GENETIC AND ENVIRONMENTAL FACTORS THAT CONTROL MERKEL CELL DEVELOPMENT AND SURVIVAL

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Merkel cells are mechanosensory cells that detect light-touch stimuli including shape, texture, and size. Merkel cells are derived from the epidermis, are innervated by SAI neurons, and require the transcription factor *Atoh1* for their development. In this dissertation, we aim to identify genetic and environmental factors that promote Merkel cell production during embryogenesis and adulthood. First, we interrogated the role of Notch signaling during Merkel cell development by manipulating elements of the Notch signaling pathway in transgenic mice. We found that canonical Notch signaling inhibits Merkel cell specification during embryogenesis. Second, we used a live animal imaging technique to track how touch domes change over time as well as how long Merkel cells survive in adult mice. We found that Merkel cells persist for longer than previously thought. Third, we used a live imaging technique to visualize the interaction between SAI neurons and Merkel cells, and found that direct contact from SAI neurons is not required for Merkel cell production. Finally, we tested how skin abrasions affect Merkel cell number, and we found that skin abrasions decrease Merkel cell number in hairless mice, but not hairy mice; suggesting the hair follicle is required to restore or maintain Merkel cell number after injury. Together these findings give insight into how Merkel cells develop and how they are replaced in adult mice. Understanding the environmental and genetic factors that promote Merkel cell development can be helpful for studying Merkel cell carcinoma, a rare but deadly cancer which is derived from skin progenitors and shares characteristics of Merkel cells.

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PREFACE

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
ANOVA	Analysis of variance
Atoh1	Atonal homolog 1
BDNF	Brain-derived neurotrophic factor
β-gal	β-galactosidase
bHLH	Basic helix-loop-helix
BMP	Bone morphogenetic protein
CKO	Conditional knockout
CNS	Central nervous system
Cre	Cre recombinase protein
CreER	Tamoxifen-inducible Cre recombinase
Cy3	Cyanine dye
D	Day, *refers to days elapsed since the start of a trial*
DAPI	4’,6-diamidino-2-phenylindole
ddH2O	Double distilled water
DTA	Diphtheria toxin
E	Embryonic day, *refers to embryonic age*
Eda	Ectodysplasin A
Edar	Ectodysplasin A receptor
EdU	5-ethynyl-2’-deoxyuridine
En1	Engrailed-1 protein

ET	Epidermal thickness
EYFP	Enhanced yellow florescent protein
Ezh1/2	Enhancer of zeste protein 1/2
flox	LoxP flanked
FGF	Fibroblast growth factor
Fz6	Frizzled 6
Gli1	GLI family zinc finger 1
HCl	Hydrocloric acid
H&E	Hematoxylin and eosin stain
Hes1	Hes family bHLH transcription factor 1
Hr^{Hr}	Hairless gene
HTMR	High-threshold mechanoreceptor
Isl1	ISL LIM homeobox 1
K	Keratin
KT	Keratinized layer thickness
LacZ	Gene coding for the protein β-galactosidase
LoxP	Locus of X(cross)-over in P1, *recognized by Cre recombinase*
LTMR	Low-threshold mechanoreceptor
MBP	Myelin basic protein
MC	Merkel cell
MCC	Merkel cell carcinoma
NICD	Notch intracellular domain
NF200	Neurofilament 200

NFH	Neurofilament H
NT	Neurotrophin
OCT	Optimum cutting temperature
OE	Overexpressing
P	Postnatal day, *refers to age*
Pax6	Paired box 6
PBS	Phosphate buffered saline solution
PBS-T	Phosphate buffered saline solution with 0.3% Triton-X
PCR	PCR polymerase chain reaction
PFA	Paraformaldehyde
PNS	Peripheral nervous system
PRC2	Polycomb repressive complex 2
RA	Rapidly adapting
Rb	Retinoblastoma protein
Rbpj	Recombining binding protein suppressor of hairless
RT-PCR	Reverse transcriptase polymerase chain reaction
SAI	Slowly adapting, type 1
SEM	Standard error of the mean
Shh	Sonic hedgehog protein
Sox2	SRY-Box 2
SLICK	Single neuron labeling with inducible cre knockout
TD	Touch dome
tdTomato	tandem dimeric red florescent protein

VGLUT2	Vesicular glutamate transporter 2
WF	Whisker follicle
WFD	Whisker follicle depth
WFW	Whisker follicle width
YFP	Yellow florescent protein

1.0 INTRODUCTION

1.1 THE BIOLOGY OF THE SKIN

1.1.1 Anatomy and function

The skin is a complex and dynamic organ that covers the entire body surface (Robbins 1997). There are three layers that make up the skin. The outermost layer is the epidermis, an avascular layer made up of stratified epithelial keratinocytes. The middle layer is the dermis, which is comprised of vascularized dense connective tissue with sensory neurons terminating at and within the epidermis. The subcutaneous layer is the deepest layer, comprised of mostly fatty tissue.

The epidermis is the most superficial layer of the skin, directly exposed to the environment. It is made up of a stratified squamous epithelium of specialized skin cells. These cells are called keratinocytes because of their high expression of keratin proteins (Robbins 1997). Epidermal stem cells reside at the basal layer. These stem cells constantly generate new keratinocytes, which migrate upward and keratinize as they differentiate. Mature keratinocytes make up the majority of the epidermis, which is 75-150μm thick in humans, though even thicker in areas that receive additional friction, such as the palms of the hands and soles of the feet (Wong et al. 2016). The most superficial keratinocytes die, leaving a barrier of keratin protein filled cells on the outside of the skin. The epidermis also contains pigmented keratinocytes and hair follicles, sweat glands, and

sebaceous gland appendages. These structures contribute to the role of the skin as a regulator of body temperature as well as a barrier against chemical, biological, and physical injury.

The second outermost layer is the dermis, primarily made of dense connective tissue with blood vessels and nerve fibers (Robbins 1997). Fibroblasts secrete a dense, yet flexible matrix of collagen and elastin. Blood vessels are abundant in the dermis with smaller capillaries residing close to the epidermal-dermal border. These blood vessels are capable of dilating and contracting to regulate body temperature and bring resident immune components to the epidermis to protect from potential infection. Lastly, a variety of peripheral nervous system components permeate the dermis to relay sensory information to the central nervous system. The most internal layer of the skin, the subcutaneous layer, is made of fatty tissue to insulate the internal body cavities.

1.1.2 Somatosensation

The skin is sensitive to a variety of physical stimuli, including temperature, pain, itch, and touch (Lederman 1997; Abraira and Ginty 2013; Zimmerman et al. 2014). These sensations are detected by free nerve endings and specialized mechanoreceptors in the skin. Mechanoreceptors have distinct localization, adaptability, transmission speed, and structure; and they each encode unique stimuli (summarized in Table 1). Low-threshold mechanoreceptors (LTMRs) are highly sensitive and encode the sense of touch, while high-threshold mechanoreceptors (HTMRs) respond only to high-force stimuli that is often perceived as pain. LTMRs are classified as Aβ, Aδ, or C fibers based on their degree of myelination and conduction velocity (Abraira and Ginty 2013). Afferent fibers can be slowly adapting (SA), meaning they respond to long term, static stimuli; or they can be rapidly adapting (RA), responding only to onset and offset of indentation. Certain

mechanoreceptors localize specifically to hairy skin and/or glabrous, non-hairy, skin. The variety of mechanoreceptors in the skin allows for perception of a vast array of tactical stimuli.

Table 1- Summary of specialized Nerve Endings

Mechanoreceptor	Specialized ending	Adaptability	Location	Type of Sensation
Aβ-SAI LTMR	Merkel cell-Neurite	Slow-adapting	Basal epidermis; hairy and glabrous skin	Static touch
Aβ-SAII LTMR	Ruffini Ending	Slow-adapting	Dermis; glabrous skin	Skin stretch
Aβ-RAI LTMR	Meissner Corpuscle	Rapidly-adapting	Superficial dermis; glabrous skin	Movement
Aβ-RAII LTMR	Pacinian Corpuscle	Rapidly-adapting	Dermis, Subcutaneous; glabrous skin	High frequency vibration
Aδ-LTMR	Hair follicle	Rapidly-adapting	Hair follicles; hairy skin	Movement, low-frequency vibration
C- LTMR	Hair follicle	Slow-adapting	Hair follicles; hairy skin	Light touch
HTMR	Free nerve ending	Slow-adapting	Epidermis; hairy and glabrous skin	Pain

The focus of this dissertation is on the Merkel cell, which is part of the Aβ-SAI LTMR. The Merkel cell-neurite complex is a low-threshold, slowly adapting mechanoreceptor with a high conduction velocity. This mechanoreceptor is most sensitive to static, light touch stimuli (Zimmerman et al. 2014) and is important for detection of texture, shape, and size. Despite the importance of Merkel cells for perception, the genetic and environmental factors that promote Merkel cell development and survival are poorly understood.

1.2 MERKEL CELL DISCOVERY AND CHARACTERIZATION

1.2.1 Historical Perspective

Merkel cells were described by Friedrich Sigmund Merkel in 1875 and again in 1880 (Merkel 1875; Merkel 1880). He described them as specialized epidermal cells closely associated with sensory neurites. Merkel called these cells *Tastzellen*, or sense cells, and postulated that these cells transduce mechanical stimuli. Near the turn of the century, researchers adopted the term "Merkel Cell" to describe these cells (Tretjakoff 1902).

For most of the 20th century, reports about Merkel cells were descriptive in nature, using electron microscopy to describe their location and morphology. Merkel cells were most frequently described in mammals, including opossums (Munger 1965), cats (Munger 1965), rats (Munger 1965; Nikai et al. 1971), guinea pigs (Munger 1965), and non-human primates (Iggo and Muir 1969). They were also detected in amphibians (Tweedle 1978), reptiles (Landmann and Halata 1980), birds (Andersen and Nafstad 1968; Saxod 1970), and even invertebrate species (Whitear and Lane 1981; Whitear 1989). Merkel cell structure remains consistent between different species (Smith 1970).

Through the 20th century two major questions were debated about Merkel cells: 1- What is the function of the Merkel cell? and 2- Are Merkel cells derived from the epidermis or the neural crest? These questions were largely untestable without the use of transgenic mouse models; thus, much of our knowledge of Merkel cell physiology has been discovered relatively recently.

1.2.2 Merkel cell localization

Merkel cells reside at the basal side of the epidermis, and depending on the location in the body, they cluster in different patterns and with differing densities. This dissertation will focus on three primary structures where Merkel cells are found in mice: 1- Glabrous, non-hairy skin, 2- Touch domes of hairy skin, and 3- whisker follicles (Lederman 1997; Abraira and Ginty 2013). A schematic of these structures is shown in Figure 1. Merkel cells have also been detected in the oral cavity and other mucosal tissue, but these areas will not be discussed in this dissertation.

In glabrous skin (non-hairy skin), Merkel cells are detected in rete ridges, where the epidermis penetrates deeper into the dermis (Figure 1A) (Halata et al. 2003). Up to 10 Merkel cells can be found in a single rete ridge, distributed in an irregular pattern. The density of Merkel cells is greater in highly sensitive areas such as the glabrous skin of the hands and soles of the feet and the oral mucosa (Nikai et al. 1971; Hashimoto 1973; Watanabe 1980). Merkel cells are more disperse in less sensitive areas like human abdominal skin (Smith 1970).

In hairy skin, Merkel cells cluster in organized structures called touch domes or *Haarscheibe* (Pinkus 1902; Iggo 1961; Iggo 1963). Touch domes are elevated structures within the epidermis containing columnar basal keratinocytes and a cluster of Merkel cells (Smith 1970). Touch dome Merkel cells organize into a characteristic crescent shape surrounding guard hair follicles (Figure 1B', 3H) (Vielkind et al. 1995). The number of Merkel cells per touch dome varies between different species, ages, and location in the body (Smith 1970). In adult mice, touch domes of the back skin contain on average 20 Merkel cells per touch dome (Wright et al. 2015), while touch domes in abdominal skin contain closer to 25 Merkel cells (unpublished observations). Touch domes are surrounded on the dermal side by highly vascularized connective tissue and are innervated by myelinated SA1 neurons (Smith 1970). The earliest electrophysiological studies of

Merkel cell function focused on touch domes (Tapper 1965; Werner and Mountcastle 1965; Iggo 1968). These studies showed that touch domes are sensitive to skin indentation, but not temperature or stretch. Touch domes are the most studied cluster of Merkel cells because, 1- they are ubiquitous in hairy skin, well organized, and easy to detect and, 2- most research is done on hairy animal models.

Merkel cells are found at highest concentration in the outer root sheath of whisker follicles (also known as vibrissae or sinus hairs) (Halata et al. 2003). These large, specialized hair follicles are embedded deep into the dermis and contain many mechanoreceptors, including Merkel cell-neurite complexes and free nerve endings (Gottschaldt et al. 1973; Halata 1975). They exist in most mammals, including rodents, and are important for detecting spatial information (Woolsey and Van der Loos 1970). Merkel cells cluster at the base of the whisker follicle, creating a dense appearance that has been described to resemble scales on a pine cone (Figure 1C, 3B) (Halata et al. 2003).

Morphologically, Merkel cells are similar across different locations of the skin. However, some reports have observed physiological differences between these compartments, especially in glabrous skin. Merkel cells in rat foot pads do not require innervation for development like they do in touch domes (Mills et al. 1989). Merkel cells also develop later in in foot pads of mice (Reed-Geaghan et al. 2016). These reports suggest that Merkel cell development is regulated differently in glabrous skin. It is probable that regulation of Merkel cell development and turnover varies in different parts of the body; however, these differences are not well understood.

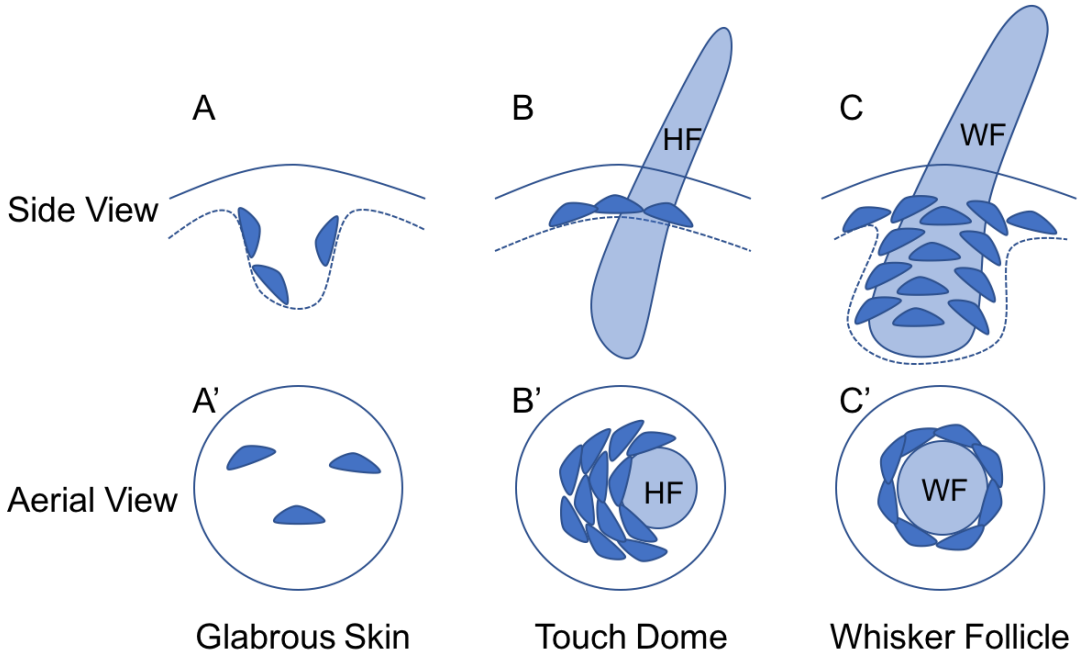

Figure 1- Schematic of Merkel cell distribution in skin

Merkel cells (dark blue shapes) are detected at the dermal-epidermal border (dashed line) in three areas in mammalian skin: (A,A') sporadically distributed in rete ridges of glabrous skin, (B,B') organized in crescent-shaped touch domes around hair follicles (HF), or (C,C') surrounding the base of whisker follicles (WF).

