et al., 2014a). While these recent studies have greatly advanced our understanding of the genetic controls underlying Merkel cell formation, a great deal is still unknown about the signaling pathways involved in allocating the Merkel cell lineage from other skin lineages.

1.4 SKIN AS A SENSORY ORGAN

Skin is our largest organ, working simultaneously to regulate our body temperature, protect us from environmental assaults, and inform us about the tactile world around us. The Merkel cell-neurite complex is one of a range of sensory receptors present in the skin responsible for detecting tactile stimuli. Each type of somatosensory receptor is specially tuned to respond optimally to specific forms of physical stimuli ranging from innocuous forms of skin stretch, vibration, and indentations to mechanically-induced pain (Abraira & Ginty, 2013). Somatosensory receptors transduce mechanical stimuli into electrical signals propagated along the peripheral axons of sensory neurons. The cell bodies of these somatosensory afferents reside in the dorsal root ganglia (DRG) and their central axons project into the dorsal horn of the spinal cord where they split and synapse on local interneurons and projection neurons. The electrical summation of these peripheral mechanical stimuli is sent to the brain, which then creates a percept of our environment. Somatosensory receptors can be classified based on a number of criteria, including the thickness of their myelination, their corresponding conduction velocities, peripheral end organs, response properties to varying stimuli, and cell soma size. The mammalian DRG houses the cell bodies of all these afferents in a seemingly-random order, making it exceedingly difficult to capture the full anatomical, physiological, and innervation

profile of individual cutaneous afferents. The advent of transgenic mice has become a powerful tool with which to label and study specific somatosensory afferent subtypes, greatly enhancing our understanding of the circuitry for cutaneous touch and pain. This enhanced knowledge of afferent identity is assisting with the creation of tools for further manipulation and understanding of how these receptors develop, function, and are maintained.

Mechanically sensitive sensory afferents are split into two categories based upon their response thresholds. High threshold mechanoreceptors (HTMRs) are thought to transduce noxious mechanical stimuli, while low threshold mechanoreceptors (LTMRs) transduce innocuous stimuli (Abraira & Ginty, 2013). Conduction velocities are used to further classify LTMRs into C (slow), A δ (intermediate), and A β (fast) fibers. C and A δ fibers innervate hairy skin and form lanceolate endings primed to detect deflection of awl-auchene and zigzag hair follicles (Horch et al., 1977; Li et al., 2011). The A β fibers comprise rapidly and slowly adapting somatosensory receptors that have particularly unique end organs and response properties specific to different forms of stimuli. The rapidly adapting (RA) LTMRs include Meissner corpuscles, lanceolate endings around guard hair follicles, and Pacinian corpuscles. These RA-LTMRs respond preferentially to skin movement, hair follicle deflection, and vibration, respectively, and fire action potentials exclusively at the onset and removal of stimuli (Knibestöl, 1973; Vallbo & Johansson, 1984). In contrast, slowly adapting (SA) LTMRs fire throughout the duration of stimulus presence and include the Merkel cell-neurite complex and Ruffini endings. The SA response is composed of an early dynamic phase at the onset of stimulus presentation followed by a static phase that lasts until stimulus removal (Iggo & Muir, 1969). SA2 fibers are thought to innervate Ruffini endings (though this has not been directly demonstrated) and have a regular firing pattern during the static phase (Chambers, Andres, Duering, & Iggo, 1972). SA1 fibers are those that contact Merkel cells, and are characterized by an irregular firing pattern during the static phase and preferential activation to skin indentation (Wellnitz et al., 2010).

Similar to the uncertainty concerning Merkel cell origin, considerable debate was held surrounding the cellular site of mechanotransduction within the Merkel cell-neurite complex. It was thought that the SA1 fiber, the Merkel cell, or both were responsible for receiving and transducing mechanical stimuli. Cell ablation by a UV laser led to conflicting results, as it was difficult to determine if either the SA1 afferent terminals were truly left undamaged and/or if Merkel cells were completely ablated in these models (Diamond et al., 1988; Ikeda et al., 1994;

Senok et al., 1996). Transgenic mouse models provided the tools necessary to accurately test this question. The first clear demonstration of Merkel cell requirement for SA1 signaling occurred in 2009, when *Hoxb1^{Cre};Atoh1^{CKO}* mice that lack Merkel cells in their body skin were shown to have no canonical SA1 responses (Maricich et al., 2009). Moreover, *K14^{Cre};Atoh1^{CKO}* mice which similarly lack Merkel cells in all body regions have deficiencies in their ability to discriminate between textured surfaces, indicating a functionally significant requirement for Merkel cells (Maricich et al., 2012). A triad of recent papers illustrated that both components are independently mechanosensitive and likely contribute to the unique firing pattern of SA1 afferents (Ikeda et al., 2014; Maksimovic et al., 2014; Woo et al., 2014). Moreover, it has been shown that cells of the keratinocyte lineage are able to stimulate firing of somatosensory afferents (Baumbauer et al., 2015). The current working model of Merkel cell-neurite mechanotransduction is that mechanically activated channels on Merkel cells are activated, causing Merkel cell depolarization and subsequent opening of voltage gated calcium channels. This calcium influx stimulates the release of a heretofore unknown neurotransmitter from Merkel cells that binds to appropriate ligand-gated channels on the SA1 terminal. Concurrently, mechanosensitive channels on the SA1 afferent are also stimulated to open. It is thought that Merkel cells continue to fire for the duration of stimuli presence, resulting in the continued SA1 response (Maksimovic et al., 2014). The fast initial, dynamic phase is therefore a result of the mechanically gated channels on the SA1 afferent, while the later static phase is a result of Merkel cell firing and continuous activation of the SA1 fiber.

However, a confounding factor to the work done by Maksimovic et al. is that in their *K14^{Cre};Atoh1^{CKO}* transgenic mouse model, the SA1 afferent was not allowed to develop normally before Merkel cell removal. While the SA1 afferent innervates touch domes and survives even in the absence of Merkel cells in these *K14^{Cre};Atoh1^{CKO}* mice, recent data demonstrates a critical role for Merkel cells in the development of SA1 molecular identity and function (Reed-Geaghan, et al. in press). A more accurate approach would have been to genetically ablate Merkel cells during adulthood, after the Merkel cell-neurite complex had been formed and matured. Therefore, the response properties of unmanipulated SA1 fibers permitted to properly develop is still up for investigation.

Merkel cell-neurite complexes are found in both glabrous and hairy skin, and though their organization in these locations differs, their response properties are largely similar. The

organization and density of this mechanoreceptor also varies between mice and humans, likely reflecting the different needs for tactile acuity in varying body areas between these species. Worth noting though, is that the morphology of individual Merkel cells look remarkably similar between these species. In the hairy skin of mice, Merkel cells are most frequently found at the epidermal-dermal border of guard hairs within touch domes, though they are also found around hair follicles in the dorsal hairy skin of paws and surrounding the outer root sheath of whisker follicles (Moll et al., 1995). Guard hairs are regularly spaced throughout the body skin and each associated touch dome has between ~3-80 Merkel cells (Cheng et al., 2014). Individual SA1 fibers innervate either one or multiple touch domes, and each touch dome is typically innervated by a single SA1 fiber, though perhaps not exclusively (Iggo & Muir, 1969; Lesniak et al., 2014; Reinisch & Tschachler, 2005; Woodbury & Koerber, 2007). Many innervated Merkel cells are also found in the glabrous skin of paws either singly or in small clusters with the greatest density on the fingertips and touch pads. In mice, the largest density of Merkel cells though is found in the buccal pad, where hundreds of Merkel cells wrap around the outer root sheath of individual whisker follicles. This high density likely reflects the significance of whisking for mouse tactile acuity and the use of this structure for awareness of and interaction with their environment.

While mice are most apt to explore their tactile environment using their whiskers, humans predominantly interact with our physical environment with our hands. A single human hand has an average of over 460 Merkel cells per mm², an incredible density when compared to the hairy regions of the body (~12 Merkel cells/mm², which varies by location) or even the entire sole of a foot (~220 Merkel cells/mm²) (Lacour et al., 1991). Other body regions with high tactile acuity (skin of the face, lips, oral mucosa) also have relatively high Merkel cell density. Contrary to what is observed in mouse skin, Merkel cells are not believed to be primarily located within touch domes of human skin, but instead are much more loosely arranged. It should be noted that these counts were performed on epidermal sheets of cadaver skin 65-91 years of age. Therefore, these may be lower estimates, as Merkel cell number is thought to decrease with age (Ulfhak et al., 2002), and Merkel cells may have adhered to the dermal side of skin during tissue preparation.

Touch dome density is irregular in human samples of hairy skin and not always found in association with hair follicles (Smith, 1970). Merkel cells in human hairy skin are not exclusively associated with touch dome structures, but instead tend to be interfollicular or

associated with the outer root sheath or hair bulb (Lacour et al., 1991). Some mention has been given to dermally located Merkel cells, which are readily present during embryogenesis, but not as well described in adulthood (Moll et al., 1986;1984). In humans, Merkel cells are first seen in glabrous and hairy skin as early as gestational week 8 where their density increases until gestational weeks 24-30 and then decreases by birth, though it is unclear if this is due to Merkel cell loss or tissue expansion without addition of more Merkel cells (Moll & Moll, 1992; Moll et al., 1984).

Merkel cells in hairy skin of mice form as early as E14.5 and begin to be innervated by E16.5, with essentially full innervation achieved by P0 (Pasche et al., 1990). It is unclear what percentage of Merkel cells remain innervated in animal maturity, though recent data estimate ~85% (Lesniak et al., 2014). It is also unclear if this innervation status is static or dynamic during an animal's lifespan, rendering possible a pliability of the SA1 afferent to remodel and innervate different Merkel cells over time. The ability of SA1s to reinnervate touch domes after injury indicates a certain degree of flexibility within the mechanosensory unit, but how this regeneration and targeting occurs has yet to be comprehensively studied. The specific organization of Merkel cells to branched SA1 neurites can drastically influence the sensitivity and firing pattern of individual touch domes (Lesniak et al., 2014). Whether or not individual touch domes tune this organization throughout development and maturity has yet to be investigated, but may provide an additional layer of complexity and plasticity to this somatosensory receptor.

1.5 MERKEL CELL CARCINOMA

For the first century after Merkel cells were initially discovered and described, analysis of their function and development was focused on their probable somatosensory and/or neuroendocrine functions. However, the potential relevance of Merkel cells to the skin cancer Merkel cell carcinoma (MCC) has provided a new perspective and incentive to understanding their cellular maintenance. The incidence rate of MCC increased by ~8% between 1986 and 2001, and though this rise could be an artifact of greater awareness and more prevalent diagnosis, the prognosis for

patients with advanced disease is still very poor (PDQ Adult Treatment Editorial Board, 2002; Saavedra & Batich, 2010). Current treatment options involve surgical excision followed by adjuvant radiation therapy, though recurrence is frequent and correlative of a poorer prognosis for survival. While MCC is still relatively rare, the severity of this carcinoma combined with limited therapeutic options underscores the incredible need for more targeted approaches in combating this cancer. It is hoped that a greater understanding of Merkel cell development and maintenance may help in the identification of these desired targeted therapeutic approaches.

Originally described by Cyril Toker in 1972, MCC skin tumors were initially termed trabecular carcinoma of the skin (Toker, 1972). These forms of tumors have since gone by many different names, owing to their heterogeneity and unknown cell of origin, including the Toker tumor, primary small cell carcinoma of the skin, primary cutaneous neuroendocrine carcinoma, and malignant trichodiscoma, though MCC is used most commonly now (Schwartz & Lambert, 2004). The most appropriate name for these tumors is still unclear and the subject of debate, as much is still unknown about the cellular origin and progression of these skin tumors (Schwartz & Lambert, 2004; Toker, 1972).

The gross morphology of MCC tumors is widely variable, though most tumors are nodular, red to violet in coloring, and their primary sites occur most frequently in UV-exposed areas of skin on older, immunocompromised individuals (PDQ Adult Treatment Editorial Board, 2002). Biopsy of the lesion is required for a positive diagnosis, and though no single specific marker of MCC is known, the presence of dense core neurosecretory granules, cytoplasmic processes, and intermediate filaments are positive indicators (Eng et al., 2007). MCC can be distinguished from other metastasizing small round cell tumors such as melanomas and small cell carcinoma of the lung (SCLC) by a trending host of distinct expression patterns including an absence of S100 and thyroid transcription factor-1 (TTF-1) expression, and the presence of K20, BDNF, and neuron specific enolase (Bobos et al., 2006; Gele et al., 2004; Kuwamoto, 2011). However, the presence or absence of any one of these markers are insufficient for diagnosis, as a minority of MCC and SCLC tumors express TTF-1 and CK20, respectively (Buresh et al., 2008; Byrd-Gloster et al., 2000; Hanly et al., 2000).

Recently the discovery of Merkel cell polyomavirus (MCPyV) and its frequent association with MCC tumors has provided additional potential insight into the etiology of some forms of MCC (Feng et al., 2008). A high viral load of MCPyV is found in ~70-80% of MCC

tumors where it is often monoclonally integrated into the host genome, indicating clonal expansion early after integration. Though almost the entire adult human population has detectable levels of MCPyV in their skin microbiome, even the highest viral loads are 60X less than that seen in MCC biopsies (Loyo et al., 2010; Schowalter et al., 2010). Interestingly, MCPyV+ tumors are associated with decreased levels of p53 and a better prognosis (Bhatia et al., 2010a; 2010b; Laude et al., 2010; Sihto et al., 2009), while MCPyV- tumors have a higher rate of regional metastasis (Sihto et al., 2009). Slight histological differences may also exist, with MCPyV+ tumors having more round nuclei compared to the polygonal nuclei found in MCPyV- tumors that also contain a qualitatively lighter and larger cytoplasm (Becker et al., 2009; Katano et al., 2009). It is thought that these two types of MCC have different etiologies, leading to differing prognoses, though their cell of origin is believed to be the same.

Many MCC tumors have expression profiles resembling neuroendocrine cells of the skin, and Toker was the first to hypothesize that Merkel cells and/or their derivatives may in fact be their cell of origin (Tang & Toker, 1978). This prediction gained further support when the human ortholog of *Atoh1* (*Hath1*) was found in a subset of MCC tumors (Leonard et al., 2002), leading to the thought that resident Merkel cell progenitors in mammalian skin may be the MCC site of origin. This hypothesis has been under a great deal of scrutiny, as proliferative Merkel cells have not been detected in adult skin (Mérot & Saurat, 1988; Moll et al., 1996b; Vaigot et al., 1987), many MCC tumors are found mixed with other carcinomas (Gould et al., 1988; Iacocca et al., 1998; Szadowska et al., 1989), and the expression profiles of the MCC and normal Merkel cells, while similar, have many discrepancies (Tilling & Moll, 2012). Nonetheless, identification and characterization of resident skin cells capable of producing cells with Merkel cell phenotypes may provide insight their formation and maintenance, potentially lending new therapeutic targets for MCC tumors.

2.0 MERKEL CELLS ARISE FROM AN *ATOHI*+ PROGENITOR IN EMBRYOGENESIS

The experiments described in this section are part of a published manuscript co-first authored by Dr. Erin Reed-Geaghan and myself (Wright et al., 2015).

My contributions:

- Discussing experimental design and data analysis with Drs. Reed-Geaghan and Maricich.
- Writing the manuscript, with edits from Drs. Reed-Geaghan and Maricich.
- Design and production of all figures, including acquisition of the majority of the images.
- Generating animals, tissue processing, and quantifications in Figures 2, 3, and 5.

Contributions from other authors:

- Figure 1 - Dr. Reed-Geaghan generated animals, processed tissue, and performed cell counts.
- Figure 4 - Alexa Bolock processed tissue and performed cell counts.
- Drs. Fujiyama and Hoshino generated and kindly provided the *Atoh1*^{CreER-T2/+} mice.

2.1 INTRODUCTION

Mammalian hairy skin comprises multiple sub-structures including hair follicles, sebaceous glands, sweat glands, interfollicular epithelia, and touch domes. The complex interplay of many signaling cascades during embryogenesis sum together to generate these unique cell populations from a single layer of homogeneous and multipotent epithelial progenitors (Fuchs, 2007). One of these sub-structures, the touch dome, is found exclusively around guard hairs and can be identified by distinct columnar epithelial cells called touch dome keratinocytes. Touch domes also house the Merkel cell-neurite complex, a mechanoreceptor responsible for light touch

detection.

Merkel cells have a complex and unique array of characteristics, leading to their common classification as a ‘neuroendocrine’ cell and inspiring a century long debate into their developmental origin. Several independent lines of evidence in the last decade have clarified this controversy, with newly available genetic techniques demonstrating an epidermal origin for mammalian Merkel cells (Bardot et al., 2013; Morrison et al., 2009; Van Keymeulen et al., 2009). These and other studies have started to detail the genetic cascades that lead to Merkel cell specification and maturation in embryogenesis. However, the identity of the specific progenitor cell responsible for generating and maintaining Merkel cells was not yet determined.

The basic helix-loop-helix transcription factor *Atoh1* is required for Merkel cell specification and is the earliest known marker of the Merkel cell lineage (Ben-Arie et al., 2000; Maricich et al., 2009). As other post-mitotic *Atoh1*+ cell populations in the body are generated by *Atoh1*+ progenitors (Akazawa et al., 1995; Helms & Johnson, 1998; Yang et al., 2001), we wondered if this was the same for Merkel cells as well. Using transgenic mice that allow us to identify and fate-map the *Atoh1*+ skin lineage, we sought to investigate the proliferative capacity of *Atoh1*+ skin cells in embryogenesis.

2.1.1 Mice

Atoh1^{GFP} (JAX 013593; The Jackson Laboratory; (Lumpkin et al., 2003)), *ROSA*^{tdTomato} (JAX 007914; (Madisen et al., 2010)), *K14*^{CreER} (JAX 005107; (Vasioukhin, Degenstein, Wise, & Fuchs, 1999)), and *Atoh1*^{CreER-T2} (Fujiyama et al., 2009) mice were maintained in accordance with International Animal Care and Use Committee guidelines at the Case Western Reserve University and the Children’s Hospital of Pittsburgh of the University of Pittsburgh Medical Center. For embryonic ages, the plug date was designated as E0.5.

2.1.2 Tamoxifen and EdU Administration

Tamoxifen (Sigma-Aldrich) was dissolved in a 9:1 corn oil/ethanol solution at a 1% or 5% concentration. Mice were briefly anesthetized with isoflurane, and tamoxifen was administered by oral gavage. For lineage tracing, tamoxifen was administered as a single dose of 0.4 mg (~10mg/kg; low-dose), or 250mg/kg (high dose) on either a consecutive 1 or 3 days. EdU (5-ethynyl-2'-deoxyuridine, Invitrogen) was dissolved in sterile PBS at a 10mM concentration and administered as a single 50mg/kg dose by intra-peritoneal injection to pregnant females.

2.1.3 Tissue processing

Mice were euthanized by cervical dislocation, and skin was dissected into cold PBS. Embryos were removed from pregnant dams and decapitated before tissue dissection. Skin processed for immunohistochemistry was fixed in 4% paraformaldehyde (PFA) for 30 min (dissected embryonic skin) or overnight (whole embryos), washed in PBS, and cryopreserved in 30% sucrose/PBS. Skin for Xgal staining was fixed in cold 4% PFA for 15 min, washed in cold PBS, and stained in Xgal overnight at 37°C. Embryonic skin and whisker follicles were dissected before incubation in Xgal. Tissue was washed and post-fixed for 2 hrs in 4% PFA before imaging. Tissue was also prepared for cryosectioning and counterstained with Nuclear Fast Red (Sigma-Aldrich).

2.1.4 Histology

Tissue was embedded in optimum cutting temperature (O.C.T.; Thermo Fisher Scientific) and serially sectioned on a cryostat (1950M; Leica) at 25µm. Slides were vacuum dried, rehydrated in PBS, and blocked with 5% normal donkey serum or 3% nonfat dry milk in 0.3% PBS-T (PBS with Triton X-100). EdU was detected with an imaging kit (Click-iT EdU; Invitrogen). Slides were incubated overnight in blocking solution containing dilutions of the following primary antibodies: chicken anti-GFP (1:1,000; GFP-1010; Aves Labs), rabbit anti-Ki67 (1:500; RM-9106-S1; Thermo Fisher Scientific), rat anti-K8 (1:20; TROMA-1; Developmental Studies

Hybridoma Bank), chicken anti- β Gal (1:1,000; BGL-1010; Aves Labs), rabbit anti-K14 (1:1,000; PRB- 155P; Covance), and rabbit anti-phospho-histone H3 (1:500; 06-570; EMD Millipore). Antigen retrieval was performed before Ki67 immunostaining by either heat-induced epitope retrieval with citrate buffer or proteolytic-induced epitope retrieval with trypsin. For heat-induced epitope retrieval, rehydrated tissue sections were incubated in sub-boiling 10mM citrate buffer solution for 7 min followed by 10 min at room temperature. For proteolytic-induced epitope retrieval, rehydrated tissue sections were incubated at 37°C for 10 min in pre-warmed 0.0125% trypsin in PBS. After primary antibody incubation, sections were washed and incubated for 30 min at room temperature in blocking solution containing the appropriate secondary antibodies obtained from Jackson ImmunoResearch Laboratories, Inc. (1:500): Alexa Fluor 488-conjugated donkey anti-rat (712-545-150), Alexa Fluor 488-conjugated donkey anti-chicken (703-545-155), Cy3-conjugated donkey anti-rabbit (711-165-152), Cy3-conjugated donkey anti-rat (712-165-150), Alexa Fluor 647-conjugated donkey anti-rabbit (711-605-152), and Alexa Fluor 647-conjugated donkey anti-rat (712- 605-150). Sections were stained with the nuclear probe DAPI (1:1,000; Thermo Fisher Scientific) to visualize nuclei and mounted in ProLong gold (Invitrogen). Whole-mount immunostaining was performed by modifying previously published protocols (Li et al., 2011) for hairy skin. Fixed skin was dissected into small pieces, and the underlying adipose tissue was removed and washed for 5-8hrs in 0.3% PBS-T. Tissue was incubated with primary antibodies for 4 days, washed for 5-8hrs in 0.3% PBS-T, and then incubated with secondary antibodies for 2 days, all at room temperature. Antibodies were diluted in 20% dimethyl sulfoxide/5% normal donkey serum/0.3% PBS-T. Confocal images were acquired with an inverted microscope (Axio Observer; Carl Zeiss) on a spinning-disc confocal (UltraVIEW VoX; PerkinElmer) with a C-Apochromat 40X, 1.1 NA water immersion objective, a camera (C9100-13; Hamamatsu Photonics), and Volocity software (PerkinElmer). Images presented here are maximum intensity projections of a z-series or single z-slices (as noted in the figures) consisting of 1 μ m optical slices collected every 0.35 μ m. Non-confocal images were acquired with a fluorescent scope (DM5500 B; Leica) using an HCX Plan Apochromat 40X, 1.25 NA and an HC Plan Apochromat 10X, 0.4 NA objective, camera (DFC420; Leica), and Leica Acquisition Software v4.2. Images were cropped, and brightness and contrast were enhanced for publication quality with Photoshop and/or Illustrator (Adobe).

2.1.5 Cell counts

Cell counts of *Atoh1*^{GFP} mice ($n = 2/\text{age}$) were performed on a microscope (DM5000 B) with a dual red/green fluorescent filter using an HCX Plan Apochromat 40X, 0.75 NA objective at room temperature. All GFP+ cells from one whisker pad were counted per animal, amounting to >500, >1,700, >6,000, >1,000, and >1,500 GFP+ cells for each mouse at E14.5, E15.5, E16.5, E17.5, P0, and P21, respectively. GFP+ cells from hairy skin were counted from a total of 15 slides/mouse from three different body levels, amounting to >40, >75, >2,500, >3,500, >3,000, and >250 GFP+ cells for each mouse at E14.5, E15.5, E16.5, 17.5, P0, and P21, respectively. Greater than 1,500 and >250 GFP+ cells were counted in the whisker follicles and hairy skin, respectively, for each *Atoh1*^{GFP} mouse at 3 and 6 mo of age. Together, we analyzed >95,000 GFP+ cells in these experiments. Cell counts from all other mice were performed at room temperature on a fluorescent microscope (DM5500 B) using an HCX Plan Apochromat 40X, 1.25 NA objective. 20 touch domes from 3–6 mice/time point were counted in embryonic *Atoh1*^{CreER-T2/+}; *ROSA*^{tdTomato} mice, amounting to >2,500 and >2,900 tdTomato+ cells at E16.5 and E18.5, respectively. In our embryonic EdU incorporation experiment, >200 tdTomato+ cells/mouse were counted each in the body skin and whisker follicles ($n = 2$ mice), amounting to ~1,500 tdTomato+ cells. 20 touch domes from two mice per age were counted in embryonic *K14*^{CreER}; *ROSA*^{tdTomato} mice, amounting to >1,000 K8+ cells. Cell counts were compared using independent sample two-tailed *t*-tests (Excel; Microsoft) or one- or two-way ANOVA with post-hoc Tukey's multiple comparisons test (Prism 6; GraphPad Software).

2.2 RESULTS

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

The transcription factor *Atoh1* is the earliest known marker of the Merkel cell lineage (Ben-Arie et al., 2000). To determine if any *Atoh1*-expressing cells in the skin were proliferative, we analyzed the Merkel cell population for presence of the proliferative marker, Ki67 (Scholzen &

Gerdes, 2000), in *Atoh1^{GFP}* mice. These mice express green fluorescent protein (GFP) under the control of *Atoh1* promoter elements, faithfully recapitulating *Atoh1* expression in the skin and allowing for us to easily visualize the Merkel cell lineage (Lumpkin et al., 2003). Hairy skin and whisker follicles from E14.5, E15.5, E16.5, E17.5, P0, and P21 *Atoh1^{GFP}* mice was immunostained for GFP and Ki67, and GFP+Ki67+ cells were present at all ages. The percentage of GFP+ cells that were also Ki67+ peaked at E14.5 in whisker follicles and at E16.5 in hairy skin and then decreased as the animals aged, reaching ~1% at P21 (>500 cells/region/mouse, n=2 mice/age; Figure 1A-D).

To determine where proliferative *Atoh1*+ cells were located, whisker follicles were divided into four equal segments and the number of GFP+/Ki67+ cells counted in each quadrant (Figure 1E). The vast majority of GFP+/Ki67+ cells were found in the most superficial 25% of the whisker follicle and never in the bottom 50% (Figure 1F). Similarly, GFP+/Ki67+ cells in guard hair follicles of the body skin were found mostly in the infundibulum (Figure 1C' and C''). These data suggest that proliferative *Atoh1*+ cells are located in specific hair follicle regions.

To expand upon these findings the *Atoh1*+ population was analyzed for a second marker of proliferation, the M-phase marker phosphohistone 3 (PH3). A subset of *GFP*+ cells in *Atoh1^{GFP}* mice was found to also express PH3 (Figure 2A-A'''). To further confirm these markers, tamoxifen was administered to *Atoh1^{CreER-T2/+};ROSA^{tdTomato}* embryos at E15.5 (250mg/kg) to label all *Atoh1* lineage cells with tdTomato, then the nucleoside analogue 5-ethynyl-2'-deoxyuridine (EdU; 50mg/kg) administered at E16.5 and skin harvested 4hrs later to identify actively dividing cells. In the whisker follicles and body skin $0.9 \pm 0.1\%$ and $1.1 \pm 1.1\%$ of tdTomato+/K8+ cells incorporated EdU, respectively (>200 tdTomato+ cells/region/mouse, ~1,500 tdTomato+ cells total; n=2 mice; Figure 2B-B'''). These data indicate that embryonic *Atoh1*+ cells are mitotically active.

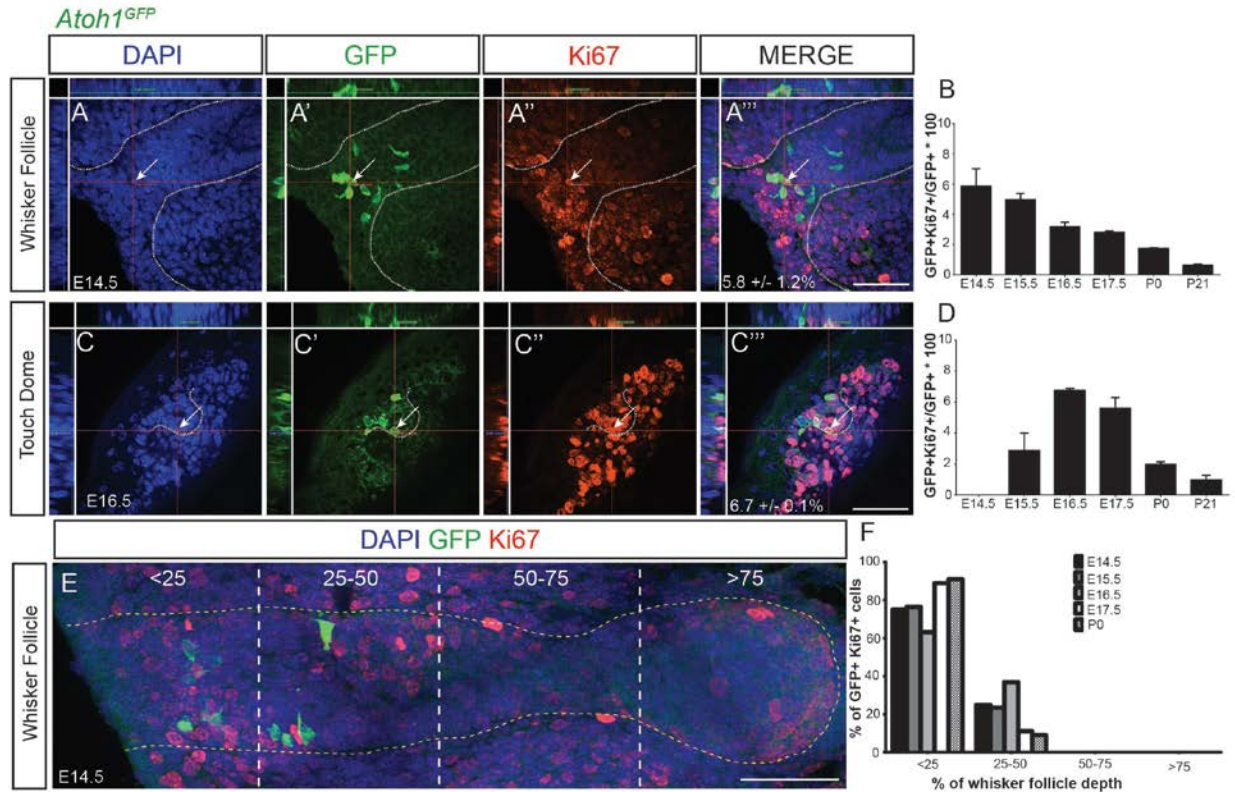


Figure 1. A subset of *Atoh1*⁺ cells in hairy skin express the proliferative marker Ki67. (A-C'') Confocal images of an E14.5 whisker follicle (A-A'') and E16.5 touch dome (C-C'') from *Atoh1*^{GFP} mice immunostained for GFP (A' and C', green) and Ki67 (A'' and C'', red), counterstained with DAPI (A and C). Whisker (A-A'') and guard hair (C-C'') follicles are outlined with dashed lines. Crosshairs are over double-labeled cells, which are also indicated by arrows. Percentages ± SEM of GFP+Ki67+ cells are shown in A'' and C''. (B and D) The percentages of GFP+Ki67+ cells within the GFP+ population changed from E14.5 to P21. Error bars show SEM. (N=2 mice/age.) (E) Cross section of E14.5 *Atoh1*^{GFP} whisker follicle illustrating how follicles were divided into quadrants. The dotted yellow line outlines a single whisker follicle. (F) GFP+Ki67+ cells are clustered at the top of whisker follicles (n=2 mice/age). Scale bars: 50µm.

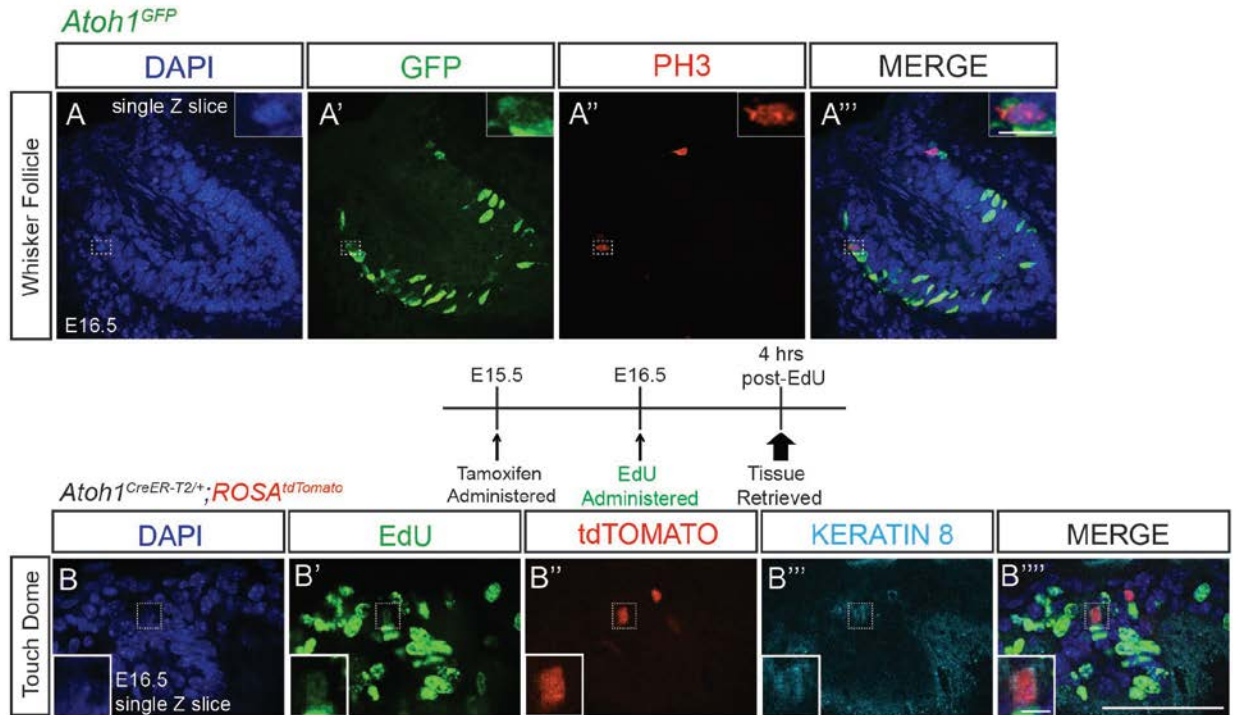


Figure 2. A subset of *Atoh1*⁺ cells express the proliferative marker PH3 and incorporate EdU. (A-A''') Single confocal z-slice image of an *Atoh1*^{GFP} E16.5 whisker follicle immunostained for GFP (A', green) and PH3 (A'', red), counterstained with DAPI. Inset shows double-labeled cell. (B-B''') Single confocal z-slice of a touch dome from a E16.5 *Atoh1*^{CreER-T2/+};*ROSA*^{tdTomato} mouse given tamoxifen at E15.5, EdU at E16.5, and tissue retrieved 4 hrs after EdU administration. EdU (B', green), tdTomato (B'', red), K8 (B''', cyan), and merge (B'''' are shown. Insets show a K8+tdTomato+K8+ cell. Scale bars: (main images) 50 μ m; (A-A''' insets) 10 μ m; (B-B'''' insets) 5 μ m.