1.2.3 Merkel cell-neurite complex structure

Structurally, Merkel cells share qualities of both keratinocytes and neurons. Like basal keratinocytes, Merkel cells border the basement membrane of the epidermis and form desmosomal junctions with neighboring keratinocytes (Figure 2) (Smith 1970). They also express keratinocyte specific markers, including Keratin 8, 18, 19, and 20 (Roland Moll et al. 1984). Conversely, Merkel cells display characteristics of neural-derived cells, forming synapse-like contacts with afferent nerves near the basement membrane. Merkel cells have opaque cytoplasmic granules near the Merkel cell-neurite junction. These granules resemble presynaptic vesicles and stain positive for

markers of synaptic vesicles (Iggo and Muir 1969). Merkel cells also contain ion channels including Piezo2, which is detected in other mechanically sensitive cells (Ranade et al. 2014).

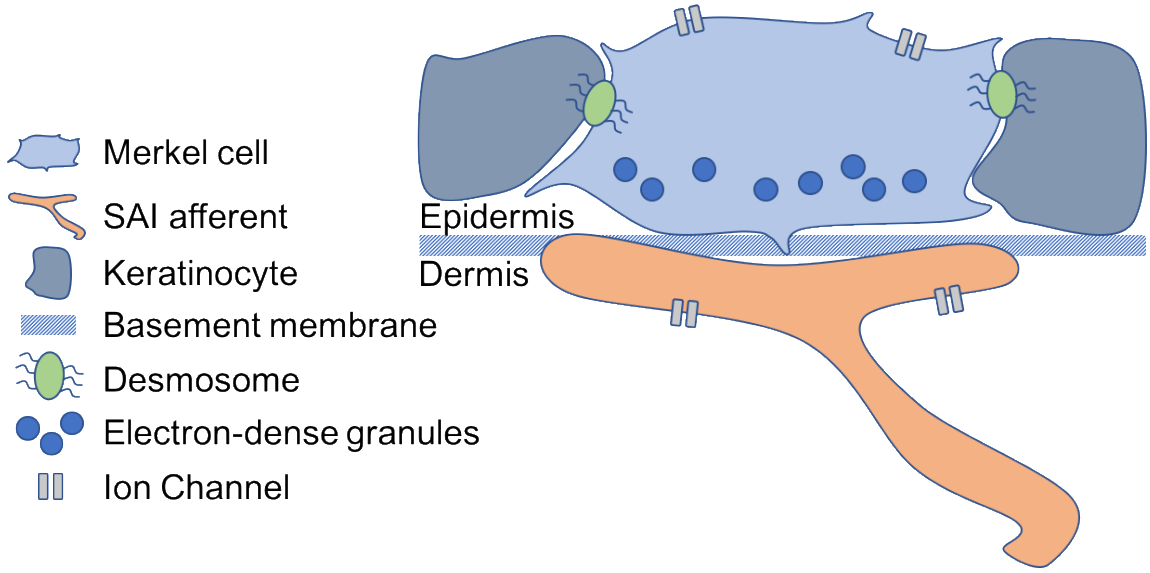

Figure 2- Diagram of Merkel cell-neurite complex

The Merkel cell forms a synapse-like contact with a SAI afferent nerve at the basement membrane. Merkel cells form desmosomal junctions with basal keratinocytes. Electron-dense granules are found near the Merkel cell-neurite junction. Ion channels are expressed by both the Merkel cell and SAI neuron.

Historically, detection of electron-dense granules in the cytoplasm by electron microscopy was the best way to distinguish Merkel cells from other keratinocytes (Halata et al. 2003). However, modern immunohistochemistry has allowed us to distinguish Merkel cells using specific antibodies to molecular markers of Merkel cells. Most commonly keratins 8 and 20 are used to immunostain Merkel cells due to their high expression levels and ease of staining (Roland Moll et al. 1984). Merkel cells also express high levels of specific mechanosensitive ion channels and neuropeptides including Rab3C, VGLUT2, Synaptophysin, Chromogranin A, methionine-enkephalin, and Vasoactive Intestinal Peptide (Gu et al. 1981; Hartschuh et al. 1983; Gauweiler et al. 1988; Ortonne et al. 1988; Hartschuh, Weihe, and Yanaihara 1989; Hartschuh, Weihe, and

Egner 1989; Oro and Higgins 2003; Haeberle et al. 2004). Merkel cells also incorporate quinacrine and FM dyes (such as FM1-43), which label and can be used to assess vesicular cycling of Merkel cells (Nurse et al. 1983; Fukuda et al. 2003).

SAI neurons innervate Merkel cells from the dermal side. The SAI neuron forms a cup-shaped calyx that partially envelops the basal side of the Merkel cell (Hartschuh and Weihe 1980), and expresses NF200 (also called NFH) and Thy1, both of which can be used to immunostain SAI afferents (Taylor-Clark et al. 2015). Merkel cell-neurite synapses resemble a typical synapse with a concentration of mitochondria near the junction (Munger 1965).

1.2.4 Merkel cell function

A long-standing debate existed over whether Merkel cells function as a true mechanoreceptor or as a neuroendocrine cell, regulating the functions of the SAI neuron. Only recently has the function of Merkel cells been tested with modern scientific techniques.

Early researchers thought Merkel cells were not required for the SAI neuron to function (Diamond et al. 1986). This hypothesis was based on several observations: 1- the SAI afferent nerve terminal shares features of a free nerve ending, which does not require an accessory cell to function, 2- Many neurites in touch domes do not contact Merkel cells, and 3- Merkel cells have granules whether or not they are innervated, suggesting that granules do not function to initiate nerve transduction (Gottschaldt and Vahle-Hinz 1981). However, these observations are not based on empirical data, and were later disproven with modern research tools (Ochiai and Suzuki 1981).

Merkel cells make synapse-like contacts with neighboring SAI neurons (Hartschuh and Weihe 1980), expressing synaptic markers including methionine-enkephalin, cholinesterase, vasoactive intestinal polypeptide, alkaline phosphatase, and 4-methoxylucine aminopeptidase

(Winkelmann 1982; Hartschuh et al. 1983). Molecular profiling of Merkel cells identified many ion channels and neurotransmitters expressed in Merkel cells (Haeberle et al. 2004; Lumpkin and Caterina 2007; Kwan et al. 2009; Maksimovic et al. 2013). Together these markers suggest that Merkel cells contain the necessary machinery to transduce mechanical stimuli.

We now know that Merkel cells function to transduce the sense of light touch (Maricich et al. 2009; Wellnitz et al. 2010). When Merkel cells are conditionally deleted in the skin, the static response of the SAI is attenuated, but the dynamic response at the onset of the stimulus is still detected (Maksimovic et al. 2014). When the mechanically sensitive ion channel, Piezo2, is conditionally deleted in the skin the static SAI response is also attenuated. This suggests that Merkel cells regulate SAI response in a Piezo2 dependent mechanism (Maksimovic et al. 2014). Taken together, these data suggest that the SAI neuron can detect the onset of light-touch stimuli independently of Merkel cells; however, the Merkel cell is required for static response.

A possible mechanism that allows the Merkel cell-neurite complex to transduce a physical stimulus into a neural message has recently been described (W. Chang et al. 2016). Upon physical stimulation, mechanically sensitive Piezo2 channels on the SAI neuron depolarize the neuron and initiate a dynamic SAI response (Ikeda et al. 2014; Maksimovic et al. 2014; Ranade et al. 2014; Woo et al. 2014). Simultaneously, Piezo2 channels polarize the Merkel cell causing release of neurotransmitters from the Merkel cell. Among several neurotransmitters, serotonin binds to 5-HT receptors on the SAI neuron, depolarizing the neuron and sustaining the SAI response (Maksimovic et al. 2014; W. Chang et al. 2016). This mechanism allows the Merkel cell-neurite complex to transmit a quick and sustained signal which is necessary for detecting light touch stimuli.

1.3 MERKEL CELL DEVELOPMENT AND MAINTENANCE

1.3.1 Timeline of Merkel cell development and turnover

In hairy skin, Merkel cell development occurs simultaneously with the development of primary hair follicles. At E14.5, signals from the dermis induce the formation of the hair placode (Duverger and Morasso 2009). The placode produces signals that promote the development of both the hair follicle and Merkel cells. Secondary and tertiary hair follicles are produced later in development (E16.5 and E18.5, respectively), but Merkel cells do not develop around these follicles. Merkel cell production can be detected as early as E14.5 and continues until birth (Wright et al. 2015). The rate of Merkel cell production, measured by percent of Ki67-positive Merkel cells, is at a maximum at E16.5 and slowly decreases. The date of Merkel cell innervation is debated. Some say innervation occurs as early as E15.5 (Pasche et al. 1990); others observe onset of innervation at E18.5 (Cheng Chew and Leung 1994; Peters et al. 2002); and some data suggests innervation occurs postnatally (Ochiai and Suzuki 1981; Vielkind et al. 1995). All of these reports have shown that a fraction of Merkel cells are innervated during embryonic development. The present data suggest that innervation begins during late embryogenesis (between E15.5 and E18.5), and continues past birth; however, more studies are necessary to understand when Merkel cell innervation occurs.

In whisker follicles, Merkel cells are detected as early as E13.5 (Pasche et al. 1990), with the rate of Merkel cell production at its maximum at E16.5 (Wright et al. 2015). 95% Merkel cells are innervated by E17.5, suggesting that maturation occurs earlier in whisker follicles than in touch domes (Pasche et al. 1990).

Postnatally, Merkel cell production occurs less often (Wright et al. 2015); however, the frequency of Merkels cell turnover is still largely debated. Our lab has shown that Merkel cells are long-lived cells (Wright et al. 2015), but other labs hypothesize that touch dome Merkel cells are completely replaced in a single hair cycle (Moll, Paus, et al. 1996; Nakafusa et al. 2006). Hair follicles regenerate every 3-5 weeks with three phases of the hair cycle: the resting phase (telogen), the growth phase (anagen), and the retraction phase (catagen) (Müller-Röver et al. 2001; Plikus et al. 2008). Some reports have suggested that touch dome Merkel cells are produced during anagen and lost during catagen (Moll, Paus, et al. 1996; Nakafusa et al. 2006). This debate will be discussed in more depth in Chapter 6 of this dissertation.

1.3.2 Developmental biology techniques

This dissertation is primarily focuses on locating when and where Merkel cells develop. There are a few common techniques that have enabled scientists to trace where cells are derived during embryogenesis. Here I will describe the tools that we use to manipulate the genetics of the mouse models in the subsequent chapters. Fate mapping and lineage tracing are tools used to identify the progeny of cells and tissues, and it is one of the most helpful techniques for studying development (Kretzschmar and Watt 2012). This technique often uses an inducible recombinase enzyme "driver" to promote the expression of a "reporter" gene.

Mouse lineage tracing and fate mapping is usually performed using the *Cre-loxP* system (Nagy 2000). In this system, a Cre recombinase enzyme is expressed using a tissue- or cell-specific promotor. Cre-induced recombination occurs at two loxP sites; therefore, a flanked loxP site ("floxed") can be removed from the mouse genome. In the experiments described in this dissertation we used this system to remove functional genes and to induce the expression of genes

preceded by a floxed stop codon. Cre function can be regulated temporally by driving expression of *CreER* a chimera protein of the estrogen receptor (ER) and Cre. This protein can be activated at specific timepoints by administering a dose of tamoxifen. Similarlly *CrePR*, a chimera protein of the progesterone receptor and Cre can be activated by administering RU846.

As stated above, the *Cre-LoxP* system can be used to delete a functional gene or it can be used to activate expression. To activate expression of a gene, we take advantage of the *ROSA26* (or simply *ROSA*) allele, which is ubiquitously expressed in most cells and tissues of the mouse (Soriano 1999). We generate mice that contain a modified *ROSA* allele followed by a floxed stop codon and a reporter gene such as *LacZ*, *tdTomato*, or *YFP*. Upon expression of a Cre protein, the stop codon will be removed from the allele and the reporter gene will be constitutively expressed under the *ROSA* promotor.

With this tool, we can irreversibly express a chromogenic or fluorescent marker in a cell- or tissue-specific location and at a specific age (if we employ an inducible Cre). This is helpful for the study of developmental biology because we can label cells of embryonic tissues and track them as they migrate, proliferate, and differentiate.

1.3.3 Tissue of origin: neural crest or epidermis?

The earliest descriptions of Merkel cell development occurred in the early 1970s and immediately stirred up controversy in the field over where the cells originated. Most early researchers thought Merkel cells were epidermal due to the formation of desmosomes between Merkel cells and neighboring keratinocytes as well as the expression of keratin proteins (Munger 1965; Smith 1970; Lyne and Hollins 1971; Roland Moll et al. 1984; Saurat et al. 1984; Saurat and Didierjean 1984). However, the electron dense granulates, which are often associated with neural tissue, led some

researchers to conclude that Merkel cells were derived from the neural crest (Brethnach and Robins 1970; Brethnach 1971). Furthermore, some scientists observed Merkel cell-like cells in the dermis of early developing tissue (Brethnach and Robins 1970; Lyne and Hollins 1971; Hashimoto 1972). Finally, chick-quail grafting of the branchial neural tube showed that Merkel cells were derived from the neural crest (Halata et al. 1990). These experiments and observations led to the formation of a hypothesis that Merkel cells were derived from migrating cells of the neural crest.

With the advent of Cre-loxP technology, this hypothesis could be further tested. In 2003, Szeder et al. used lineage tracing to determine if Merkel cells were derived from a *Wnt1*-positive, neural crest progenitor (Szeder et al. 2003). A $Wnt1^{Cre}$ driver was used to induce *Cre* expression in neural crest cells with a $ROSA^{LacZ}$ reporter. With this method, a cell that expresses *Wnt1* will irreversibly express *β-galactosidase*, allowing for detection of all cells derived from *Wnt1* positive progenitors. Szeder found that Merkel cells were positive for β-galactosidase, concluding that Merkel cells were derived from the neural crest. However, because of the proximity of Merkel cells to SAI neurons, it is hard to say whether the positive stain is from Merkel cells or the neighboring neuron. This hypothesis could be definitively tested by specifically knocking out a gene that promotes Merkel cell development in either the epidermis or the neural crest.

In 2000, a lab studying the basic helix-loop-helix transcription factor, *Atoh1* (also known as *Math1*), created an $Atoh1^{LacZ}$ mouse, which could label all *Atoh1*-positive cells in the developing mouse embryo (Ben-arie et al. 2000). They expected its expression in the CNS, but were surprised to also observe *Atoh1* expression in hair cells of the inner ear and in Merkel cells. Later it was found that when *Atoh1* is conditionally deleted in the mouse, Merkel cells fail to develop (Maricich et al. 2009). This suggests that Merkel cells are derived from an *Atoh1*-positive lineage.

In 2009, two independent labs showed that Merkel cells were derived from an epidermal lineage (Van Keymeulen et al. 2009; Morrison et al. 2009). Van Keymeulen repeated the experiment that Szeder performed in 2003 with $Wnt^{Cre};ROSA^{YFP}$ mice. Van Keymeulen did not observe YFP-positive Merkel cells, refuting the hypothesis that Merkel cells were derived from the neural crest. Both Morrison and Van Keymeulen then used a $Wnt^{Cre};Atoh1^{flox/flox}$ mice to conditionally knockout *Atoh1* in the neural crest. They found that this had no effect on Merkel cell production, which further refutes that Merkel cells arise from the neural crest. However, when *Atoh1* is conditionally deleted from the epidermis in $K14^{Cre};Atoh1^{flox/flox}$ mice, touch domes still form, but no Merkel cells are produced. Furthermore, when *Atoh1* is overexpressed in the developing mouse epidermis, ectopic Merkel cells develop (Ostrowski et al. 2015). These experiments have confirmed that Merkel cells are derived from epidermis during development.