2.2.2 Proliferative *Atoh1*⁺ cells give rise only to Merkel cells

While it was clear that a subset of *Atoh1*⁺ cells were proliferating, we wanted to confirm that they were actively generating new cells, and identify who those new cells were. To determine this, *Atoh1*⁺ cells were lineage traced using *Atoh1*^{CreER-T2/+};*ROSA*^{tdTomato} embryos. Recombination was limited to the day of tamoxifen administration by administering a single low dose (10mg/kg) to pregnant dams at E15.5 and then tissue harvested 1 (E16.5) or 3 (E18.5) days later. We found ~71% more tdTomato⁺ cells/touch dome at E18.5 than at E16.5 (18.0 ± 1.2 vs. 10.5 ± 0.6 ; n=20–30 touch domes/embryo from 3–6 embryos/age; $P=4 \times 10^{-4}$, *t*-test; Figure 3B–

D), suggesting that *Atoh1*⁺ cells proliferated between these ages (Figure 3A). As expected, immunostaining for K8 demonstrated that the mean number of Merkel cells per touch dome also increased between E16.5 and E18.5 (13.8 ± 0.7 and 21.3 ± 0.8 , respectively; $P=5.9 \times 10^{-6}$, *t*-test). The proportion of K8⁺ cells co-expressing K20 also increased between E16.5 and E18.5 ($22.9 \pm 0.7\%$ and $46.8 \pm 2.6\%$, respectively; $n=3$ mice/age; $P=9.3 \times 10^{-4}$, *t*-test; Figure 4B–C’’’). Importantly, all tdTomato⁺ cells were also K8⁺ and/or K20⁺, demonstrating that proliferative *Atoh1*⁺ cells are unipotent and only give rise to Merkel cells. These data demonstrate that some *Atoh1*⁺ cells present at E15.5 are mitotically active and continue to divide after E16.5.

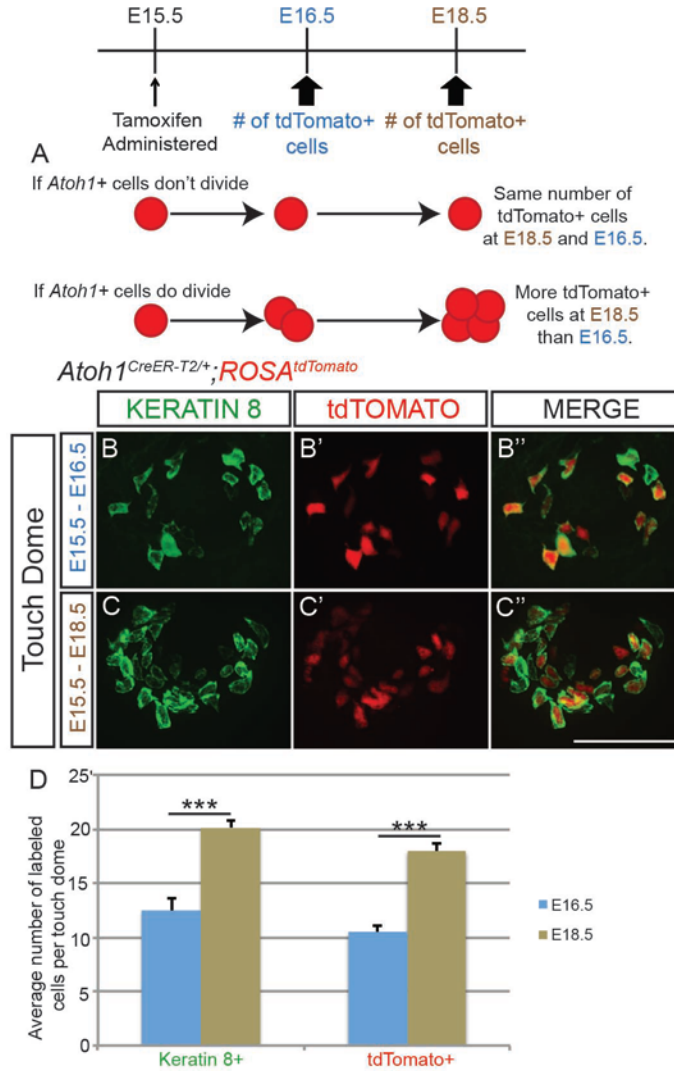


Figure 3. Embryonic Merkel cell precursors express *Atoh1* and are unipotent. (A)

Experimental paradigm, outcomes, and interpretations. (B-C'') Whole-mount immunostaining for K8 (B and C, green), endogenous tdTomato signal (B' and C', red), and merged images (B'' and C'') in E16.5 (B-B'') and E18.5 (C-C'') *Atoh1*CreER-T2/+;ROSAtdTomato embryonic body skin. (D) K8+ and tdTomato+ cell numbers increased from E16.5 to E18.5 (n=3-6 mice/age, t test). Error bars show SEM. Scale bars: 50µm.

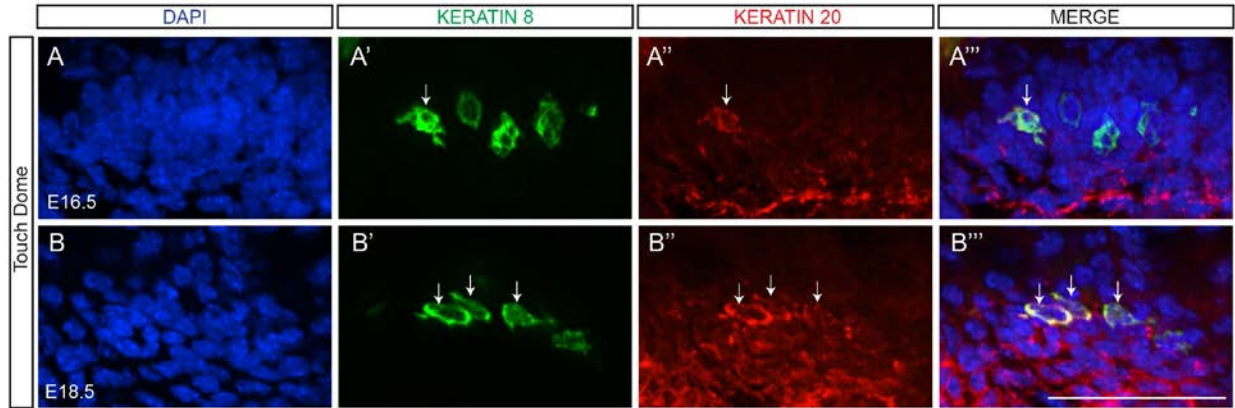


Figure 4. Embryonic K8+ Merkel cells co-express K20. Back skin from E16.5 (A-A''') and E18.5 (B-B''') mice immunostained for K8 (A', B', green) and K20 (A'', B'', red), demonstrating co-expression (arrows). Scale bar: 50 μ m.

2.2.3 The *Atoh1*+ lineage separates from K14+ skin lineages in late embryogenesis

Embryonic *Atoh1*+ cells are derived from the *K14* lineage (Morrison et al., 2009; Van Keymeulen et al., 2009). Very soon after their specification, *Atoh1*+ Merkel cells lack K14 and begin to express the cytokeratins 8, 18, and 20 (Perdigoto et al., 2014b). Given our data suggesting that the *Atoh1*+ population expanded between E16.5 and E18.5, we wondered when *Atoh1*- skin precursor cells stopped producing *Atoh1*+ Merkel cell precursors. To verify the absence of K14 in *Atoh1*+ cells, I immunostained for K14 in the body skin and whisker follicles of E16.5 *Atoh1*^{GFP} mice and found no GFP+K14+ cells (Figure 5A-A'''). To determine if *Atoh1*-K14+ cells contributed to Merkel cell generation after E16.5, tamoxifen was administered to E16.5 and E17.5 *K14*^{CreER}; *ROSA*^{tdTomato} embryos and the Merkel cell population analyzed at E18.5. No K8+tdTomato+ cells were found (>250 hairy skin and >500 whisker follicle K8+ cells counted/mouse, n=2 mice/age; Figure 5B-B''). Tamoxifen administration at E14.5, when *Atoh1*+ cells first arise from the K14 lineage, did yield a subset of K8+/tdTomato+ cells at E18.5 (Figure 5C-C''). These data suggest that the full complement of *Atoh1*+ Merkel cell progenitors are created in a 2–3 day period beginning with the appearance of the first *Atoh1*+ cells in the skin at E14.5.

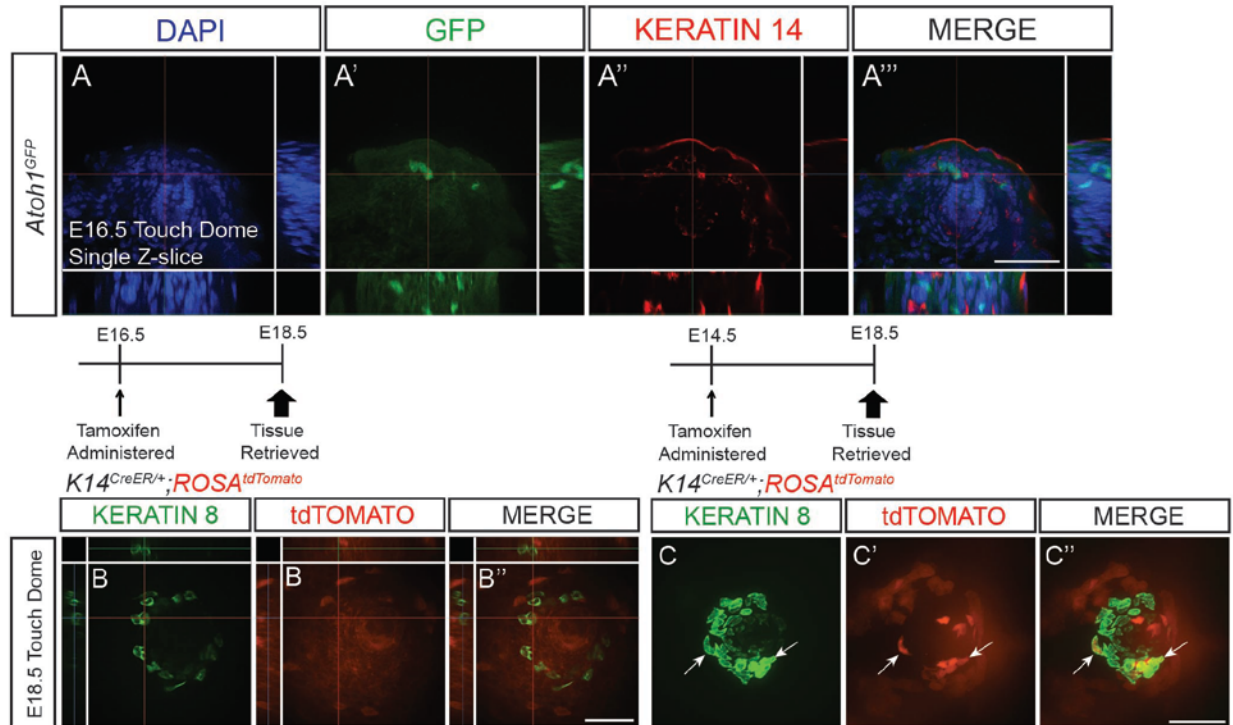


Figure 5. Embryonic *Atoh1*⁺ cells separate from the K14 lineage very early. (A-A''') Single confocal z-slice of a touch dome immunostained for GFP (A', green) and K14 (A'', red) in the body skin of an E16.5 *Atoh1*^{GFP} mouse. (B-B'') Single confocal z-slice of touch dome whole-mount preparation from an E18.5 *K14*^{CreER/+}; *ROSA*^{tdTomato} mouse given tamoxifen at E16.5 and immunostained for K8 (B) shows that the two signals are not co-localized. (C-C'') Confocal image of a whole mount touch dome from an E18.5 *K14*^{CreER/+}; *ROSA*^{tdTomato} mouse given tamoxifen at E14.5 and immunostained for K8 (C). Arrows mark double-labeled cells. Scale bars: 50µm.

2.3 DISCUSSION

We have identified a novel, *Atoh1*+ progenitor population located in the infundibulum of guard hairs and whisker follicles that produces only Merkel cells during embryogenesis. These progenitors are allocated from the *K14*+ lineage very early in skin development and are found at all embryonic ages after E14.5 in both whisker follicles and body skin.

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

Our group and many others have clearly demonstrated that Merkel cells derive from *K14*+ progenitors (Bardot et al., 2013; Morrison et al., 2009; Van Keymeulen et al., 2009). We show here that *K14* expression is immediately down-regulated in newly specified *Atoh1*+ Merkel cells and their progenitors, both by fate-mapping in *K14^{CreER};ROSA^{tdTomato}* mice and with immunostaining in *Atoh1^{GFP}* and *Atoh1^{CreER-T2};ROSA^{tdTomato}* mice. As predicted, we did find K8+tdTomato+ cells in *K14^{CreER};ROSA^{tdTomato}* mice treated with tamoxifen at E14.5 when *Atoh1*+ cells first arise from the *K14* lineage (Morrison et al., 2009; Vielkind et al., 1995), suggesting that our findings were not secondary to a technical issue. Notably, a microarray experiment conducted on early postnatal skin also failed to detect *K14* expression in purified Merkel cells (Haeberle et al., 2004). Other labs have reported *K14* protein in *Atoh1*+ cells by co-immunostaining (Van Keymeulen et al., 2009), which we were unable to replicate. It is likely that either *K14* signal from an adjacent *Atoh1-K14*+ cell was mistaken for *K14*-expression in the

Atoh1⁺ cell, or that K14 protein persists for a short time after its expression has ended. These possibilities are not mutually exclusive.

Our study does not identify the factors that control the initial specification of *Atoh1*⁺ cells from the *K14* lineage. Recent work suggests that the transcription factor *Sox2* is a direct, positive regulator of *Atoh1*, whereas the Polycomb repressive complex negatively regulates Merkel cell specification through repression of *Sox2* (Bardot et al., 2013; Lesko et al., 2013). Deletion of *Sox2* in the developing skin reduces the number of Merkel cells in embryonic mice but does not preclude their production nor their expression of multiple canonical markers (Bardot et al., 2013; Lesko et al., 2013). *Sox2* and *Atoh1* are expressed concomitantly in the epidermis starting at E14.5; however, the genetic cascades and signaling molecules necessary for the initiation of *Atoh1* and *Sox2* expression are unknown. Future experiments are required to determine which *Atoh1*⁻ cells in the developing skin ultimately give rise to the Merkel cell lineage.

3.0 MOST MATURE MERKEL CELLS ARE MADE IN EMBRYOGENESIS AND ARE INFREQUENTLY REPLACED IN ADULTHOOD

The experiments described in this section are part of a published manuscript co-first authored by Dr. Erin Reed-Geaghan and myself (Wright et al., 2015) and a second manuscript currently in preparation.

My contributions:

- Discussing experimental design and data analysis with Drs. Reed-Geaghan, Maricich and Greg Logan.
- Writing the manuscript, with edits from Drs. Reed-Geaghan and Maricich.
- Design and production of all figures, including acquisition of the majority of the images (with the exception of Figures 19, 20, and 21).
- Generating animals, tissue processing, and quantifications in Figures 6, 8, 9, 10, 13, 14, 15, 17, 18, 22, 23.

Contributions from other authors:

- Figure 7 - Alexa Bolock processed tissue and performed quantifications.
- Figure 11 - Dr. Reed-Geaghan generated the mice for and processed the tissue. I performed the cell counts.
- Figure 12 – Alexa Bolock processed tissue and performed cell counts for embryonic and early postnatal time points. I generated all mice, processed tissue and performed cell counts for adult time points.
- Figure 16 – I generated all mice; Alexa Bolock and Julie Hemphill processed the tissue; Julie Hemphill performed cell counts; Alexa Bolock took images.
- Figures 19, 20, 21 – Greg Logan imaged all mice; Greg and I each did 50% of the quantifications; Greg performed the statistical analysis and made the figures.

3.1 INTRODUCTION

Mammalian skin is a dynamic organ that provides protection against a variety of environmental insults. Damage to the skin caused by these stressors must be repaired through constant skin cell replacement in adulthood. Skin integrity is maintained by a heterogeneous population of resident progenitor cells capable of self-renewal and production of diverse cell types that make up hair follicles, glands, and interfollicular epidermis (Breathnach, 1978; Fuchs, 2007; Ghazizadeh & Taichman, 2001; Mayumi Ito et al., 2005; Jaks et al., 2010; Saxod, 1978; Solanas & Benitah, 2013).

In addition to its role as a barrier, skin also houses multiple somatosensory receptors, each tuned to detect different forms of mechanical stimuli. The Merkel cell–neurite complex is one such receptor located at the epidermal–dermal border of mammalian skin around whisker follicles, in hairy skin within specialized structures called touch domes and in glabrous (non-hairy) skin of the hands and feet (Halata et al., 2003; Taira et al., 2002; Winkelmann, 1977). This somatosensory receptor is unique in its composition of two distinct cell types; an epidermally-derived Merkel cell and a neural crest-derived afferent.

Adult Merkel cells are postmitotic (Boulais & Misery, 2007; Moll et al., 1995). However, quantitative, morphological, and fate-mapping studies suggest that Merkel cell numbers in adult hairy skin oscillate with the hair cycle and are entirely replaced multiple times over throughout an animal's lifespan (Moll et al., 1996a; Nafstad, 1987; Nakafusa et al., 2006; Tweedle, 1978; Van Keymeulen et al., 2009). This ‘Merkel cell renewal hypothesis’ has led to an exhaustive search to identify the elusive Merkel cell progenitor in adult mice. Mitotically active progenitors are the likely source of new Merkel cells, as a small percentage of Merkel cells are labeled several days after administration of nucleotide analogues (Mérot & Saurat, 1988; Mérot et al., 1987; Moll et al., 1990; Vaigot et al., 1987; Woo et al., 2010). Recent work in hairy skin has suggested that these progenitors are either multipotent stem cells located in the hair follicle bulge region or bipotent progenitors found among the touch dome keratinocytes (Compton et al., 1990; Doucet et al., 2013; Van Keymeulen et al., 2009; Woo et al., 2010). Accurate identification of Merkel cell progenitors is crucial because of the potential for these cells to act as the cellular origin of Merkel cell carcinoma (MCC), a rare but potentially devastating disease that currently

has no targeted therapies (Kuwamoto, 2011; Sidhu et al., 2005; Tilling & Moll, 2012).

Here, we sought to test the central hypothesis that Merkel cells are frequently and entirely replaced in adult mice during normal skin homeostasis. We used multiple techniques to investigate the kinetics of Merkel cells replacement during adulthood and to quantify the lifespan of individual Merkel cells. We found that touch dome Merkel cell number is constant throughout maturity, that Merkel cells born in embryogenesis persist into late adulthood, that new Merkel cells are infrequently generated during adult skin homeostasis, and that these few nascent Merkel cells are derived from an *Atoh1-K14*- progenitor. These experiments also uncovered a phenomenon by which repeated skin shaving induces an increased production of new Merkel cells.

3.2 MATERIALS AND METHODS

3.2.1 Mice

C57BL/6J (JAX 000664; The Jackson Laboratory), *Atoh1^{CreER-T2}* (Fujiyama et al., 2009), *Atoh1^{GFP}* (JAX 013593; The Jackson Laboratory; (Lumpkin et al., 2003)) *ROSA^{LacZ}* (JAX 003474; The Jackson Laboratory; (Soriano, 1999)), *ROSA^{tdTomato}* (JAX 007914; The Jackson Laboratory; (Madisen et al., 2010)), *ROSA^{YFP}* (JAX 006148; The Jackson Laboratory; (Srinivas et al., 2001)), *ROSA^{DTA}* (JAX 009669; The Jackson Laboratory; (Voehringer et al., 2008)), *K14^{CreER}* (JAX 005107; The Jackson Laboratory; (Vasioukhin et al., 1999)), and *K18^{CreER-T2}* (JAX 017948; The Jackson Laboratory; (Van Keymeulen et al., 2009)) mice were maintained in accordance with International Animal Care and Use Committee guidelines at the Case Western Reserve University and the Children's Hospital of Pittsburgh of the University of Pittsburgh Medical Center. For embryonic ages, the plug date was designated as E0.5.

3.2.2 Tamoxifen and EdU administration

Tamoxifen (Sigma-Aldrich) was dissolved in a 9:1 corn oil/ethanol solution at a 1% or 5% concentration. Mice were briefly anesthetized with isoflurane and tamoxifen administered by oral gavage. For lineage tracing, tamoxifen was administered at a dose of 250mg/kg once daily for three consecutive days or as a single dose of 0.1mg (5mg/kg; low-dose). For embryonic administration, EdU (Invitrogen) was dissolved in sterile PBS at a 10mM concentration and administered by intraperitoneal injection at a dose of 10mg/kg to pregnant females. For adult administration, EdU was dissolved in ddH₂O water at a 0.2mg/ml concentration and provided *ad libitum* for five weeks.

3.2.3 Tissue processing

Adult mice were euthanized by cervical dislocation, their skin shaved with an electric razor, depilated with surgicream, and dissected into cold PBS. Embryos were removed from pregnant dams and decapitated prior to tissue dissection. Skin processed for immunohistochemistry was fixed in 4% PFA for 30-60 minutes (adult tissue) or overnight (whole embryos, P0, and P3 mice) and washed in PBS. Tissue for cryosectioning was cryopreserved in 30% sucrose/PBS.

3.2.4 Histology

Tissue was embedded in optimum cutting temperature (O.C.T.; Thermo Fisher Scientific) and serially sectioned on a cryostat (1950M; Leica) at 25µm. Hair cycle stage was determined in hematoxylin and eosin–stained sectioned skin by analyzing hair follicle dimensions, depth into the subcutaneous tissue, and the shape and size of the dermal papilla and hair bulb as previously described (Maricich et al., 2009; Müller-Röver et al., 2001). Slides were vacuum dried, rehydrated in PBS, and blocked with 5% normal donkey serum in 0.3% PBS-T (PBS with Triton X-100). EdU was detected with an imaging kit (Click-iT EdU; Invitrogen). Slides were incubated overnight in blocking solution containing dilutions of the following primary antibodies: chicken anti-β-Gal (1:1000; BGL-1010; Aves Labs), chicken anti-GFP (1:1,000;

GFP-1010; Aves Labs), goat anti-TrkB (1:200; AF1494; R&D Systems), mouse anti-cytokeratin 20 (1:100; 182200; Life Technologies), rabbit anti-Ki67 (1:500; RM-9106-S1; Thermo Fisher Scientific), rat anti-K8 (1:20; TROMA-1; Developmental Studies Hybridoma Bank), rabbit anti-K14 (1:1,000; PRB- 155P; Covance), and rabbit anti-cytokeratin 17 (1:1,000; ab53707; Abcam). Antigen retrieval was performed before Ki67 immunostaining by either heat-induced epitope retrieval with citrate buffer or proteolytic-induced epitope retrieval with trypsin. For heat-induced epitope retrieval, rehydrated tissue sections were incubated in sub-boiling 10mM citrate buffer solution for 7 min followed by 10 min at room temperature. For proteolytic-induced epitope retrieval, rehydrated tissue sections were incubated at 37°C for 10 min in pre-warmed 0.0125% trypsin in PBS. After primary antibody incubation, sections were washed and incubated for 30 min at room temperature in blocking solution containing the appropriate secondary antibodies obtained from Jackson ImmunoResearch Laboratories, Inc. (1:500): Alexa Fluor 488-conjugated donkey anti-rabbit (711-545-152), Alexa Fluor 488-conjugated donkey anti-rat (712-545-150), Alexa Fluor 488-conjugated donkey anti-chicken (703-545-155), Cy3-conjugated donkey anti-chicken (703-095-155), Cy3-conjugated donkey anti-goat (705-165-003), Cy3-conjugated donkey anti-mouse (715-165-150), Cy3-conjugated donkey anti-rabbit (711-165-152), and Cy3-conjugated donkey anti-rat (712-165-150). Sections were stained with the nuclear probe DAPI (1:1,000; Thermo Fisher Scientific) to visualize nuclei and mounted in ProLong gold (Invitrogen). Whole-mount immunostaining was performed by modifying previously published protocols (Li et al., 2011) to use hairy skin. Fixed skin was dissected into small pieces, the underlying adipose tissue removed, and the skin washed for 5–8 hrs in 0.3% PBS-T. Tissue was incubated with primary antibodies for 4 days, washed for 5–8 hrs in 0.3% PBS-T, and then incubated with secondary antibodies for 2 days, all at room temperature. Antibodies were diluted in 20% dimethyl sulfoxide/5% normal donkey serum/0.3% PBS-T. Confocal images for *Atoh1*^{CreER-T2/+}; *ROSA*^{LacZ} adult fate-mapping experiments were acquired with a laser-scanning confocal microscope (LSM 510 META; Carl Zeiss) using a 40X C-Apochromat, NA 1.2, water immersion objective. Images presented here are maximum intensity projections of a z-series consisting of 1µm optical slices collected every 0.5µm (optimal interval setting determined by LSM 510 software, AIM 4.2). All other confocal images were acquired with an inverted microscope (Axio Observer; Carl Zeiss) on a spinning-disc confocal (UltraVIEW VoX; PerkinElmer) with a C-Apochromat 40X, 1.1 NA water immersion objective,

a camera (C9100-13; Hamamatsu Photonics), and Volocity software (PerkinElmer). Images presented here are maximum intensity projections of a z-series or single z-slices (as noted in the figures) consisting of 1 μ m optical slices collected every 0.35 μ m. Images in Figure 8G–H''' are stitched from z-stacked 4X images. Multiple 3D images were acquired for the desired region of interest with 10% overlap between adjacent images. Images were compiled and stitched using Volocity software with the standard brightness correction. For *in vivo* imaging of *Atoh1*^{GFP} touch domes, mice were placed on a specially designed platform with their belly skin on a coverslip. A 10X objective with 1.6 optivar was used to capture Z-stacks of 120 μ m thickness. Presented images are projections of the entire Z-stack. Non-confocal images were acquired with a fluorescent scope (DM5500 B; Leica) using an HCX Plan Apochromat 40X, 1.25 NA and an HC Plan Apochromat 10X, 0.4 NA objective, camera (DFC420; Leica), and Leica Acquisition Software v4.2. Images were cropped, and brightness and contrast were enhanced for publication quality with Photoshop and/or Illustrator (Adobe).

3.3 RESULTS

3.3.1 The *Atoh1* skin lineage is maintained into late adulthood

Several lines of evidence suggested that mature Merkel cells have a finite lifespan, implying their replacement by precursor cells located in the skin (Doucet et al., 2013; Moll et al., 1996a; Nakafusa et al., 2006; Van Keymeulen et al., 2009). To more accurately estimate the lifespan of *Atoh1*⁺ cells in the skin, *Atoh1*⁺ cells were lineage traced in postnatal day 21–28 (P21–P28) *Atoh1*^{CreER-T2/+; ROSA^{LacZ}} mice by administering tamoxifen (250mg/kg) for 3 consecutive days during the growth phase (anagen) of the first hair cycle and analyzing the K8⁺ population 3 and 9 mo later. Xgal⁺ (5-bromo-4-chloro-indolyl- β -d-galactopyranoside) cells were found only in the expected locations for Merkel cells in the hairy skin and whisker pads both 3 (n=3) and 9 (n=1) mo after tamoxifen administration (Figure 6A–B'), allowing for the completion of multiple hair cycles (Alonso & Fuchs, 2006). To confirm that these β -galactosidase (β -Gal)⁺ cells were Merkel cells, we co-immunostained for β -Gal and the Merkel cell marker K8 (Figure 6C–D''');

(Vielkind et al., 1995)). Three months after tamoxifen administration, $93.5 \pm 1.7\%$ and $99.2 \pm 0.4\%$ of K8+ cells in hairy skin and whisker follicles co-expressed β -Gal, respectively; these percentages were 91.5% and 98.1% at 9 mo (≥ 200 hairy skin and ≥ 500 whisker follicle K8+ cells counted/ mouse; Figure 6E). All β -Gal+ cells were also K8+, and nearly all K8+ cells ($99.0 \pm 0.4\%$, ≥ 150 K8+ cells/mouse, $n = 3$ mice) were also Keratin 20+ (K20; Figure 7), in agreement with other studies (Eispert et al., 2009; Lesko et al., 2013).

As a complementary approach to assay Merkel cell turnover, we next assayed how frequently new Merkel cells were made. The *Atoh1*+ population was genetically ablated by administering tamoxifen (250mg/kg) for 3 consecutive days to P28 *Atoh1*^{CreER-T2/+};*ROSA*^{DTA} mice and *Atoh1*^{CreER-T2/+};*ROSA*⁺ littermate controls. We used only *Atoh1*^{CreER-T2/+};*ROSA*⁺ mice as controls because they have slightly fewer K8+ cells per touch dome than their *Atoh1*^{+/+} siblings (18.1 ± 1.0 and 25.3 ± 1.8 , respectively; >600 K8+ cells/ mouse; $n = 5$ mice/genotype; $P = 0.007$, *t*-test). Back and belly skin and whisker pads were harvested 1, 3, and 6 mo after tamoxifen administration ($n=2$ mice/genotype/time point). Touch domes in the hairy skin were identified by immunostaining for TrkB, which we serendipitously found to be a reliable marker of touch dome keratinocytes (Figure 8A–B''). Skin from *Atoh1*^{CreER-T2/+};*ROSA*^{DTA} mice and *Atoh1*^{CreER-T2/+};*ROSA*⁺ littermate controls had similar densities of touch domes (56.5 ± 2.8 vs. 53.7 ± 3.4 touch domes per 1cm^2 of hairy skin, respectively; $F(2,6)=0.55$, $P = 0.6$, two-way ANOVA; Figure 8C).

Ablation of the K8+ Merkel cell population was confirmed at 1 mo post-tamoxifen in *Atoh1*^{CreER-T2/+};*ROSA*^{DTA} mice, as 64% of their touch domes lacked any Merkel cells. This was verified on tissue sections double labeled with K8 and K17 (Figure 8E and F). The average number of K8+ cells per touch dome was significantly lower in *Atoh1*^{CreER-T2/+};*ROSA*^{DTA} mice relative to their control littermates (0.6 ± 0.4 vs. 14.4 ± 1.5 K8+ cells/touch dome, respectively, $P = 6.0 \times 10^{-4}$). At 3 and 6 mo post-tamoxifen administration, still 65% and 60% of touch domes in *Atoh1*^{CreER-T2/+};*ROSA*^{DTA} mice had no Merkel cells, respectively; Mean numbers of K8+ cells per touch dome were also still significantly decreased ($F(1,6) = 183.7$, $P < 0.0001$, two-way ANOVA) 3 (0.6 ± 0.2 vs. 10.1 ± 0.01 , $P = 4.5 \times 10^{-3}$) and 6 (0.8 ± 0.1 vs. 11.3 ± 2.0 , $P = 2.6 \times 10^{-3}$) mo after tamoxifen administration (Figure 8D). The same was true in whisker follicles, in which *Atoh1*^{CreER-T2/+};*ROSA*^{DTA} mice had fewer K8+ cells/follicle than *Atoh1*^{CreER-T2/+};*ROSA*⁺ littermate controls (≥ 6 follicles/mouse; $F(2,6) = 15.45$, $P = 4.0 \times 10^{-3}$, two-way ANOVA) 1 (127.4 ± 14.6 vs.

499 \pm 24.3; $P = 5.8 \times 10^{-3}$, Tukey's post-hoc multiple comparisons test), 3 (1.6 \pm 1.0 vs .530 \pm 12.9; $P=6.0 \times 10^{-4}$), and 6 (8.37 \pm 7.6 vs. 529.8 \pm 22.2; $P=2.0 \times 10^{-3}$) mo later (Figure 8G–I). Thus, deletion of *Atoh1*⁺ cells led to a persistent deficit in Merkel cells numbers. This suggests one of two possibilities. Either a unipotent *Atoh1*⁺ progenitor is regularly dividing to maintain the Merkel cell population at a still unknown rate, or Merkel cells are replaced exceedingly infrequently during normal tissue homeostasis and persist through to late adulthood.

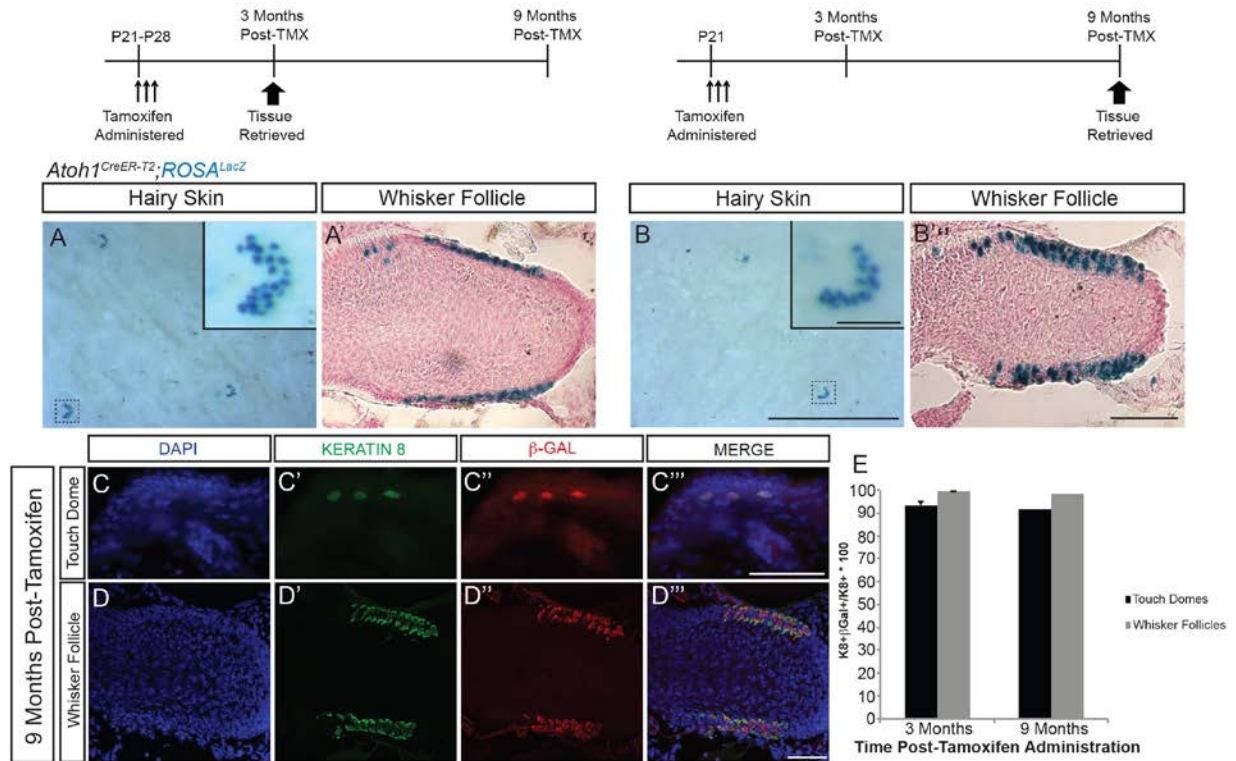


Figure 6. The *Atoh1*+ skin lineage is continuous in adulthood (A-B') Xgal staining of hairy skin (A and B) and whisker follicles (A' and B') shows the presence of labeled cells 3 (A and A'; n=3 mice) and 9 (B and B'; n=1 mouse) mo after tamoxifen. Insets in A and B are individual touch domes. (A' and B') Counterstain is Nuclear Fast Red. (C-D''') Touch domes (C-C''') and whisker follicles (D-D''') immunostained for K8 (C', D', green) and β -Gal (C'', D'', red). (E) Percentages of K8+ cells that co express β -Gal at 3 (n=3) and 9 (n=1) mo after tamoxifen (TMX). Error bars show SEM. Scale bars: (A and B, main images) 1mm; (A and B, insets) 100 μ m; (C-D'') 50 μ m.