1.3.4 Merkel cell progenitors

Because Merkel cells are post mitotic (Moll, Zieger, et al. 1996), they are probably replenished by a population of progenitors in the epidermis. Several labs have attempted to identify the progenitor that replaces Merkel cells in adult mice.

In 2010, a lab run by David Owens identified touch dome keratinocytes as possible Merkel cell progenitors (Woo et al. 2010). Touch dome keratinocytes express α-integrin, sca1, and CD200. These markers were used to isolated touch dome keratinocytes from $K14^{Cre};ROSA^{LacZ}$ mice using FACS sorting. The tissue was reconstituted by injecting the sorted cells in the dorsal fascia of nude mice. Four weeks later the grafts were collected, and found to contain β-gal+/K8+ Merkel cells. These experiments suggest CD200-positive touch dome keratinocytes are the progenitors for

Merkel cells; however, since the experiments involved skin grafts, it was unclear if the conclusions could be applied to non-injured Merkel cell homeostasis.

David Owens' lab followed up these experiments, characterizing the touch dome keratinocyte population during homeostasis (Doucet et al. 2013). K17 is expressed specifically in the touch dome, but not in Merkel cells (Moll, Paus, et al. 1996). Doucet et al. created a tamoxifen inducible $K17^{CreER}$ mouse to determine if Merkel cells are replaced by a *K17* positive lineage, and at what rate. Using $K17^{CreER}$;$ROSA^{EYFP}$ mice, they did a pulse-chase experiment by administering tamoxifen in adult mice and quantified how many Merkel cells expressed EYFP between 24 hours and 12 weeks after tamoxifen treatment. They found that while few Merkel cells are EYFP-positive 24 hours later, almost all Merkel cells were EYFP-positive by 12 weeks after tamoxifen treatment. They observed similar results in whisker follicles and glabrous skin. These experiments suggested two things: 1- Merkel cells are maintained by a K17 positive lineage and 2- All Merkel cells are replaced by K17 positive cells in a 7-12 week timeframe.

In 2015, our lab showed that Merkel cells are derived from *Atoh1*-positive progenitors (Wright et al. 2015). *Atoh1* expression is required in precursors of other *Atoh1*-lineal cell types including hair cells of the inner ear, cerebellar granule cells, and gut secretory cells (Kelley 2006; Gerbe et al. 2011; Kim and Shivdasani 2011; Zheng et al. 2011; Chonko et al. 2013). Wright et al. interrogated whether Merkel cells are similarly derived from *Atoh1* positive precursors. To test this hypothesis, Wright et al. did lineage tracing using $Atoh1^{CreER}$;$ROSA^{LacZ}$ and $Atoh1^{CreER}$;$ROSA^{tdTomato}$ mice. They administered tamoxifen in adolescent mice and collected skin and whisker follicles 3 and 9 months later. At both of these time points, the majority (>90%) the labeled cells were positive for K8 and β-gal. To determine if Merkel cells could be replaced by a *Atoh1*-negative lineage, Wright used a $Atoh1^{CreER}$;$ROSA^{DTA}$ mouse which will express diphtheria

toxin (DTA) in *Atoh1* positive cells upon tamoxifen administration. Few Merkel cells were detected in this model 1, 3, or 6 months after tamoxifen administration. Together these findings suggest threefold: 1- All Merkel cells are derived from an *Atoh1*-positive lineage 2- *Atoh1*-positive progenitors are unipotent, only differentiating into Merkel cells, and 3- Merkel cells are surprisingly long-lived.

In 2015, the Isaac Brownell lab identified a population of *Gli1*-positive epidermal cells, which they claim replenish touch dome keratinocytes and Merkel cells (Xiao et al. 2015). They performed lineage tracing experiments, by administering tamoxifen to adult $Gli1^{CreER}$;$ROSA^{LacZ}$ mice and collecting hairy skin 9 days later. They found a subset of Merkel cells (<10%) were *β-gal*-positive, suggesting that Merkel cells are derived from a *Gli1*-positive progenitor. Since *Gli1* is a component of the Shh signaling pathway, this also suggests that Merkel cell development could be regulated by Shh. This will be discussed in more depth in section 1.3.5.

In summary, the literature has multiple hypotheses about which epidermal progenitors replenishing the Merkel cell population. Our lab has proposed that Merkel cells are derived from a unipotent *Atoh1*-positive progenitor (Wright et al. 2015). Other labs suggest that Merkel cells are replaced by touch dome progenitors, namely *K17*-positive and *Gli1*-positive touch dome keratinocytes (Doucet et al. 2013; Xiao et al. 2015). These reports are somewhat contradictory and are still being understood. Further, the reports discussed in this section have inconsistent conclusions on how frequently Merkel cells are replaced and what induces their production.

1.3.5 Genetic regulators of Merkel cell development

Molecular profiling has revealed several transcription factors that are expressed in Merkel cells at higher levels than in the surrounding epidermis (Haeberle et al. 2004). A few of these transcription factors are required for development and maturation of Merkel cells.

As mentioned above, the transcription factor *Atoh1* is required for Merkel cell development (Maricich et al. 2009). *Atoh1* has a basic helix-loop-helix (bHLH) domain which allows it to heterodimerize and bind to E-box DNA consensus sequences (Cai and Groves 2014). The *Atoh1* gene itself has an E-box domain, which allows *Atoh1* to autoregulate its expression (Helms et al. 2000). *Atoh1* is highly conserved among vertebrates, and plays a role in the development of many cell types in the body, including cerebellar precursors, hair cells of the inner ear, and secretory cells of the gut (Kelley 2006; Gerbe et al. 2011; Kim and Shivdasani 2011; Zheng et al. 2011; Chonko et al. 2013). *Atoh1* is one of the most important transcription factors for regulating Merkel cell development, as its expression is required for Merkel cell specification (Van Keymeulen et al. 2009; Maricich et al. 2009; Morrison et al. 2009; Ostrowski et al. 2015).

Several other transcription factors are important for Merkel cell development. Merkel cells express *Sox2* as early as E14 (Haeberle et al. 2004; Lesko et al. 2013), which led to the hypothesis that it promotes Merkel cell development. When *Sox2* is removed from the epidermis of $K14^{Cre};Sox2^{flox/flox}$ mice, the number of K8-positive Merkel cells decreases by 50% (Lesko et al. 2013). Interestingly, K18 and K20 positive Merkel cells decrease to near zero in *Sox2* knockout mice (Bardot et al. 2013). This suggests that *Sox2* is not required for Merkel cell development; rather, it is important for complete maturation of Merkel cells.

Isl1 also promotes Merkel cell maturation. As mentioned earlier, *Sox2* knockout mice only have a partial decrease in the number of K8 positive cells, this led some to believe that there is

some redundancy in the transcription factors that promote Merkel cell maturation (Perdigoto et al. 2014). Perdigo et al. found that when they knockout *Isl1* in the epidermis of $K14^{Cre};Isl1^{flox/flox}$ mice. There was no change in the number of K8 and Atoh1-positive Merkel cells. However, they found that when you knockout both *Isl1* and *Sox2* in $K14^{Cre};Isl^{flox/flox};Sox2^{flox/flox}$ mice, they observed an additive loss in the number of K8 and Atoh1-positive Merkel cells. These findings suggest that *Isl1* and *Sox2* co-regulate *Atoh1* to promote Merkel cell development and maturation.

The transcription factor *Pax6* plays a minor role in Merkel cell development (Parisi and Martin Collinson 2012). E16.5 $Pax6^{-/-}$ mice have normal number of K8 and K20 positive Merkel cells, but at E18.5 Merkel cells lose K8 expression. This suggest that *Pax6* is important for the survival of Merkel cells, but more experiments would be needed to fully understand the role of *Pax6* in Merkel cell development.

1.3.6 Genetic and environmental regulators of Merkel cell patterning

For the epidermis to develop properly it must receive signals from surrounding tissues. Signals come from the dermis to promote keratinocyte differentiation and hair follicle cycling (Liu et al. 2013; Morgan 2014). Subcutaneous fat, differentiated keratinocytes, and nerves can also affect epidermal development (Hsu et al. 2010; Brownell et al. 2011; Festa et al. 2011). Over the past decade, scientists have begun to interrogate the signals that promote Merkel cell development.

The best described pathway that regulates touch dome Merkel cell development is a cascade of Wnt, Eda, and Shh signaling that is initiated in the dermis and promotes development of the hair follicle, touch dome, and Merkel cells (Xiao et al. 2016). Xiao et al. first showed that blocking dermal Wnt signaling in $En1^{Cre};\beta\text{-}catenin^{flox/flox}$ mice prevented placode development. Since Wnt signaling induces *Edar* expression in the placode (Chen et al. 2012), they hypothesized

that Edar/Eda promoted touch dome and Merkel cell development. They observed *Edar* knockout mice developed dermal placodes, but failed to develop touch domes. RT-PCR revealed that *Edar* knockout mice had significantly lower levels of *Sox2* and *Atoh1* expresion as well as *Shh* and *Gli1* expression. They had previously shown that *Shh* drives production of touch dome precursors (Xiao et al. 2015), so this led them to hypothesize that *Eda/Edar* signaling drives touch dome production by increasing *Shh* expression. Finally, they observed that touch domes and Merkel cells failed to develop in *Shh* knockout embryos. Together, these experiments elucidate a cascade that guides touch dome and Merkel cell development during embryogenesis: Wnt signaling initiates placode development and *Edar* expression, which subsequently induces *Shh* expression and touch dome development.

Signals in the interfollicular epidermis prevent Merkel cell production. The polycomb repressor complex 2 (PRC2) is a chromatin repressor that inhibits Merkel cell production. PRC2 is a complex of several proteins including Ezh1 and Ezh2. When PRC2 is disrupted by deletion of *Ezh1* and *Ezh2,* ectopic Merkel cells are produced in the interfollicular epidermis (Bardot et al. 2013; Dauber et al. 2016). Furthermore, PRC2 disruption results in increased *Sox2* and *Isl1* expression (Bardot et al. 2013), suggesting that PRC2 epigenetically inhibits Merkel cell production by decreasing expression of important transcription factors. Recently, a report showed that PRC2 inhibits Merkel cell production around secondary and tertiary hair follicles (Perdigoto et al. 2016). These results suggest that PRC2 inhibits Merkel cell production outside of the touch dome.

Research has shown that signals from the innervating SAI neuron promote Merkel cell survival, since when SAI neurons are chronically dennervated, Merkel cell number decreases (Krimm et al. 2004). Merkel cells and the surrounding keratinocytes can also drive the survival of

mature SAI afferents through production of neurotrophins (NTs). NT3 and BDNF are neurotrophins that are expressed in the epidermis and are shown to promote the formation of mature neurites (Airaksinen et al. 1996; Albers et al. 1996; LeMaster et al. 1999; Krimm et al. 2004; Reed-Geaghan et al. 2016). Interestingly, when NT3 and BDNF are conditionally deleted from the epidermis, innervation decreases, followed by a decrease in Merkel cell numbers. This suggests that neurons secrete factors that promote Merkel cell survival. There are likely to be multiple neural-derived factors that promote survival, but to date, only one potential factor has been identified: neural-derived Shh is necessary for production of touch dome progenitors (Brownell et al. 2011; Xiao et al. 2015).

The polarity of the hair follicle and the touch dome is regulated by Frizzled (Fz)6 signaling (H. Chang et al. 2016). The top of the hair follicle normally orients toward the caudal end of the mouse. Interestingly, Chang et al. found that hair follicles in $Fz6^{-/-}$ mice did not consistently orient in any direction. Further, they found that touch domes surrounded 360° around the hair follicle, instead of forming their characteristic crescent shape (Figure 1B'). This phenomenon is probably not dependent on Merkel cells because when *Fz6* was conditionally deleted in Merkel cells of $Atoh1^{Cre};Fz6^{CKO}$ mice, they touch domes were distributed normally. This suggests that *Fz6* establishes polarity of hair follicles during development, and in turn regulates the patterning of Merkel cells.

It is unlikely that the pathways above are the only signals that regulate Merkel cell development and patterning. Bone morphogenic protein (BMP) and fibroblast growth factor (FGF) pathways promote hair cycling and skin development (Hébert et al. 1994; Suzuki et al. 2000; Scarlato et al. 2001; Kobielak et al. 2007; Plikus et al. 2008; Bhattacherjee et al. 2013). These pathways, as well as others could play a role in Merkel cell development.

1.4 MERKEL CELL CARCINOMA

In 1972 Cyril Toker described an aggressive neuroendocrine carcinoma called trabecular carcinoma (Toker 1972; Tang and Toker 1978). In the following years, researchers discovered that trabecular carcinoma cells had electron-dense granules and expressed Merkel cell markers (R Moll et al. 1984; Sidhu et al. 2009). This led to the hypothesis that this particular cancer subtype was derived from Merkel cells, soon the name Merkel cell carcinoma (MCC) became the accepted term for the disease. MCC is a deadly carcinoma, but its cell of origin as well as the processes that drive its development are poorly understood (Albores-Saavedra et al. 2010).

MCC is a rare, but deadly carcinoma. According to the Surveillance, Epidemiology and End Results (SEER) program at the National Institutes of Health only 3870 cases have been reported between 1973 and 2006 (Albores-Saavedra et al. 2010). This number is probably an underestimate due to unreported or misdiagnosed cases, but it remains a rare disease. On average, women with MCC have a 10-year survival of 64.8%, while men have a poorer 10-year survival rate of 50.5%. Poor survival from the disease can be attributed to the aggressiveness of the carcinoma as well as the lack of reliable treatments for the disease (Schrama et al. 2012).

MCC is identified by its location and the markers it expresses. MCC can originate within the dermis, epidermis, or subdermis, and neoplasms are most commonly found on head or neck (Sibley et al. 1985). MCC cells are round with spinous processes and contain vesicular nuclei with multiple small nucleoli. The cytoplasm of MCC cells is granulated, amphophilic (sensitive to both hematoxylin and eosin), and contains complex cellular junctions. MCC has several biomarkers including—most commonly—Neuron specific enolase, synaptophysin, chromogranin A, and K20 (Kuwamoto 2011). Other MCC markers include neurofilaments, CD56, CD57, and microtubule associate protein (MAP) 2.

The signals that drive its progression are only beginning to be understood. In 2008, a polyomavirus was found in association with many MCC tumors (Feng et al. 2008). This Merkel cell polyomavirus (MCV or MCPyV) integrates its DNA into the genome of skin cells in 80% of healthy individuals and 93% of patients with MCC (Schrama et al. 2012). Higher levels of virus are detected in patients with MCC. MCPyV is thought to drive progression of MCC (Pipas 2009), but needs additional oncogenic "hits" such as mutations in *Atoh1* and *PIK3CA* (Bossuyt et al. 2009; Nardi et al. 2012).

The cell of origin for MCC is still debated. Originally, researchers hypothesized that MCC was derived from the Merkel cell due to the resemblance and the overlapping markers; however, since Merkel cells are considered terminally differentiated and postmitotic, it is unlikely that Merkel cells acquire a highly proliferative phenotype (Tilling and Moll 2012). Instead, it is more likely that MCC is derived from an epidermal or dermal progenitor, which acquires oncogenic mutations as well as infection with MCPyV to drive carcinogenesis.