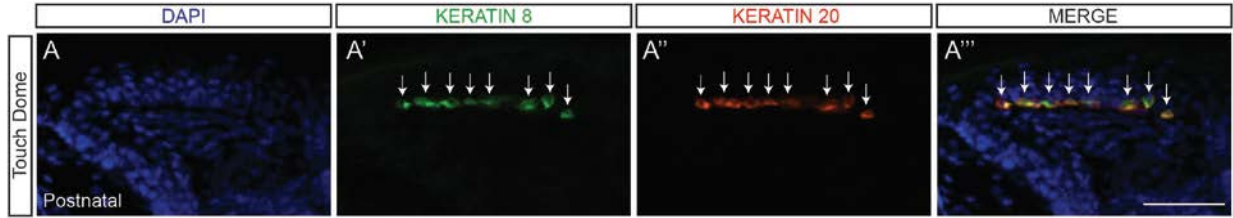


Figure 7. All postnatal K8+ Merkel cells co-express K20. (A-A''') Back skin from postnatal mice immunostained for K8 (A', green) and K20 (A'', red), demonstrating co-expression (arrows) Scale bar: 50 μ m.

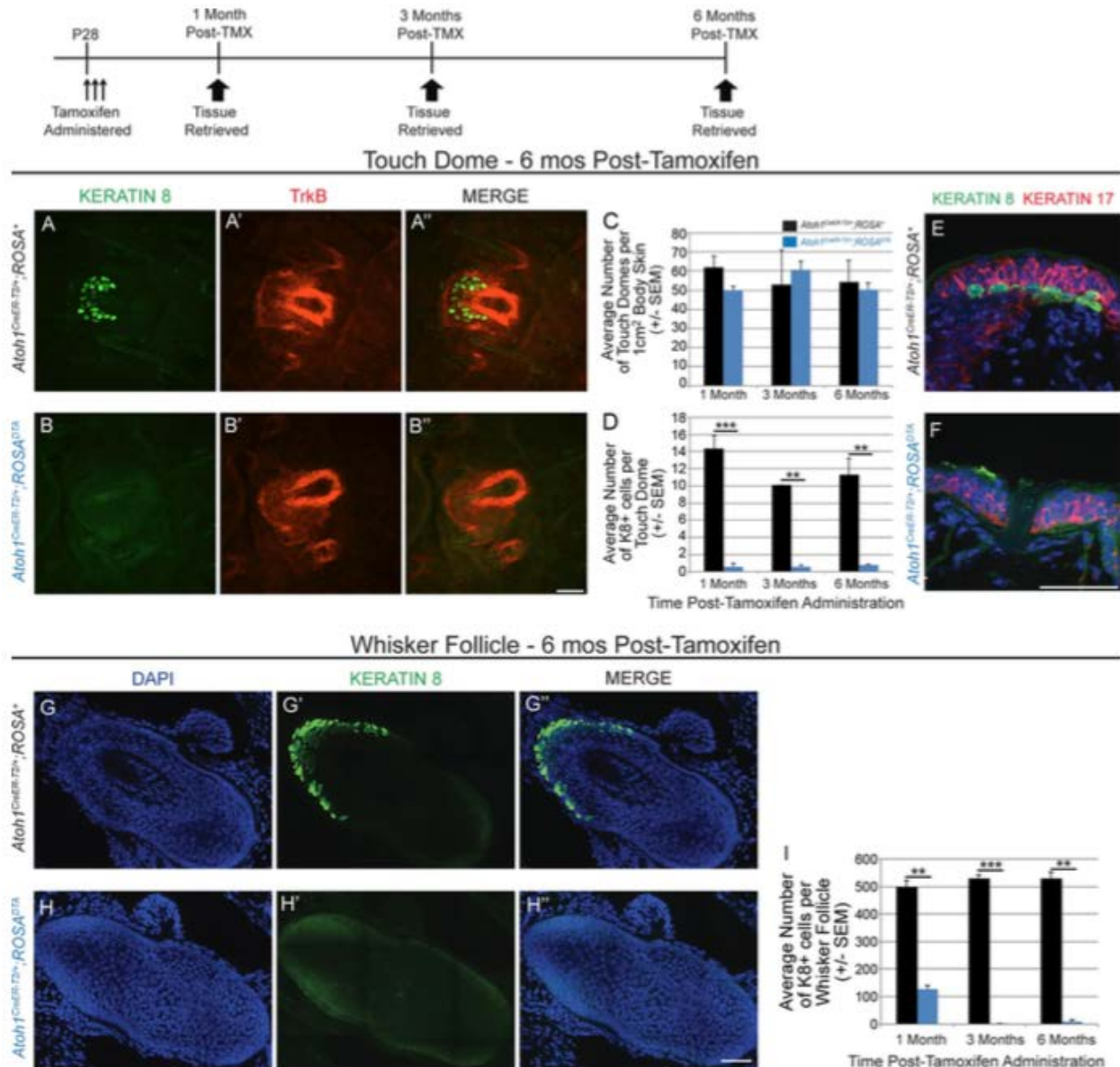


Figure 8. Merkel cells are not replaced after genetic ablation of all *Atoh1*+ skin cells (A–B'') Whole-mount skin preparations immunostained for K8 (A and B, green) and TrkB (A' and B', red) show presence of touch domes 6 mo after tamoxifen (TMX) treatment in *Atoh1^{CreER-T2/+}; ROSA⁺* and *Atoh1^{CreER-T2/+}; ROSA^{DTA}* mice. (C) Mean numbers of touch domes in *Atoh1^{CreER-T2/+}; ROSA⁺* and *Atoh1^{CreER-T2/+}; ROSA^{DTA}* mice per 1cm² of body skin (n=2 mice/genotype/time point, two- way ANOVA). (D) Mean numbers of K8+ cells per touch dome were markedly decreased in *Atoh1^{CreER-T2/+}; ROSA^{DTA}* mice relative to *Atoh1^{CreER-T2/+}; ROSA⁺* littermate controls at all time points (n=2 mice/genotype/time point, two-way ANOVA). (E and F) Cryosectioned hairy skin immunostained for K8 and K17 reveals touch domes that lack Merkel cells in

Atoh1^{CreER-T2/+};*ROSA*^{DTA} mice. (G–H’’) Stitched images of cryosectioned whisker follicles immunostained for K8. (I) Mean numbers of K8+ cells per whisker follicle were decreased in *Atoh1*^{CreER-T2/+};*ROSA*^{DTA} mice relative to *Atoh1*^{CreER-T2/+};*ROSA*⁺ littermate controls at all time points (n=2 mice/genotype/time point, two-way ANOVA). **, P < 0.01; ***, P < 0.001. Error bars show SEM. Scale bars: 50µm.

3.3.2 *Atoh1*+ cells do not express K14, but a subset does express K17

Though embryonically derived from the *K14* lineage, mature Merkel cells do not express K14 (Moll et al., 1993; Rose et al., 2009). However, other investigators reported that Merkel cells were maintained by K14+ progenitors (Akazawa et al., 1995; Ben-Arie et al., 1996; Van Keymeulen et al., 2009). To determine whether *Atoh1*+ Merkel cells and potential Merkel cell progenitors retained *K14* expression, K14 immunostaining was performed in hairy skin and whisker follicles from tamoxifen-treated adult *Atoh1*^{CreER-T2/+};*ROSA*^{tdTomato} mice. All *Atoh1*+ cells were K14 negative (>250 GFP+ or tdTomato+ hairy skin and whisker follicle cells/mouse, n=2 mice; Figure 9A–A’’’). We postulated that K14 could be expressed in the *Atoh1* lineage, but at low protein levels undetectable by immunostaining. To examine this possibility, we conditionally fate mapped the *K14* lineage in adulthood by administering high-dose tamoxifen for a consecutive 3 days to P28 *K14*^{CreER/+};*ROSA*^{tdTomato} mice and then harvested tissue 1 and 4 weeks later. Immunostaining for K8 revealed that all K8+ cells were tdTomato negative (>250 hairy skin and >500 whisker follicle K8+ cells counted/mouse, n=2 mice/age; Figure 9B–B’’). Collectively with our data from embryonic *K14*^{CreER/+};*ROSA*^{tdTomato} (Figure 5), *Atoh1*^{CreER-T2/}, this indicates that *Atoh1*+ cells do not express K14 at embryonic or postnatal ages.

A second study suggested that bipotential K17+ progenitor cells located in touch domes give rise to touch dome keratinocytes and Merkel cells (Ben-Arie et al., 2000; Doucet et al., 2013). To determine whether K17 was expressed by cells committed to the Merkel cell lineage, we co-immunostained adult C57BL/6J mouse hairy skin for K8 and K17. We found that 28.4 ± 6.0% and 9.2 ± 2.6% of K8+ cells in touch domes and whisker follicles, respectively (>250 hairy skin and >500 whisker follicle K8+ cells counted/mouse, n=2–3 mice), were also K17+ (Figure 10A–B). Because all K8+ cells are also *Atoh1*+ (Figure 10C–D’’), this demonstrates that some

Atoh1⁺ cells co-express K17, though it does not prove that potential *Atoh1*⁺ progenitors are K17⁺.

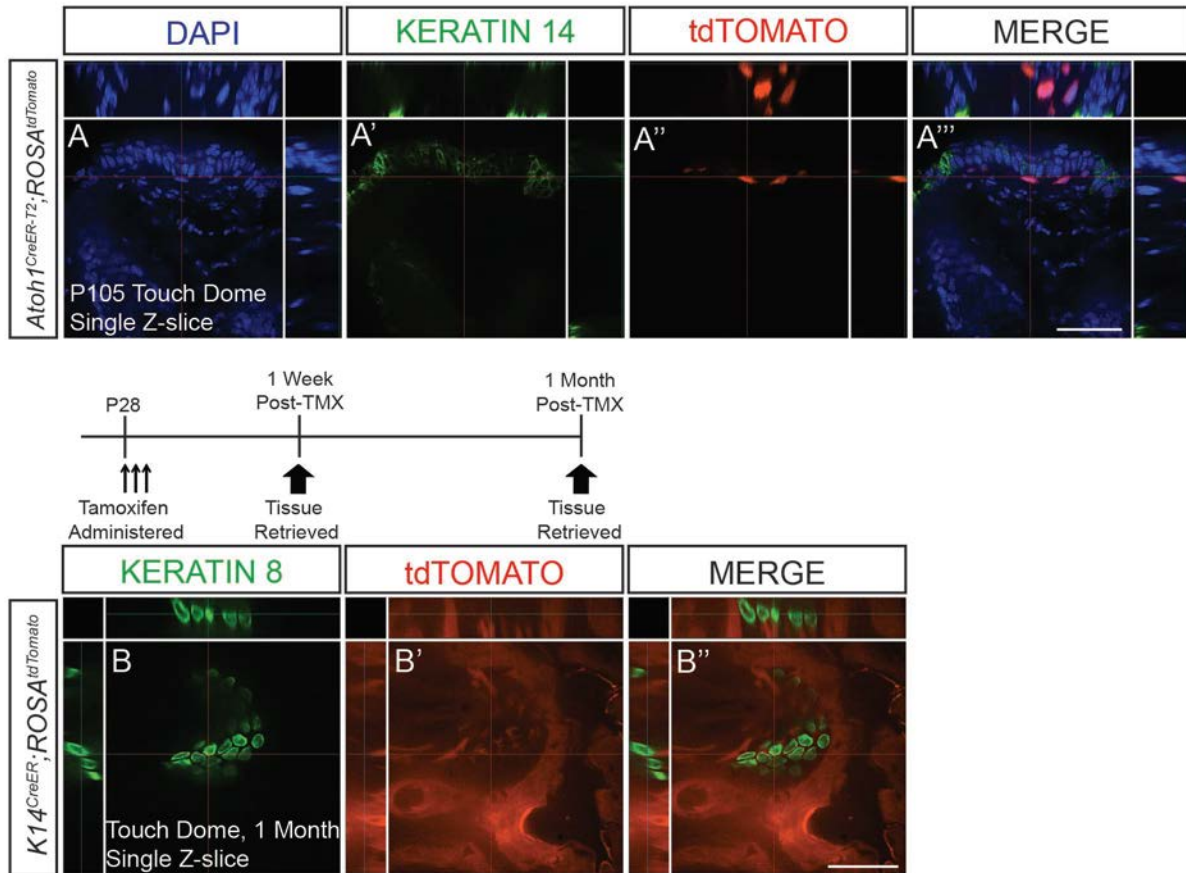


Figure 9. The adult *Atoh1*+ lineage does not express K14 (A-A'') All tdTomato+ cells in adult *Atoh1^{CreER-T2/+};ROSA^{tdTomato}* mouse touch domes are K14-. (B-B'') Single confocal z-slice image of a whole-mount touch dome preparation from a P60 *K14^{CreER};ROSA^{tdTomato}* mouse given tamoxifen (TMX) at P28 and immunostained for K8 (C). There is no signal co-localization. Scale bars: 50 μ m.

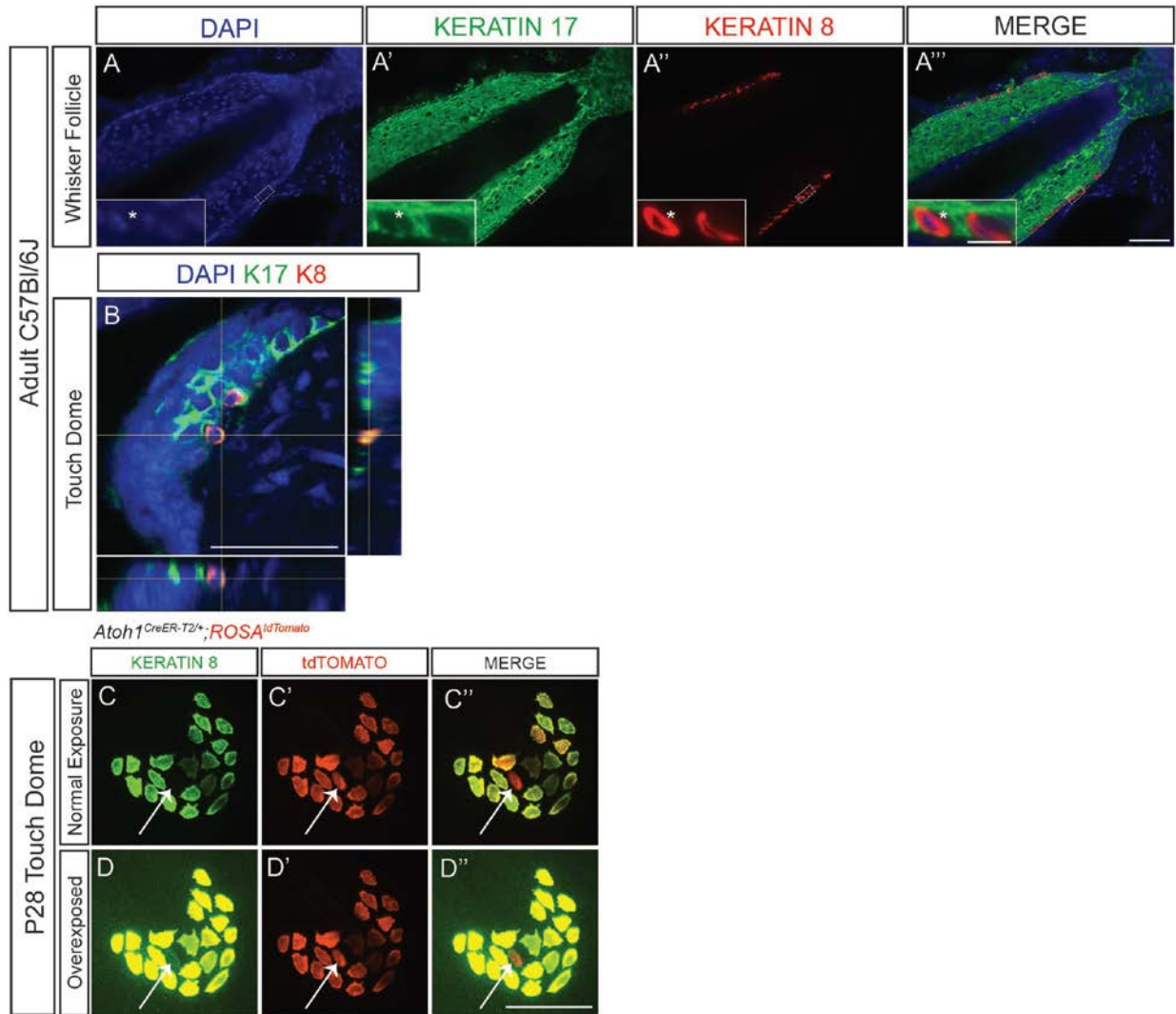


Figure 10. All *Atoh1*⁺ cells are K8⁺ and a subset of K8⁺ cells co-express K17 (A-A''')

Whisker follicles from an adult C57Bl/6J mouse immunostained for K17 (A', green) and K8 (A'', red). Insets show one K8⁺K17⁺ cell (as indicated by asterisks). (B) Single confocal z-slice images of touch dome from back skin of an adult C57Bl/6J mouse immunostained for K8 and K17 showing signal co-localization. (C-D''') Hairy skin from a tamoxifen-treated P28 *Atoh1^{CreER-T2/+}; ROSA^{tdTomato}* mouse immunostained for K8 (n=3 mice). tdTomato⁺ cell (arrows) that appears to be K8⁻ at exposure times that identify other K8⁺ cells (C-C'') in fact expresses low levels of K8 (D-D''). Scale bars: (main images) 50µm; (insets) 10µm.

3.3.3 *Atoh1*+ cells rarely express proliferative markers in adulthood

We next examined 3 (n=4) and 6 (n=2) mo old adult *Atoh1*^{GFP} mice to determine whether GFP+Ki67+ cells were present and whether the numbers of these cells changed during the natural hair cycle. Three of these mice were in the growth phase of the hair cycle (anagen), and three were in the resting phase (telogen). GFP+ cells in the body skin (250–500/mouse, ~1,700 GFP+ cells total) and whisker follicles (1,500–5,000/mouse, ~11,000 GFP+ cells total) were analyzed. We found one GFP+/Ki67+ cell in the body skin of a 3 mo old mouse whose skin was in telogen (Figure 11A-A'') and one GFP+/Ki67+ cell in the whisker follicle of a 6 mo old mouse (Figure 11B-B''). No K8+EdU+ cells were found in the whisker follicles or back skin of P19–P24 mice (n=3; >400 K8+ cells/mouse/region) after administration of EdU (50mg/kg) and tissue harvest 4 hrs later. Together with our previous data, this suggests that *Atoh1*+ progenitors are either very infrequently active or that *Atoh1*+ progenitors do not maintain the Merkel cell population.

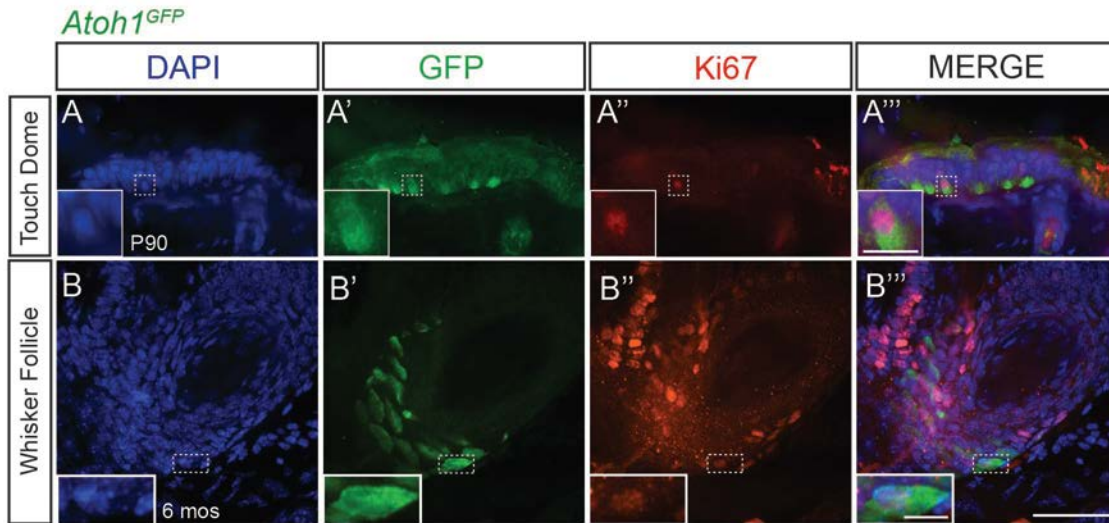


Figure 11. Very few *Atoh1*⁺ cells express mitotic markers in adulthood (A-B''') Adult *Atoh1*^{GFP} touch dome (A-A''') and whisker follicles (B-B''') immunostained for GFP (A', B', green) and Ki67 (A'', B'', red). Insets show GFP+Ki67+ cells. These were the only two K8+Ki67+ cells found in >11,000 K8+ cells examined. Scale bars: (main images) 50µm; (insets) 10µm.

3.3.4 Merkel cell number decreases over early postnatal life and does not oscillate with the adult hair cycle

Previous studies had reported a correlation of touch dome Merkel cell number with the hair cycle, showing higher Merkel cell numbers while during hair follicle growth (anagen) followed by decreases during hair follicle regression (catagen). However, those studies were performed on sectioned mouse skin after hair cycle induction or on epidermal sheets of rat skin, both techniques that can limit visualization of entire touch domes. To conclusively determine if there was a relationship between touch dome Merkel cell number and hair follicle stage in mouse skin, we immunostained for the Merkel cell marker K8 in whole-mount back and belly skin from female B6 mice during hair follicle genesis (E17- P21), the first hair cycle, (P21-P52), and after the second and third hair cycles (12 and 20 weeks of age, respectively) (>20 touch domes/mouse, n=3-5 mice/age; Figure 12). Tissue was also retrieved, sectioned, and H&E stained to validate hair cycle stage (Muller-Rover et al., 2001; Figure 13). The average number of K8+ cells per touch dome increased through embryogenesis to peak at 27.0 ± 2.2 in back and 35.0 ± 2.7 in belly

skin at E18.5, after which it decreased during early postnatal life until P14. From P14 until 20 weeks of age, there was no difference between ages in average number of K8+ cells per touch dome, as the numbers remained stable with a slow decrease over time (One Way ANOVA, Back Skin $F=2.78$, $p=0.02$, post-hoc Scheffe test- no significant difference between ages; Belly Skin $F=1.67$, $P=0.14$).

We also tested the influence of hair cycle induction on touch dome Merkel cell number. P22 female B6 mice were anesthetized with ketamine/xylene and the right half of their back skin shaved and depilated with surgicream to induce hair cycle initiation. The left half of their back skin was left unmanipulated. Skin from these mice was retrieved 0, 3, 5, 12, and 18 days post-hair cycle induction, hemotoxylin & eosin stained for hair cycle stage characterization and whole mount immunostained for K8 to quantify touch dome Merkel cell number ($n=3$ mice/time point). Hair cycle induction with this method was confirmed, as skin that had been shaved and depilated entered into anagen and cycled into catagen sooner than unmanipulated skin on the same animal (Figure 14). However, no difference in K8+ Merkel cell number within touch domes was seen at any time point between induced and naturally cycling back skin (Figure 15). Together this indicates a lack of correlation between touch dome Merkel cell number and the growth and regression phases of the natural or induced hair cycle. It also suggests that either there is no hair cycle stage at which Merkel cell progenitors are preferentially active or that Merkel cells are not replaced with great frequency in mature animals during normal tissue homeostasis.

B6 Female Mice - Natural Hair Cycle

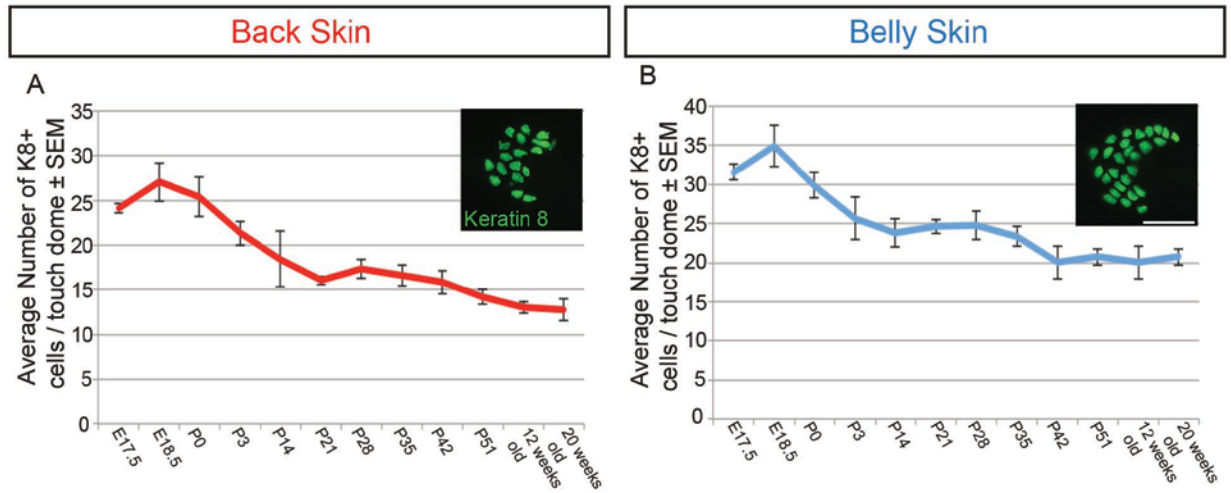


Figure 12. Mean touch dome K8+ cell number peaks at E18.5 and stays constant during adulthood Average K8+ cell number per touch dome was quantified (n=3-5 mice/age) and plotted over time in back (A) and belly (B) skin. SEMs are shown by bars. Scale bar: 50µm.

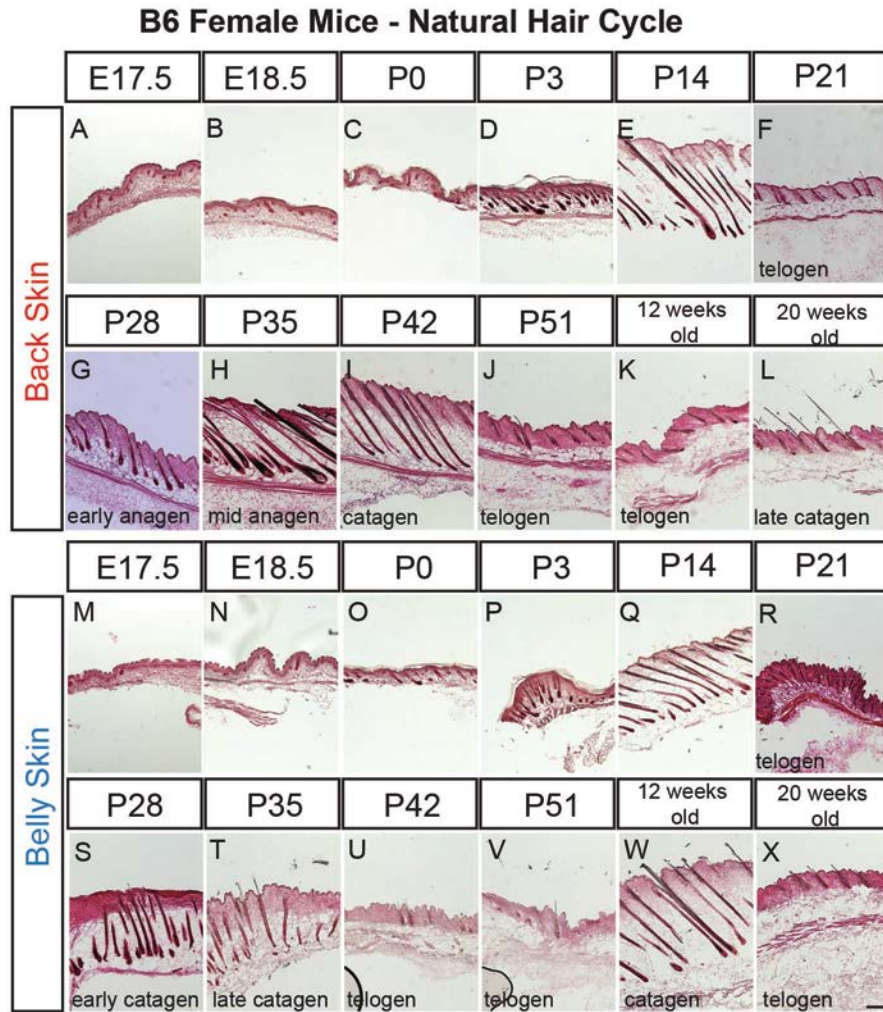


Figure 13. Confirmation of hair cycle progression in B6 mice. Cryosectioned back (A-L) and belly (M-X) skin in naturally cycling female B6 mice H&E stained and hair cycle stage categorized at E17.5, E18.5, P0, P3, P14, P21, P28, P35, P42, P51, 12 weeks of age, and 20 weeks of age. Scale bar: 50 μ m.

B6 Female Mice - Induced Hair Cycle

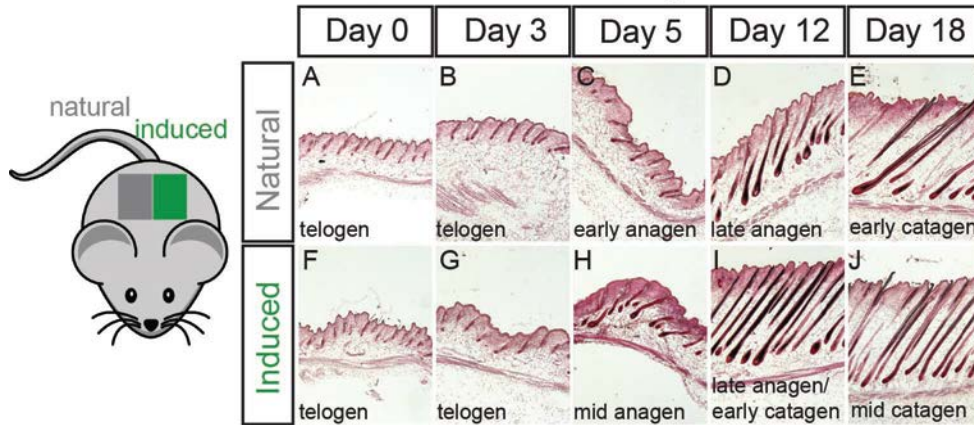


Figure 14. Hair cycle staging after induction in B6 female mice. Cryosectioned back skin during normal progression (A-E) or after hair cycle induction (F-J) H&E stained and hair cycle stage categorized for mice at 0, 3, 5, 12, and 18 days post-induction. Scale bar: 50 μ m.

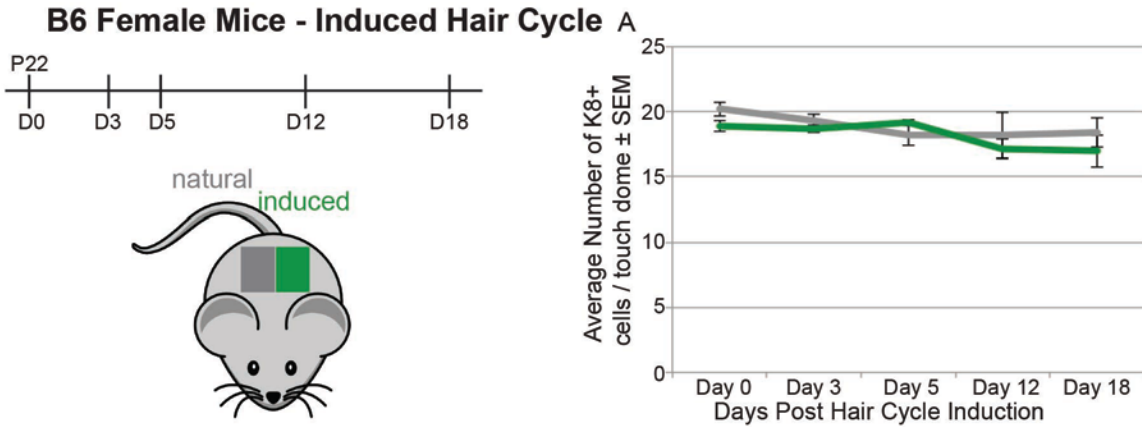


Figure 15. No change in average K8+ cell number after hair cycle induction. Average K8+ cell number per touch dome was quantified (n=3 mice/time point) and plotted over time in back skin that progressed naturally (gray line) or was induced (green line). SEMs are shown by bars.

3.3.5 *K18*+ Merkel cells of murine whisker follicles are not replaced

Another piece of evidence indicating Merkel cell turnover was fate-mapping performed in *K18^{CreER};ROSA^{YFP}* mice that demonstrated loss of labeled cells in the whisker over three weeks of pulse-chase (Van Keymeulen et al., 2009). We repeated this experiment with *K18^{CreER}* mice crossed to both a *ROSA^{YFP}* and a more sensitive *ROSA^{tdTomato}* reporter. Tamoxifen was administered as a single dose of 5mg/mouse and tissue retrieved 1, 3, or 9 weeks later (Figure 16). One week post-tamoxifen administration the recombination of K8+ Merkel cells was 5.7±1.3% YFP+K8+/K8+ and 48.4±1.3% tdTomato+K8+/K8+ (>500 K8+ cells/mouse; n=2 mice; Figure 16). This proportion was maintained at 3 (5.9±2.4% YFP+K8+/K8+ and 43.0±4.9% tdTomato+K8+/K8+) and 9 (6.1±1.2% YFP+K8+/K8+ and 35.1±0.7% tdTomato+K8+/K8+) weeks post-tamoxifen (>500 K8+ cells/mouse; n=2-3 mice/time point; YFP: F=0.01, p=0.99; tdTomato: F=3.23, p=0.1255 Figure 16). We were therefore unable to independently provide evidence of Merkel cell turnover in the whisker follicles with this model. We were also unable to perform this experiment in body skin, as this mouse demonstrated robust recombination in K18-cells of the skin. This was also seen in the whisker follicles, but more sparsely.

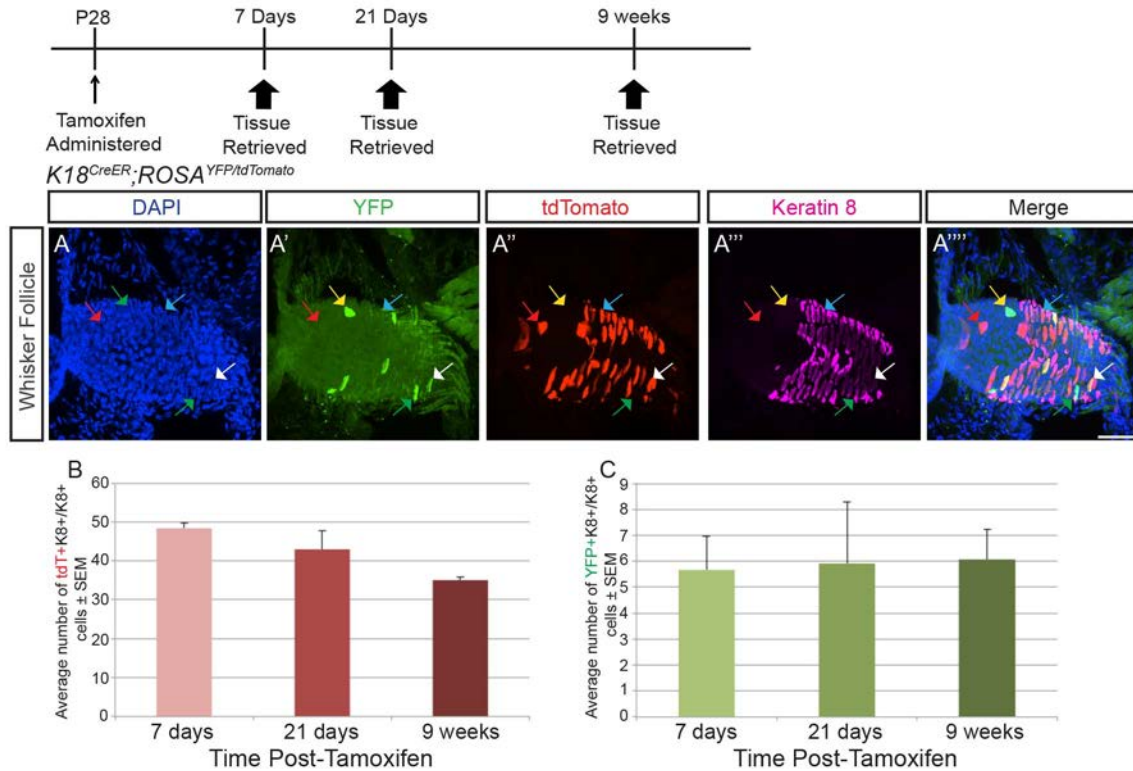


Figure 16. *K18*⁺ cells are not replaced by *K18*⁻ cells in the murine whisker follicle

K18^{CreER};ROSA^{YFP/tdTomato} mice were generated and administered tamoxifen at P28. (A-A''') Whisker follicles 9 weeks after tamoxifen administration immunostained for, YFP (A', green), K8 (A''', magenta), endogenous tdTomato (A'', red), and counter stained with DAPI (A, blue). Green arrow indicates a YFP+K8+ cell; blue arrow indicates a tdTomato+K8+ cell; white arrow indicates a tdTomato+YFP+K8+ cell; red arrow indicates a tdTomato+K8- cell; yellow arrow indicates a YFP+K8- cell. (B) Quantification of the average percent of tdTomato+K8+/K8+ cells (\pm SEM). (C) Quantification of the average percent of YFP+K8+/K8+ cells (\pm SEM). Scale bar: 50 μ m.

3.3.6 Embryonic Merkel cells persist into late adulthood and nascent Merkel cells are rarely made during adult skin homeostasis

We employed a birth-dating approach with the modified nucleoside 5-ethynyl-2'-deoxyuridine (EdU) to estimate the lifespan of embryonic-born Merkel cells. This nucleoside is incorporated into cells during DNA synthesis and is retained for the lifespan of the cell. Cells lose EdU

positivity either by cell death or signal dilution after multiple cell divisions. We administered 10mg/kg EdU by intraperitoneal injection once daily to B6 pregnant females at E14.5, E15.5, and E16.5, the ages at which most Merkel cells are generated. EdU given at higher concentrations (50mg/kg) resulted in a greater proportion of K8+ cells incorporating EdU but led to animal mortality (data not shown). Therefore, this 10mg/kg once-daily dose is not intended to label all Merkel cells born between E14.5-E16.5, but instead to label a subset whose proportion can be followed over time. Mice were retrieved at P0 and 9 mo of age to determine the proportion of K8+ cells that initially incorporated EdU (P0) and persisted into maturity (9 mo) (n=4 mice/age). At P0 42.3±4.9 %, 42.2±0.6 %, and 46.3±3.1 % of K8+ cells had incorporated EdU in the body skin, whisker follicles, and forepaws, respectively (Figure 17A-A''', C-C''', E-E'''). In the whisker follicles of 9 mo old animals this proportion was maintained (42.5±4.7% of K8+EdU+/K8+ cells, P=0.95 *t*-test). The percentage of EdU+K8+ cells in body skin and glabrous skin of the paw decreased slightly at 9 mo of age, though importantly a large proportion of K8+ cells retained EdU incorporation (21.1±3.1% and 20.7±3.2% of K8+EdU+/K8+ cells, respectively). This indicates a lack of complete Merkel cell turnover in these regions.

To determine if new Merkel cells were formed in adulthood in body skin, whisker follicles, and/or glabrous paw skin, we performed a more chronic paradigm of EdU exposure by administering 0.2mg/mL EdU in the drinking water of mice beginning at P21 and extending for 5 weeks, at which time tissue was retrieved and processed for K8 and EdU (Figure 18; n=3 mice). Consistent with the lack of K8+ turnover in the whisker follicles suggested in our prior experiment, no K8+ cells were found to have incorporated EdU in the whisker follicles, indicating a lack of Merkel cell generation during this time (Figure 18B-B'''; >250 K8+ cells/mouse; n=3 mice). In body skin and glabrous paw skin only a very small proportion of K8+ cells were EdU+ after 5 weeks of EdU exposure (1.8±0.5% and 1.1±0.2%, respectively, >100 K8+ cells/mouse, n=3 mice, Figure 18A-A'''). This small population of nascent Merkel cells indicates that while new Merkel cells are generated as part of normal skin homeostasis, it is at an incredibly slow rate.

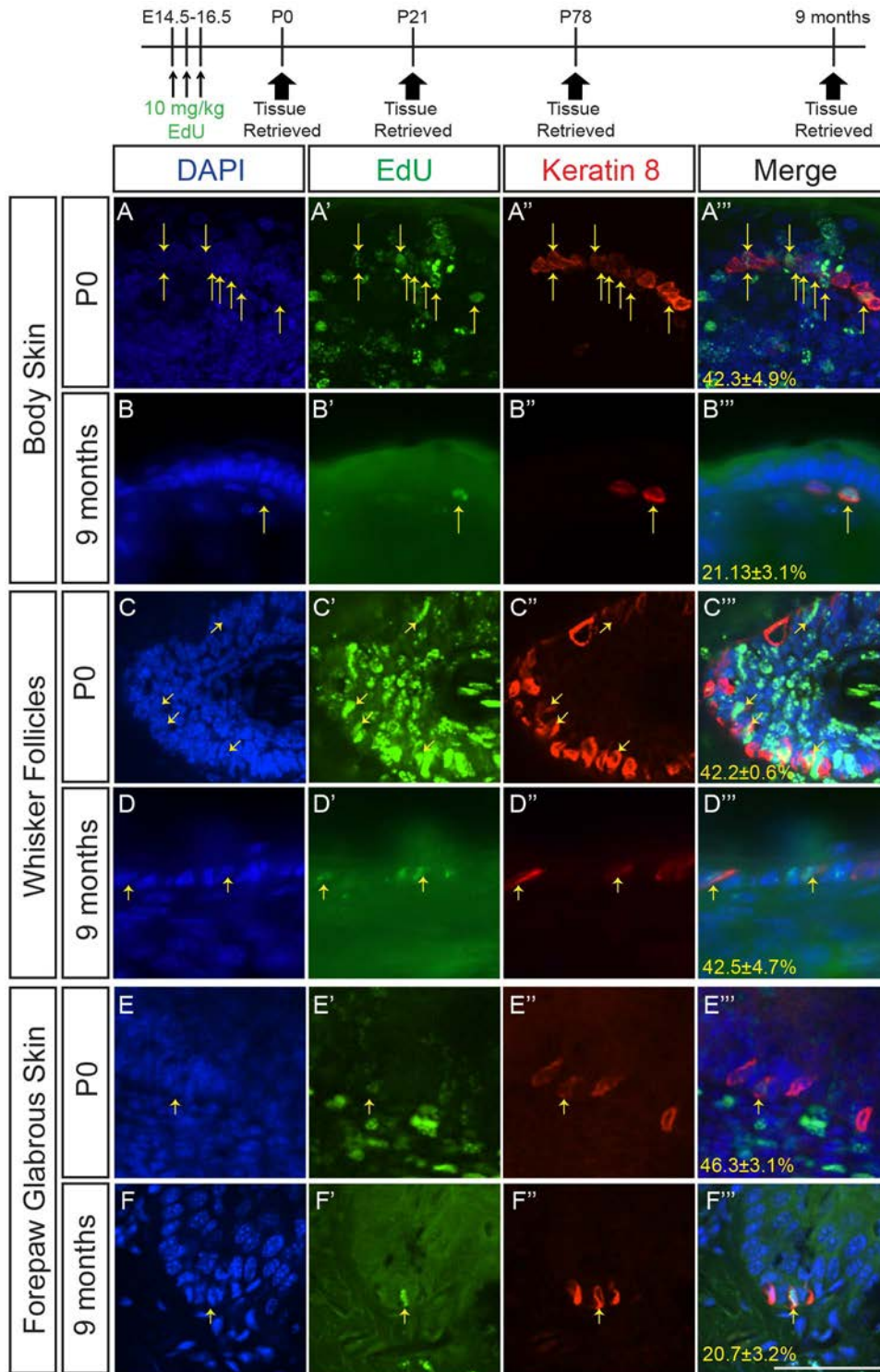


Figure 17. K8⁺ cells born in embryogenesis persist to 9 months of age. Sectioned body skin (A-B'''), whisker follicles (C-D'''), and glabrous forepaw skin (E-F''') from B6 mice that received EdU at E14.5, 15.5, and 16.5 and were retrieved at P0 (A-A''', C-C''', E-E''') or 9 months of age (B-B''', D-D''', F-F''') processed for EdU detection (A', B', C', D', E', F',

green) and K8 immunostaining (A'', B'', C'', D'', E'', F'', red). Yellow arrows indicate EdU+K8+ cells; red arrows indicate EdU-K8+ cells. The proportion of K8+EdU+/K8+ cells within each tissue at each age is given in the bottom left hand corner of all merge panels (n=4/age). Scale bar: 50µm.

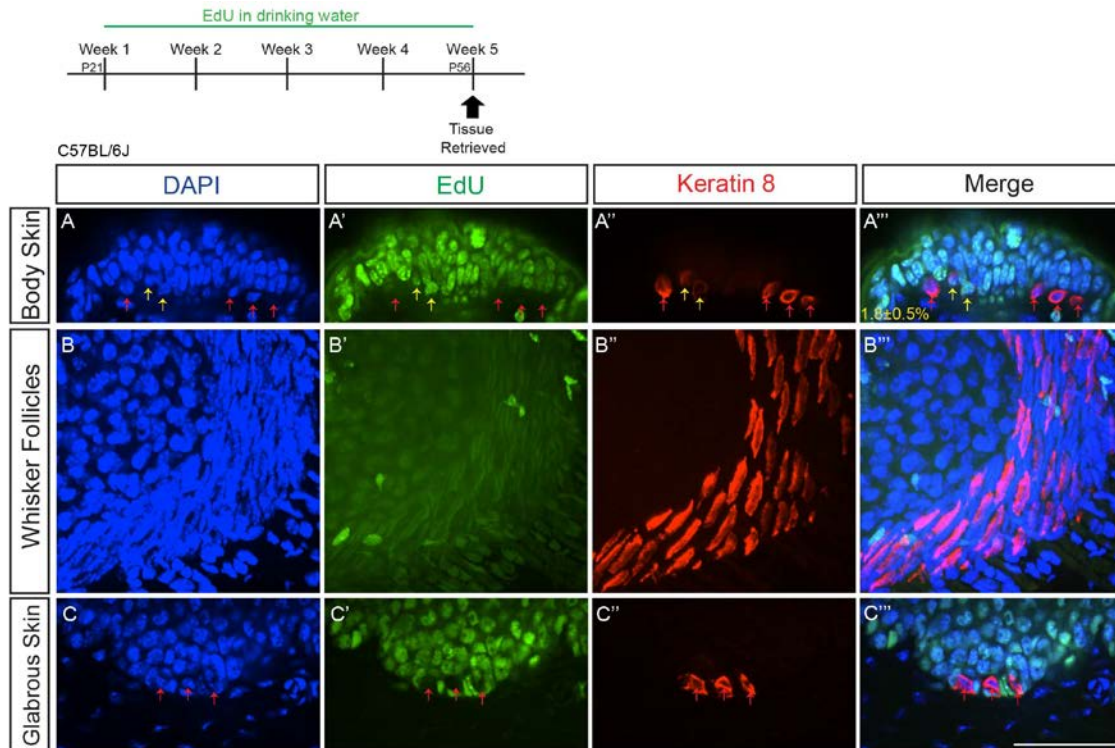


Figure 18. Very few nascent Merkel cells are made during skin homeostasis Sectioned body skin (A-A'''), whisker follicles (B-B''') and glabrous forepaw skin (C-C''') from P56 B6 mice that received 0.2mg/mL EdU in their drinking water for five weeks processed for EdU detection (A', B', C', green) and K8 immunostaining (A'', B'', C'', red). Yellow arrows indicate EdU+K8+ cells; red arrows indicate EdU-K8+ cells. Scale bar: 50µm.

3.3.7 *In vivo* visualization of touch dome maintenance

To visualize the dynamics of touch dome homeostasis we devised a strategy to repeatedly image the same touch domes over many months in living adult animals. Young *Atoh1^{GFP}* mice were anesthetized with ketamine/xylazine (100mg/kg and 10mg/kg) and their belly skin shaved with a straight razor to permit visualization of the skin. A square was drawn with India Ink on the belly

skin to provide landmarks allowing us to return to the same touch domes over time (Figure 19A). Mice were placed on a specially-designed platform with their belly skin over a cover slip and imaged on an inverted spinning disc confocal microscope. Z-stacks of individual touch domes were taken and mapped within the previously drawn square. Mice were repeatedly shaved and imaged once a week for 13-21 weeks, at which time they were sacrificed, tissue retrieved and immunostained for GFP and NF200 (week 13) or GFP and K8 (week 21).

Between 7 and 12 touch domes were mapped per mouse (n=4 mice, n=37 total touch domes, n=2,530 total GFP+ cells) and individual Merkel cells traced within these touch domes from week to week (**Figure 20A-G'**). We found that during the first 8 weeks of imaging (the previously estimated Merkel cell lifespan) $64.3 \pm 3.6\%$ of Merkel cells that were found at the first week of imaging (original Merkel cells) remained stable. This demonstrates that mature Merkel cells persist for at least two months and the entire cell population does not turn over (**Figure 20H**). However, we were surprised to observe the generation of many new GFP+ cells during this time. While overall the average number of Merkel cells per touch dome stayed constant (**Figure 19B, C, D, E; Figure 20I**), an average of 3.14 ± 0.6 nascent cells were added per touch dome each week (**Figure 20J**). No GFP+ cells were observed outside of touch domes. Cells arose consistently throughout the imaging period, with no clear temporal correlation with age or hair cycle stage as judged by skin appearance (**Figure 19B', C', D', E'**). These nascent cells had a lower probability of survival relative to original cells that had been imaged from the very first week prior to skin shaving (median survival, original: 84 days, nascent: 21 days)(**Figure 20K, Figure 21**). About half of all nascent cells were gone within the first two weeks after being born, but the ones that survived after 2 weeks appeared to have an equal likelihood of survival relative to original cells (**Figure 20K**). At the time of tissue retrieval, fewer nascent cells were innervated by NF200+ nerve endings than original cells (75.9% vs 91.0%, respectively; **Figure 20L**). Within those nascent cells, there was a trend for nascent cells that had persisted for >2 weeks to have a higher likelihood of being innervated than those that had been arisen <2 weeks prior ($84.9 \pm 5.2\%$ vs $71.1 \pm 7.5\%$, respectively, $p=0.11$). Together this suggests a positive correlation of cell survival with innervation status. After 21 weeks of imaging 100% and 92% of original and nascent GFP+ cells were K8+, respectively, confirming Merkel cell identity. This *in vivo* analysis illustrates the persistence of Merkel cells during adulthood.

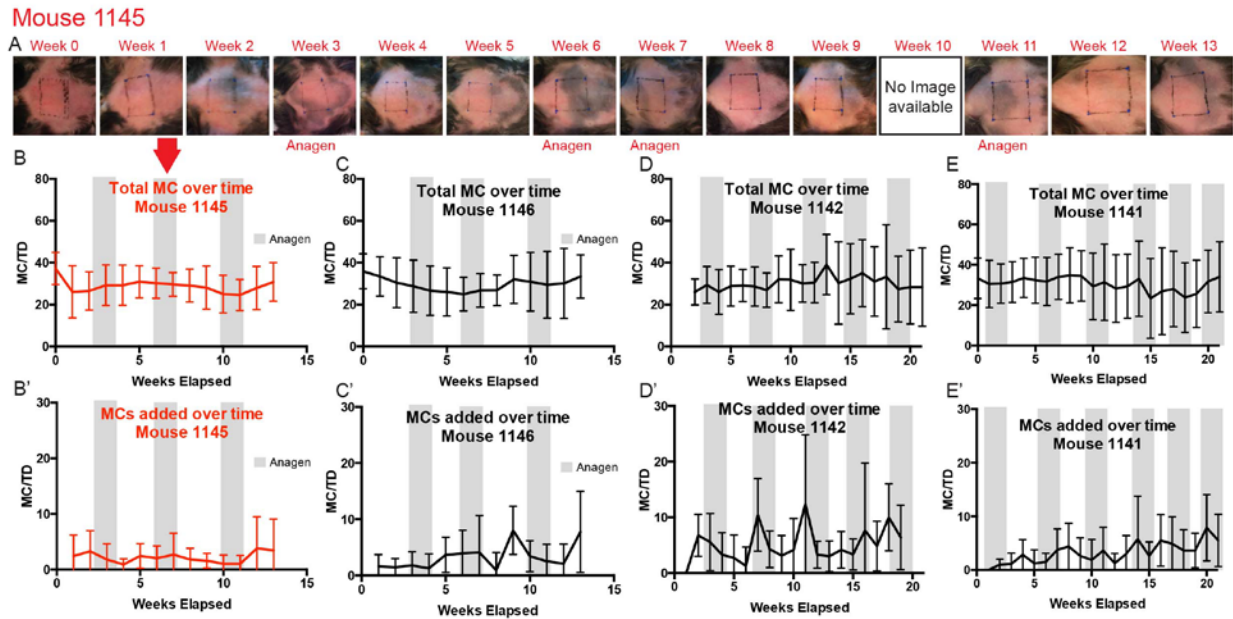


Figure 19. Average number of Merkel cells in touch domes for individual mice (A) Image of belly skin for mouse #1145 prior to imaging each week for 13 weeks showing hair regrowth during anagen. (B, C, D, E) Average number (\pm SEM) of Merkel cells per touch dome through the imaging period. Gray bars denote anagen. Red graph is the same mouse illustrated in panel A. (B', C', D', E') Average number (\pm SEM) of nascent Merkel cells added to touch domes over time.

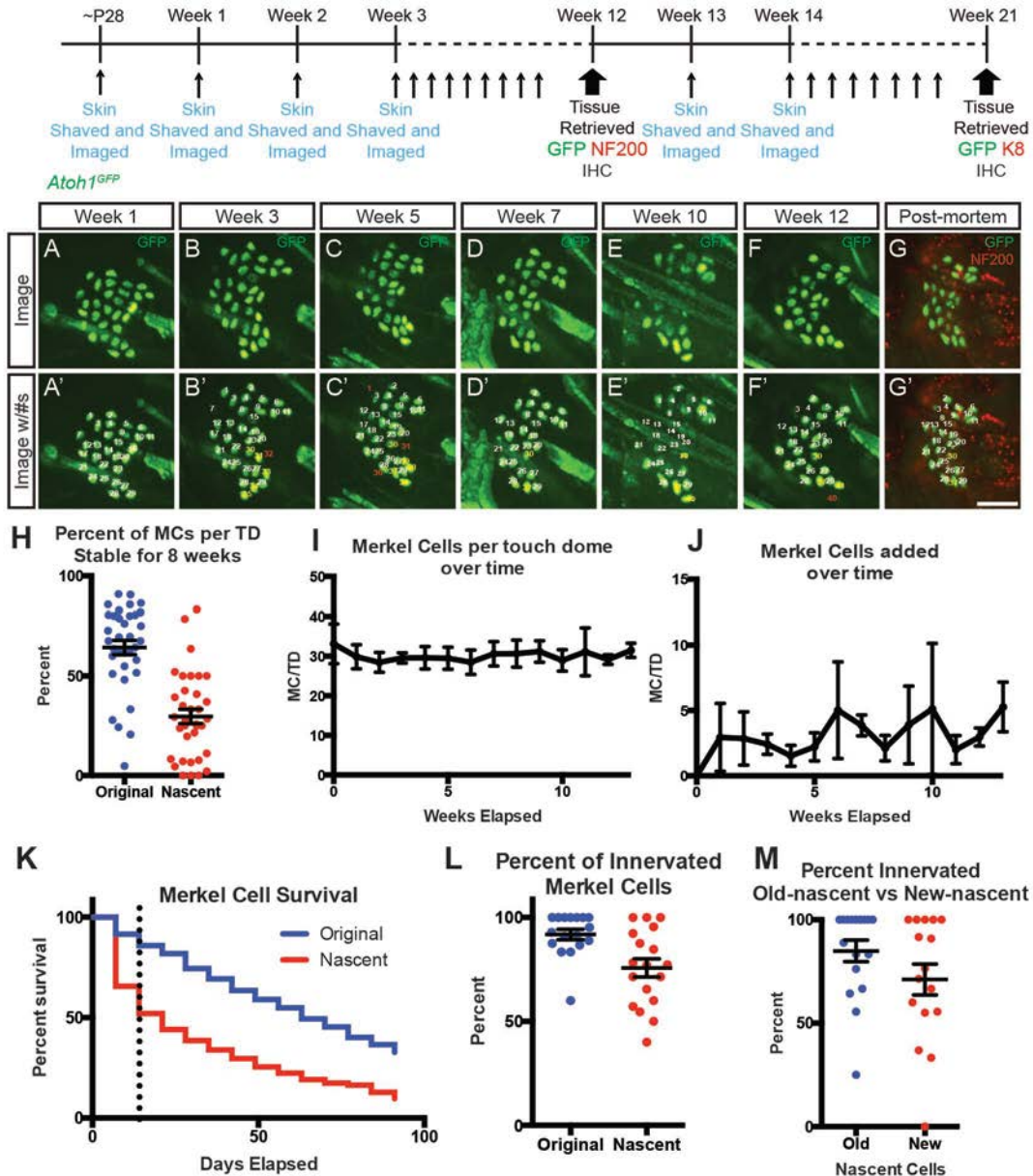


Figure 20. *In vivo* imaging of touch domes in *Atoh1^{GFP}* mice over months Adolescent *Atoh1^{GFP}* mice were shaved and imaged once weekly to enable tracking of individual touch domes over time. (A-G') *Atoh1^{GFP}*+ cells of the same touch dome at Week 1 (A, A'), Week 3 (B, B'), Week 5 (C, C'), Week 7 (D, D'), Week 10 (E, E'), Week 12 (F, F') and post-mortem (G, G') immunostained for GFP (green) and NF200 (red). Panels A', B', C', D', E', F', and G' indicate individual cells tracked over these weeks. (H) The percent of stable original *Atoh1^{GFP}*+ cells (blue) and stable nascent cells (red) over 8 weeks of imaging. (I) The average number (\pm SEM) of GFP+ cells per touch dome over 13 weeks of imaging. (J) The average number (\pm SEM) of GFP+ cells added per touch dome over the 13 week imaging period. (K) Average

survival curves for original (blue) and nascent (red) *Atoh1*^{GFP}+ cells. (L) Percent of original (blue) and nascent (red) Atoh1-GFP+ cells contacted by NF200+ nerve terminals at the end of the imaging period. (M) Percent of >2 week old nascent (old nascent) and <2 week old nascent (new nascent) cells contacted by NF200+ nerve terminals at the end of the imaging period. Scale bar: 60µm.

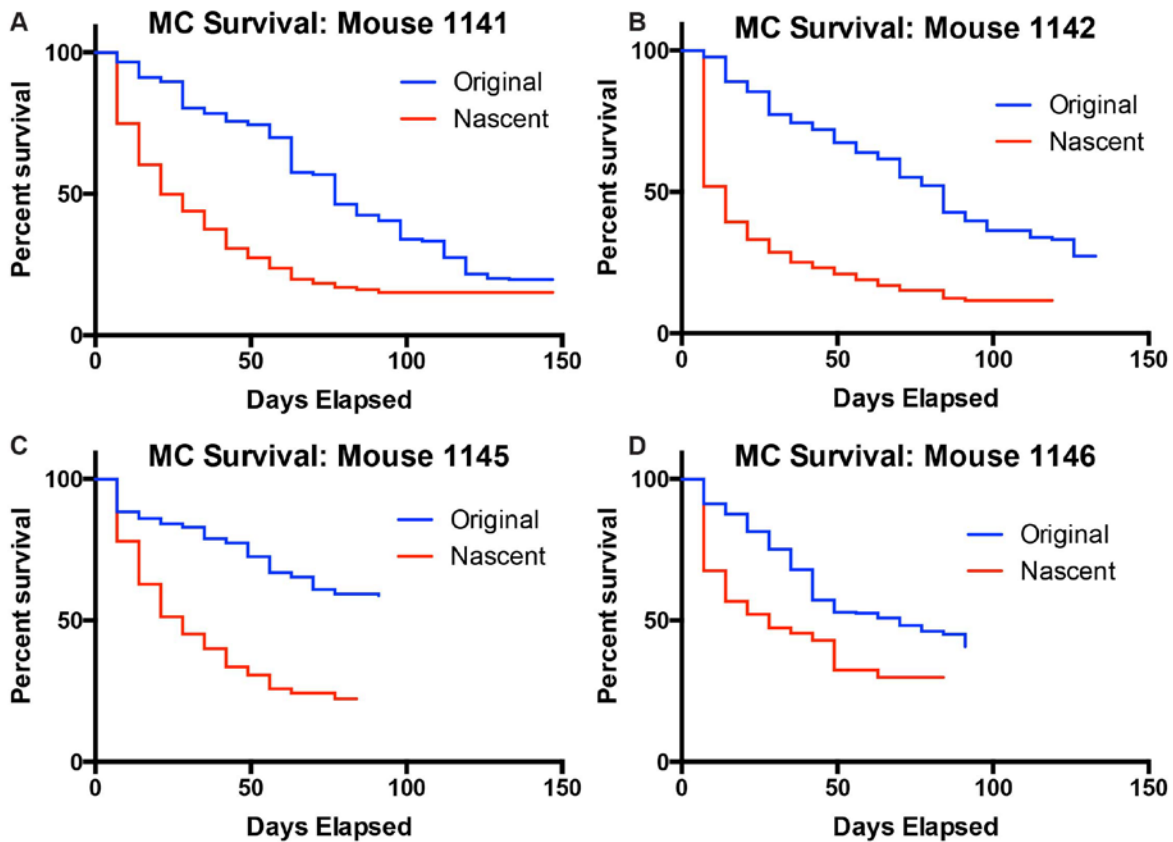


Figure 21. Survival curves for original and nascent Merkel cells in *Atoh1*^{GFP} mice.

Individual survival curves for original (blue) and nascent (red) Atoh1-GFP+ cells over the imaging period. (A, B) Mice #1141 and #1142 were imaged for 21 weeks. (C, D) Mice #1145 and #1146 were imaged for 13 weeks.

3.3.8 Repeated shaving induces production of new Merkel cells

The high number of nascent Merkel cells observed in imaged *Atoh1^{GFP}* mice was not easily accounted for in our EdU birth-dating studies. We were concerned that the repeated shaving performed for touch dome visualization had induced non-homeostatic skin conditions not representative of typical touch dome maintenance. To test if new Merkel cells arose at the same rate in shaved and unmanipulated skin, we administered EdU in the drinking water (0.2mg/mL) to B6 female mice that were shaved once weekly, mirroring the protocol to expose skin for live imaging (Figure 22). Beginning at P21, a section of back and belly skin was shaved with a straight razor once weekly for four weeks, followed by tissue retrieval and processing for EdU and K8 one week after the last skin shave (P56). This length of EdU exposure is also identical to that of the unmanipulated B6 mice in Figure 18. Shaved back and belly skin had $7.75 \pm 2.7\%$ of K8+ cells present that had incorporated EdU, a much higher proportion than in unmanipulated skin (Figure 22B-D; $p=0.0325$; $n=2$ mice; >150 K8+ cells/mouse). Therefore, shaving of murine body skin stimulated an increased production of touch dome-associated Merkel cells relative to the very low rate during typical skin homeostasis. Our *in vivo* imaging studies of *Atoh1^{GFP}* mice therefore illustrate touch dome maintenance in a non-homeostatic environment and serendipitously exposed a mechanism driving Merkel cell production in adult mice.

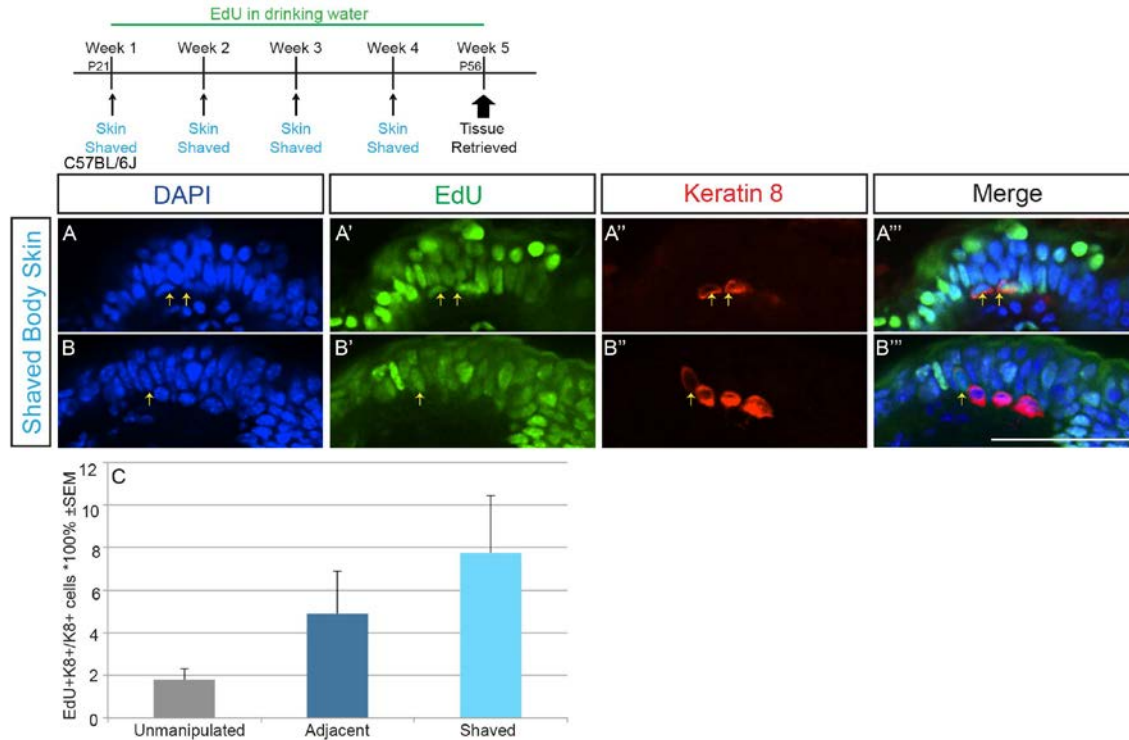


Figure 22. Shaving stimulates additional Merkel cell production Single z-slice confocal images of sectioned body skin from B6 mice that were shaved once weekly or left unmanipulated while receiving EdU for five weeks processed for EdU detection (A', B', green) and K8 immunostaining (A'', B'', red). Yellow arrows indicate EdU+K8+ cells. (C) Average percentage of K8+EdU+/K8+ cells per mouse (mean±SEM). Scale bar: 50µm.

3.3.9 Nascent Merkel cells come from neither *Atoh1*+ or *K14*+ progenitors

To determine if these nascent cells came from *Atoh1*+ progenitors, we ablated the *Atoh1*+ population by administering tamoxifen (250mg/kg for 3 consecutive days) to *Atoh1*^{CreER-T2/+};*ROSA*^{DTA} mice as has been done previously (Figure 8). Consistent with our previous experiments, 28 days after tamoxifen administration *Atoh1*^{CreER-T2/+};*ROSA*^{DTA} mice (n=2) had far fewer K8+ cells per TrkB+ touch dome than *Atoh1*^{CreER-T2/+};*ROSA*^{DTA} mice that did not receive tamoxifen (0.42±0.1 vs 16.53±1.5 K8+ cells/TD, respectively; p=0.002 *t*-test; n=2-4 mice/genotype). With this experimental paradigm, there is no recovery of K8+ cell number out to six months post-tamoxifen administration (Figure 8). To test if nascent K8+ cells were derived from an *Atoh1*+ progenitor, or required the presence of mature Merkel cells for their production,

littermates from this cohort of *Atoh1*^{CreER-T2/+};*ROSA*^{DTA} mice treated with tamoxifen (n=3 mice) had their back and belly skin shaved once a week for four weeks (as described above) and skin retrieved one week after the last day of shaving. The average number of K8+ cells per touch dome was significantly elevated relative to mice that were unmanipulated (3.29±0.5 K8+ cells/TD; p=0.015, t-test) (Figure 5A-C). The presence of these new cells indicates that they arose from an *Atoh1*- progenitor, and that signals from original Merkel cells are not necessary for the production of new cells.

We next asked whether these nascent Merkel cells arose from *K14*+ epidermal cells. P28 *K14*^{CreER};*ROSA*^{tdTomato} mice were administered tamoxifen (250mg/kg for 3 consecutive days) and their back and belly skin shaved as described above or left unmanipulated (n=3 mice/treatment). Skin was retrieved, immunostained for K8, and imaged for K8 and tdTomato. Since there was such a high density of tdTomato positivity in the skin with this paradigm, Z-stack confocal images were taken to accurately determine whether tdTomato was in the K8+ cells. If nascent Merkel cells arose from *K14*+ progenitors, we would expect to see K8+tdTomato+ cells. Ten touch domes from each back and belly skin were analyzed per mouse, and no K8+ cells were found to be tdTomato+ in either shaved or unmanipulated skin (Figure 5D-D''; n=6 mice, >400 K8+ cells/mouse). Therefore, no adult-born K8+ cells in adult mice derive from *K14*+ progenitors.