Our understanding of the genes and signals that drive MCC progression is not clear. Since Merkel cells and MCC tumor cells share biomarkers, it is possible that they progress and differentiate through similar mechanisms. Thus, studying development of Merkel cells could provide insight to how MCC progresses. Furthermore, A major challenge of studying MCC as well as other cancers is a lack of reliable *in vivo* and *in vitro* models which replicate human disease (Cagan and Meyer 2017). This challenge underscores the importance of doing basic biological science research. Understanding the basic mechanisms that promote normal Merkel cell differentiation could contribute to our understanding of MCC.

2.0 NOTCH SIGNALING ANTAGONIZES DEVELOPMENT OF MERKEL CELLS

The experiments described in this chapter are in submission at Developmental Biology:

My contributions:

-Discussing experimental design with Dr. Maricich

-Generating mice, processing tissues, and performing cell counts on Figures 1 and 2

-Analyzing all data and performing statistical analyses on figures.

-Acquiring images and assembling figures for publication

-Writing the manuscript with edits from Drs. Wright and Maricich

Contributions from other Authors:

-Figure 3- Mice were generated by Dr. Nadean Brown of UC Davis

-Figure3- Dr. Wright processed tissues and performed cell counts

2.1 INTRODUCTION

Merkel cells are mechanosensitive skin cells derived from the epidermal lineage (Morrison et al. 2009) that require the basic helix-loop-helix transcription factor Atoh1 for their development (Maricich et al. 2009; Wright et al. 2015; Ostrowski et al. 2015). Genes and signaling pathways that regulate *Atoh1* expression during Merkel cell specification are poorly understood.

Two pathways have recently been shown to regulate the development of Merkel cells around first-wave hair follicles. First, a cascade of Wnt, Eda, and Shh signaling drives development of first-wave hair follicles, otherwise known as guard hairs and initiates specification

of surrounding Merkel cells (Xiao et al. 2016). Second, the polycomb repressor complex 2 (PRC2) inhibits development of Merkel cells around second-wave hair follicles (Bardot et al. 2013; Dauber et al. 2016; Perdigoto et al. 2016).

Notch signaling is vital for embryonic development and maintenance of many tissues including the skin (Massi and Panelos 2012). In the epidermis, Notch signaling acts as a fate switch to induce differentiation of basal epidermal stem cells into mature keratinocytes (Blanpain, William E Lowry, et al. 2006). Canonical Notch signaling is initiated when a membrane-bound Notch ligand binds a Notch receptor on an adjacent cell, triggering cleavage of the Notch intracellular domain (NICD). NICD translocates into the nucleus, where it pairs with RBPj (recombining binding protein suppressor of hairless) to promote transcription of downstream targets. In the epidermis, Notch promotes production of Hes1 (hairy and enhancer of split-1). Interestingly, both Notch signaling and Hes1 expression directly inhibit *Atoh1* transcription in the inner ear, gut, and cerebellum (Kelley 2006; Gerbe et al. 2011; Kim and Shivdasani 2011; Zheng et al. 2011; Chonko et al. 2013).

We previously demonstrated that loss of Notch signaling in adult skin causes increased Merkel cell production (Ostrowski et al. 2015). Here, we investigated the role of Notch signaling on Merkel cell formation using transgenic mice that permit manipulation of Notch signaling components. We found that mice overexpressing NICD developed fewer Merkel cells in touch domes and whisker follicles. Mice that lack RBPj produced more whisker follicle Merkel cells and developed ectopic Merkel cells in the hairy skin. Further, we found that *Hes1*$^{-/-}$ mice had more Merkel cells in whisker follicles. Together, these data demonstrate that Notch signaling antagonizes Merkel cell specification in the developing epidermis.

2.2 MATERIALS AND METHODS

2.2.1 Mice

Mice were housed per University of Pittsburgh Institutional Animal Care and Use Committee guidelines. *K14*$^{Cre/+}$ mice (Dassule, Lewis, Bei, Maas, & Mcmahon, 2000, Jax#004782) were bred to *ROSA*$^{NICD/+}$ mice (Murtaugh, Stanger, Kwan, & Melton, 2003, Jax#004782) to produce *NICD-OE* mice and littermate controls. *RBPJ*$^{flox/+}$ mice (Han et al. 2002) were bred with *K14*$^{Cre/+}$*; RBPj*$^{flox/+}$ to generate *RBPj-CKO* mice. *K14*$^{Cre/+}$; *RBPj*$^{flox/+}$ mice were bred to *RBPj*$^{flox/flox}$; *ROSA*$^{NICD/+}$ mice to generate *RBPj-CKO*; *NICD-OE* mice. *Hes1*$^{-/-}$ mouse embryos were generously provided by Dr. Nadean Brown (UC Davis, Davis, CA).

2.2.2 Tissue processing

Adult mice were sacrificed by cervical dislocation under anesthesia with isoflurane. For embryonic ages, the day of plug detection was designated E0.5. E15.5 mice were dissected out of pregnant dams. Embryos were decapitated before processing. Fresh frozen tissue was collected for all E15.5 tissue by embedding in OCT compound (Tissue-Tek) and kept at -80°C. To obtain mid-sagittal sections of whisker follicles, E15.5 heads were sectioned at 25μm through the horizontal plane. Tissue sections were fixed with acetone for 10 minutes before immunostaining. P0 embryonic tissue was drop-fixed in 4% paraformaldehyde overnight.

2.2.3 Histology

Sectioned tissues were stained on glass slides using shandon coverplates (Thermo Scientific). Tissues were rehydrated in 1xPBS for 2 minutes and then incubated in PBS/0.3%H_2O_2 for 15 minutes at room temperature. Slides were then blocked in PBS with 0.3% Triton-X and 5% normal donkey serum (Millipore) for 30 minutes. Primary antibodies were diluted in blocking buffer and tissue sections were incubated for one hour at room temperature with the following antibodies: rat anti-keratin 8 (1:20; TROMA-1; Developmental Studies Hybridization Bank) and rabbit anti-Hes1 (gift of Ben Stanger, Zong 2009). After primary incubation, slides were washed 3x5 minutes at room temperature and incubated for 30 minutes in secondary antibodies: Cy3 conjugated anti-rat and cy3 conjugated anti-rabbit (1:250). Nuclei were stained with DAPI (1:1000; Thermo Fisher Scientific). P0 tissue was processed and stained by wholemount with a four day primary incubation and a two day secondary incubation, as described previously (Wright et al. 2016). All tissues were mounted in Prolong Gold (Invitrogen) on glass coverslips.

2.2.4 Whisker follicle morphology parameters

To assess whisker follicle morphology, we measured four parameters from H&E stained tissue of whisker follicle sections. Each of the following parameters were measured from 5 whisker follicles per mouse (3 mice/genotype). 1) Epidermal thickness (ET) was measured as distance from the basal lamina to the keratinized layer, with the keratinized layer being defined as the denucleated layer on the superficial side of the epidermis. 2) Keratinized thickness (KT) was measured as the distance from the superficial side of the nucleated epidermis to the most superficial side of the keratinized layer. 3) Whisker follicle width (WFW) was measured at the base of the interfollicular

epidermis. 4) Whisker follicle depth (WFD) was measured from the base of the interfollicular epidermis to the deepest point of the whisker follicle. One way t-tests were used to compare transgenic mice to controls for each of the parameters.

2.2.5 Cell counts

To quantify the number of Merkel cells per whisker follicle, intact embryonic heads were serial sectioned and 7-10 whisker follicles per mouse (n=3 mice/genotype) reconstructed based on location and morphology. To determine the number of Merkel cells per touch dome in wholemount tissue, K8+ cells were counted in 25 touch domes per mouse (n=4 mice/genotype). Touch dome and ectopic cell density was measured by total number of touch domes and ectopic cells in a 5x5mm area of skin (n=4 mice/genotype). To determine the central angle of touch domes, 10 images of touch domes were captured from each mouse (n=4 mice/genotype), and ImageJ used to measure the angle formed from the crescent-shaped touch dome. One-way ANOVAs were performed for all measurements in wholemount tissue. All parameters were measured with the investigator blinded to the genotypes of the mice. Images were adjusted for contrast and brightness for publication.

2.3 RESULTS

2.3.1 Epidermal NICD overexpression decreases Merkel cell number

To test the effect of Notch signaling on Merkel cell development, we generated *K14^{Cre}; ROSA^{NICD}* (hereafter referred to as NICD-OE) mice that overexpress the Notch intracellular domain (NICD) in epidermal cells of the developing mouse (Blanpain, William E Lowry, et al. 2006). We first analyzed the whisker follicles of embryonic day E15.5 mice, when rapid Merkel cell production occurs (Morrison et al. 2009; Wright et al. 2015). Though epidermal NICD overexpression has been shown to alter epidermal morphology in body skin (Blanpain, William E Lowry, et al. 2006), its effect on whisker follicle morphology has not been described. To determine if NICD overexpression altered whisker follicle structure we measured four morphological parameters of H&E stained whisker follicle sections: 1) epidermal thickness (ET), 2) keratinized layer thickness (KT), 3) whisker follicle width (WFW), and 4) whisker follicle depth (WFD) (Fig 3A-D). We found that ET in E15.5 *NICD-OE* mice was greater than that in control mice (36.8 ± 4.2μm vs. 27.2 ± 1.0μm, p=0.044, t-test; N=3 mice/genotype, N≥7 follicles/mouse). The average KT of NICD-OE mice was 49% greater than control mice, though the difference was not significant (22.7 ± 8.4μm vs. 11.6 ± 3.8μm, p=0.150, t-test; N=3 mice/genotype, N≥7 follicles/mouse). WFW was larger (96.8 ± 4.5μm vs. 75.5 ± 7.9μm, p=0.039, t-test; N=3 mice/genotype N≥7 follicles/mouse) and WFD was decreased (74.5 ± 10.1μm vs. 113.6 ± 12.9μm, p=0.038, t-test; N=3 mice/genotype N≥7 follicles/mouse) in NICD-OE mice. To determine whether NICD overexpression altered Merkel cell numbers, we immunostained E15.5 whisker follicle sections for the Merkel cell marker Keratin 8 (K8) (Fig 3E-F). Whisker follicles were reconstructed from serial sections, and total numbers of Merkel cells per whisker follicle counted (Fig 3G). Whisker follicles from *NICD-OE*

mice had fewer K8+ cells per follicle than whisker follicles of control mice (52.4 ± 44 vs. 157.4 ± 35.2, p=0.037, paired t-test; n=3 mice/genotype, n≥7 follicles/mouse). To investigate if NICD overexpression similarly altered Merkel cell number in body skin, we wholemount immunostained the body skin of P0 NICD-OE mice and control siblings for K8. We found that NICD-OE mice had significantly fewer K8+ cells per touch dome than their control littermates (3.8 ± 0.7 vs 22.0 ± 0.7 $p<0.0001$, t-test) (Fig 3H-J). Together, these results indicate that epidermal Notch signaling inhibits Merkel cell development in whisker follicles and touch domes.

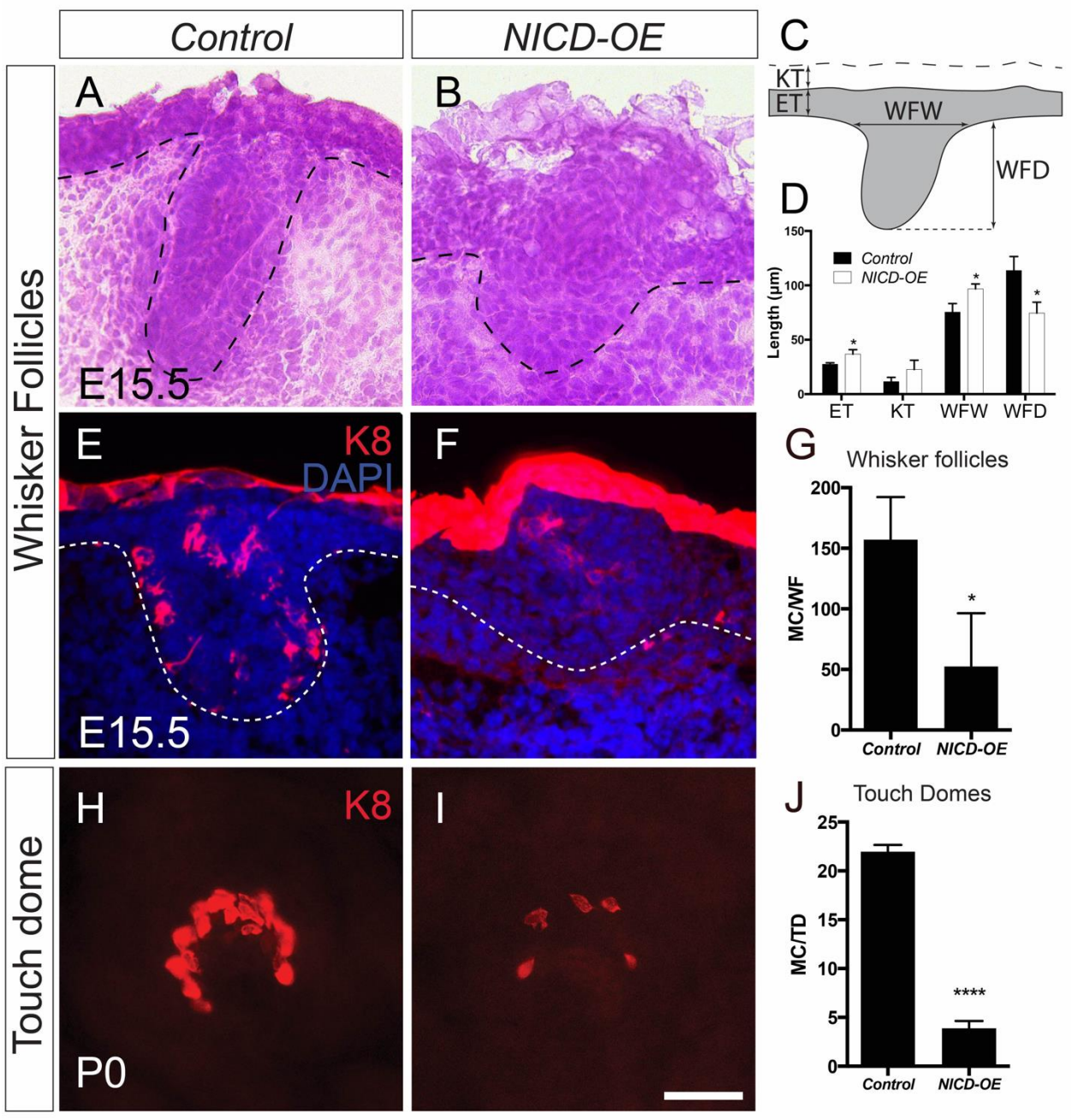

Figure 3- Epidermal *NICD* overexpression decreases Merkel cell numbers in developing whisker follicles and touch domes.

(A,B) H&E stained whisker follicle sections from E15.5 control (A) and *K14^{cre};ROSA^{NICD}* (B, hereafter referred to as *NICD-OE*) mice. Dashed line indicates the epidermal-dermal border. (C) Diagram of four whisker follicle morphology parameters: 1) epidermal thickness (ET), 2) keratin thickness (KT), 3) whisker follicle width (WFW), and 4) whisker follicle depth (WFD). (D) Quantification of E15.5 whisker follicle morphology. *NICD-OE*

mice have an increased ET (N=3 mice/genotype, N≥7 follicles per mouse, p=0.044, t-test) and WFW (p=0.039, t-test), and a decreased WFD (p=0.038, t-test). KT was not significantly different (p=0.150, t-test). (E,F) K8 immunostaining of whisker follicle sections from E15.5 *NICD-OE* mice. (G) Average number of K8+ Merkel cells per whisker follicle (N=3 mice/genotype, N≥7 follicles/mouse; p=0.037, paired t-test). (H,I) K8 wholemount immunostaining of touch domes from P0 control and *NICD-OE* mice. (J) Average number of K8+ Merkel cells per touch dome (N=3 mice/genotype, N=25 touch domes/mouse; t-test). N=3 mice/genotype. K8 expression in K8+ cells was qualitatively lower in whisker follicles and touch domes of *NICD-OE* mice compared to controls. Error bars are ±SEM. Scale bar = 50μm. *p<0.05, ****p<0.0001.