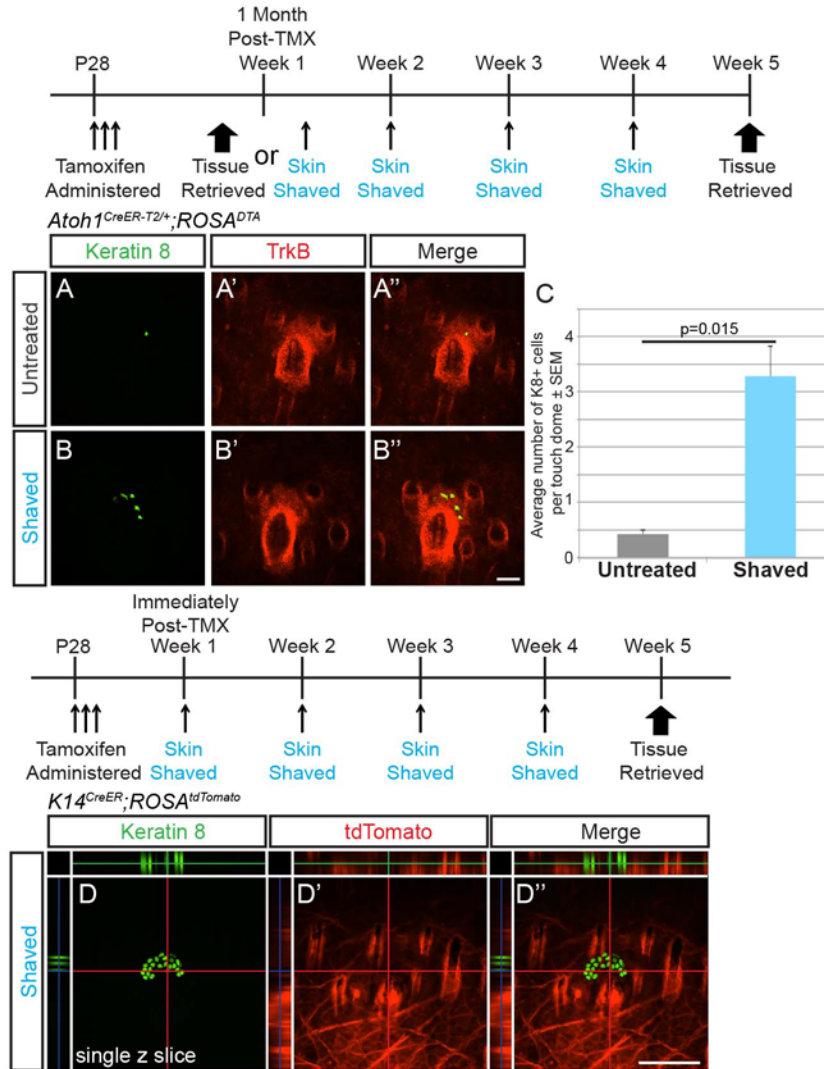


Figure 23. Nascent Merkel cells arise from neither *Atoh1*⁺, or *K14*⁺ progenitors (A-B'')

Confocal z-stack projection images of whole mount body skin from tamoxifen-treated *Atoh1*^{CreER-T2/+};ROSA^{DTA} mice that were shaved once weekly or left unmanipulated immunostained for K8 (A, B, green) and TrkB (A', B', red). (C) Average number of K8+ cells per TrkB+ touch dome quantified (mean±SEM). (D-D'') Single z-slice confocal image of *K14*^{CreER};ROSA^{tdTomato} mouse treated with tamoxifen at P28 and shaved once weekly immunostained for K8 and imaged for endogenous tdTomato. Scale bars: 50µm.

3.3.10 Shaving induces production of nascent Merkel cells adjacent to the manipulated region

To test if the increased production of nascent Merkel cells was limited only to the area of direct manipulation, we counted the number of EdU+K8+/K8+ cells present in body skin adjacent to the shaved area in B6 mice shaved once weekly for 5 weeks (Figure 22). We found that $4.9\pm 2.0\%$ of K8+ cells in adjacent body skin were EdU+, a proportion intermediate to that of fully unmanipulated (1.8 ± 0.5) and directly shaved (7.7 ± 2.7) skin. Furthermore, adjacent body skin in *Atoh1*^{CreER-T2/+};*ROSA*^{DTA} mice shaved once weekly for five weeks also had a recovery of K8+ cells (0.9 ± 0.3) intermediate to that of fully unmanipulated (0.4 ± 0.1) and directly shaved (3.3 ± 0.5) skin (Figure 23). This indicates that the effect of increased Merkel cell production is not limited to the directly shaved area.

3.4 DISCUSSION

Here we present multiple lines of evidence opposing the prevailing view of ‘Merkel cell renewal’ in adult skin homeostasis. We show that touch dome Merkel cell number does not oscillate during natural or induced hair cycles, Merkel cells born in embryogenesis persist out into late adulthood, and new Merkel cells are infrequently generated during normal skin homeostasis. Moreover, we serendipitously revealed that the mechanical influence of repeatedly shaving mouse body skin stimulates an increased production of nascent Merkel cells, an effect not limited to the area of manipulation. These cells arise from a progenitor population that is neither *Atoh1*+ nor *K14*+. This is the also first study to utilize live imaging for repeated visualization of touch domes *in vivo*, providing a clear illustration of their persistence through adulthood.

Merkel cells are first seen in embryonic skin at E14.5, where they are identifiable by *Atoh1*, *Sox2*, and *K8* expression (Lesko et al., 2013). Their number steadily increases through late embryogenesis and peaks prior to birth, with the highest number of K8+ Merkel cells per touch dome occurring at E18.5. This correlates well with our prior analysis of *Atoh1*+*Ki67*+ cells in embryonic body skin in which the highest proportion of proliferative *Atoh1*+ progenitors is at E16.5 and E17.5 (Figure 2). Interestingly, this population of Merkel cells is not fully

maintained, as average touch dome K8+ cell number decreases from P0 through early postnatal life. Therefore, *Atoh1*+ embryonic Merkel cell progenitors appear to produce more K8+ Merkel cells than the touch dome compartment can either sustain or require. It is likely that K8+ cell attrition is a result of Merkel cell competition for nerve-derived trophic support. Consistent with this hypothesis, mice that have increased branching of the SA1 afferent fiber (and therefore likely more opportunity for trophic support) secondary to cutaneous overexpression of neurotrophin-3 (NT3-OE) maintain a higher average number of Merkel cells per touch dome (Albers, 1996; Krimm et al., 2004). This increased number of adult Merkel cells in adult NT3-OE mice is similar to the number of Merkel cells we see in E18.5 wildtype mice. It is therefore likely that these supernumerary Merkel cells are the result of increased Merkel cell survival, and not increased Merkel cell production. Moreover, denervation in both wildtype and NT3-OE mice leads to drastic touch dome Merkel cell loss to the same level, indicating that both normal and supernumerary Merkel cells are dependent on the nerve for survival, not cutaneous NT3 expression (Krimm et al., 2004). Further experiments are necessary to test this hypothesis. This study further defines the period of Merkel cell genesis, though it is still unclear what signaling cascades initiate and halt Merkel cell progenitor proliferation at this time. Epigenetic regulation of *Atoh1* and *Sox2* chromatin structure is likely involved, though additional experiments are required to more comprehensively describe how this is initiated (Bardot et al., 2013; Lesko et al., 2013).

The oscillation of touch dome Merkel cell number with the growth and regression phases of the adult hair cycle has been the strongest pillar of evidence for adult Merkel cell turnover (Moll et al., 1996a; Nakafusa et al., 2006). However, technical limitations likely prevented analysis of full touch domes on whole skin pelts in those previous studies. Moll, 1996 performed their counts on a small amount of serially sectioned mouse skin, a technique that can easily provide a skewed representation of Merkel cell number (Lacour et al., 1991). Nakafusa, 2006 dissected rat skin into epidermal sheets prior to immunostaining for the Merkel cell marker K8. It is possible that the high and low numbers of Merkel cells on the epidermal side from which they counted was an artifact of stronger Merkel cell adhesion to either the epidermis or basement membrane at varying hair cycle stages. Our analysis in five mice per age at multiple stages of the first hair cycle and into late adulthood provides a clear demonstration of average touch dome Merkel cell number remaining constant throughout adulthood. We also saw no change in touch

dome Merkel cell number after the hair cycle had been experimentally induced by chemical depilation, indicating that Merkel cell maintenance is independent of hair follicle stem cell activation. These data are consistent with experiments in hairless mice in which hair follicles form but degenerate during the first hair cycle. In these mice, Merkel cells are maintained throughout adulthood, indicating a lack of dependence on cycling hair follicles for their homeostasis (Xiao et al., 2015).

As a skin cell, Merkel cells are remarkably distinct from surrounding keratinocytes in their expression profiles, innervation by afferent neurons, and extended lifespans (Haeberle et al., 2004; Halata et al., 2003; Moll et al., 1995). The exact amount of time that Merkel cells persist after their genesis and the identity and proliferative capacity of their progenitor has been the subject of multiple independent investigations. Our fate-mapping and conditional cell ablation experiments indicate that these cells either all persist through late adulthood and are rarely replaced, or that *Atoh1*⁺ progenitors are responsible for their adult maintenance (Figure 6, Figure 8). To estimate lifespan, we therefore had to go back to Merkel cell lineage formation in embryogenesis, where we labeled cells with a modified nucleoside as they were born. We found that these label-retaining embryonic-born Merkel cells continued to persist into late adulthood, far beyond the expected lifespan of any other post-mitotic epidermal cell, and therefore many embryonic-born Merkel cells likely exist for the lifetime of the mouse.

To directly visualize Merkel cell homeostasis, we developed a system to image the belly skin of *Atoh1*^{GFP} mice *in vivo* and used it to image the same touch domes from week to week. This powerful approach is the first sequential analysis of Merkel cells in single specific touch domes over multiple months. Consistent with our previous experiments, many *Atoh1*^{GFP}⁺ Merkel cells were present throughout the entire imaging period (13-21 weeks), indicating that these cells lived for much longer than had been previously projected. However, there was a great deal of Merkel cell turnover in this system not reflected in our fate-mapping and birth-dating experiments. We confirmed that this was an effect of repeated skin shaving, as mice whose skin was shaved had 4.3 times more new K8+EdU⁺ cells than mice whose skin had been unmanipulated. We are currently testing approaches to enable touch dome visualization without altering normal skin homeostasis.

The mechanical influence of repeated skin shaving caused the touch dome compartment to generate more Merkel cells than during normal skin homeostasis (Figure 20, Figure 22). It is

likely that this approach manipulates the skin similarly to the common methods for mild abrasion such as sandpaper-rubbing or tape-stripping, in which the epidermal barrier is compromised while the dermis is left unaffected (Bertsch et al., 1976; Hardy et al., 2003). In these mild wound systems, keratinocyte proliferation peaks 24-72 hours post-injury and returns to baseline at 7 days (Ekanayake-Mudiyanselage et al., 1998; Hardy et al., 2003). This is also characterized by a transient increase in keratin 6 (K6) and involucrin expression paired with a decrease in K5 and K14 expression in the areas affected by abrasion (Hardy et al., 2003) We are currently testing if these expression profiles and proliferative alterations occur after our shaving protocol. We are also currently working to identify the signaling pathways stimulating Merkel cell progenitor activity in this wounding paradigm.

We were surprised that this effect on Merkel cell number from shaving was not limited to directly shaved skin, as skin adjacent to the shaved region also had an increased number of nascent Merkel cells. This phenomenon of secondary effects on adjacent, unmanipulated touch domes has been described before, in which the skin of cats was exposed to small tattoos that injured touch domes (English, 1974). Many touch domes that were not tattooed, but within 3-7mm, or even as far way as 1cm, from tattooed touch domes had profound morphological changes. These changes were similar to that seen in denervation studies and included loss of both Merkel cells and their overlying touch dome keratinocytes. It is unclear how these signals are propagated between touch domes, as it could be transmitted through keratinocytes, circulating immune cells, or the SA1 afferent. Further studies are required to test which signaling pathways instruct this change in Merkel cell number across the skin, and particularly to test the involvement of the SA1 afferent in disseminating this signal.

In this study we were limited in our ability to distinguish between mechanically-induced nascent Merkel cells and Merkel cells that arose through normal tissue homeostasis. Therefore, it is unclear if there are any differences in the gene expression profiles, function, and/or lifespan of these two nascent Merkel cell types. Moreover, we cannot determine if shaving induces expanded proliferation of the same progenitor responsible for Merkel cell generation during normal homeostasis, or if an alternative progenitor population was recruited to adopt a Merkel cell fate. This fate-switching of skin progenitor populations has been shown to occur after skin wounding, in which hair follicle bulge progenitor cells migrate and differentiate as part of the interfollicular epidermis instead of contributing solely to the hair follicle (Levy et al., 2007).

Some K8+EdU+ cells appeared columnar and within the boundaries of touch dome keratinocytes (Figure 22B-B'''), suggesting that they may arise from K17+Gli1+ touch dome progenitors, as has been previously suggested (Doucet et al., 2013; Woo et al., 2010; Xiao et al., 2015). We can say with certainty that neither type of nascent Merkel cells derive from a *K14*+ progenitor, as fate-mapping the *K14*+ lineage in shaved and unmanipulated *K14^{CreER};ROSA^{tdTomato}* mice resulted in no tdTomato+K8+ cells (>1,200 K8+ cells from 3 mice; Figure 23). However, it is possible that the *Atoh1*+ progenitors responsible for Merkel cell generation in embryogenesis continue generate new Merkel cells during tissue homeostasis, but that a separate progenitor is stimulated to produce Merkel cells in this wounding paradigm. We are currently testing this possibility.

Our findings have potential implications for understanding the genesis of MCC, a rare and potentially devastating form of skin cancer for which there are no truly effective treatments aside from surgical excision. Although the cell type of origin of MCC tumors is unknown (Tilling and Moll, 2012), evidence that MCC arises from Merkel cells or their precursors comes from expression of *Hath1*, the human *Atoh1* homologue, in MCC tumor lines and primary tumor cells, along with other Merkel cell markers such as K20, chromogranin A, synaptophysin, and neuron-specific enolase (Leonard et al., 2002; Heiskala et al., 2010). Here we demonstrate that although touch dome Merkel cells are infrequently generated, their production is increased following mild superficial skin wounds. It is therefore possible that the stimulation of Merkel cell progenitor proliferation, combined with other factors such as Merkel cell polyomavirus and/or UV radiation may trigger unregulated cell proliferation. Identification and further characterization of the progenitors responsible for Merkel cell production will therefore continue to lend potential insight into the pathogenesis of MCC.

4.0 ECTOPIC *ATOHI* EXPRESSION DRIVES MERKEL CELL PRODUCTION IN EMBRYONIC, POSTNATAL, AND ADULT EPIDERMIS

The experiments described in this section are part of a published manuscript co-first authored by Dr. Stephen Ostrowski and myself (Ostrowski et al., 2015).

My contributions:

- Design and production of all main figures and some supplementary figures, including acquisition of the majority of the images.
- Discussing experimental design and data analysis with Drs. Maricich and Ostrowski.
- Editing and some writing of the manuscript.
- Tissue processing and quantifications in Figure 27A-B''', E-H'''.
- Generating animals, tissue processing, and quantification in Figure 31.
- Generating animals, tissue processing, and classification of hair cycle stage in Figure 32.
- Tissue processing and quantifications in Figure 33E.
- Tissue processing and quantifications in Figure 34B-I.

4.1 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 (Ikeda et al., 2014; Maksimovic et al., 2014; Maricich et al., 2009; 2012). Merkel cells express the primitive epithelial intermediate filament proteins K8, K18 and K20; also known as Krt8, Krt18, and Krt20, respectively (Moll et al., 1995; Moll et al., 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 & Leung, 1991; English et al., 1992; Fantini & Johansson, 1995; Garcia-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, 1973; 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., 1996; 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 & Samuelson, 2010; Zheng & 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 & 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.

4.2 MATERIALS AND METHODS

4.2.1 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/6J 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 3 mice/genotype were analyzed for each of the experiments unless otherwise stated.

4.2.2 Tamoxifen Administration

For induction of *Rpbj* deletion in the keratinocyte lineage, tamoxifen (250mg/kg; Sigma) dissolved in a 1:9 ethanol:corn oil solution was administered to P18-P24 mice by oral gavage every other day for 3 doses, followed by doxycycline administration for indicated durations (see below).

4.2.3 Doxycycline Administration

Doxycycline-containing chow (200mg/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 hours for adolescent/adult experiments. For early postnatal ages, doxycycline (1mg in 100mL of 100% ethanol) was applied to the entire back and flank skin of pups twice per day for 3 days (P2-P4).

4.2.4 FM dye injections

Fixable FM1-43 dye (4mg/kg; FM® 1-43FX; Life Technologies) was injected intraperitoneally and mice were sacrificed 24 hrs later (Meyers et al., 2003).

4.2.5 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 OCT or immersion fixed for 1 hr in ice-cold 4% paraformaldehyde. Skin for wholemound immunostaining was washed and stored in 1X PBS, whereas skin for sectioning was cryoprotected in 30% sucrose, embedded in OCT, and cryosectioned onto Fisher Superfrost Plus slides at 10 or 20 μ m using a Leica CM1950 cryostat.

4.2.6 Immunostaining

We used the following primary antibodies: rat anti-keratin 8 (TROMA1, DSHB) 1:20, mouse anti-keratin 18 (clone RGE53, Millipore) 1:200, mouse anti-keratin 20 (clone Ks20.8, Life Technologies) 1:100, rabbit anti-VGLUT2 (Synaptic Systems, cat #135402) 1:3000, rabbit anti-Rab3c (Genetex, cat #GTX13047) 1:1000, rabbit anti-keratin 14 (Covance, PRB-155P) 1:1000, rabbit anti-Islet 1 (Abcam, cat #AB109517) 1:200, rabbit anti-Sox2 (Millipore, cat #AB5603) 1:400, and chicken anti-chicken Atoh1 (generous gift of Drs. Tom Coates and Matthew Kelley, NIDCD/NIH; 1:10,000). Secondary antibodies (Jackson ImmunoResearch) were used at a dilution of 1:250.

Wholemound immunostaining was carried out using a published protocol (Li et al., 2011) with modifications. Adipose tissue was removed and the skin cut into 2x2mm pieces, rinsed in 1X PBS, washed with 1X PBS/0.3% Triton X-100 (0.3% PBST) every 30 minutes for 5-8 hrs, 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 minutes for 5-8 hrs, 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 minutes for 5-8 hrs then mounted on Fisher Superfrost Plus slides using Prolong Gold (Invitrogen).

For sectioned tissue, slides were washed in 1XPBS, blocked in 5%NDS for 30 minutes, and incubated in primary antibody diluted in blocking solution overnight at 4°C. Slides were washed in 1XPBS, incubated in secondary antibody diluted in blocking solution for 30 minutes

at room temperature, washed in 1XPBS, counterstained with DAPI (4'6'-diamidino-2-phenylindole dihydrochloride; Fisher, 1:1000), and mounted in Prolong Gold.

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

4.2.7 Image acquisition

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

4.2.8 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 (1cm² 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 200µm² 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 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 20X 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 Scheffe pairwise comparison testing or Students t-test as indicated.

4.3 RESULTS

4.3.1 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 (Figure 24B-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 (Figure 24A).

Adolescent [postnatal(P)22-26] *K14^{Cre};ROSA^{rtTa};Tet^{Atoh1}* mice that received doxycycline for 24 hrs prior to sacrifice produced Atoh1 protein throughout the foot pad epidermis, hairy skin follicular and interfollicular epidermis, and in epidermal cells within whisker follicles (Figure 24. C', 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) (Figure 24C'', D'', I'', J'', N''). Doxycycline administration for 96 hours resulted in greater numbers of ectopic Atoh1+ cells in all regions (Figure 24. E-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 (Figure 24E'', F'', K'', L'', O''). We never found ectopic Atoh1+ or K8+ cells in any skin region in control littermates (Figure 24B-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 hrs experienced severe weight loss, probably secondary

to degeneration of the tongue epithelium causing decrease oral intake (Figure 25A-C). Therefore, we used the 24 hrs 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 hrs to adolescent *K14^{Cre};ROSA^{rtTa};Tet^{Atoh1}* mice and harvesting skin 4 days, 2 weeks, 6 weeks and 3 mo after doxycycline was withdrawn (Figure 26). In glabrous paw skin and whisker follicles, ectopic K8+ cells were present 4 days after doxycycline administration, but very few remained 2 weeks after doxycycline administration (Figure 26A-F’). These cells were not studied further. By contrast, ectopic K8+ cells were found in body skin hair follicle epidermis at all time points, but their numbers decreased between 4 days and 6 weeks post-doxycycline, then remained constant through 3 mo post-doxycycline (Figure 26G-K). Co-immunostaining for K8 and cleaved caspase-3 4 days post-doxycycline revealed that $1.3\pm 0.8\%$ of ectopic K8+ cells were caspase-3+, suggesting that the decline in ectopic K8+ cell number occurred secondarily to apoptosis (Figure 27A-A’’) Rare *Atoh1*+K8- cells were also found at these time points, but the vast majority of *Atoh1*+ cells co-expressed K8 (Figure 26L-M’’’). At 4 days post-doxycycline, ectopic K8+ cells were found throughout the hair follicle epidermis (Figure 26L-L’’’) but were restricted to hair follicle bulb and bulge regions from 2 weeks post-doxycycline onward (Figure 26M-M’’’; data not shown). These data indicate that the majority of ectopic K8+ cells disappear over time, but that a small subset survives for at least 3 mo post-*Atoh1* induction.

Embryonic *Atoh1*+ cells undergo mitosis (Figure 2, Figure 3) so we wondered whether ectopic *Atoh1*-expressing cells were proliferative. No ectopic K8+ cells expressed the proliferation marker Ki67 in *K14^{Cre};ROSA^{rtTa};Tet^{Atoh1}* mice 4 days post-doxycycline, indicating that ectopic K8+ cells were not mitotically active (Figure 27B-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/20X field of view, $p=0.7$; follicular: 34.3 ± 4.4 versus 25.9 ± 0.4 Ki67+ cells/20X 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 (Figure 28). 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 to 2 weeks post-doxycycline, and these cells had qualitatively stronger Atoh1 immunofluorescence at the 4 day time point (Figure 27C-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.

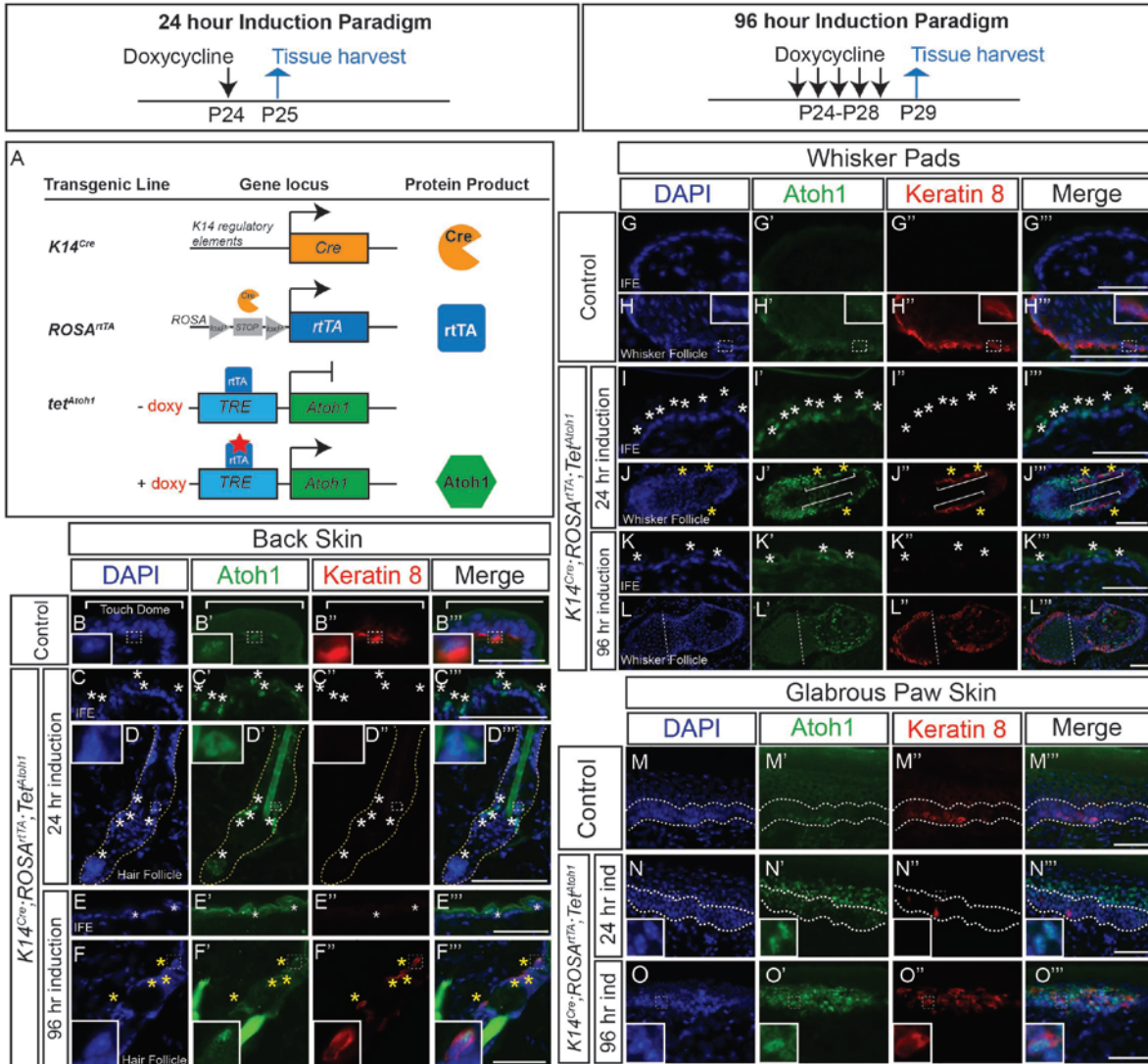


Figure 24. Inducible *Atoh1* expression produces ectopic K8+ cells in glabrous and hairy skin of adolescent $K14^{Cre};ROSA^{rtTa};Tet^{Atoh1}$ mice Experimental induction paradigms are shown at the top of the figure. (A) Schematic of $K14^{Cre};ROSA^{rtTa};Tet^{Atoh1}$ mouse alleles. Cre is produced 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 hrs. 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: 50µm.

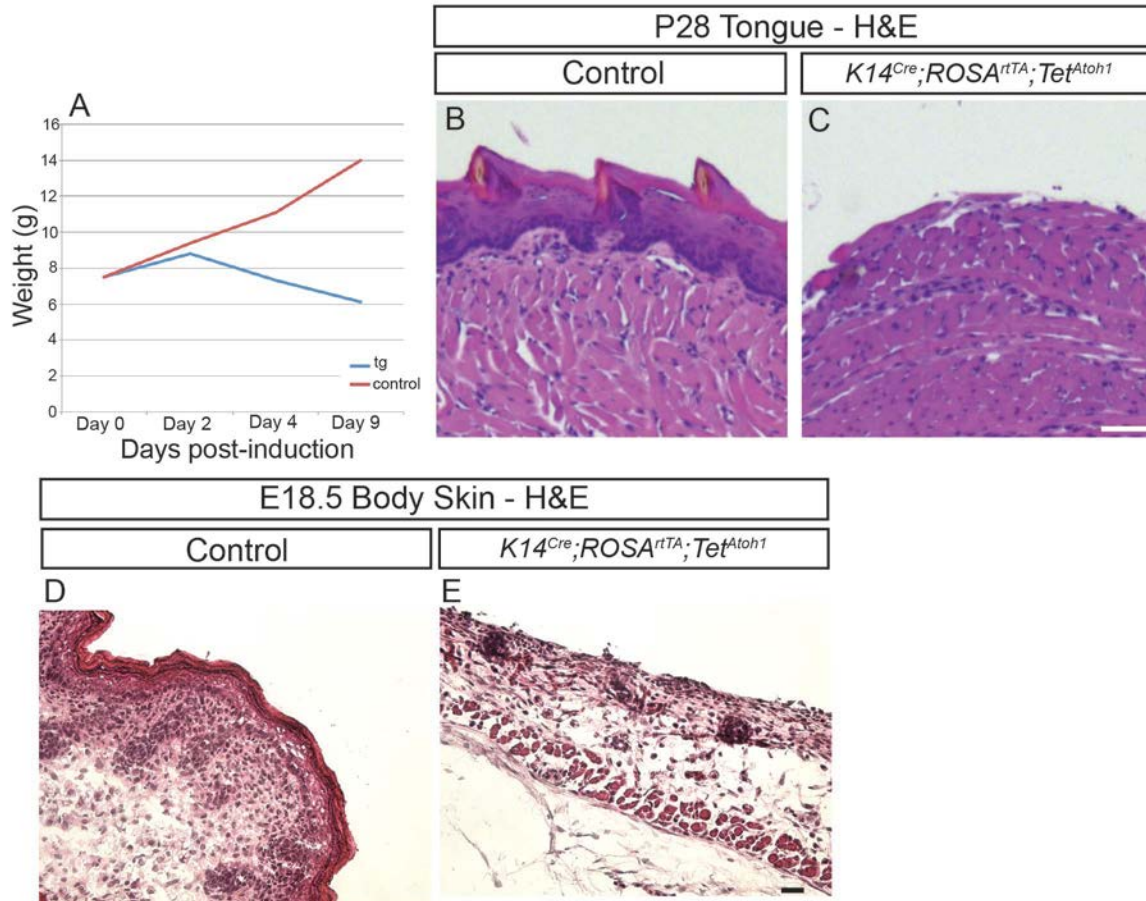


Figure 25. Excessive ectopic *Atoh1* over-expression damages the skin. (A) Weight of P24-P28 *K14^{Cre};ROSA^{rtTa};Tet^{Atoh1}* (n=7) and control (n=7) mice treated for 2 or 4 days with doxycycline. *K14^{Cre};ROSA^{rtTa};Tet^{Atoh1}* (n=2) mice but not littermate controls (n=2) allowed to age past day 4 required euthanasia on day 9 due to emaciation. (B, C) Hematoxylin and eosin (H&E)-stained tongue tissue sections from P28 control littermate (B) and *K14^{Cre};ROSA^{rtTa};Tet^{Atoh1}* mice (C) that received doxycycline for 4 days reveals acantholysis and epidermal loss in the latter. (D, E) H&E staining of skin from E18.5 control littermate (D) and *K14^{Cre};ROSA^{rtTa};Tet^{Atoh1}* (E) embryos given doxycycline from E14.5-E18.5 demonstrates acantholysis and epidermal loss in the latter. Scale bars: 50 μ m.

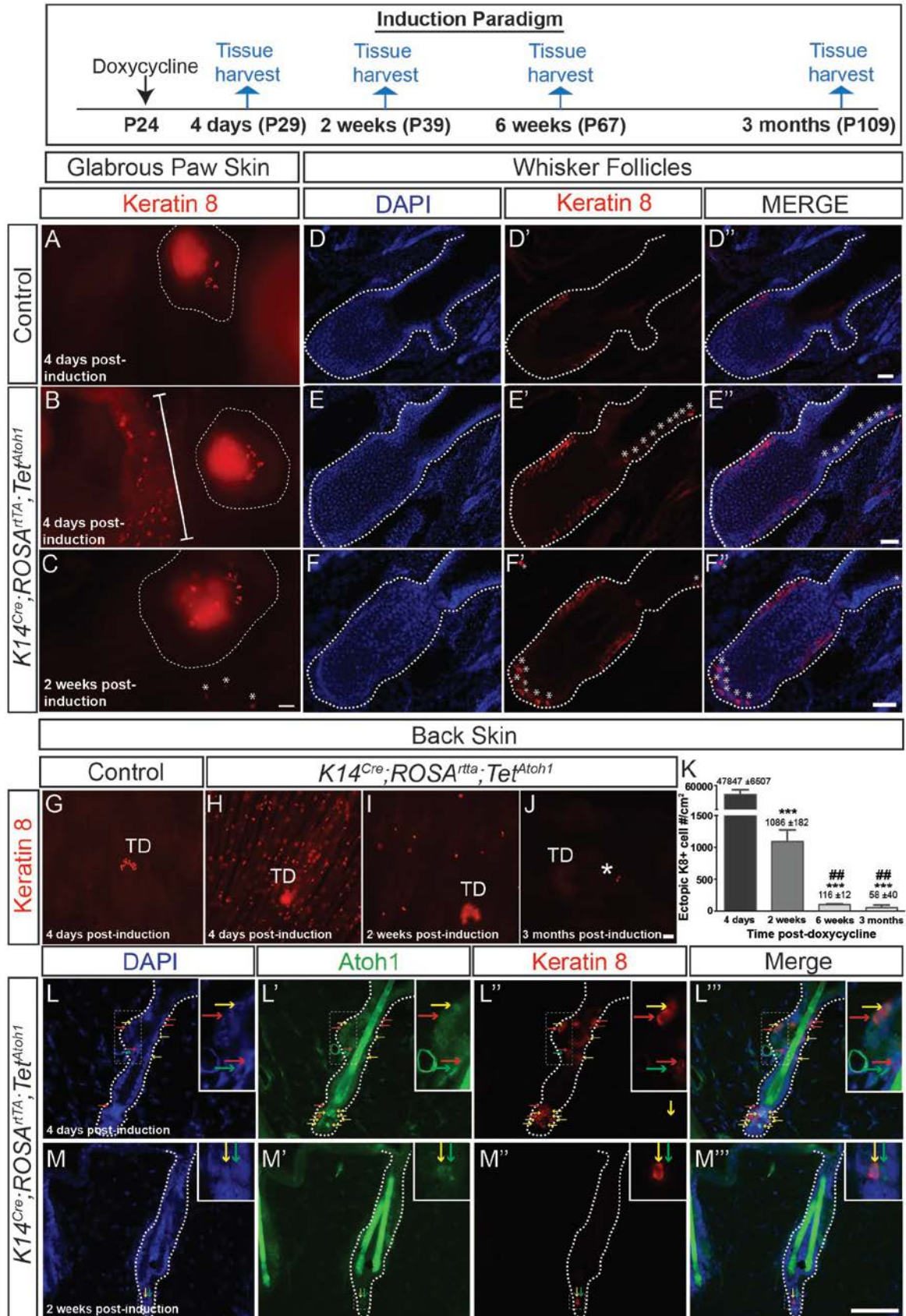


Figure 26. Ectopic K8+ cells persist in glabrous and hairy skin of *K14^{Cre};ROSA^{rtTa};Tet^{Atoh1}* mice Experimental induction paradigm is shown at the top of the figure. (A-J) Wholemout glabrous paw skin (A-C), sectioned whisker follicles (D-F'') and wholemount back skin (G-J) of control (A,D-D'',G) and *K14^{Cre};ROSA^{rtTa};Tet^{Atoh1}* mice four days (B,E-E'',H), two weeks (C,F-F'',I) and three mo (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±SEM ***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: 50µm.

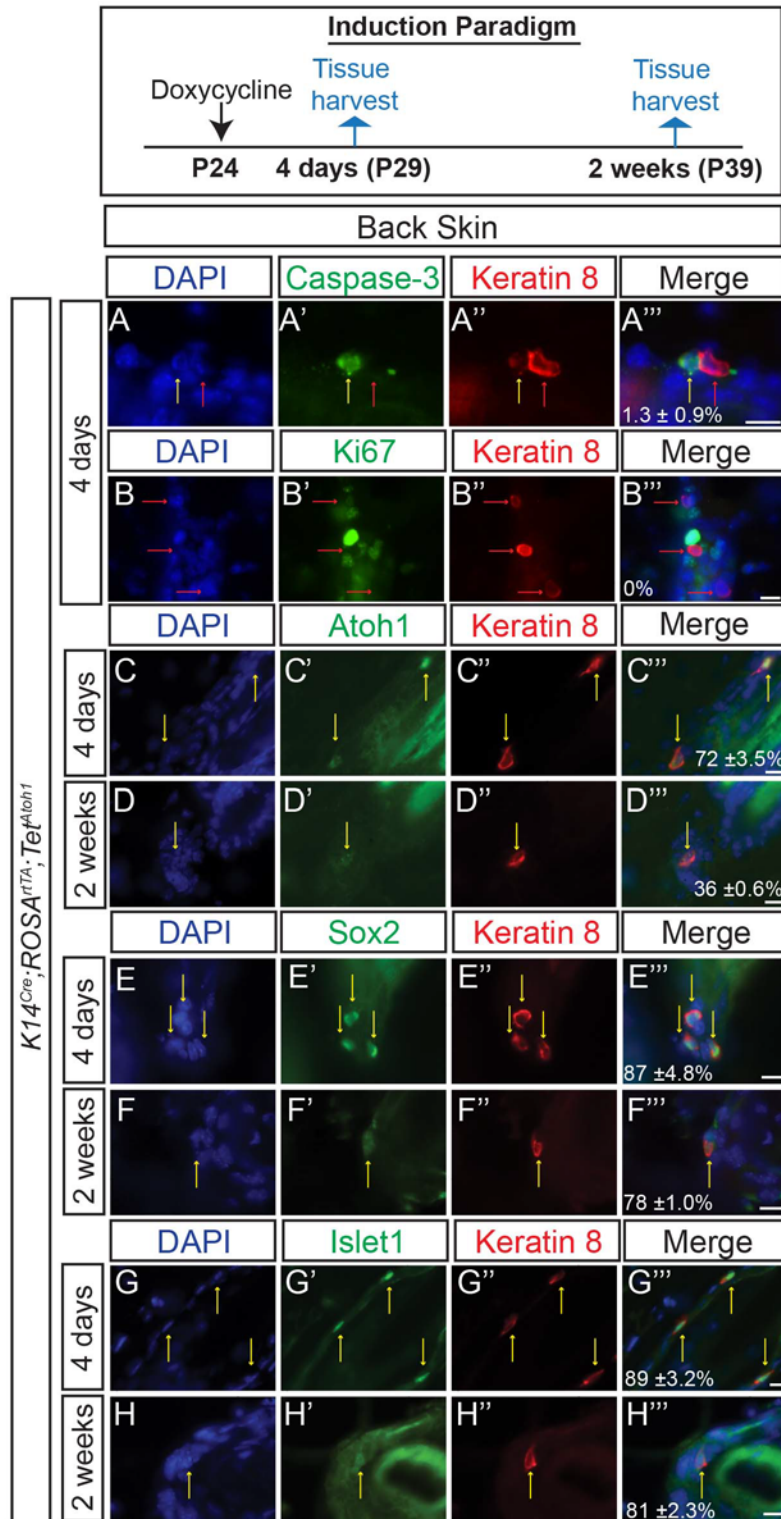


Figure 27. Ectopic K8+ cells express Atoh1, Sox2 and Isl1, some express caspase-3, but none are mitotically active Experimental induction paradigm is shown at the top of the figure. (A-H''') Sectioned back skin of adolescent *K14^{Cre}; ROSA^{rtTa}; Tet^{Atoh1}* mice showing ectopic K8+

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 (\pm SEM) co-expressing each marker is shown (n=100-200 K8+ cells from each of three mice). Scale bars: 10 μ m.

4.3.2 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 (Figure 28). 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., 2014a). The vast majority of ectopic K8+ cells co-expressed Sox2 and Isl1 at 4 days and 2 weeks post-doxycycline (Figure 27E-H'''), 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 hrs doxycycline pulse nearly all ectopic K8+ cells co-expressed the Merkel cell markers K18 and K20 (Figure 29B-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 (Figure 29E-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 (Figure 29D-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), 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* (Figure 29A-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 14.5 (E14.5) (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 (Figure 30A-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 (Figure 30D-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.

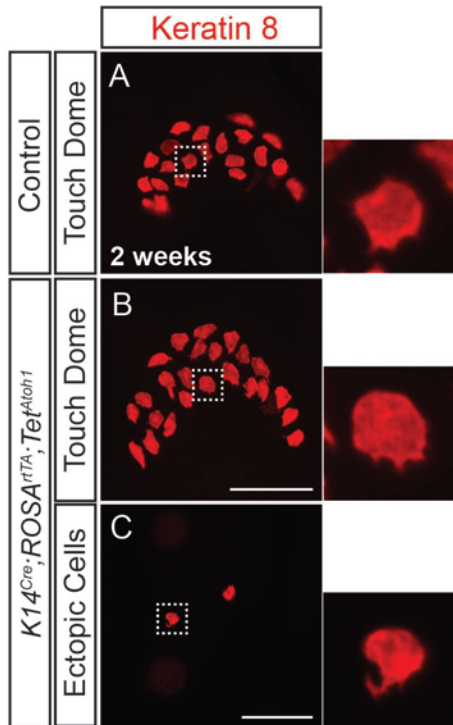


Figure 28. *Atoh1* induction does not affect Merkel cell morphology or number in touch domes, ectopic K8+ cells have typical Merkel cell morphology K8 immunostaining in wholemount back skin 2 weeks post-doxycycline shows touch dome (A, B) and ectopic (C) K8+ cells in adult littermate control (A) and *K14^{Cre}; ROSA^{rtTa}; Tet^{Atoh1}* (B, C) mice. Boxed regions are shown at higher magnification to the right of each panel. Scale bars: 50 μ m.

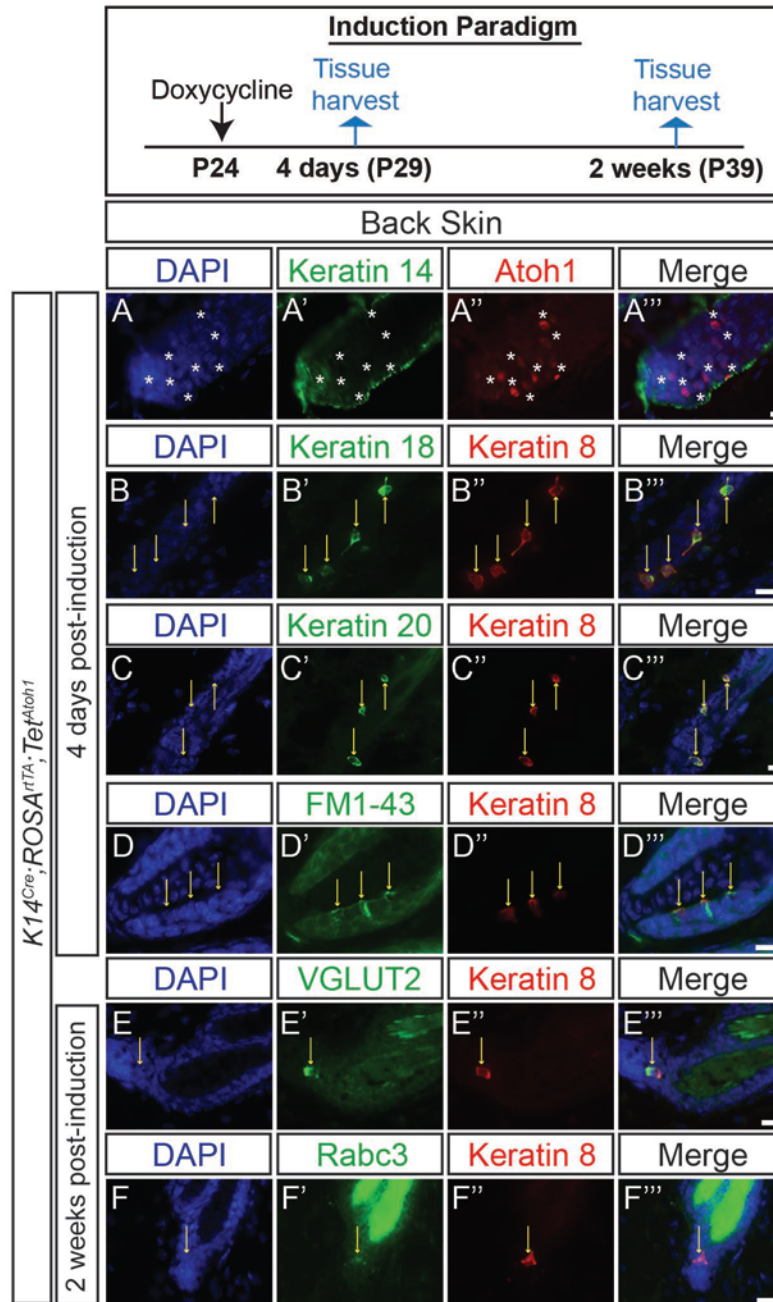


Figure 29. Ectopic K8+ cells express mature Merkel cell markers but not Keratin 14

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: 10µm.

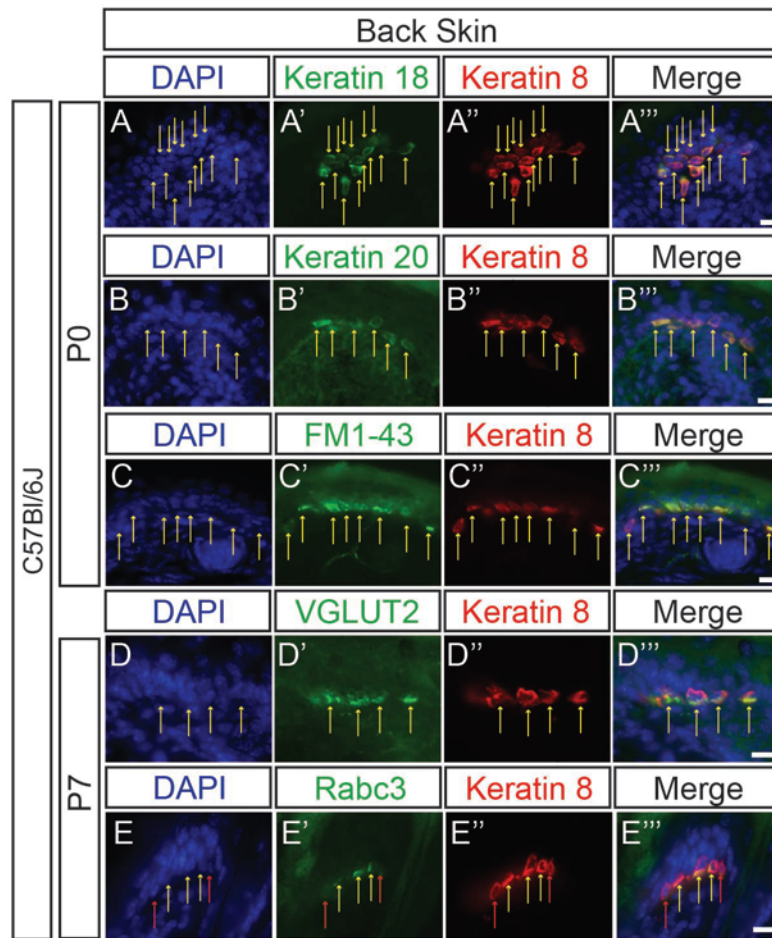


Figure 30. Developmental time course of touch dome Merkel cell marker expression

(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 μ m.

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 development

	Ectopic K8+ cells in <i>K14^{Cre};ROSA^{rtTa};Tet^{Atoh1}</i> 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

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

Previous reports suggest that Merkel cell number changes during the hair cycle in rodents (Moll et al., 1996a; Nakafusa et al., 2006) so we wondered whether hair cycle stage might affect the competency of keratinocytes to respond to *Atoh1* induction. *Atoh1* induction for 24 hrs during anagen I at P24-26 followed by tissue harvest 4 days or 2 weeks later resulted in production of ~55 times (p=0.011, t-test) and ~100 times (p=0.004, t-test) more ectopic K8+ cells, respectively, than induction at P19-20 during telogen I (Figure 31). We verified hair cycle stage at time of induction and time of collection on Hematoxylin and Eosin-stained tissue sections (Figure 32). These data suggest that factors that vary during the hair cycle control responsiveness of keratinocytes to ectopic *Atoh1* expression.

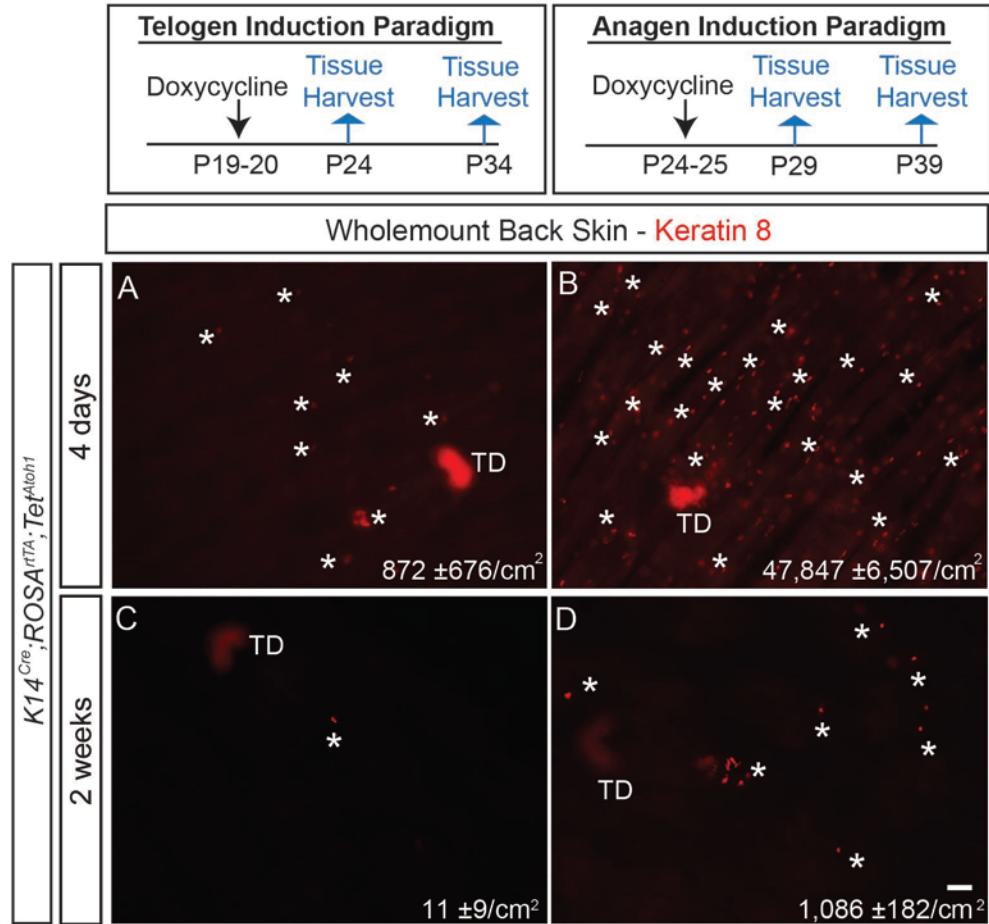


Figure 31. Keratinocyte competency to respond to ectopic *Atoh1* varies by hair cycle stage

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 (\pm SEM; n=3 mice/hair cycle stage). TD, touch dome. Scale bar: 50 μ m.

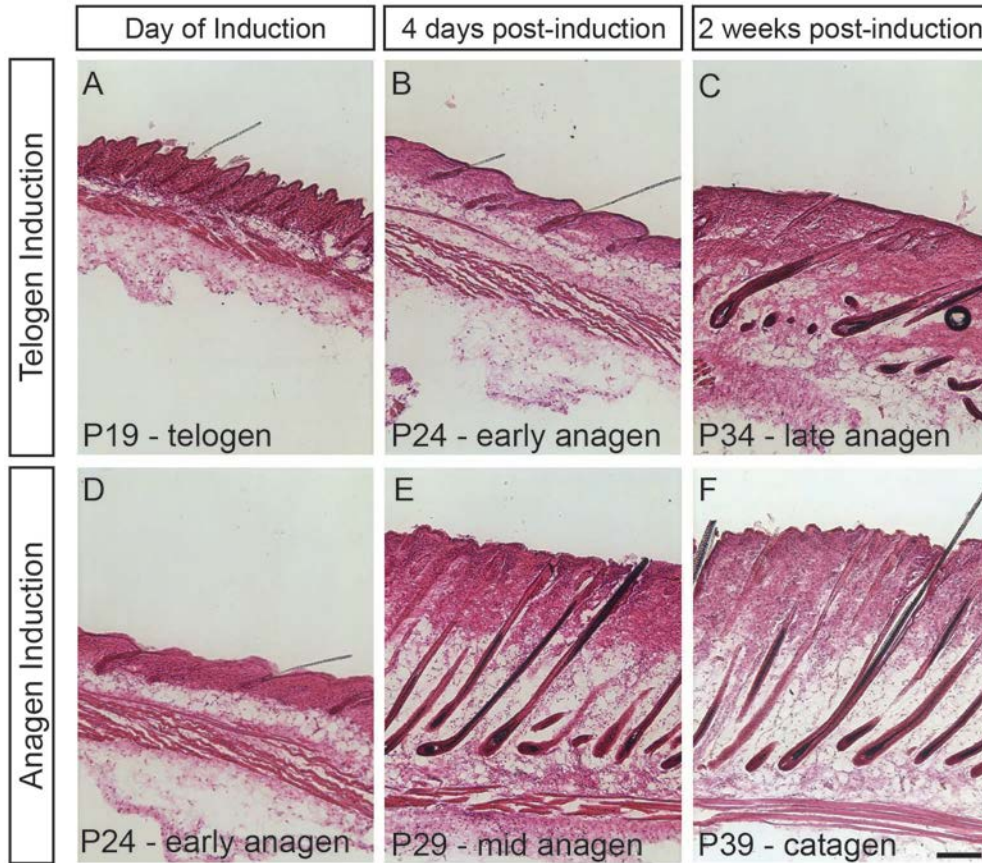


Figure 32. $K14^{Cre};ROSA^{rtTa};Tet^{Atoh1}$ mouse hair cycle stages following induction during telogen I and anagen I. H&E staining of sectioned back skin from $K14^{Cre};ROSA^{rtTa};Tet^{Atoh1}$ mice at the time of (A, D), four days (B, E) and two weeks (C, F) post- doxycycline administration. Hair cycle stage and age at time of tissue harvest are indicated in each panel. Scale bar: 100 μ m.

4.3.4 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-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, while *Atoh1* and

K8 were detected only in touch domes of littermate controls (Figure 33A, B, E-E’’). 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 (Figure 25D, E). Induction of *Atoh1 in utero*, even for periods as short as 24 hrs, resulted in embryonic or early postnatal lethality of transgenic pups, and this prevented 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 P2-4 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 only within the follicular epidermis and the associated infundibulum (Figure 33D, E, G-G’’). *Atoh1*⁺ and K8⁺ cells were confined to touch domes of P4 control littermates (Figure 33C). 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 Scheffe 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 restricted to hair follicle epidermis shortly after birth.

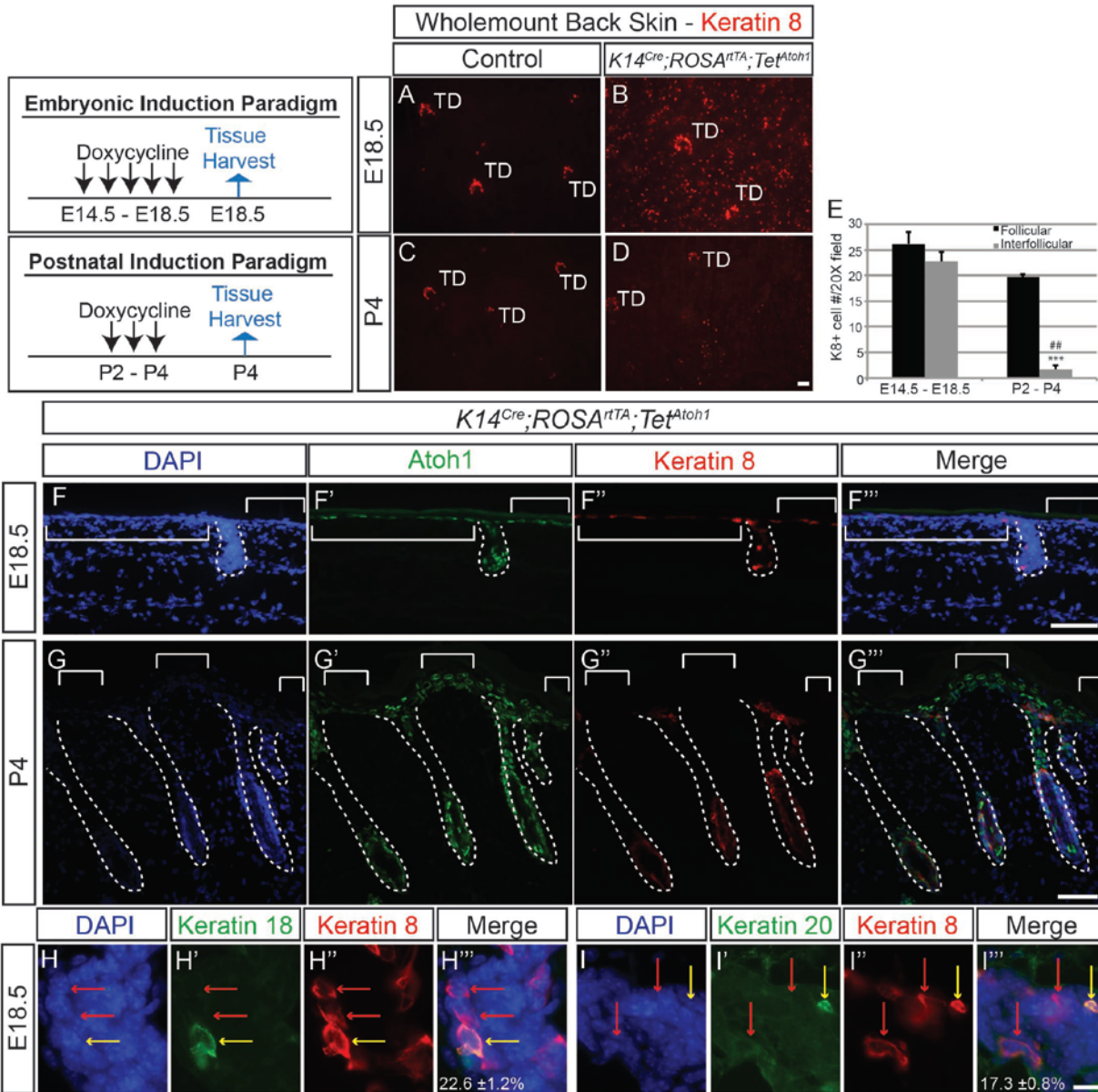


Figure 33. Keratinocyte competency to respond to ectopic *Atoh1* decreases as animals age
 Experimental induction paradigms are shown at the top of the figure. (A-I''') Wholemount (A-D) and sectioned (F-I''') back skin immunostained for K8 (A-D, F'', G'', H'', I'') and Atoh1 (F', G'), K18 (H') or K20 (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 20X field ±SEM; 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 (\pm SEM) of double-labeled cells are shown (n=100-200 K8+ cells from each of three mice). TD, touch dome. Scale bars: (A-G''') 50 μ m; (H-I''') 10 μ m.

4.3.5 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 the 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 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 & Reiter, 2011; Zhang et al., 2009) (Figure 34A-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 (n=2-3/genotype/age) were given 3 doses (250 mg/kg) of tamoxifen via oral gavage between P19 and P24 to activate *K14^{CreER}*, followed by *Atoh1* induction with doxycycline for 4 days from P24-28 and tissue harvested on P28 or 3 mo 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 (Figure 34B). Because mosaic recombination occurred in hair follicles, at P28 only relatively small numbers of K8+ cells were present in the hairy skin of *K14^{CreER};ROSA^{rtTa};Tet^{Atoh1};RBPj^{+/+}* mice (Figure 34C). By contrast, *K14^{CreER};ROSA^{rtTa};Tet^{Atoh1};RBPj^{flox/+}* and *K14^{CreER};ROSA^{rtTa};Tet^{Atoh1};RBPj^{flox/flox}* had larger numbers of K8+ cells in follicular epidermis (Figure 34D, E), suggesting that acute ablation of Notch signaling greatly enhanced the 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

Scheffe tests: $K14^{CreER};ROSA^{rtTa};RBPj^{flox/flox}$ versus $K14^{CreER};ROSA^{rtTa};Tet^{Atoh1};RBPj^{flox/flox}$ $p=0.05$ and $K14^{CreER};ROSA^{rtTa};Tet^{Atoh1};RBPj^{+/+}$ versus $K14^{CreER};ROSA^{rtTa};Tet^{Atoh1};RBPj^{flox/flox}$ $p=0.042$). 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 $K14^{CreER};ROSA^{rtTa};RBPj^{flox/flox}$, $K14^{CreER};ROSA^{rtTa};Tet^{Atoh1};RBPj^{+/+}$, $K14^{CreER};ROSA^{rtTa};Tet^{Atoh1};RBPj^{flox/+}$, $K14^{CreER};ROSA^{rtTa};Tet^{Atoh1};RBPj^{flox/flox}$ mice (19 ± 2.0 , 17 ± 0.4 , 17 ± 0.4 , and 17 ± 0.2 K8+ cells/touch dome, respectively; one-way ANOVA $F=0.21$, $P=0.8873$). Three mo post-induction (P140), $K14^{CreER};ROSA^{rtTa};RBPj^{flox/flox}$ and $K14^{CreER};ROSA^{rtTa};Tet^{Atoh1};RBPj^{flox/flox}$ 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 (Figure 34G,H). These data demonstrate that Notch signaling regulates competency to respond to ectopic *Atoh1* expression in the skin of juvenile mice.

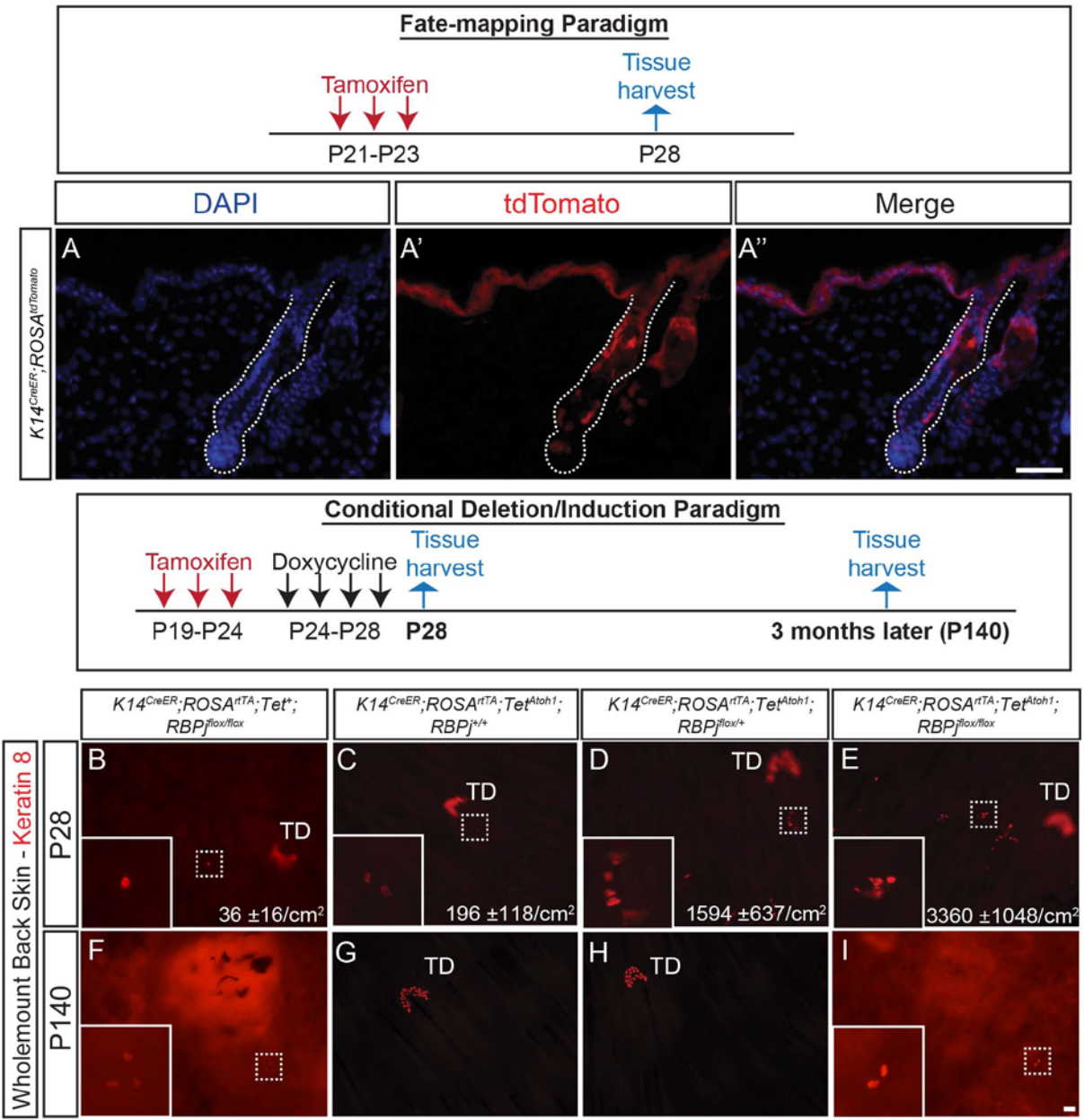


Figure 34. Loss of Notch signaling enhances epidermal competency to respond to *Atoh1*

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}; RBP^{flox/flox}*, *K14^{CreER}; ROSA^{rtTa}; Tet^{Atoh1}; RBP^{+/+}*, *K14^{CreER}; ROSA^{rtTa}; Tet^{Atoh1}; RBP^{flox/+}* and *K14^{CreER}; ROSA^{rtTa}; Tet^{Atoh1}; RBP^{flox/flox}* mice were dosed with tamoxifen and doxycycline as indicated, then back skin harvested and immunostained for

K8 immediately (B-E) or 3 mo later (F-I). Boxes indicate areas shown at higher magnification in insets. Quantifications indicate Merkel cell density. TD, touch dome. Scale bars: 50µm.

4.4 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 & Samuelson, 2010).

Cellular competency to respond to the robust doxycycline-induced ectopic *Atoh1* expression seen in *K14^{Cre};ROSA^{rtTa};Ter^{Atoh1}* mice varied by age, location and hair cycle stage (Figure 24, Figure 26, Figure 31, Figure 33). 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, co-expression 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;ROSA^{rtTa};Tet^{Atoh1};RBPj^{fllox/fllox}* mice caused a marked increase in the number of ectopic K8+ cells (Figure 34), 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 & 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 & 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 mo post-induction, probably secondary to apoptotic cell death (Figure 26, Figure 27A-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 mo (Figure 26K). 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 SA1 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,; 1998b; 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., 1996a; 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 (Figure 2, Figure 3). 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 (Figure 27B-B'''). Second, ectopic K8+ cell numbers remained constant between 6 weeks and 3 mo post-induction, suggesting that there was neither cell death nor replication (Figure 26K). Third, clusters of K8+ cells were never observed at these time points, suggesting that clonal expansion of individual cells did not occur (Figure 26). 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., 2014a). Upregulation of *Sox2* and *Isl1* combined with *Atoh1* autoregulation 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* (Gele et al., 2004; Leonard et al., 2002), and other lines of evidence suggest that MCC might arise from keratinocytes, skin stem cells or even immune B cells (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.

5.0 GENERAL DISCUSSION

5.1 SUMMARY AND INTERPRETATION

Our interaction with the world around us stems from our ability to detect, transmit, and perceive stimuli in our surrounding environment. This process is accomplished by our sensory systems, enabling us to see, hear, smell, taste, and touch the world around us. These sensory systems each translate their specific received environmental stimuli into the electrical language of our nervous system, providing our brain the information with which to generate an accurate and complete percept of our surroundings. Somatosensation is unique among the sensory systems in its multimodality, being able to detect temperature, noxious chemicals, and mechanical stimuli. Our perception of touch, in particular, is the summation of the multiple somatosensory receptors, each tuned to detect particular forms of stimuli from their peripheral endings that innervate the skin. Of these, the SA1 afferent is distinctive in that it innervates a specific type of skin cell, the Merkel cell, as its end organ. The relationship between and distinguishing characteristics of SA1 afferents and Merkel cells has been an enigmatic question discussed and studied by scientists for well over a century. The experiments outlined in this dissertation complement and expand upon the work pioneered by those scientists, bringing greater clarity to the developmental characteristics of this mechanoreceptor.

Specifically, the central focus of my dissertation has been to characterize how and when Merkel cells are made. To investigate these elements surrounding Merkel cell homeostasis, my thesis work has focused on two specific hypotheses:

Hypothesis 1: Mature Merkel cells are formed by *Atoh1*+ embryonic progenitors and can persist for the lifetime of the animal.

Hypothesis 2: Atoh1 in keratinocytes is sufficient to induce Merkel cell fate.

In Chapter 2, we assayed the proliferative capability of *Atoh1*⁺ cells in the body skin and whisker follicles during embryogenesis. These experiments were performed in *Atoh1*^{GFP} mice to enable our visualization of all *Atoh1*-expressing cells, as antibody labeling for this transcription factor has proven notoriously difficult. Our results demonstrated that:

1. A subset of embryonic *Atoh1*^{GFP}⁺ cells expressed the mitotic markers Ki67 and PH3, as well as incorporated the modified nucleoside EdU.
2. These proliferative *Atoh1*⁺ cells only gave rise to Merkel cells.
3. While originally derived from the *K14*⁺ lineage, the *Atoh1*⁺ lineage is entirely separate by late embryogenesis.