2.3.2 RBPj deletion in the epidermis increases Merkel cell numbers

We wondered whether disruption in Notch signaling would lead to opposite effects on Merkel cell numbers. We therefore conditionally deleted RBPj, an obligate binding partner of NICD, by generating $K14^{Cre};RBPj^{flox/flox}$ mice as previously described (Blanpain, William E Lowry, et al. 2006). $K14^{Cre};RBPj^{flox/flox}$ (hereafter referred to as *RBPj-CKO*) mice have decreased canonical Notch activity, shown by decreased expression of epidermal HES1, a downstream target of canonical Notch signaling (Blanpain, William E. Lowry, et al. 2006). We immunostained whisker follicle sections from E15.5 mice for K8 and reconstructed whisker follicles to determine total Merkel cell numbers per follicle (Fig 4A-C). We found that whisker follicles of E15.5 *RBPj-CKO* mice had significantly more Merkel cells per whisker follicle than control mice (156.3 ± 24.75 vs. 127.3 ± 24.44, p=0.0026, t-test). In contrast to *NICD-OE* mice, E15.5 *RBPj-CKO* mice did not demonstrate changes in whisker follicle morphology (ET p=0.288, KT p=0.817, WFW p=0.673, WFD p=0.725, Fig 4C-D). These data demonstrate that disruption of canonical Notch signaling increases Merkel cell numbers in whisker follicles. In contrast, wholemount K8 immunostaining of back skin from P0 *RBPj-CKO*, $K14^{Cre};RBPj^{flox/+}$ mice (hereafter referred to as *RBPj-HET*) and

K14^{Cre};RBP$^{+/+}$ (hereafter referred to as control) mice demonstrated equivalent touch dome densities and Merkel cell numbers/touch dome in all three genotypes (Table 2, Fig 4E-G, I, J). This demonstrates that disruption of Notch pathway signaling does not lead to changes in touch dome Merkel cell numbers.

The Notch pathway can also signal through non-canonical mechanisms, where cytosolic NICD antagonizes the Wnt/β-catenin pathway by titrating active β-catenin (Andersen et al. 2012). Given that Wnt/β-catenin promotes Merkel cell specification (Xiao et al. 2016), NICD overexpression decreased hairy skin Merkel cell numbers and that RBPj deletion had no effect on Merkel cell numbers, we wondered whether non-canonical Notch signaling might explain the observed overexpression phenotype. We therefore generated *K14^{Cre};RBPj$^{flox/flox}$;ROSA^{NICD}* (*RBPj-CKO;NICD-OE*) mice to determine if the effects of overexpressing NICD depend on RBPj (Fig 4H). All values measured from wholemount immunostaining are summarized in Table 1. P0 *RBPj-CKO; NICD-OE*, *RBPj-CKO*, *RBPj-HET* and control mice had equivalent touch dome densities and numbers of Merkel cells/touch dome (Fig 4H-J). These data suggest that Notch effects on Merkel cell production operate through canonical Notch signaling pathways.

Although numbers of Merkel cells/touch dome were normal in P0 *RBPj-CKO*, *RBPj-HET* and *RBPj-CKO; NICD-OE* mice, we noticed two key differences between them and control mice. First, increased numbers of ectopic, interfollicular K8+ cells were present outside of touch domes in hairy skin of all three genotypes (Table 2, Fig 4E-H, K), similar to what was previously described in adult *K14^{CreER};RBPj$^{flox/flox}$* mice (Ostrowski et al. 2015). The largest numbers of ectopic Merkel cells were seen in *RBPj-CKO* mice, with intermediate numbers present in *RBPj-HET* and *RBPj-CKO; NICD-OE* mice. These results demonstrate that RBPj-dependent Notch signaling inhibits epidermal Merkel cell production outside of hairy skin touch domes. Second,

patterning of Merkel cells in touch domes of P0 *RBPj-CKO* and *RBPj-CKO; NICD-OE* mice was disrupted. Specifically, we found that touch dome Merkel cells in control and *RBPj-HET* mice were restricted to the characteristic crescent occupying 281 ± 4.3º and 273 ± 6.1°, respectively, around the guard hair follicles (Fig 4E, F, L). In contrast, Merkel cells of *RBPj-CKO* and *RBPj-CKO; NICD-OE* mice occupied 331 ± 6.2° and 328 ± 5.7°, respectively, around guard hairs (Table 2, Fig 4G, H, L). These data demonstrate that Notch signaling patterns Merkel cell location in touch domes by restricting Merkel cell position.

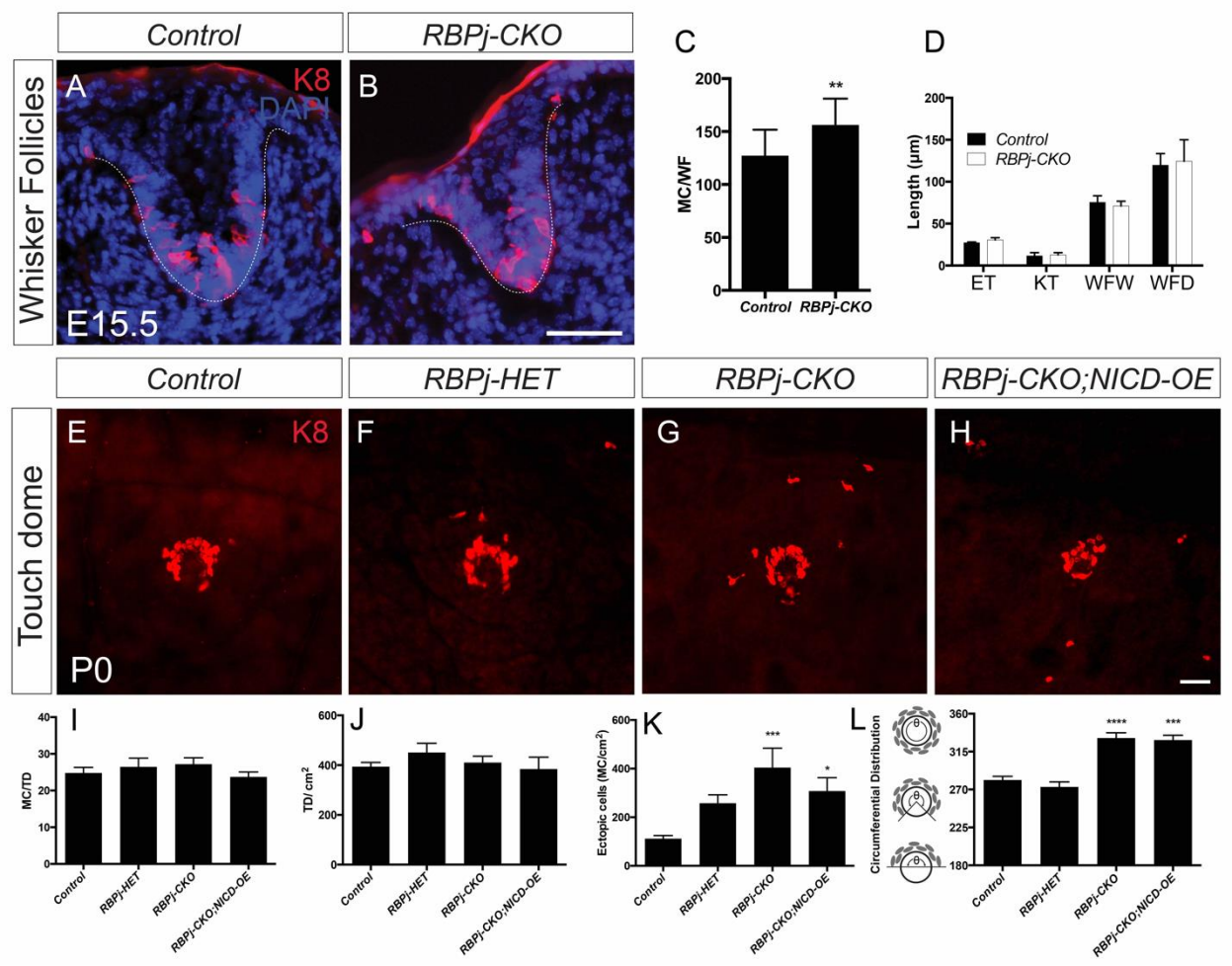

Figure 4- Disruption of Notch signaling increases Merkel cell numbers in whisker follicles and body skin.

(A,B) K8 immunostaining in whisker follicle sections of control and *K14^{cre}; RBPJ$^{flox/flox}$* mice (hereafter referred to as *RBPj-CKO* mice). Dashed line labels epidermal-dermal border. (C) Average numbers of K8+ Merkel cells in reconstructed whisker follicles. *RBPj-CKO* mice have more Merkel cells per whisker follicle than control mice (N=3 mice/genotype, N≥7 follicles/mouse; p=0.0026, paired t-test) (D) No differences in quantitative measures of whisker follicle morphology were seen between *RBPj-CKO* and control mice (ET p=0.288, KT p=0.817, WFW p=0.673, WFD p=0.725). (E-H) K8 immunostaining of wholemount back skin of control, *K14^{cre}; RBPj$^{flox/+}$* (hereafter referred to as *RBPj-HET*), *RBPj-CKO*, and *RBPj-CKO;NICD-OE* mice. (I) Average numbers of Merkel cells per touch dome (N=4 mice/genotype; N=25 touch domes/mouse; p=0.589, one-way ANOVA). (J) Touch dome density (N=4 mice/genotype, N=25 touch domes/mouse; p=0.659, one-way ANOVA). (K) Density of ectopic Merkel cells. *RBPj-CKO* and *RBPj-CKO;NICD-OE* mice have significantly more ectopic Merkel cells per cm^2 than control mice (N=4 mice/genotype, N=25 touch domes/mouse; ANOVA p=0.0005, control vs. *RBPj-CKO* p=0.0002, control vs. *RBPj-CKO;NICD-OE* p=0.013, Dunnett's post-hoc comparison). (L) Average circumferential distribution of Merkel cells in touch domes. Touch domes from control and *RBPj-HET* mice surround 281.3° ± 4.343 of the hair follicle. Touch domes from *RBPJ-CKO* and *RBPJ-CKO;NICD-OE* mice have a significantly larger central angle than control mice (N=4 mice/genotype, N=10 touch domes/mouse, ANOVA p<0.0001, *RBPj-CKO* vs. control p<0.0001, *RBPJ-CKO;NICD-OE* vs. control p=0.0001). N=4 mice/genotype for entire figure. Error bars are ±SEM. Scale bars = 50μm. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Table 2- Summary of data from Figure 2I-L

	MC/TD		TD/cm^2		Ectopic cells/cm^2		Circumferential Distribution	
	Average ± SEM	p-value†	Average ± SEM	p-value†	Average ± SEM	p-value†	Average ± SEM	p-value†
Control	24.8 ± 1.5		394 ± 17		112 ± 12		281 ± 4.3°	
RBPJ-HET	26.5 ± 2.4	0.8404	450 ± 37	0.3313	258 ± 35	0.0503	273 ± 6.1°	0.5674
RBPJ-CKO	27.2 ± 1.8	0.6202	410 ± 26	0.9488	404 ± 80	0.0002***	331 ± 6.2°	<0.0001****
RBPJ-CKO;NICD-OE	23.7 ± 1.4	0.9561	384 ± 48	0.9911	308 ± 55	0.0133*	328 ± 5.7°	<0.0001****
ANOVA		0.5888		0.6593		0.0005***		<0.0001****

*p<0.01, **p<0.05, ***p<0.001, ****p<0.0001, † compared to control

2.3.3 The Notch downstream target Hes1 inhibits Merkel cell specification

In the epidermis, Notch signaling promotes expression of *Hes1*, a transcription factor that inhibits *Atoh1* expression in the inner ear, in the cerebellum, and in secretory cells of the gut (Kelley 2006; Gerbe et al. 2011; Kim and Shivdasani 2011; Zheng et al. 2011; Chonko et al. 2013). Since Atoh1 is required for Merkel cell production (Maricich et al. 2009), we hypothesized that Notch-induced Hes1 expression might antagonize Atoh1 expression in the epidermis and subsequently inhibit Merkel cell production.

Hes1 expression is increased in the epidermis of hairy skin of *NICD-OE* mice and decreased in the hairy skin of *RBPj-CKO* mice (Blanpain, William E Lowry, et al. 2006); however Hes1 expression has not been described in whisker follicles. Whisker follicles of control *RBPj-CKO,* and *NICD-OE* littermates were immunostained for Hes1 (Fig 5A-C'). We observed qualitatively stronger nuclear Hes1 staining throughout the whisker follicles of *NICD-OE* mice and qualitatively weaker nuclear Hes1 staining in whisker follicles of *RBPj-CKO* mice, both relative to control littermates. These data demonstrate that Hes1 expression is driven by Notch signaling in a similar fashion in whisker follicles and hairy skin.

To determine whether epidermal Hes1 expression regulated Merkel cell production, we counted numbers of K8+ cells in whisker follicles of E15.5 Hes1-/- mice and control littermates (Fig 5D-F). *Hes1*$^{-/-}$ mice had significantly more Merkel cells per whisker follicle than control mice (238.5 ± 18.46 vs. 175.5 ± 17.47, p=0.0234, t-test). These results suggest that Hes1 is the downstream Notch effector in the epidermis that regulates Merkel cell production during embryogenesis. Unfortunately, *Hes1*$^{-/-}$ mice do not survive past E16.5, so we were unable to measure Merkel cell numbers in body skin of these mice.

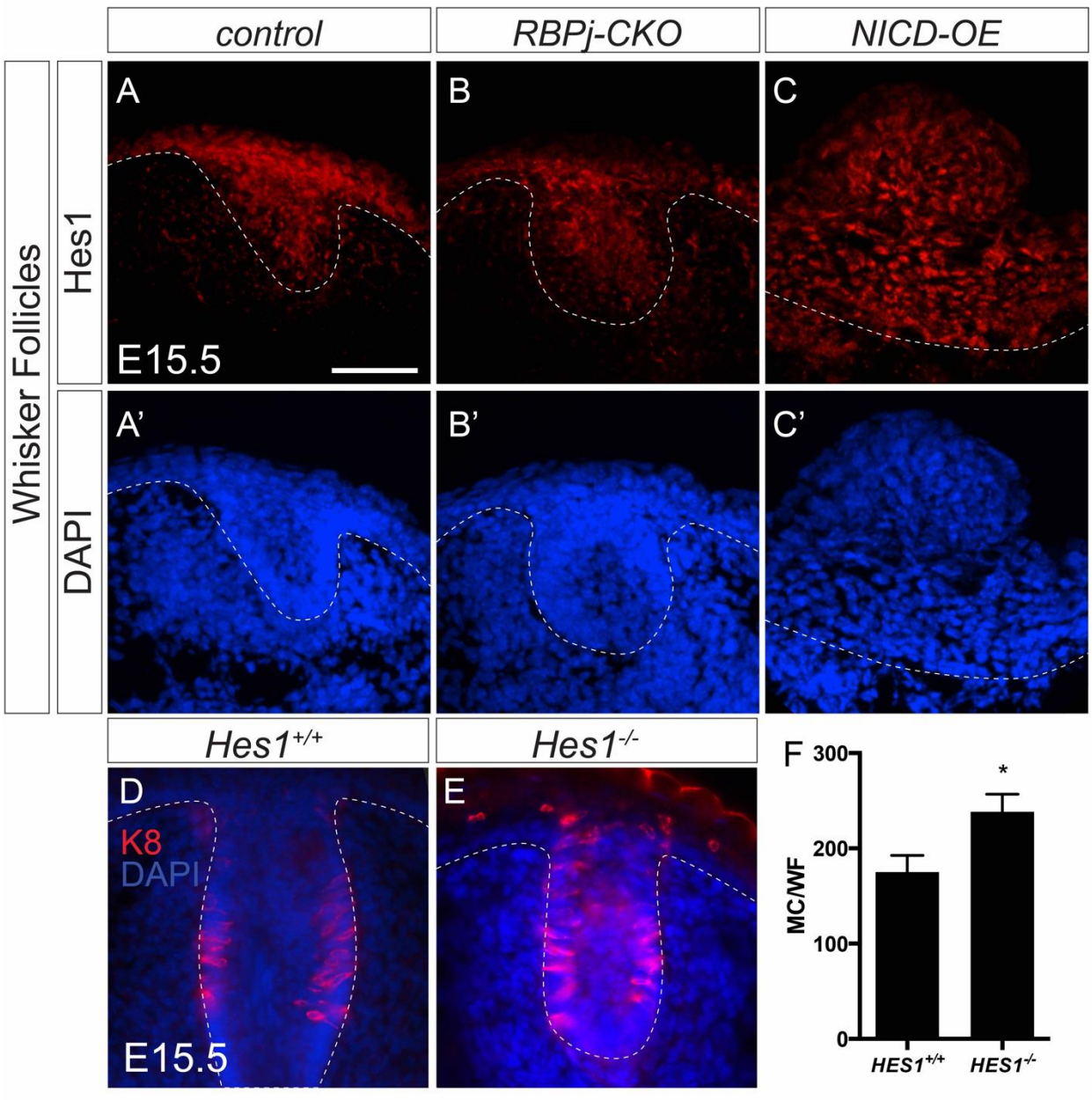

Figure 5- Hes1-null mice have more whisker follicle associated-Merkel cells.

(A,B) (A-C) Hes1 immunostaining of whisker follicles from E15.5 control (A), *RBPj-CKO* (B), and *NICD-OE* (C) mice. (A'-C') Corresponding DAPI staining. Images are representative of n=3 mice/genotype. (D,E) K8 immunostaining of whisker follicle sections from control and *Hes1*$^{-/-}$ mice. Dashed line labels the epidermal-dermal border. (F) Average numbers ±SEM of K8 positive Merkel cells from reconstructed whisker follicles (n=4 mice/genotype, p=0.023, *p<0.05) Scale bar = 50μm.

2.4 DISCUSSION

Our results identify Notch signaling as an important pathway that regulates Merkel cell production during embryogenesis. Fewer Merkel cells are produced in mice that overexpress NICD, while more Merkel cells are produced in the whisker follicle of embryonic *RBPj-CKO* mice, and ectopic Merkel cells are produced in the body skin of *RBPj-CKO* mice. Epidermal Notch signaling promotes Hes1 expression, and *Hes1*$^{-/-}$ mice produce more Merkel cells in embryonic whisker follicles. Together, these data demonstrate that Notch-induced Hes1 expression regulates Merkel cell production during embryogenesis.

Notch signals through a juxtacrine mechanism, whereby a cell with a Notch ligand binds to an adjacent cell with a Notch receptor to initiate the signaling cascade (Guruharsha et al. 2012). Mammals have four isoforms of the Notch receptor (Notch1-4) and 5 isoforms of the Notch ligand (Jagged1-2 and Delta-like1,3, and 4), each with different potential to activate the Notch signaling cascade (Gordon et al. 2009; Andersson et al. 2011). Notch signaling can promote lateral inhibition, in which the fate of two adjacent cells become defined when one cell expresses high levels of a Notch receptor and the other expresses high levels of a Notch ligand. Through this mechanism Notch can regulate embryonic patterning (Perrimon et al. 2015). In *RBPj-CKO* mice, the presence of ectopic Merkel cells and loss of the typical crescent-shaped distribution within touch domes suggests that Notch plays a role in Merkel cell patterning (Fig 4). Interestingly, a circular distribution of Merkel cells around the hair follicles is also observed in Frizzled6 knockout (*Fz6*$^{-/-}$) mice, where disruption of the Wnt signaling pathway leads to loss of hair follicle polarity (H. Chang et al. 2016). Hair follicle polarity was not altered in *RBPj-CKO* mice (data not shown), suggesting that this mechanism does not explain touch dome disruption in these mice. Further

experiments are necessary to determine if the Notch pathway interacts with the Wnt signaling pathway in other ways that might affect Merkel cell patterning.

Several Notch receptors and ligands such as Notch 1-3, Jagged 1-2, and Delta-like 1 are expressed in the epidermis and are involved in regulating differentiation of basal epidermal stem cells, but their expression in touch domes and whisker follicles has not been described (Watt et al. 2008). The differentiation of other *Atoh1*-positive progenitors into secretory cells of the gut or hair cells of the inner ear requires the expression of Notch ligands. Gut secretory cell differentiation is promoted by Jagged-1 (Kim and Shivdasani 2011; Gomi et al. 2016), and inner ear hair cell differentiation is promoted by Delta-like1 and Jagged-2 (Kelley 2006; Kiernan 2013). We predict that Merkel cell differentiation requires expression of Notch ligand(s); further experiments are necessary to understand which ligands regulate this process.

A previous study by our lab showed that the inhibitory role of Notch on Merkel cell production persists into adulthood (Ostrowski et al. 2015). In that study, epidermal RBPj deletion in adult mice led to the appearance of a modest number of ectopic, interfollicular Merkel cells. Here, we observed around 10-fold greater density of ectopic Merkel cells following epidermal RBPj deletion (404±80 vs. 36±16 Merkel cells/cm^2). One possibility for this discrepancy could be secondary to increased competence of epidermal cells in younger animals to become Merkel cells. A second possibility is that additional genes and/or signaling pathways that promote Merkel cell specification during embryogenesis are downregulated in postnatal animals.

Recently, a cascade of Wnt, Eda, and Shh signaling was shown to be essential for touch dome formation (Xiao et al. 2016). Wnt initiates the cascade by promoting hair follicle development and inducing expression of Eda and Shh. Shh subsequently promotes formation of touch dome Merkel cells. Hair follicles of *NICD-OE* mice appear to develop normally (Blanpain,

William E Lowry, et al. 2006), indicating that Notch must be downstream of Wnt signaling. Notch and Shh interact cooperatively to promote differentiation of neural progenitors (Dave et al. 2011; Kong et al. 2015), but it is unlikely that they work synergistically to regulate Merkel cell development since the two pathways have opposing effects on Merkel cell specification (Xiao et al. 2016). Further experiments are needed to clarify how Notch signaling interacts with the Wnt/Eda/SHH cascade.

The PRC2 has been suggested to restrict Merkel cell generation to first-wave primary hair follicles by preventing Merkel cell creation in secondary hair follicles that develop later in embryogenesis (Bardot et al. 2013; Dauber et al. 2016; Perdigoto et al. 2016). Our data raise the possibility that Notch signaling and PRC2 may act cooperatively to restrict Merkel cell formation and patterning during skin development, similar to the way that Notch and Polycomb proteins synergize to inhibit Rb expression in Drosophila (Ferres-Marco et al. 2006). Notch/PRC2 synergy may also play a role in the modest increases seen when RBPJ is deleted in adult mice (Ostrowski et al. 2015). Further exploration of the relationship between Notch and PRC signaling is warranted.

Understanding the intricacies of the multiple signaling pathways regulating Merkel cell development could provide insight into the biology of Merkel cell carcinoma (MCC), a deadly skin cancer (Tang and Toker 1978; Leonard et al. 2002; Eng et al. 2007; Tilling and Moll 2012). We postulate that disrupting Notch inhibition of Merkel cell formation could contribute to MCC initiation and/or progression. In support of this hypothesis, Notch1 is expressed by most MCC tumors (Panelos et al. 2009), and miR-375, the most highly expressed micro RNA in MCC tumors, post-transcriptionally represses Rbpj and Notch2 (Abraham et al. 2016). More research is needed to understand what role Notch signaling plays in MCC progression.

3.0 MERKEL CELLS ARE LONG-LIVED CELLS WHOSE PRODUCTION IS STIMULATED BY SKIN INJURY

The experiments described in this chapter are from a publication by Margaret Wright (MCW) (Wright et al. 206). We contributed to a part of the document as described below. To provide the proper context for my contributions, the paper is presented in its entirety.

My Contributions:

GL designed experiments for figures 10, 12, and 13 with help from MCW and Stephen Maricich (SMM)

GL did experiments for figures 10, 12, and 13 with help from Adam Kubicki

GL did analysis for figures 10, 12, and 13 with help from MCW

GL assisted MCW with cell counts for figures 11 and 14E-F

Contributions from other authors:

MCW wrote the manuscript

MCW and SMM designed the overarching study

MCW did experiments for figures 6-9, 11, and 14

MCW did analysis for figures 6-9, 11, and 14

Alexa Bolock, Julie Hemphill, and Tim Sanders helped with experiments

3.1 INTRODUCTION

A primary function of mammalian skin is to provide a protective barrier against environmental insults. Trauma to skin cells necessitates their frequent replacement by resident skin progenitors to maintain skin integrity (Levy et al. 2005; Page et al. 2013). These progenitor cells maintain the barrier function of the skin and insure that skin appendages such as hair follicles, sebaceous and sweat glands continue to function (Jensen et al. 2009; Lu et al. 2012). Skin cell turnover occurs on a regular schedule (for instance, as part of the hair cycle) and as needed following injury (Jaks et al. 2008; Hsu et al. 2010). Different progenitor cell populations located in different regions of the skin participate in these processes (Ito et al. 2005; Levy et al. 2005; Horsley et al. 2006; Levy et al. 2007).

Merkel cells are mechanosensitive cells found in mammalian hairy skin, whisker follicles and glabrous (non-hairy) skin of the hands and feet (Halata et al. 2003). Merkel cells are innervated by slowly-adapting type 1 (SA1) afferent neurons, and these Merkel cell-neurite complexes detect certain light touch stimuli (Iggo and Muir 1969; Johnson and Lamb 1981; Johnson and Hsiao 1992; Maricich et al. 2009; Maricich et al. 2012). Reported variations in Merkel cell numbers during the hair cycle (Moll et al. 1994; Nakafusa et al. 2006) and genetic lineage tracing studies (Van Keymeulen et al. 2009; Doucet et al. 2013; Wright et al. 2015; Xiao et al. 2015) suggest that, like other skin cells, adult Merkel cells are regularly replaced. However, the frequency of Merkel cell replacement and identities of Merkel cell progenitors remain unclear.

We analyzed Merkel cell lifespan by EdU birthdating studies beginning in embryogenesis, leading to the unexpected discovery that they persisted into late adulthood. This prompted us to perform a multifaceted analysis to investigate the kinetics of Merkel cell production, survival and replacement. Surprisingly, we found that touch dome Merkel cell numbers are constant throughout

the hair cycle and that new Merkel cells are infrequently generated during adult skin homeostasis. We repeatedly visualized the same touch domes over many months using confocal microscopy in living adult transgenic mice. Consistent with our EdU birthdating studies, we observed that a significant number of Merkel cells lived for longer than 5 months. Furthermore, we illustrate that large numbers of new Merkel cells were generated only in the setting of Merkel cell loss induced by repeated shaving and confirm that these new Merkel cells arise from touch dome keratinocytes. These data reveal important insights into Merkel cell biology that have potential relevance for understanding peripheral somatosensation and the development of Merkel cell carcinoma.

3.2 MATERIALS AND METHODS

3.2.1 Mice

Female C57BL/6J (JAX 000664), *Atoh1*^{GFP} (JAX 013593;(Lumpkin et al. 2003)), *Atoh1*$^{CreER\text{-}T2}$ ((Fujiyama et al. 2009)), Hairless (Charles River Crl:SKH1-*Hr*^{hr}), *K14*^{CreER} (JAX 005107; (Vasioukhin et al. 1999)), *ROSA*^{tdTomato} (JAX 007914; (Madisen et al. 2009)), and *ROSA*^{DTA} (JAX 009669; (Voehringer et al. 2008)) mice were maintained in accordance with International Animal Care and Use Committee guidelines at the Children's Hospital of Pittsburgh of the University of Pittsburgh Medical Center. For embryonic ages, the plug date was designated as E0.5. Mice for live imaging were anesthetized with 100mg/kg ketamine, 10mg/kg xylazine mixture. Embryonic and early postnatal mice were determined to be female by analyzing internal reproductive structures post-mortem.

3.2.2 Tamoxifen and EdU administration

Tamoxifen (Sigma-Aldrich) was dissolved in a 9:1 corn oil/ethanol solution at a 5% concentration. Mice were briefly anesthetized with isoflurane and tamoxifen administered by oral gavage at a dose of 250mg/kg once daily for three consecutive days. For embryonic administration, EdU (Invitrogen) was dissolved in sterile phosphate buffered saline (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 0.2 mg/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 dissected from pregnant dams and decapitated before tissue dissection. Skin processed for immunohistochemistry was fixed in 4% paraformaldehyde 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. 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) and slides pre-treated with 2N HCl for 15 minutes. Slides were incubated overnight in blocking solution containing dilutions of the following primary antibodies: chicken anti-GFP (1:1,000; GFP-1010 Aves Labs), goat anti-TrkB (1:200; AF1494; R&D Systems), rabbit anti-NF200 (Sigma- Aldrich, NF142; 1:500), and rat anti-keratin 8 (1:20; TROMA-1; Developmental Studies Hybridization Bank). After primary antibody incubation, sections were washed and incubated for 30 minutes 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, Alexa Fluor 488-conjugated donkey anti-chicken, Cy3-conjugated donkey anti-chicken, Cy3-conjugated donkey anti-goat, Cy3-conjugated donkey anti-rabbit, and/or Cy3-conjugated donkey anti-rat. Sections were stained with the nuclear probe DAPI (1:1000; Thermo Fisher Scientific) to visualize nuclei and mounted in ProLong Gold (Invitrogen). Whole-mount immunostaining was performed on pelts of hairy skin. Fixed skin was dissected, underlying adipose tissue removed, and washed for 5-8 hours in 0.3% PBS-T. Tissue was incubated with primary antibodies for 3 (embryonic skin) or 4 (adult skin) days, washed for 5-8 hours in 0.3% PBS-T, and then incubated with secondary antibodies for 1 (embryonic skin) or 2 (adult skin) days, all at room temperature. Antibodies were diluted in 20% dimethyl sulfoxide/5% normal donkey serum/0.3% PBS-T.

3.2.5 Imaging

Confocal images for live imaging were acquired with spinning-disc confocal imaging system (UltraVIEW VoX; PerkinElmer) utilizing a sensitive EM-CCD camera (C9100-13; Hamamatsu Photonics) allowing for minimal light exposure and photoxicity. The system was coupled to an inverted microscope (Axio Observer; Carl Zeiss) with a C-Apochromat 40X, 1.1 NA water

immersion objective. Images were obtained minimizing light exposure and resulting photoxicity and analyzed with the Volocity (Perkin Elmer) Acquisition and Analysis software. Images presented here are maximum intensity projections of a z-series or single axial slices (as noted in the Figures) consisting of 10μm optical slices collected every 0.45μm. 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 1.6x optivar was used to capture Z-stacks of 120μm thickness with single images taken every 3μm. Presented images are projections of the entire Z-stack. 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). Touch domes were identified from week to week based on their location to the square drawn on their bellies and their proximity to other touch domes (Figure 10A). Cells were classified as original or new based on positioning relative to the hair follicle and other cells from week to week. Non-confocal images were acquired with a Leica DM5500B fluorescent microscope using HCX Plan Apochromat 40X, 1.25 NA and HC Plan Apochromat 10X, 0.4 NA objectives, Leica DFC420 camera and Leica Acquisition Software v4.2. Images were cropped and brightness and contrast enhanced for publication quality with Adobe Photoshop and/or Illustrator.