These experiments demonstrate an early allocation of the *Atoh1*⁺ Merkel cell lineage from their surrounding keratinocytes, making them among the very first fully differentiated skin cells. While likely only dividing a small number of times after *Atoh1* expression is initiated, these progenitors only ever gave rise to cells that expressed the Merkel cell marker K8, indicating their unipotent capacity. This led us to hypothesize that a subpopulation of *Atoh1*⁺ cells may continue to proliferate in adulthood. We also wondered for how long these embryonic-born Merkel cells persisted. With these two questions in mind, our results in Chapters 2 and 3 demonstrated that:

1. The *Atoh1*⁺ lineage remained by-and-large separate from that of the surrounding keratinocytes through late adulthood.
2. While *Atoh1*⁺ cells did not express K14, a subset was found to express K17, suggesting a genetic distinction from interfollicular keratinocytes and slight overlap with touch dome keratinocytes.
3. Adult *Atoh1*⁺ cells very rarely expressed proliferative markers.
4. Average touch dome Merkel cell number peaks at the end of embryogenesis, decreases in early postnatal life, and remains stable throughout adulthood, regardless of natural or induced hair cycle stages.
5. Many embryonic-born Merkel cells persist into late adulthood.
6. Few Merkel cells are made during unmanipulated skin homeostasis.

These results were hugely important in demonstrating the limited turnover of Merkel cells during adulthood and indicate that Merkel cell progenitors are mitotically active very infrequently. Prior to these experiments, murine Merkel cells had a predicted lifespan of 7-8 weeks, significantly less than what we demonstrated here. While 9 months of age is the oldest we analyzed for persistence of embryonic-born Merkel cells, I suspect that the lifespan of these cells would extend to the lifetime of the animal. Consistent with this, very few *Atoh1*⁺ cells were found to express proliferative markers when examined at multiple postnatal ages. However, fate-mapping and genetic ablation experiments suggested that the *Atoh1* lineage remained distinct from that of other keratinocytes, again up to 9 mo of age. The tamoxifen-inducible Cre model used for these experiments has a recombination rate of ~97%, which was the percentage of cells labeled 9 mo post-tamoxifen administration in *Atoh1*^{CreER-T2/+};*ROSA*^{LacZ} mice and the approximate percentage of cells remaining in *Atoh1*^{CreER-T2/+};*ROSA*^{DTA} mice 6 mo post tamoxifen administration. It is likely that the addition of adult-born Merkel cells after tamoxifen administration in these models was not detected due to their infrequent generation and decreased likelihood of survival. These genetic and nucleotide-incorporation models provided snapshots of time that demonstrated the persistence of embryonic-born Merkel cells, which was further supported by our chronic *in vivo* live imaging experiments. These experiments also demonstrated that:

1. Many Merkel cells persisted over the entire imaging period.
2. Many Merkel cells were generated and lost over this imaging period, unrelated to hair cycle stage.
3. The repeated shaving performed for visualization of the skin induced a superficial wound that stimulated the production of more nascent Merkel cells than arise through normal skin homeostasis.
4. These nascent Merkel cells arose from neither *Atoh1*- or *K14*-expressing progenitors.

The powerful approach provided by *in vivo* confocal imaging clearly illustrated the persistence of many *Atoh1*⁺ touch dome Merkel cells through once-weekly imaging. Both unfortunately and serendipitously, the necessity for repeated skin shavings to expose skin for imaging prompted the touch dome environment to produce more nascent Merkel cells and lose more original Merkel cells than unmanipulated skin. This wounding paradigm is similar to others previously described (Hardy, 2003; Bertsch, 1976; Ekanayake 1998) and presumably stimulates

the activity of capable Merkel cell progenitors. The identity of this progenitor and its potential activity and contributions during homeostatic conditions has yet to be determined.

In closing on *Hypothesis 1*, our experiments have demonstrated that *Atoh1*⁺ progenitors are active in embryogenesis and the mature Merkel cells they generated persist into late adulthood. Merkel cells do continue to be made in postnatal life, though very infrequently and by a still unidentified progenitor population. Repeated skin shavings activate either these or other skin progenitors to produce more nascent Merkel cells than during normal skin homeostasis.

In Chapter 4 we tested the hypothesis that the transcription factor *Atoh1* is sufficient for Merkel cell production. To test this, we expressed *Atoh1* conditionally within the skin by administering doxycycline to *K14^{Cre};ROSA^{rtTa};Tet^{Atoh1}* mice (Figure 24). Results demonstrated that:

1. Ectopic K8⁺ cells were produced after *Atoh1* expression was induced in glabrous and hairy skin.
2. These ectopic K8⁺ cells acquired Merkel cell markers in a time course similar to that of normally developing Merkel cells.
3. Competency of keratinocytes to respond to *Atoh1* expression was linked to hair cycle stage, developmental age, and the influence of Notch signaling.

Together these experiments confirmed our hypothesis that *Atoh1* expression is sufficient to transform keratinocytes into Merkel-like cells. While the vast majority of these cells died or lost expression of Merkel cell markers within weeks, a small subset in the hair follicle bulge persisted in this permissive niche for at least 3 months. We predict that these permissive niches provide Merkel cells with a trophic factor that enables their survival. It is likely that this trophic factor works similarly on these ectopic Merkel cells as the SA1 maintains Merkel cell survival in touch domes. When the SA1 is removed by surgical ligation, many (though not all) Merkel cells die as a result, leading to the hypothesis that the SA1 releases a trophic factor necessary for Merkel cell survival (Nurse et al., 1984). It would be intriguing to know the identity of the trophic factors in these two scenarios.

The transformation of keratinocytes into ectopic Merkel cells was led by a step-wise inheritance of Merkel cell-specific factors in a time course similar to that of embryonic Merkel cells. The developmental program of embryonic Merkel cell specification therefore seems to be led primarily by *Atoh1* expression. In this model, *Atoh1* was only expressed transiently for 24

hours, which was sufficient for Merkel cell production. The subsequent activation of transcription factors *Sox2* and *Isl1* provide further developmental instruction for Merkel cell maturation in embryogenesis (Perdigoto et al., 2014a), a phenomenon recapitulated in the ectopically induced cells in this model.

While touch dome-associated Merkel cell production is independent of hair follicle cycling, ectopic Merkel cells were more readily produced when the hair follicles were actively growing during anagen than during hair follicle stasis in telogen. This could be attributed to greater keratinocyte number present during anagen, when the hair follicle is longer, greater competency of keratinocytes to respond to *Atoh1* signaling, or a combination of the two. Given the greater responsiveness of keratinocytes to *Atoh1* during earlier ages, it seems likely that cells are more able to respond to genetic instruction and change fate during the hair growth.

Notch signaling was also found to impact keratinocyte responsiveness, regardless of animal age. It is possible that natural changes in Notch signaling factor into cell responsiveness, as discussed above. However, it is also possible that Notch signaling is not active in normal touch dome development and maintenance, but that the result of its manipulation is revealing no physiologically relevant developmental or maintenance pathways. Further investigation into Notch signaling during embryonic development will best answer this possibility.

5.2 INTERPRETING DISCREPANCIES BETWEEN PUBLISHED STUDIES: WHO IS THE ADULT MERKEL CELL PROGENITOR?

Within the past eight years at least five independent groups of investigators have committed an incredible amount of time and resources to identifying and characterizing the activity of adult Merkel cell progenitors. The identification of Merkel cells as an epidermally-derived, *Atoh1*-lineal cell population (Maricich et al., 2009; Morrison et al., 2009; Van Keymeulen et al., 2009) together with the expansion of novel genetic techniques for lineage tracing and selective gene excision provided the necessary springboard for this search. A seemingly straightforward question has turned into a cacophony of potentially contradictory experimental outcomes and interpretations that I will outline and bring into greater clarity (Table 2).

Table 2. Overview of studies investigating the identity of adult Merkel cell progenitors

Manuscript	Tissue Analyzed	Brief Relevant Experimental Summary	Authors Interpretation
Van Keymeulen et al., 2009	Body Skin	n/a	n/a
	Whiskers	<i>K18^{CreER};ROSA^{YFP}</i> mice given pulse chase of tamoxifen. Percent of labeled YFP+K8+/K8+ cells decreases from 7-21 days post-tamoxifen	The K18+ Merkel cell population is replaced by a <i>K18</i> - progenitor.
		<i>K15^{CrePR};ROSA^{YFP}</i> mice given RU486 for 5 or 21 days. The percent of YFP+K8+/K8+ cells increases from 5 to 21 days post-tamoxifen	The K8+ Merkel cell population is replaced by a <i>K15</i> + progenitor.
	Paws	BrdU administered for 10 days. All Merkel cells are BrdU-.	K8+ cells are quiescent during homeostasis.
BrdU administered for 10 days. All Merkel cells are BrdU-.			
Woo et al, 2010	Body Skin	1 or 6 EdU administrations for 1hr, 24 hrs, or 3-8 days. K8+ cells only EdU+ with 6 EdU injections after 3-8 days.	K8+ cells arise from a resident multipotent touch dome progenitor.
		Touch dome versus interfollicular keratinocyte isolation and transplant into nude mice. Only TD transplants made new K8+ cells.	The touch dome compartment houses a progenitor uniquely capable of producing Merkel cells.
	Whiskers	n/a	n/a
	Paws	n/a	n/a
Doucet et al., 2013	Body Skin	<i>K17^{CreER};ROSA^{YFP}</i> mice administered tamoxifen in adolescence. The percent of YFP+K8+/K8+ cells increases over time to ~80% at 7 weeks post-TMX.	K17+ progenitors maintain adult Merkel cells and the entire K8+ Merkel cell population turns over in ~7-8 weeks.
		<i>K17^{CreER};ROSA^{ΔDTR}</i> mice administered tamoxifen followed by diphtheria toxin. K17+ cells were ablated 24 hrs after diphtheria toxin administration ended, while Merkel cells were still present, though their innervating afferents were degenerating.	
	Whiskers	<i>K17^{CreER};ROSA^{YFP}</i> mice administered tamoxifen in adolescence. No K8+YFP+ cells at 24 hours, by 12 weeks post-TMX many YFP+K8+ cells were found.	
Paws	<i>K17^{CreER};ROSA^{YFP}</i> mice administered tamoxifen in adolescence. No K8+YFP+ cells at 24 hours, by 7 weeks post-TMX many YFP+K8+ cells were found.	K17+ progenitors maintain adult Merkel cells.	
Xiao et al., 2015	Body Skin	<i>Gli1^{CreER};ROSA^{YFP}</i> mice administered tamoxifen at P26. <10% of K8+ cells were YFP+ at both 9 days and 2 months post-tamoxifen.	A <i>Gli1</i> + progenitor replaces Merkel cells only during anagen. Merkel cell population turnover dependent on hair cycle.
		<i>Gli1^{CreER};ROSA^{YFP}</i> mice were depilated and tamoxifen administered in adolescence. >90% of K8+ cells were YFP+ 3 months post-tamoxifen.	
	Whiskers	n/a	n/a
Paws	n/a	n/a	

5.2.1 Van Keymeulen et al., 2009 - *Epidermal progenitors give rise to Merkel cells during embryonic development and adult homeostasis*

The first group to perform fate-mapping experiments aimed at identifying resident adult Merkel cell progenitors was led Dr. Cedric Blanpain (Van Keymeulen et al., 2009). Published almost concomitantly with a paper from our lab (Morrison et al., 2009), experiments outlined in Blanpain's manuscript used complementary approaches to ours that demonstrated an epidermal origin for Merkel cells. While his embryonic fate-mapping and conditional gene deletion studies are consistent with ones we conducted, I have been unable to replicate their adult fate-mapping experiments (Figure 5, Figure 9, Figure 16, Figure 23).

Merkel cells of adult whisker follicles and paws were the primary focus of their fate-mapping studies, though their interpretations are also extended to Merkel cells of the body skin. In an attempt to estimate Merkel cell turnover, this group generated $K18^{CreER}$ mice and crossed them to a $ROSA^{YFP}$ reporter. Adolescent mice from this cross were subjected to a tamoxifen-induced pulse-chase study in which tamoxifen was administered once at day 0 and tissue analyzed 7 or 21 days later. K18 is a cytokeratin that in adult skin is exclusively expressed in Merkel cells where it overlaps ~100% with K8+ (Figure 30; Table 1) (Perdigoto et al., 2014a). In this way, the experiment was designed to label as many mature Merkel cells as possible and then estimate how long these Merkel cells persisted, providing an approximate rate of Merkel cell replacement. Recombination is inefficient in this system, with only 17% of whisker follicle K8+ cells expressing YFP 7 days after administration of a relatively high dose of tamoxifen (15 mg/mouse). Twenty-one days after tamoxifen was administered, the authors quantified a decrease in the proportion of K8+YFP+/K8+ cells (9%) with no change in overall Merkel cell number. Their ensuing interpretation was that K18+ whisker follicle Merkel cells are lost and replaced by a K18-progenitor. I did not observe this replacement with either my $Atoh1^{CreER-T2/+};ROSA^{LacZ}$ fate-mapping or $Atoh1^{CreER-T2/+};ROSA^{DTA}$ cell ablation experiments (Figure 6, Figure 8), and this rate of replacement is inconsistent with the EdU incorporation studies I performed (Figure 17; Figure 22). These mice became commercially available, allowing us to directly test this inconsistency by crossing them to both $ROSA^{YFP}$ and $ROSA^{tdTomato}$ reporters. I quickly noted an aberrant expression of both YFP and tdTomato reporter in this system; many K18- cells were YFP+ and/or tdTomato+, respectively. This can actually be seen in images

presented by Van Keymeulen et al., as two YFP+ cells can be seen outside the Merkel cell boundaries, contrary to the author's claim. Expression of these reporters was dependent on tamoxifen administration, indicating that Cre expression is unfaithful to its intended driver. In the body skin this expression was robust enough to preclude distinguishing apart individual cells in *K18^{CreER};ROSA^{tdTomato}* mice, limiting my analysis to the whisker follicles. Even so, I did not register a statistically significant decrease in K8+tdTomato+/K8+ or K8+YFP+/K8+ cells at the time points they performed (7 and 21 days) or even when extended to 9 weeks (Figure 16). Incredible variability between mice may have attributed to the significant difference they noted, as well as incomplete quantification. Their analysis involved grouping all K8+ cells from multiple mice together, instead of quantifying the proportion of reporter-expressing K8+ cells for each individual and taking that average. For these reasons, most significant of which is the unfaithful *K18-Cre* expression in this model, this experiment is insufficient to demonstrate turnover of whisker follicle K18+ cells.

Hair follicle bulge stem cells were hypothesized by Van Keymeulen et al. to be the likely progenitor population replacing whisker follicle Merkel cells, which they tested by fate-mapping *K15^{CrePR};ROSA^{YFP}* mice. Unlike their experimental approach with *K18^{CreER}* mice, application of the Cre-inducer RU486 was given chronically for either 5 or 21 days, not as a pulse chase. An increase in K8+YFP+/K8+ cells was noted from 5 days (4.5%) to 21 days (22.3%) during this exposure to RU486. While K15 is not expressed at significant levels in Merkel cells (Haeberle et al., 2004; Moll et al., 1993; Van Keymeulen et al., 2009), it is possible that small amounts of the transcript are made, inducing recombination and YFP expression. This would be difficult to detect by K15 immunostaining and could easily be missed in gene expression studies. If this is the case, the increase in YFP+K8+ cells in *K15^{CrePR};ROSA^{YFP}* mice over time could be attributed to recombination of more original K8+ Merkel cells, instead of their replacement. A pulse-chase study of *K15^{CrePR};ROSA^{YFP}* mice and/or a more sensitive gene expression analysis for *K15* transcript in Merkel cells would be required to verify this possibility.

In support of this possibility are results from modified nucleoside incorporation experiments performed in this same manuscript by Van Keymeulen et al. BrdU was administered to mice for 10 days and Merkel cells of the whisker follicle and glabrous skin of the paw analyzed for its incorporation. No K8+BrdU+ cells were found after this exposure paradigm, consistent with my EdU incorporation data (Figure 17; Figure 22) and indicating that not only

are K8+ cells quiescent, they were not replaced by any progenitor during this period of time. Therefore, the 4.5% of K8+YFP+/K8+ cells observed in *K15^{CrePR};ROSA^{YFP}* mice only 5 days post-initial RU486 exposure cannot be the result of their replacement by K15+K8- cells.

As K15+ hair follicle progenitors are not present in glabrous skin of the paw, Van Keymeulen et al. hypothesized that the replacement of these Merkel cells is the responsibility of resident K14+ epidermal progenitors. Similar to their approach with *K15^{CrePR};ROSA^{YFP}* mice, *K14^{CreER};ROSA^{YFP}* mice were generated and given persistent exposure to tamoxifen, specifically by its injection once every 3 days for 30 days. Within the glabrous skin of the paws, 25% of K8+ Merkel cells were noted to be YFP+ after this extended tamoxifen treatment. I have performed extensive fate-mapping with these *K14^{CreER}* mice crossed to the more sensitive and robust *ROSA^{tdTomato}* reporter and have been unable to demonstrate the presence of any tdTomato expression in K8+ Merkel cells past E14.5 (Figure 5; Figure 9; Figure 23). However, my analysis has been primarily focused on Merkel cells of the hairy skin. While it is possible that the maintenance of glabrous paw Merkel cells is different than that of hairy skin, this is unlikely considering the infrequency of Merkel cell production in adulthood (Figure 22). A careful analysis of the image presented by Van Keymeulen et al. illustrating a K8+YFP+ cell raises concern and a possible explanation. K8+ Merkel cells are entirely surrounded by K14+ epidermal cells and their close cellular membrane association may lead to the false appearance of YFP+K8+ co-localization. This is brought into greatest relief when the most basal side of the K8+ Merkel cell depicted is scrutinized, as it is not directly adjacent to any K14+ cell and is significantly less yellow (which denotes K8+Cy3 and YFP+ co-localization) than the other cellular boundaries. I am concerned that the double-labeled cells quantified may therefore be inaccurate. Repeating this experiment with *K14^{CreER}* mice crossed to a nuclear-localized reporter would provide a much cleaner and crisp image to demonstrate cellular recombination.

While the embryonic fate-mapping and conditional gene deletion studies performed in this manuscript were of great importance to assist with our understanding of Merkel cell genesis, the experimental approaches and interpretations performed in adulthood are less satisfying. Touch dome Merkel cells were analyzed only in embryonic studies and are conspicuously and unfortunately absent in their adult experiments. For the reasons described above, I am unconvinced that adult Merkel cells arise from either K15+ or K14+ progenitors or are replaced with the frequency they suggest.

5.2.2 Woo et al., 2010 - *Identification of epidermal progenitors for the Merkel cell lineage*

In contrast to the focus of whisker follicle and paw Merkel cells in Van Keymeulen et al., this paper from Dr. Owens' lab studied the contribution of epidermal progenitors to maintenance of touch dome Merkel cells (Woo et al., 2010). Here, Woo et al. performed skin grafting experiments combined with K14-lineage tracing to compare the skin regeneration contributions of touch dome keratinocytes and their progenitors to that of interfollicular keratinocytes. Their findings are the first of a small handful that demonstrate the unique potential of keratinocyte progenitors within the touch dome compartment.

Expanding on modified nucleoside incorporation experiments performed by Van Keymeulen et al., these authors performed a variety of different EdU incorporation experimental paradigms to assay proliferation in the touch dome compartment. The first very quick pulse of EdU (taken 1hr after a single administered dose) resulted in labeling of only 'basal' touch dome keratinocytes (TDKCs), with no 'suprabasal' TDKCs or Merkel cells being EdU+. The authors do not illustrate their classification of 'basal' versus 'suprabasal', it is fair to presume that basal includes only keratinocytes directly above Merkel cells, while suprabasal refers to keratinocytes of the touch dome epithelia not in contact with Merkel cells. EdU is found in subrabasal TDKCs starting at three days after this single injection, though all Merkel cells are still BrdU-. Only after six EdU injections over three days followed by a 3-8 day chase are any K8+ Merkel cells found, which the authors attribute to proliferation of basal TDKCs. The actual number of Merkel cells counted in these analysis is unclear, as cell counts are presented as number of touch domes that have BrdU incorporation, without indicating the number of individual cells or animals represented at each time point. Between 30-100+ touch domes were counted at each time point, but since this is in sectioned skin and not in whole mount it is unclear if single touch domes are represented multiple times in these counts or how many cells were labeled in each touch dome. These data therefore make it difficult to interpret the frequency with which basal TDKCs proliferate. Also, since touch dome keratinocytes are not the only actively proliferating skin cell, it is possible that these nascent Merkel cells migrated from elsewhere, potentially the hair follicle bulge. Though the raw number and proportion of EdU+K8+/K8+ cells is not stated, it is likely that only 2 K8+ cells were found to incorporate EdU in all of these counts. All said, this set of EdU incorporation experiments supports the very slow rate of touch dome Merkel cell turnover I

propose, but does not independently pinpoint the progenitor responsible for Merkel cell generation.

Using a variety of touch dome and interfollicular epidermis (IFE) markers, the authors next sorted keratinocytes from *K14^{Cre};ROSA^{LacZ}* mice into TD (touch dome) and IFE^{ΔTD} (interfollicular epidermis minus touch dome) populations and then implanted them into the dorsal fascia of Nude mice. This approach allowed for the identification of graft (β-Gal+) versus host (β-Gal-) derived cells and analysis of touch dome specific progenitor contributions to skin regeneration. Both TD and IFE^{ΔTD} populations contained proliferative progenitors and were therefore able to reconstitute a grafted epidermis. Only grafts from TD populations contained β-Gal+K8+ Merkel cells, indicating that general IFE^{ΔTD} skin populations were not instructed to form Merkel cells as part of their regeneration. The authors concede that Merkel cells could have been included as part of the TD population grafts, and may not have been newly generated. Again, their presented quantifications complicate independent interpretations as they only report the number of touch domes seen in a certain number of sections, not the raw number of K8+ cells observed or the number of animals represented. Based on my EdU incorporation studies I find their interpretation likely, in that touch dome keratinocytes have a unique capacity to generate Merkel cells. Their experimental paradigms are probably much more reminiscent of a wounding response than of skin homeostasis, similarly to the shaving paradigm I described earlier.

These experiments illustrate the unique potential of touch dome keratinocyte progenitors to form Merkel cells in skin grafts after 4 weeks of transplantation. However, it remains unanswered whether these cells are also responsible for producing Merkel cells as part of skin homeostasis. Also unclear are how many progenitor populations exist within the boundaries of a touch dome and whether or not their generative potentials vary between homeostatic and injury states. There could be a handful of unipotent progenitors, each designated to produce a specific cell type; a bipotent progenitor, generating Merkel cells and touch dome keratinocytes; or multipotent progenitor(s), generating touch dome keratinocytes, Merkel cells, and interfollicular and/or follicular keratinocytes. These possibilities are not mutually exclusive. More targeted fate-mapping with touch dome-specific Cre-drivers would be necessary to distinguish between these possibilities.

5.2.3 Doucet et al., 2013 - *The touch dome defines an epidermal niche specialized for mechanosensory signaling*

This second paper from Dr. Owens' lab sought to answer the question of touch dome keratinocyte contribution to Merkel cell maintenance during skin homeostasis. His group generated *K17^{CreER-T2}* mice that allowed for selective manipulation of the touch dome keratinocyte lineage. Very similarly to the fate-mapping and selective cell ablation experiments I performed with the *Atoh1*+ Merkel cell lineage, Doucet et al. performed pulse-chase lineage tracing and selective cell ablation experiments with these mice, analyzing the contribution of K17+ cells to Merkel cell maintenance in body skin, whisker follicles, and glabrous paw skin.

Doucet et al. were unable to find appreciable levels of K17 protein in K8+ Merkel cells by immunostaining and performed their experiments with the understanding that no K8+ cells co-expressed K17. *K17^{CreER-T2};ROSA^{YFP}* mice were administered 2mg of tamoxifen once daily for four days and tissue retrieved 24 hrs, 1, 3, 7, or 12 weeks later. Twenty four hours after the last day of tamoxifen administration, 10.7% (3/28) of K8+ cells were already YFP+. This percentage increased incrementally to 33%, 50%, 81%, and 95%, respectively, at the proceeding time points. As 80% is the approximate recombination rate that they quantified in their K17+ touch dome keratinocytes, the authors concluded that Merkel cells persist for approximately 7-8 weeks, after which the entire Merkel cell population has been replaced. This linear increase in recombination also suggests a constant rate of replacement, independent of age or hair cycle stage. Moreover, the authors state that while few to no K8+YFP+ cells were found in the whisker follicles or glabrous paw skin of tamoxifen-treated *K17^{CreER-T2};ROSA^{YFP}* mice at 24 hrs, many were 'routinely found' at 7-12 weeks.

The stark contrast of the interpretations from Doucet et al. to the conclusions drawn in this dissertation is confounding. The regular and complete turnover of K8+ Merkel cells in 7-8 weeks is inconsistent with my EdU incorporation studies demonstrating limited loss of embryonic-born Merkel cells and only slight production of nascent Merkel cells during adult skin homeostasis. Personal communications with authors of this manuscript confirm that there was no experimental manipulation (ex. skin shaving, depilation, or wounding) that could easily reconcile these differences. *K17^{CreER-T2}* mice are not commercially available, so I have unfortunately been unable to independently repeat this experiment.

It seems most likely that these differences arose from technical issues. As stated above, it can be difficult to distinguish YFP expression as being present in adjacent keratinocytes or within K8+ cells. The images presented in this manuscript make clear the difficulty that was likely had while imaging and quantifying this sectioned tissue. Moreover, the quantifications themselves are relatively small, with only between 28-92 total K8+ Merkel cells quantified at each time point (n=3 mice/time point). This is far below the standard 100-500 K8+ Merkel cells per mouse I routinely quantify for co-localization experiments (n=2-4 mice/time point). If there were variable recombination efficiencies between mice, this may easily skew their data. A nuclear-localized reporter would assist with easier cell counts.

Prior to my EdU incorporation studies we interpreted our *Atoh1* fate-mapping data under the lens of this experiment. With an expected lifespan of 7-8 weeks, this suggested that Merkel cells were replaced in adulthood by *Atoh1*+ progenitors, and indeed I found a subpopulation of K8+ Merkel cells that co-expressed K17, albeit at much lower levels than the overlying keratinocytes (Figure 10). We inferred that a unipotent, likely *Atoh1*+*K17*+, progenitor was responsible for Merkel cell homeostasis. The recently acquired EdU birth-dating studies now make this an unlikely possibility. It is possible that all K8+ Merkel cells express K17, albeit at low protein levels that are difficult to detect, possibly lending a potential explanation if any leak is present in these *K17^{CreER-T2};ROSA^{YFP}* mice. Vehicle-treated mice were analyzed for YFP expression in the absence of tamoxifen only at the 24 hrs time point, where no YFP+ cells were reported. These mice may become more prone to aberrant YFP expression as the animals age, an untested possibility that could account for the incremental increase in recombined cells if reporter leak was present.

5.2.4 Xiao et al., 2015 - *Neural Hedgehog signaling maintains stem cell renewal in the sensory touch dome epithelium*

Led by Dr. Isaac Brownell, Xiao et al. describe the touch dome as a unique Hedgehog (Hh) responsive niche in murine interfollicular epidermis. The authors use the active Hh signaling reporter *Gli1* to mark and fate map the contributions of these touch dome keratinocytes during skin homeostasis. This is remarkably similar to the experiments performed by Doucet et al., as *K17* and *Gli1* appear to display complete overlap in touch dome keratinocytes.

Gli1^{CreER};ROSA^{YFP} mice were generated and 200mg/kg tamoxifen administered for 3 days to activate YFP reporter expression.

Two different experimental paradigms were performed in this fate-mapping model. First, *Gli1^{CreER};ROSA^{YFP}* mice were administered tamoxifen during early anagen (P23-P26) and tissue retrieved 9 days or 2 months later. At both time points, less than 10% of K8+ cells were YFP+. If there is complete overlap of the *K17* and *Gli1* cell populations in the touch dome, this data contradicts that described in Doucet et al., as they quantify >80% of K8+YFP+/K8+ cells by this time. Xiao et al. attribute the lack of increase in K8+YFP+ cell proportion in *Gli1^{CreER};ROSA^{YFP}* mice in this model due to the animals not having yet reached their next anagen (mice were P82 at the 2 month retrieval time point; the next anagen begins at P84). This data is consistent with mine, demonstrating limited turnover of the K8+ population during adulthood.

In their second fate-mapping experiment, *Gli1^{CreER};ROSA^{YFP}* and *Gli1^{CreER};ROSA^{LacZ}* mice of 2 to 4 months of age were administered tamoxifen as described above in addition to having their skin depilated. Tissue was retrieved at 3 months and 2 years post-depilation and >90% of the K8+ Merkel cell population was found to be reporter positive at both of these time points. The authors interpret this to be an effect of anagen induction, with new Merkel cells arising from *Gli1*+ touch dome keratinocytes only during hair follicle growth. The depilation procedure used in this particular experiment was aggressive; hair follicles were manually plucked from the skin followed by wax-strip depilation. As this procedure happened concomitantly with tamoxifen administration, it's likely that this wounding response either stimulated the production of K8+ Merkel cells from *Gli1*+ progenitors, consistent with what I observe with shaved skin, and/or this manipulation activated Hh signaling in Merkel cells, causing them to be *Gli1*+ without being replaced. If intermediate time points prior to 3 months post-induction had been analyzed, these possibilities could be distinguished. Likewise, the authors' interpretation of anagen induction initiating Merkel cell production could have been more cleanly tested by allowing *Gli1^{CreER};ROSA^{YFP}* mice that had not undergone depilation to pass through their next natural anagen. I would predict that the ~10% of K8+YFP+/K8+ cells stays constant all through adulthood, regardless of natural hair cycle stage. These experiments support the limited turnover of K8+ touch dome Merkel cells during early adulthood and potentially illustrate the contribution of touch dome keratinocytes to Merkel cell replacement after superficial skin injury.

5.2.5 Summary

The manuscripts described above performed a wide variety of experiments aimed to characterize progenitors replacing Merkel cells during adult skin homeostasis. Many inconsistencies existed between these reports, as Merkel cell progenitors have been described as *K14*-, *K15*-, *K17*-, *Gli1*- and/or *Atoh1*-expressing, but no epidermal cell type in the skin (yet found) has been shown to express all these factors. Though the full complement of reasons why these inconsistencies arose is not entirely apparent, a partial contributing factor was likely the central supposition that Merkel cells have finite lifespans. This hypothesis was supported by data suggesting oscillation of Merkel cell number with the hair cycle, a phenomenon that would presuppose the addition and subtraction of a large proportion of the Merkel cell population.

Having challenged and refuted the premise of Merkel cell number oscillation in adulthood, the work described here in my thesis demonstrates instead a relative stasis of Merkel cells within mammalian whisker follicles, paws, and body skin. Moreover, discovering that this rate of replacement can be increased following even mild skin injury lends some insight into how these discrepancies arose in the literature. Two specific variables should be carefully controlled for future experiments seeking insight into Merkel cell maintenance. First, investigators should be cautious with the manipulations done to the skin of their transgenic mouse models, as we show that even the slight injury of repeated skin shaving changes Merkel cell responsiveness in a way that no longer models normal skin homeostasis. Secondly, fate-mapping experiments performed on tissues whose cell bodies lie in very close apposition, such as skin, would benefit from reporters whose localization permits clearer boundaries to more easily distinguish between labeled and non-labeled cells. For example, a nuclear-localized reporter would enable easier discrimination of fluorescent signal between adjacent cells, a difficult task for even confocal microscopy when fluorescent reporters permeate entire cell bodies.

5.3 LOSS OF PROLIFERATIVE POTENTIAL IN *ATOHI1*+ LINEAL CELLS

Virtually all *Atoh1*⁺, K8⁺, K18⁺, and K20⁺ cells of the adult skin can be defined as fully differentiated and mature Merkel cells, which have been repeatedly demonstrated to be non-proliferative (Mérot & Saurat, 1988; Moll et al., 1996b; Vaigot et al., 1987; Woo et al., 2010). During embryogenesis however there exists a heterogeneity of skin *Atoh1*-lineal cells, which contain both mature and proliferative cell populations. Our embryonic fate-mapping and proliferative marker analyses are the first to demonstrate a proliferative capability of some *Atoh1*-lineal cells in the skin. It is still unclear whether all or only a subpopulation of *Atoh1*⁺ cells proliferate during embryogenesis, as well as the signaling pathways that initiate and terminate this capability.

Instrumental to our fate-mapping of *Atoh1*⁺ embryonic progenitors was titrating a dose of tamoxifen to persist for only 24 hrs after administration. This was tested by administering low doses of tamoxifen to E13.5 or E14.5 (prior to and at the onset of *Atoh1*⁺ expression in the skin, respectively) *Atoh1*^{CreER-T2/+}; *ROSA*^{LacZ} mice and analyzing the Merkel cell population for *LacZ* expression at E18.5. No K8+βGal⁺ cells were found in mice that received tamoxifen at E13.5 with a 10mg/kg dose of tamoxifen, confirming that this paradigm only labeled cells present within 24 hrs of exposure. Interestingly, many K8+βGal⁺ cells were found in the body skin of mice that received tamoxifen at E14.5, but in low densities. Labeled touch domes only had between 1-4 βGal⁺ cells per group, all of which were K8⁺. This suggests that the proliferative capacity of embryonic *Atoh1*⁺ progenitors is low, and possibly limited to only one or two cell divisions at most, but exclusively giving rise to Merkel cells. It is possible that these proliferative *Atoh1*⁺ cells act as a transit-amplifying population and not as true progenitors, though those classifications may be a semantic argument.

Our analysis using *Atoh1*^{GFP} mice to label embryonic *Atoh1*-expressing cells was critical in the identification of this proliferative subpopulation, as experiments by other investigators utilizing more mature Merkel cell markers (ex. K18) were unable to identify them (Bardot et al., 2013). It is likely that the initiation of Merkel cell differentiation markers (ex. K18, K20) occur in a similar time frame as factors that inhibit cell cycle progression. Expression of the cyclin-dependent kinase inhibitor p27^{Kip1} has been associated with withdrawal from the cell cycle and differentiation in progenitors of the organ of Corti that form *Atoh1*-lineal inner ear hair cells

(Chen & Segil, 1999). It is probable that *Atoh1*+ Merkel cell-lineal progenitors undergo a similar expression pattern of initiating cell cycle inhibitors soon after lineage specification, thereby permitting only limited proliferation of early-specified cells prior to their differentiation.

The loss of proliferative capacity in adult Merkel cells is likely a result of their lineage commitment and cell cycle withdrawal during embryogenesis, preventing any further transit amplifying activity. This is supported by experiments with *Atoh1*^{CreER-T2/+};*ROSA*sT mice in which the oncogenic small T (sT) antigen of MCPyV is expressed in *Atoh1*-lineal cells following tamoxifen administration (Shuda et al., 2015). The subsequent induction of sT expression in *Atoh1*-lineal cells only stimulated ectopic production of K8+ cells during embryogenesis, independently demonstrating a difference in proliferative potential between embryogenesis and adulthood. Whether or not a difference in environment may also contribute to the proliferative capacity of these *Atoh1* lineal cells is unclear, though would not be a mutually exclusive possibility. Ectopic expression of *Atoh1* did not appear to initiate the proliferation of ectopic *Atoh1*-expressing cells in our transgenic *K14*^{Cre};*Tet*^{Atoh1};*ROSA*^{rtTa} model, indicating that even in environmental niches that allow for Merkel cell persistence, proliferation is not induced.

Our most recent data and that from other labs reinforce the improbability that *Atoh1*+ progenitors persist in adult murine skin. If *Atoh1*+ cells were mitotically active, one would predict that sT expression in *Atoh1*^{CreER-T2/+};*ROSA*sT mice would have resulted in the production of many K8+ cells. Even removal of the cell cycle inhibitor p53 was insufficient to induce ectopic *Atoh1*-lineal cell production in combination with sT overexpression (Shuda et al., 2015). Nascent Merkel cells made during typical homeostasis in this model therefore likely arose from an *Atoh1*- progenitor. If these nascent cells follow a developmental pathway similar to that of embryonic-born Merkel cells, then there is likely a short window of time in which they have yet to withdraw from the cell cycle and may be susceptible to sT oncogenic influence. It would be interesting to overexpress the sT antigen in a homeostatic Merkel cell progenitor to determine if early differentiating Merkel cells retain proliferative potential, similarly to their embryonic counterparts. If they are proliferatively capable, a comparison of their progeny with cells of MCC tumors would be a noteworthy endeavor, as these cells could hold the potential to be a cell of origin for MCC.

5.4 IMPORTANCE OF TOUCH DOME ULTRASTRUCTURE

Touch domes can be readily identified both in sectioned and/or whole mount skin of multiple species, generally due to the comparatively large size of their associated guard hairs and columnar touch dome keratinocytes. These structures cause touch domes to protrude slightly from the plane of the skin, a facet that has been exploited in early research methods to study Merkel cell function (Burgess et al., 1974; Smith, 1967). Though guard hairs are the first hair type to be specified and their placodes are seen as early as E14.5, touch dome keratinocytes do not take their distinctive shape until after birth (Duverger & Morasso, 2009). The genetic and signaling cascades required for touch dome keratinocyte development have not been thoroughly studied, though more is becoming known about their development and function (Doucet et al., 2013; Woo et al., 2010; Xiao et al., 2014).

Touch dome keratinocytes are distinct from interfollicular and follicular keratinocytes as well as Merkel cells by their robust expression of K17 in adulthood (Moll et al., 1993). Though a subset of Merkel cells also express this cytokeratin, it is at levels appreciably lower than that of the overlying keratinocytes (Figure 10). It is possible that K8+K17+ cells represent nascent Merkel cells derived from touch dome keratinocyte progenitors during adulthood, or that heterogeneous K17-expression is present in the Merkel cell population with no functional effect or developmental correlation. Given that $9.2 \pm 2.6\%$ of K8+ cells in the whisker follicle co-express K17, but I found limited evidence of their replacement during adulthood, the latter hypothesis is most likely.

Adult K17+ touch dome keratinocytes are necessary for maintenance and survival of the SA1 afferent that innervates Merkel cells within touch domes (Doucet et al., 2013). The trophic factor derived from this cell population for SA1 survival is still unknown, as is the age at which this survival dependency initiates. SA1 afferents reach the touch dome in embryogenesis around the same age as Merkel cell formation, indicating its initial guidance and targeting is independent of Merkel cells or touch dome keratinocytes, though likely specific for guard hairs. Merkel cell loss in this K17+ conditional cell ablation study was likely due to a secondary effect of SA1 afferent death. It is therefore unclear whether touch dome keratinocytes also directly provide Merkel cell trophic support.

Merkel cells begin to form long before touch dome keratinocytes mature, though their numbers stabilize around the time of touch dome keratinocyte formation. As demonstrated here, Merkel cells are likely the longest-persisting post-mitotic epidermal cell population. Environmental stressors require the epidermis to undergo constant renewal, and the exception of Merkel cells from this rule is noteworthy. Merkel cells are positioned at the epidermal-dermal border, which is necessary for their mechanosensory function but also puts them in fairly close proximity to harmful external elements. It is possible that touch dome keratinocytes serve as a supplemental barrier between Merkel cells and the environment, providing a thicker cushion from the everyday epidermal stressors that require regular turnover of interfollicular keratinocytes.

In addition to protecting Merkel cells from general wear and tear, touch dome keratinocytes may also influence the mechanosensory properties of the Merkel cell-neurite complex. Touch dome keratinocytes connect to Merkel cells very tightly through desmosomes and must necessarily be the first point of contact for tactile stimuli. Indeed, indirect stimulation of Merkel cells by displacement of adjacent cells elicits mechanically activated currents within Merkel cells with only slight differences to that of direct stimulation (Ikeda et al., 2014). Though the phenotype is not as robust in murine skin, the columnar organization of keratinocytes results in the raised nature of touch domes, potentially enhancing the sensitivity of Merkel cell-neurite complexes (Burgess et al., 1974; English, 1974; Smith, 1967).

5.5 POTENTIAL INSIGHT INTO THE MERKEL CELL CARCINOMA CELL OF ORIGIN

Though the experiments in this dissertation still leave unconfirmed the precise identity and characteristics of adult Merkel cell progenitors, they illustrate their infrequent proliferation and limited Merkel cell replacement. Most importantly, the experiments outlined in Section 4 demonstrate that expression of *Atoh1* is alone sufficient to transform keratinocytes into Merkel cell-like cells, and this appears to limit their proliferative capacity. This work also re-emphasizes conclusions from previous studies illustrating the post-mitotic nature of Merkel cells. Together, these findings support the hypothesis that MCC tumors arise not from Merkel cells, but instead

from epidermal stem cells that lack cell cycle control and aberrantly express *Atoh1* and its downstream factors.

Soon after *Atoh1* expression is initiated, the proliferative potential of *Atoh1*-lineal cells is lost. Even driving the oncogenic small T antigen of MCPyV in combination with loss of p53 cannot override the post-mitotic nature of mature Merkel cells (Shuda et al., 2015). It still remains possible though that Merkel cell progenitors, who are most likely *Atoh1*- and are potentially bi- or multi-potent, have a very small window of time after *Atoh1* expression is initiated where they maintain proliferative capacity. If the MCPyV was driven in these progenitors and during this window cell cycle withdrawal overridden, these cells could still remain potential candidates for MCC origin. The heterogeneity of MCC tumors indicate that the cells of origin either have a great deal of multipotency and/or that multiple cell of origins may exist for this skin cancer. The decreasing likelihood of Merkel cells as the cellular source of MCC may also initiate a new discussion as to the most appropriate name for this carcinoma.

6.0 FUTURE DIRECTIONS

6.1 ARE HOMEOSTATIC AND MECHANICALLY-INDUCED NASCENT MERKEL CELLS ARISING FROM THE SAME PROGENITOR?

New touch dome-associated Merkel cells are generated during normal skin homeostasis, though at a much slower rate than had been previously estimated. This proportion of nascent Merkel cells is increased following skin shaving, likely the result of a response to skin wounding. Mitotically active progenitors must be responsible for producing these cells, as they can be identified by EdU incorporation. Though a central focus of my thesis has been to identify Merkel cell progenitors, this question has been left incompletely answered. Moreover, it is unclear whether the production of Merkel cells after skin shaving is the result of increased proliferation of homeostatic Merkel cell progenitors, or if a different progenitor population is recruited to also generate Merkel cells.

6.1.1 What is the contribution of *Gli1*+ progenitors to Merkel cell production?

Nascent K8+EdU+ Merkel cells in touch domes of mice that received EdU in their drinking water for 5 weeks were often found oriented perpendicular to the skin surface, wedged between the touch dome keratinocytes that sit above K8+ cells. These cells also had appreciably lower levels of K8 expression, indicating that they were recently specified. Skin that had been shaved or left unmanipulated both had cells of this appearance, indicating their presence is not the result of a wounding response in the skin. This suggests that touch dome keratinocyte progenitors may be at least one mitotically active source for Merkel cell maintenance.

To test this possibility, I am currently generating *Gli1^{CreER};ROSA^{YFP}* mice, as had been done by Xiao et al. (see section 5.2.4). Adolescent mice (beginning at P28) will receive EdU in

their drinking water for 5 weeks. One cohort (n=3 mice) will have their belly skin shaved with a straight razor once weekly, while the second cohort (n=3 mice) will be left unmanipulated. At the end of 5 weeks, skin from these mice will be retrieved and processed for YFP and EdU.

If *Gli1*⁺ touch dome keratinocytes contribute to the generation of Merkel cells during normal skin homeostasis, YFP+EdU+K8⁺ cells should be present in both shaved and unmanipulated *Gli1*^{CreER};*ROSA*^{YFP} mice. If *Gli1*⁺ touch dome keratinocytes only contribute to the generation of Merkel cells during normal skin homeostasis, but not to mechanically-induced Merkel cell production, YFP+EdU+K8⁺ cells should be present in similar proportions in both shaved and unmanipulated *Gli1*^{CreER};*ROSA*^{YFP} mice, while a number of YFP-EdU+K8⁺ cells would only be present in shaved belly skin. However, if *Gli1*⁺ touch dome keratinocytes do not contribute to the generation of Merkel cells during normal skin homeostasis, but only to mechanically-induced Merkel cell production, YFP+EdU+K8⁺ cells should only be present in shaved belly skin, while unshaved belly skin would have YFP-EdU+K8⁺ cells.

6.1.2 What is the contribution of hair follicle bulge progenitors to Merkel cell production?

Fate-mapping results from Van Keymeulen et al. suggest that K15⁺ hair follicle bulge progenitors contribute to Merkel cell production during homeostasis (see section 5.2.1). Though their results were limited to whisker follicles, they projected their interpretations to maintenance of Merkel cells around touch domes surrounding guard hairs. We saw no evidence of K8+EdU+ cells migrating from below the touch dome during adult maintenance or in a response to skin shaving. It is possible though that K8 expression could be initiated after cell migration.

To analyze the contribution of hair follicle bulge progenitors to Merkel cell maintenance, we obtained Hairless mice that lack hair follicle bulge progenitors, resulting in their loss of cycling hair follicles. Adolescent (P28) Hairless mice were administered EdU in their drinking water for 5 weeks and one group of mice had their belly skin shaved once weekly (n=3 mice) while the second group was left unmanipulated (n=2 mice). Tissue was retrieved after 5 weeks of exposure to EdU. This portion of the experiment has been completed, though tissue not yet analyzed.

If hair follicle bulge progenitors contribute to the generation of Merkel cells during normal skin homeostasis, EdU+K8+ cells should be absent in both shaved and unmanipulated Hairless mice. If hair follicle bulge progenitors only contribute to the generation of Merkel cells during normal skin homeostasis, but not to mechanically-induced Merkel cell production, EdU+K8+ cells should be present in similar proportions in both shaved and unmanipulated Hairless mice. However, if hair follicle bulge progenitors do not contribute to the generation of Merkel cells during normal skin homeostasis, but only to mechanically-induced Merkel cell production, EdU+K8+ cells should only be present in shaved belly skin, while unshaved belly skin would have no EdU+K8+ cells. If hair follicle bulge progenitors have no contribution to Merkel cell generation or maintenance, there should be approximately the same percentage of K8+EdU+/K8+ cells in shaved and unmanipulated mice that we saw with similarly treated B6 mice, as described in Figure 22.

6.1.3 What is the contribution of *Atoh1*+ cells to homeostatic Merkel cell production?

Our original interpretations from *Atoh1*^{CreER-T2/+};*ROSA*^{LacZ} fate-mapping and *Atoh1*^{CreER-T2/+};*ROSA*^{DTA} selective cell ablation experiments indicated that *Atoh1*+ progenitors produce Merkel cells during skin homeostasis. Though it is clear from our subsequent experiments with *Atoh1*^{CreER-T2/+};*ROSA*^{DTA} mice whose skin was shaved that mechanically-induced Merkel cells do not arise from *Atoh1*+ progenitors, it is still possible that production of Merkel cells during normal skin homeostasis may be from *Atoh1*+ cells.

To test this possibility, I am generating *Atoh1*^{CreER-T2/+} mice crossed to ROSA reporter lines (either *ROSA*^{YFP}, *ROSA*^{mCherry}, or *ROSA*^{ZsGreen}) and administering EdU in the drinking water for 5 weeks. The intent with testing these three reporter lines is to determine which one is most faithful to tamoxifen-induced Cre activity and is easiest to visualize. One cohort of mice (n=3 mice) will have their belly skin shaved once weekly while a second cohort of mice will be left unmanipulated (n=3 mice). Tissue will be retrieved after 5 weeks of exposure to EdU.

If *Atoh1*+ progenitors contribute to Merkel cell maintenance during skin homeostasis, reporter+K8+EdU+ cells should be present in the belly skin of unmanipulated mice. If this is true and normal proliferative activity is maintained even in the presence of shaving, the same

proportion of reporter+K8+EdU+ cells should be present in the belly skin of unmanipulated mice, as well as a larger proportion of reporter-K8+EdU+ cells.

6.2 WHAT SIGNALING PATHWAYS ARE REGULATING MECHANICALLY-INDUCED MERKEL CELL PRODUCTION?

Though we repeatedly demonstrate that skin shaving stimulates an increased production of Merkel cells in body skin, the precise mechanism governing this event is unclear. We also have not verified that shaving skin is stimulating any inflammatory or wound response. Mild abrasive skin treatments with sandpaper and tape-stripping have been shown to initiate an inflammatory response that leaves the dermis unaffected in Hairless mice (Ekanayake-Mudiyanselage et al., 1998; Hardy et al., 2003). Specifically, investigators found an acute effect of increased proliferation, downregulation of K14, and upregulation of K6 and involucrin 24-48 hours following rubbing skin with sandpaper. To test if these expression and proliferative changes occur following our skin shaving paradigm in both B6 and Hairless mice, I took n=2 adolescent mice of each background and shaved their belly skin with a straight razor, as described previously. These mice also received EdU in their drinking water from the time of shaving until 24 hours later when mice were sacrificed and tissue retrieved. The tissue has yet to be analyzed, but I predict that shaved skin will have a higher percentage of cells incorporating EdU, lower expression of K14, and higher expression levels of involucrin and K6. This experiment will verify if skin shaving is inducing an inflammatory response.

Secondly, we hope to identify the exact signaling mechanism inducing this increase in Merkel cell production. To investigate this, a cohort of *Atoh1^{GFP}* mice will have their belly shaved once weekly for four weeks (as described previously), while a second will remain unmanipulated. Using GFP to identify Merkel cells, multiple touch domes will be microdissected from mice and digested. RNA Seq will be performed on pooled touch domes and an expression analysis done between the two cohorts. Genes upregulated in shaved skin relative to unmanipulated skin will be candidates for signaling pathways regulating Merkel cell production. Localization of the source of these factors can be done by sorting Merkel cells (*Atoh1^{GFP}* mice) or touch dome keratinocytes (*Gli1^{CreER};ROSA^{YFP}* mice) prior to digestion and RNA Seq. Gene

candidates for increasing Merkel cell production will first be verified by immunostaining or *in situ* in shaved touch domes, and then their expression can be manipulated in touch domes with available transgenic mice or viral gene silencing.

6.3 HOW CAN WE PERFORM LIVE IMAGING WHILE MAINTAINING TRUE HOMEOSTATIC SKIN CONDITIONS?

The live imaging experiments described in this dissertation all involve shaving of belly skin with a straight razor to expose touch domes for imaging. While this approach allowed for the serendipitous demonstration of putative abrasive skin wounds influencing Merkel cell production, it creates a problem for the usefulness of this tool in our *in vivo* analysis of Merkel cells and their afferents during normal skin homeostasis. Specifically, we aim to use this technique to visualize the interaction between Merkel cells and SA1 neurites after denervation and conditional gene deletion in these two cell populations. Additional attempts have been and are being made at visualizing the skin with limited interruption to skin homeostasis. Other investigators use a chemical depilatory cream to remove hair from above the ear for similar *in vivo* imaging (Pineda et al., 2015). I have tested two different brands of depilatory agents, Surgicream and Nair. Both creams are effective for hair removal and enable visualization of GFP+ cells in *Atoh1^{GFP}* mice. However repeated application resulted in robust skin inflammation and even epidermal peeling. Touch dome visualization became very difficult as a result of this skin inflammation, though experiments are ongoing to determine whether this alters Merkel cell homeostasis. Another option for these depilatory agents would be to test them at varying dilutions in water to find a concentration that was sufficient for hair removal but did not induce skin inflammation after multiple exposures.

To enable touch dome visualization with no required skin manipulation, we are currently crossing transgenic mice with fluorescent reporters in Merkel cells (*Atoh1^{GFP}*) and the SA1 afferent (*Thy1^{CreER-EYFP}*) to Hairless mice. These mice form body hair normally and then undergo progressive hair loss in adolescence, exposing the skin while hopefully presumptively undergoing normal touch dome maintenance. These mice should be generated and available for testing within the next 4 months.

7.0 CONCLUSION

A unique and intriguing population of skin cells, Merkel cells have puzzled scientists for decades. My thesis has comprehensively studied the role of *Atoh1* lineal cells in the formation and maintenance of embryonic and adult Merkel cells, culminating in my conclusion that embryonic *Atoh1*⁺ progenitors produce Merkel cells that independently persist for the lifetime of an animal. I tested many discrepancies in the literature that would refute this theory and have shown that their results and interpretations were often limited by technical approaches. The work described here provides additional and valuable insight into the developmental profile of this enigmatic cell population.

APPENDIX A

THE ROLE OF *ATOHI* IN MERKEL CELL DEVELOPMENT

Work from our lab and others have demonstrated that the transcription factor *Atoh1* plays multiple roles during its period of expression in inner ear hair cells (Cai et al., 2013; Chonko et al., 2013). Inner ear hair cells share many characteristics to Merkel cells including their mechanotransductive properties and overlapping expression of some developmentally required genes, including *Atoh1*. Though the role for *Atoh1* in hair cell specification was well established (Bermingham et al., 1999), additional and separable roles for hair cell survival and differentiation after lineage specification were also shown to exist. New genetic tools for conditional gene excision studies enabled the identification of these later roles that had previously been hidden by the earlier requirement of *Atoh1* for lineage specification. With these same tools I have begun to test the possibility of differential requirements for *Atoh1* during Merkel cell lineage specification, differentiation, and survival.

A.1 ROLE OF *ATOHI* IN MERKEL CELL DEVELOPMENT DURING EMBRYOGENESIS

To test if Merkel cells have differential requirements for *Atoh1* after their specification, I selectively ablated *Atoh1* after lineage specification with *Atoh1*^{CreER-T2/flox}; *ROSA*^{tdTomato} (*Atoh1*^{CKO}) mice. The intent with these experiments was to administer tamoxifen at later stages

of Merkel cell lineage development and selectively ablate *Atoh1* in Merkel cells after lineage specification, while Merkel cells are maturing and being maintained.

Tamoxifen was administered to pregnant females at E15.5, after *Atoh1*⁺ Merkel cells are first seen in the skin, and embryos retrieved at E18.5 or P3. Recombined *Atoh1*-lineal cells here are identifiable by expression of the *ROSA* reporter tdTomato. At E18.5 and P3, many tdTomato⁺ cells were still present in touch domes of the skin, indicating their persistence for 3 days after *Atoh1* was conditionally deleted (Figure 35B, C). *Atoh1*^{CKO} mice had fewer tdTomato⁺ cells per touch dome than their *Atoh1*^{CreER-T2/+};*ROSA*^{tdTomato} siblings (E18.5: 14.4 vs 26.4±0.5 tdTomato⁺ cells/TD; P3: 8.3±1.5 vs 18.1±2.2 tdTomato⁺ cells/TD; n=1-2 mice/genotype; Figure 35A-C). *Atoh1* is therefore not required for cell survival after specification. It is unclear whether this slight decrease in cell number is due to fewer *Atoh1*⁺ cells being specified and/or lack of proliferation of *Atoh1*⁺ progenitors. To test the hypothesis that *Atoh1*-lineal cells cannot divide without *Atoh1* expression we have similarly generated *Atoh1*^{CKO} mice that received tamoxifen at E15.5 and administered EdU at E16.5. Mice were retrieved 4 hrs later, and the percentage of tdTomato⁺EdU⁺ cells will be quantified. If *Atoh1* is required for progenitor proliferation, we should see an absence of tdTomato⁺EdU⁺ cells in *Atoh1*^{CKO} mice.

Immunostaining for the early Merkel cell marker K8 revealed that tdTomato⁺ cells in *Atoh1*^{CKO} mice were all K8⁺, similarly to their control siblings. K8⁺ signal intensity was much lower in cells of *Atoh1*^{CKO} mice and the morphology of these persisting *Atoh1*-lineal cells was noticeably different, with cells having many long cytoplasmic extensions and smaller, less rounded cell bodies (Figure 35A-B''). These morphometric differences are currently being quantified. Interestingly, the developmental transcription factor *Isl1* is still expressed in *Atoh1*-lineal cells of *Atoh1*^{CKO};*ROSA*^{tdTomato} mice at E18.5 (Figure 35D-E'''). Though a downstream target of *Atoh1*, it is possible that *Isl1* regulation becomes independent of *Atoh1* after initial specification. Together these experiments illustrate that *Atoh1* is not required for Merkel cell survival, but does play a role in cell differentiation during late embryogenesis. We have flow-sorted and performed RNA sequencing on tdTomato⁺ cells from *Atoh1*^{CKO};*ROSA*^{tdTomato} and *Atoh1*^{CreER-T2/+};*ROSA*^{tdTomato} mice to more carefully determine the genetic consequences of *Atoh1* excision during Merkel cell lineage maturation.

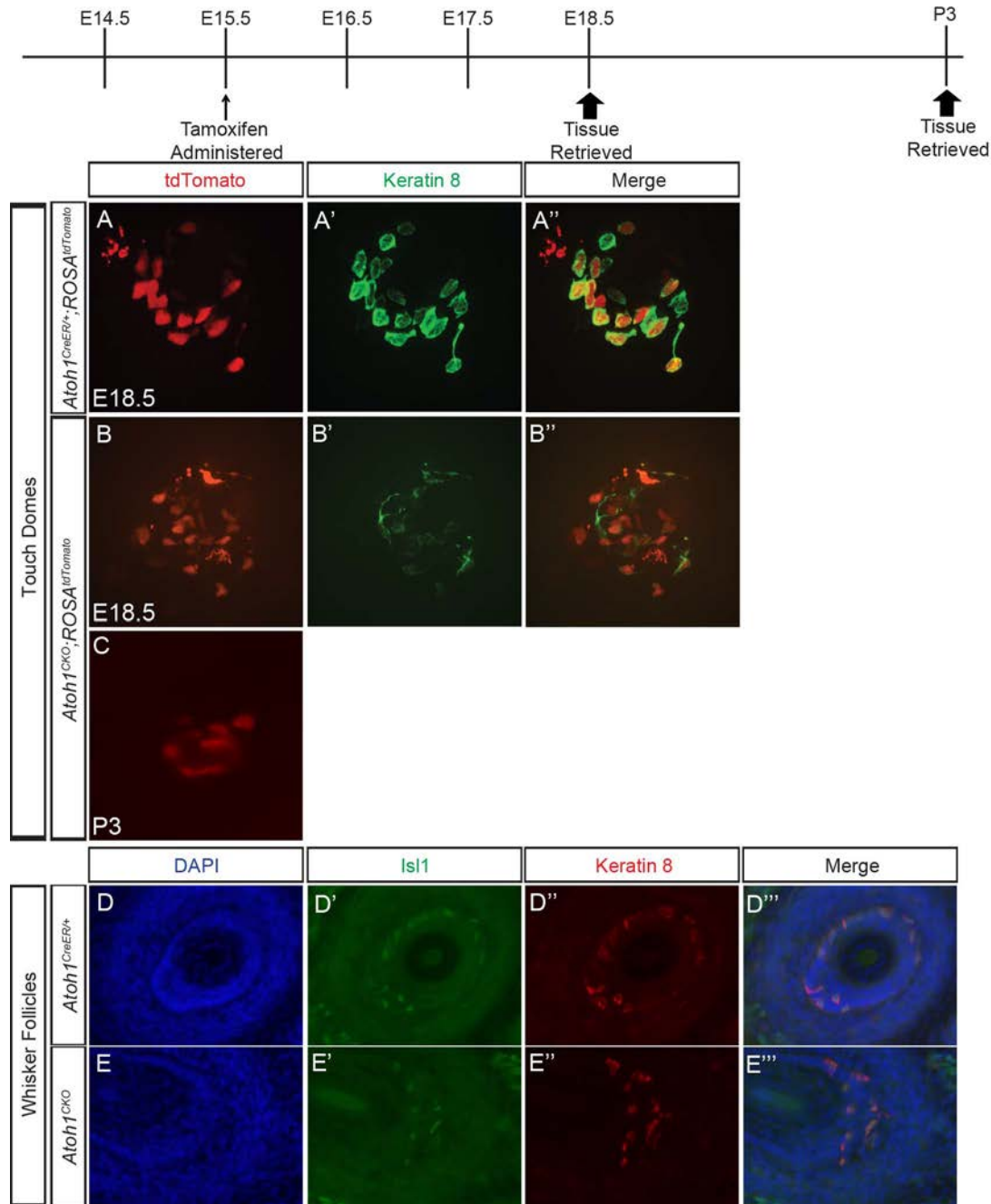


Figure 35. *Atoh1* is necessary for Merkel cell differentiation but not survival after lineage specification *Atoh1*^{CKO} and *Atoh1*^{CreER-T2/+} mice received tamoxifen at E15.5 and were retrieved at E18.5 or P3. Whole mount skin immunostained for K8 (A', B', green) merged with endogenous tdTomato fluorescence (A, B, C red) in *Atoh1*^{CreER-T2/+}; *ROSA*^{tdTomato} (A-A'', control) and *Atoh1*^{CreER-T2/flox}; *ROSA*^{tdTomato} (B-C, CKO) mice at E18.5 (A-B'') and P3 (C). Sectioned whisker follicles of E18.5 *Atoh1*^{CKO} and *Atoh1*^{CreER-T2/+} mice immunostained for Isl1 (D', E', green) and K8 (D'', E'', red) counterstained for DAPI.

A.2 ROLE OF *ATOH1* IN MERKEL CELLS DURING ADULTHOOD

To determine if *Atoh1* plays a role in Merkel cell survival and/or maintenance during adulthood we used the same conditional deletion approach with *Atoh1*^{CKO} mice. Tamoxifen was administered at P28 to CKO and control (*Atoh1*^{CreER-T2/+}) mice (250mg/kg for 3 days) and tissue retrieved one week, 6 weeks, 3 months, and 9 months post-tamoxifen administration (n=1-3 mice/genotype/time point). *Atoh1* excision was confirmed by immunostaining at 3 months post-tamoxifen administration (>100K8+ cells/mouse, n=1 mouse/genotype; Figure 36A-B’’). There was no sizeable decrease in the number of K8+ Merkel cells per touch dome out of the range of normal variability at one week post-tamoxifen administration, nor any over difference in cell morphology (Back: 11.3±0.8 vs 11.1 K8+ cells/TD; Belly: 19.7±0.8 vs 14.6 K8+ cells/TD, CKO vs control). Three months post-tamoxifen administration the average number of K8+ cells per touch dome significantly decreased to 37±5.0% and 63±1.4% of control mice in back and belly skin, respectively (Back: 4.3±0.8 vs 11.5±0.3 K8+ cells/TD; Belly: 11.7±0.3 vs 18.3±1.4 K8+ cells/TD, CKO vs control). At nine months post-tamoxifen administration this subpopulation of K8+ Merkel cells was maintained and did not undergo a further decrease, still at 38±5.7% and 72±10.9% of control mice in the back and belly skin, respectively (Back: 4.0±0.4 vs 10.4 K8+ cells/TD; Belly: 7.8 vs 5.7±0.9 K8+ cells/TD, CKO vs control). Therefore, two subpopulations of Merkel cells appear to exist that vary on their dependence for continued *Atoh1* expression.

To determine if *Atoh1* was required for the expression of canonical Merkel cell markers during adult cellular maintenance we immunostained for various markers in the body skin of CKO and control mice three months post-tamoxifen administration. K8+ Merkel cells retained expression of K18, the vesicular glutamate transporter VGLUT2, remained innervated by NF200+ afferents, and incorporated the small styryl dye FM1-43 (Figure 37). This indicates that *Atoh1* is no longer required for the expression of these markers or maintenance of innervation in adult Merkel cells. It remains to be tested whether or not these Merkel cell-neurite complexes are functional in the absence of *Atoh1*, though the decrease of Merkel cell density in touch domes may be a complicating factor in determining the role of this transcription factor in Merkel cell function as a mechanoreceptor.

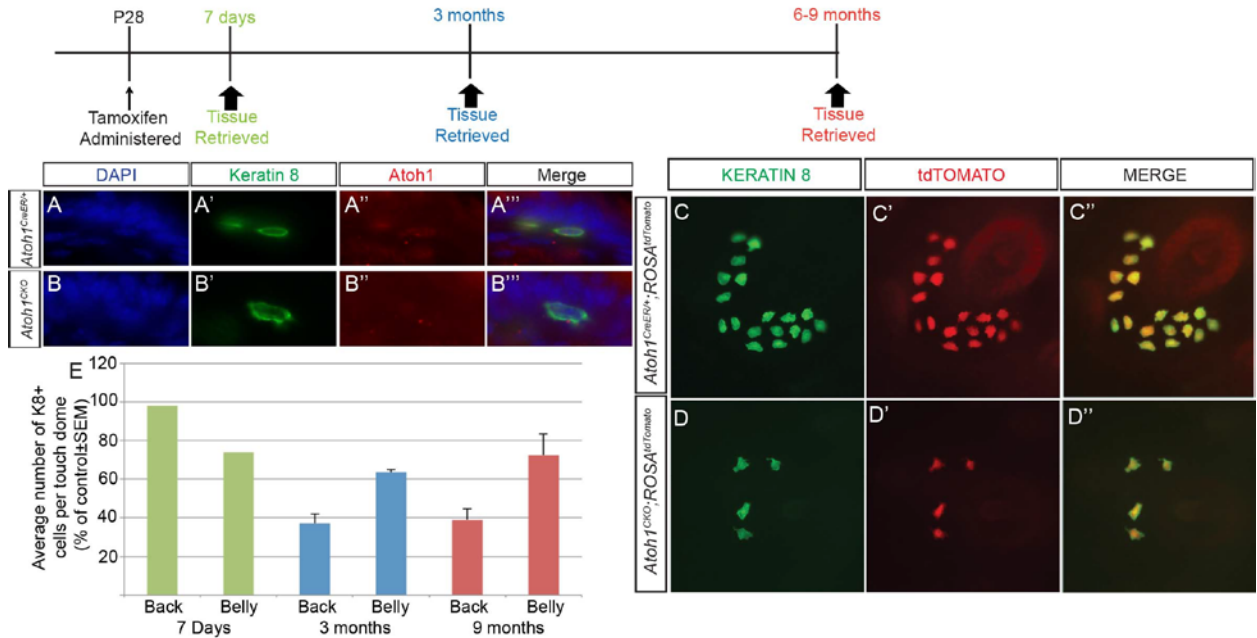


Figure 36. A subpopulation of K8+ Merkel cells persist after *Atoh1* excision P28 *Atoh1*^{CKO} and *Atoh1*^{CreER-T2/+} mice were administered tamoxifen on 3 consecutive days and tissue retrieved 7 days, 3 months, or 6-9 months later. Efficient gene excision was confirmed by immunostaining for K8 (A', B', green) and *Atoh1* (A'', B'', red) and counterstained with DAPI. (C-D'') Whole mount back and belly skin was immunostained for K8 (C, D, green) and merged with endogenous tdTomato fluorescence (C', D', red). (E) The average number of K8+ cells per touch dome was quantified and given as a percent of control mice at these time points (mean ± SEM, n=1-3 mice/genotype/age).

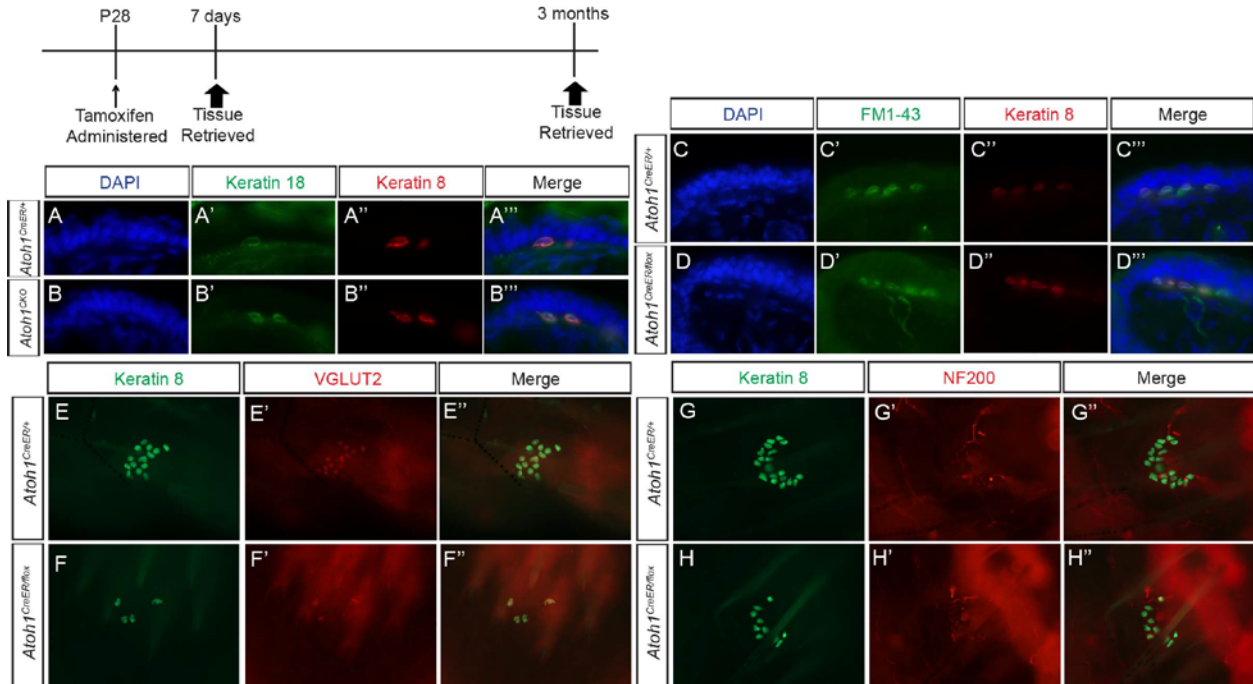


Figure 37. Persisting K8+Atoh1- cells still retain expression of multiple canonical Merkel cell markers and remain innervated Body skin from P28 *Atoh1^{CKO}* and *Atoh1^{CreER-T2/+}* mice administered tamoxifen on 3 consecutive days and tissue retrieved 7 days or 3 months later were co-immunostained for K8 (A'', B'', C'', D'', E, F, G, H) and K18 (A', B'), VGLUT2 (E', F'), and NF200 (G', H'). The styryl dye FM1-43 (C', D') was also administered and shown to be present in mice of both genotypes (C-D''').

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