3.2.6 Cell counts

All cell counts were done on the mid-back and belly skin of mice. For K8+ cell counts from E18.5 to 20 weeks of age at least 20 touch domes per mouse were counted for each back and belly (n=3-5 mice/age). Cell counts for K8+EdU+ co-label in C57BL/6J and *Hr*^{hr}/*Hr*^{hr} mice, K8+tdTomato+ co-label in *K14*^{CreER};*ROSA*^{tdTomato} mice, and K8+YFP+EdU+ co-label in *Gli1*$^{CreER-T2}$;*ROSA*^{YFP} mice were done on single slices of confocal z-stacked images (>100 K8+ cells/tissue/mouse).

Statistical tests were students t-test (Excel) or one way ANOVA followed by post-hoc Tukeys multiple comparisons testing (Prism).

3.3 RESULTS

3.3.1 Embryonic Merkel cells persist into late adulthood and new Merkel cells are rarely made during adult skin homeostasis

Keratin (K)14-expressing epidermal progenitors generate the first set of murine Merkel cells in late embryogenesis, beginning at embryonic day (E)14.5 and continuing until birth (Wright et al. 2015). However, it is unclear for how long this initial cohort of Merkel cells survives into postnatal life. To quantify the lifespan of these embryonic-born Merkel cells, we employed a birthdating approach using the modified nucleoside 5-ethynyl-2'-deoxyuridine (EdU). Incorporation of modified nucleosides like EdU into DNA occurs during S-phase; cells that become post-mitotic after incorporation retain EdU throughout their lives, while the signal is diluted until it becomes undetectable (~2-5 cell divisions) in cells that continue to divide (Kiel et al. 2007; Ganusov and De Boer 2013). To determine the lifespan of Merkel cells created during embryogenesis, we administered EdU (10mg/kg) by once daily intraperitoneal injection to pregnant C57Bl/6J female mice at E14.5, E15.5, and E16.5, the ages of peak Merkel cell generation (Wright et al. 2015). We harvested and sectioned skin from progeny mice of the same litters at postnatal day (P)0, P21 and 9 months of age (n=4 mice/age), immunostained for the Merkel cell marker Keratin 8 (K8),

visualized EdU, then calculated the percentage of K8+ cells that were also EdU+ (Figure 6; K8+EdU+/K8+ cell quantifications shown in merged panels). Qualitatively, the robustness of the EdU signal in K8+ cells was similar at all three ages, suggesting that the majority of cells labeled during embryogenesis that survived into adulthood did not continue to divide over time. Percentages of K8+EdU+/K8+ cells did not change between P0 and 9 months of age in the back skin or whisker follicles ($p=0.37$ and 0.95, t-test; Figure 6A-D’’’), nor between P21 and 9 months of age in the glabrous skin of the forepaw (Figure 6F-G’’’). A decrease in the forepaw between P0 and P21 was noted, likely secondary to continued production of Merkel cells within the glabrous skin at this age ($p=0.003$, one-way ANOVA, P0 vs. P21 $p<0.01$, P0 vs. 9 mo. $p<0.001$, P21 vs. 9 mo. $p>0.05$; Figure 6E-E’’’). These data indicate that Merkel cells born during embryogenesis survive for at least 9 months after becoming post-mitotic.

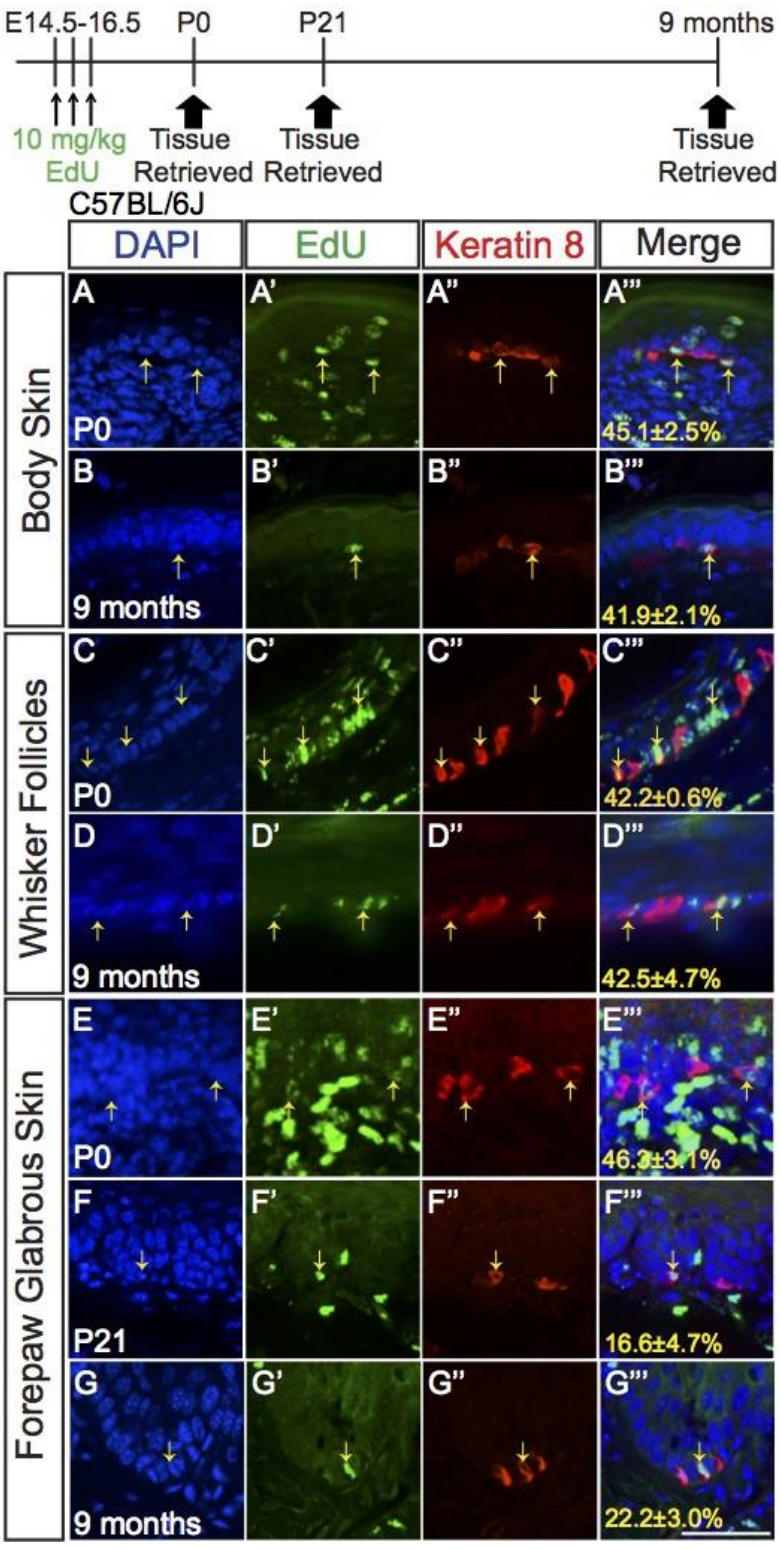

Figure 6- K8+ cells born in embryogenesis survive at least 9 months.

Single z-slice confocal images of sectioned body skin (A-B’’’), whisker follicles (C-D’’’) and glabrous forepaw skin (E-G’’’) from P0 (A-A’’’, C-C’’’, E-E’’’), P21 (F-F’’’) and 9 month-old (B-B’’’, D-D’’’, G-G’’’) female

C57Bl/6J mice that received 10mg/kg EdU at E14.5, 15.5 and 16.5. Tissues were processed for EdU (A'-G', green) and K8 immunostaining (A''-G'', red); percentages of K8+ cells that were EdU+ are shown (A'''-G''') (n=4 mice/age). Yellow arrows indicate K8+EdU+ cells. Exposure times are similar for all panels. Scale bar: 50μm.

We were surprised to see the persistence of embryonic-born Merkel cells out to 9 months of age, as previous lines of evidence suggested that this cell population should have undergone multiple rounds of complete turnover and replacement during this time (Nakafusa et al. 2006; Doucet et al. 2013; Xiao et al. 2015). We re-examined the frequency of Merkel cell production during the first hair cycle (P21-P56), a five week period of time during which approximately 50% of the Merkel cell population would be predicted to have been generated and incorporated into touch domes (Doucet et al. 2013). EdU was administered in the drinking water (0.2mg/mL) to P21 C57Bl/6J female mice for 5 weeks, after which back skin, whisker follicles, and glabrous skin of the forepaw was retrieved and processed for K8 and EdU (Figure 7A-C'''; n=3 mice). We verified that EdU exposure did not cause Merkel cell loss, as mice that received EdU and age-matched untreated mice had comparable numbers of K8+ cells/touch dome (20.3±2.5 vs 16.7±0.7, respectively, p=0.41, t-test; n=3 mice/condition). No K8+ cells in the whisker follicles or glabrous paw skin were found to have incorporated EdU (whiskers: >250 K8+ cells/mouse; paws: >70 K8+ cells/mouse; Figure 7A-B'''). In back and belly skin, a very small proportion (1.8±0.5%) of K8+ cells were EdU+ after 5 weeks of EdU exposure (>300 K8+ cells/mouse; Figure 7C-C'''). No difference was seen in the proportion of K8+EdU+/K8+ cells between back and belly skin. Together this indicates that new Merkel cells are generated and maintained as part of normal skin homeostasis during the first hair cycle, but only very infrequently and only in touch domes.

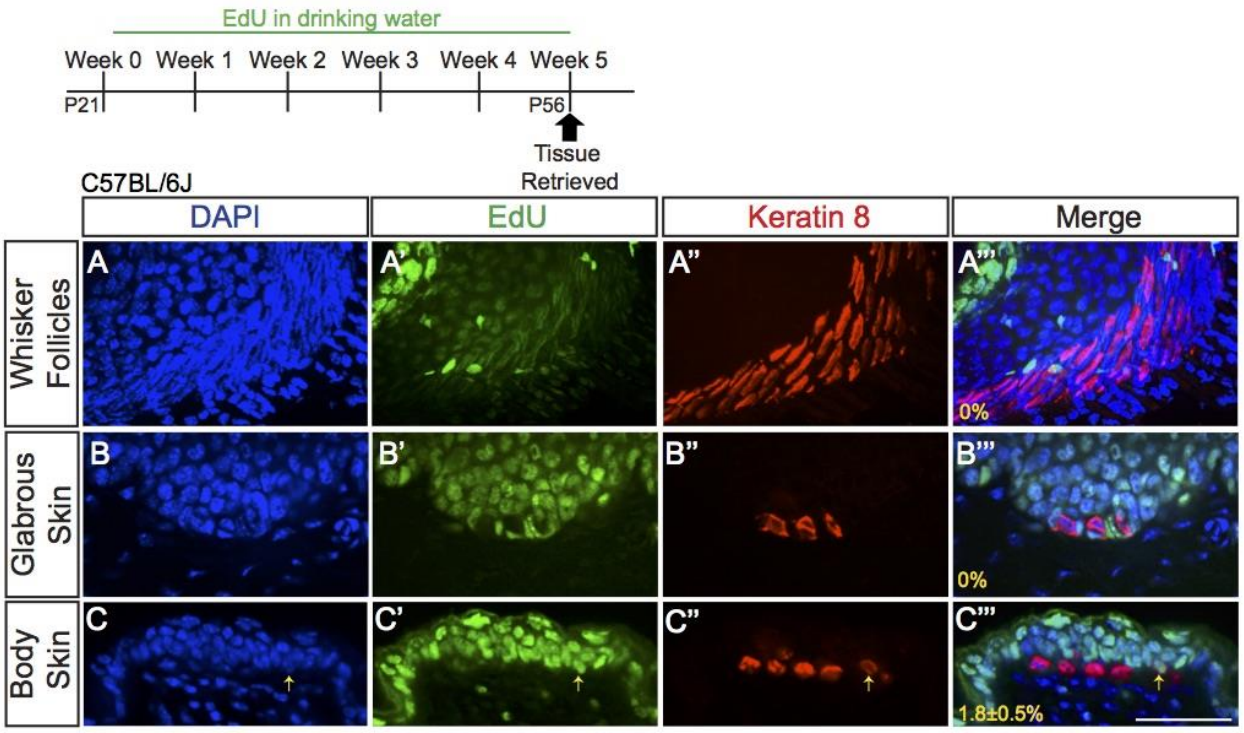

Figure 7- Few adult Merkel cells are formed, and only within touch domes.

Fig. 2. Few adult Merkel cells are formed, and only within touch domes. Sectioned whisker follicles (z-stack projection; A-A‴), glabrous forepaw skin (single z-slice; B- B‴), and back skin (single z-slice; C-C‴) from female P56 C57BL/6J mice that received 0.2 mg/mL EdU in their drinking water for five weeks. Tissues were processed for EdU (A′,B′,C′; green) and K8 immunostaining (A″,B″,C″; red). Yellow arrow (C-C‴) indicates a K8+EdU+ cell. Percentages of K8+ cells that were EdU+ are shown (A‴-C‴) (n=3 mice). Scale bar: 50 µm.

3.3.2 Merkel cell numbers decrease over early postnatal life, remain relatively constant throughout adulthood and do not oscillate with the adult hair cycle

Our data demonstrating the persistence of embryonic-born Merkel cells and infrequent generation of adult-born Merkel cells was seemingly inconsistent with previous data demonstrating oscillations of Merkel cell number with the hair cycle. To examine whether touch dome Merkel cell numbers on a C57Bl/6J background were linked to hair cycle stage, we immunostained

wholemount back skin from female mice for the Merkel cell marker K8 during hair follicle morphogenesis (E17-P21), during the first hair cycle (P21-P52) and at times after the second and third hair cycles (12 and 20 weeks of age) (>20 touch domes/mouse, n=3-5 mice/age; Figure 9A). Portions of the skin were sectioned and stained with hematoxylin and eosin (Figure 8A-L) to validate hair cycle stage using published guidelines (Müller-Röver et al. 2001). Average numbers of K8+ cells/touch dome peaked at E18.5, decreased precipitously until P21, and then decreased slightly (~20%) up to 20 weeks of age (one-way ANOVA, $F=2.80$, $p=0.02$; Figure 9A). There were no differences in K8+ cell numbers during any stage of the first hair cycle (P21-P51) (one-way ANOVA, $F=1.33$, $p=0.29$). These data show that average Merkel cell numbers do not change during natural hair cycles in the back skin of C57Bl/6J mice.

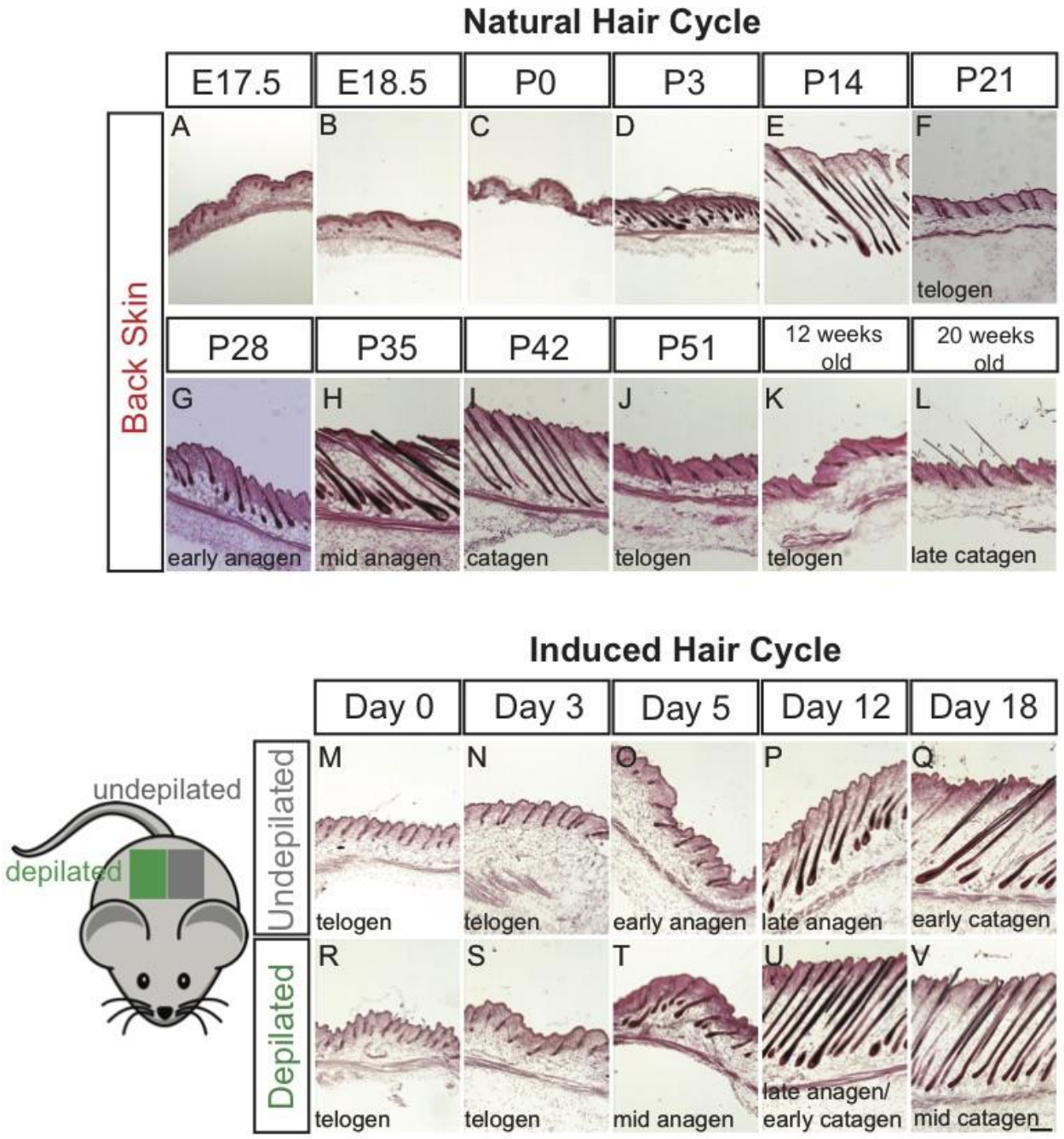

Figure 8- Confirmation of hair cycle stage in female C57Bl/6J mice.

Cryosectioned hematoxylin and eosin-stained back skin from female C57Bl/6J mice (A-L) and from undepilated (M-Q) or depilated (R-V) regions of adolescent mice. Ages (A-L) or days after depilation (M-V) and hair cycle stages are indicated in the panels. Scale bar: 50μm.

We also tested the influence of hair cycle induction on touch dome Merkel cell numbers. Back skin of P22 female C57Bl/6J mice was shaved and depilated with Surgicream (right side) or left untreated (left side), then harvested 0, 3, 5, 12 or 18 days later. These timepoints were chosen to correlate with noted Merkel cell number changes after hair cycle induction as previously published (Moll, Paus, et al. 1996). We verified that depilation induced the hair cycle on hematoxylin and eosin-stained skin sections, which showed that shaved/depilated skin entered anagen and transitioned to catagen sooner than untreated skin from the same mice (Figure 8M-V). Wholemount immunostaining revealed no differences in K8+ cell numbers between induced and naturally cycling skin at any time (two-way ANOVA $F(4, 20)=0.5848$, $p=0.7$; Figure 9B). These data demonstrate that Merkel cell numbers do not vary with induced hair cycle stage.

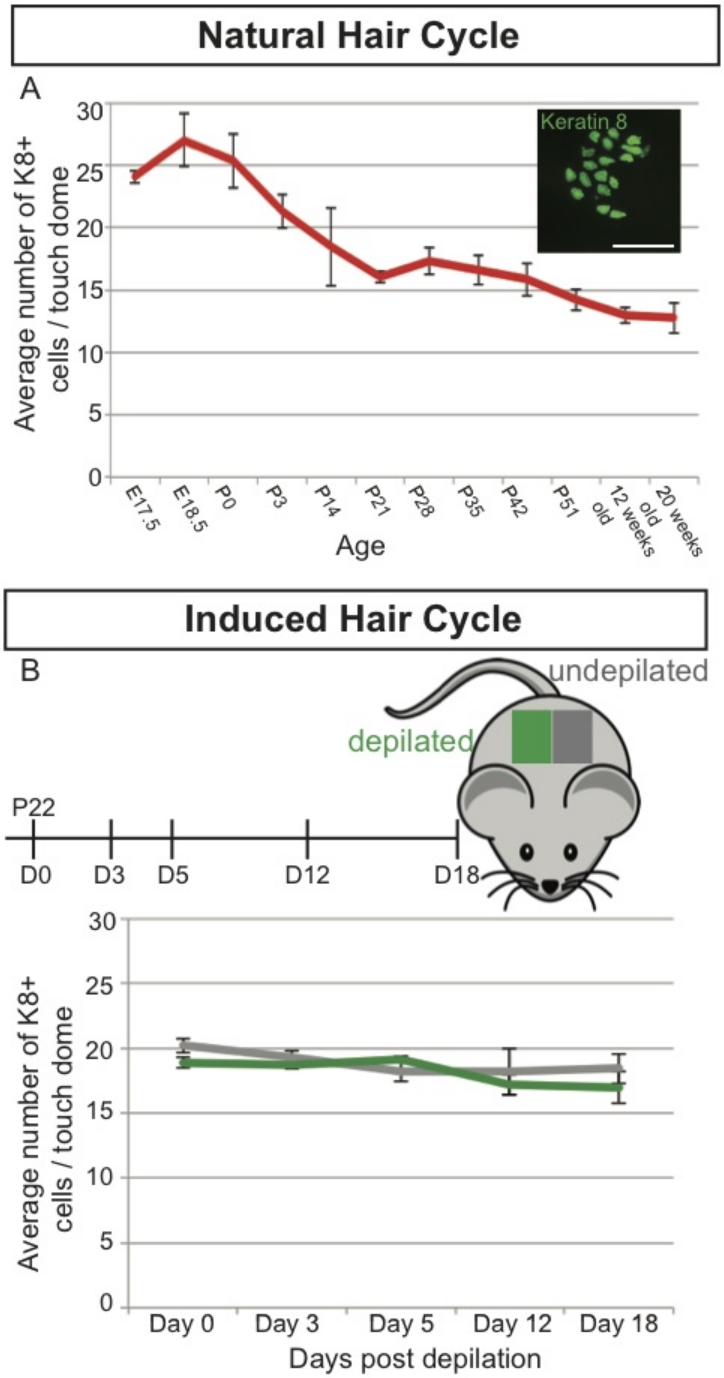

Figure 9- K8+ cell numbers in touch domes decrease over the first 3 weeks of postnatal life but remain constant throughout natural and induced hair cycles.

(A) Average K8+ cell numbers per touch dome at different stages of the natural hair cycle in back skin of C57Bl/6J female mice of varying ages (n=3-5 mice/age). Inset shows whole mount skin immunostained for Keratin

8. Scale bar: 50μm. (B) Average K8+ cell numbers per touch dome in depilated (green) vs. undepilated (gray) back skin 0, 3, 5, 12, and 18 days post-depilation (n=3 mice/timepoint). Bars on graphs are SEMs.

3.3.3 In vivo visualization of touch domes demonstrates long Merkel cell lifespan

As a final approach to investigate Merkel cell persistence in adulthood, we devised a strategy to repeatedly image the same touch domes in living adult mice over an extended period of time. Young (~P28) *Atoh1*^{GFP} mice, in which all Merkel cells are GFP+ (Lumpkin et al. 2003), were anesthetized and had the same regions of belly skin shaved once per week with a straight razor to allow repeated visualization of the same touch domes (Figure 10A). A square imaging area was marked with India Ink on the belly skin, permitting mapping and tracing of individual touch dome locations from week to week (Figure 10A). Individual touch domes were identified and mapped from week to week based on their location within the square and proximity to other touch domes. Individual touch domes (7-12/mouse from 4 mice (37 total); n=2,530 total GFP+ cells) were imaged on a spinning disc confocal microscope each week for 13-21 weeks (Figure 12A-F'). GFP+ cells were confined to touch domes and were never seen in follicular or interfollicular epidermis. Cells were considered "original" Merkel cells if they were observed at the first imaging session (week 0) or "new" if they were observed in subsequent weeks, but not at week 0. While this system did not permit unique labeling of individual cells as they were traced from week to week, their relative proximities to other GFP+ cells and the hair follicle allowed us to confidently identify and track them over time. We found that during the first 8 weeks (one estimate of Merkel cell lifespan) (Doucet et al. 2013) 64.3±3.6% of original GFP+ cells remained at week 8 (Figure 12G). Furthermore, 52±3.6% and 28±6.54% of original GFP+ cells (n=579 cells from n=4 mice) survived for 13 and 21 weeks, respectively. Touch domes retained much of their original

organization over this time, illustrating a stability of this sensory structure and verifying their position. This persistence of adult Merkel cells is consistent with our above EdU pulse-chase experiments (Figure 6) and illustrate the unique longevity of this epidermal cell population.

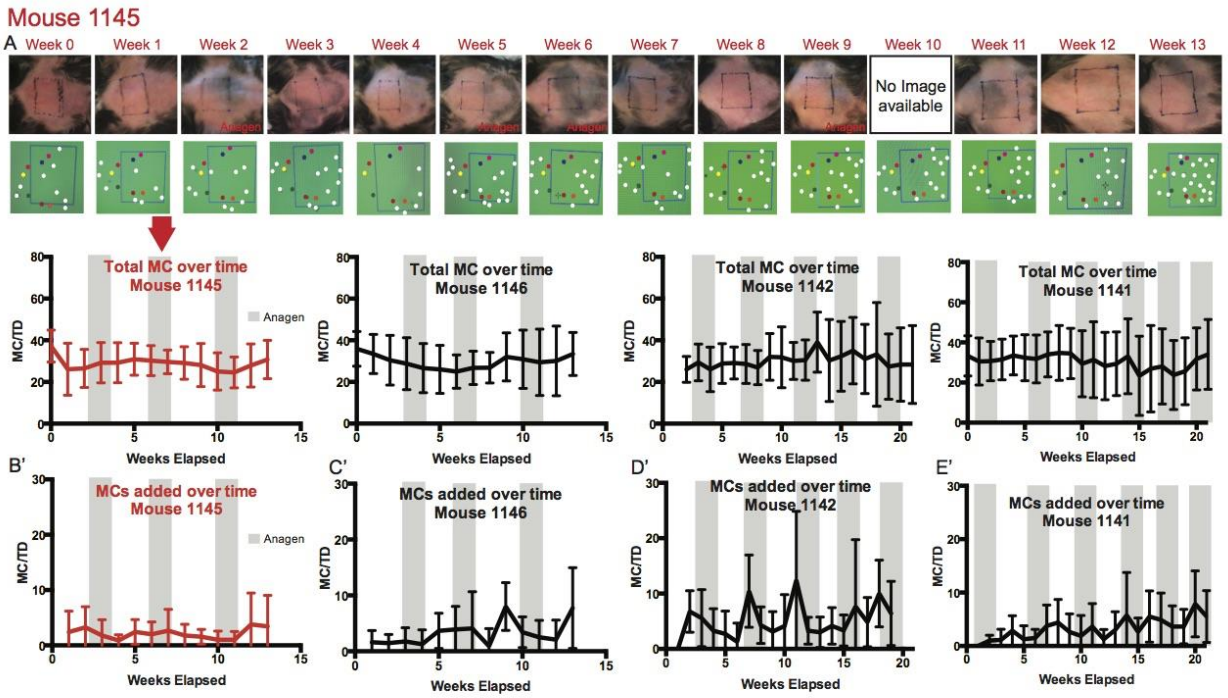

Figure 10- Skin changes and Merkel cell numbers in in vivo-imaged mice.

(A) Belly skin of mouse #1145 prior to imaging and over 13 weeks showing skin pigment changes suggestive of progression through the hair cycle. Images used to track individual touch domes (marked by unique colors) are shown below for each week. Average Merkel cell numbers per touch dome (B-E) and number of new Merkel cells added (B'-E') throughout the imaging periods. Gray bars denote anagen. Red graphs are from mouse in panel A. SEMs are shown on each graph.

Interestingly though, we noted a significant gain and loss of GFP+ cells in this model over time that was unaccounted for in our adult chronic EdU administration experiment (Figure 7C-C'''). We hypothesized that this observed increased production of Merkel cell number was somehow linked to the repeated shaving necessary for imaging and is more representative of regenerative skin conditions. To test this, we repeated the above adult EdU administration

experiment (0.2mg/mL in drinking water for 5 weeks) while shaving a section of back and belly skin with a straight razor once weekly for four weeks, as had been done to prepare the skin for live imaging, then harvested skin and stained for K8 and EdU one week after the last shave (P56). K8+EdU+ cells made up 7.8±2.7% of the K8+ population at this time, a 430% increase in Merkel cell production over that seen without shaving (Figure 11A-A’’’; p=0.03, one-tailed t-test; n=2 mice; >150 K8+ cells/mouse). Shaving therefore induces Merkel cell production as a result of cell division that is not entirely representative of normal skin homeostasis.

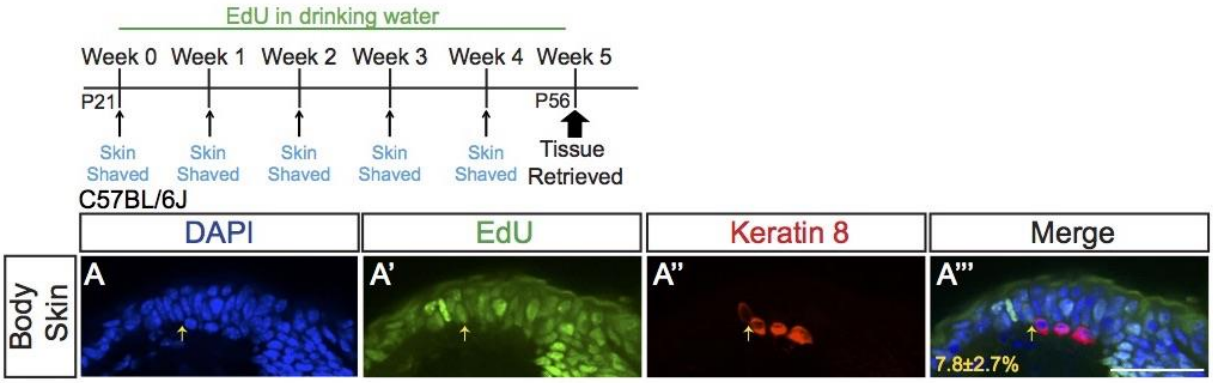

Figure 11- More Merkel cells are generated after shaving of skin

(A) Sectioned back skin (single z-slice) from female P56 C57BL/6J mice that received 0.2 mg/mL EdU in their drinking water for five weeks and were shaved once weekly. Tissue was processed for EdU (A′; green) and K8 immunostaining (A″; red). Yellow arrow indicates a K8+EdU+ cell. Percentage of K8+ cells that were EdU+ shown (A‴) (n=2 mice). (B) Average percent of K8+EdU+/K8+ cells in back and belly skin of shaved and unshaved mice (n=2–3 mice/condition). Bars on graph are SEMs. Scale bar: 50 µm.

Despite these changes, we found that average Merkel cell number/touch dome remained constant over the imaging period (Figure 13H). An average of of 3.14±0.6 new GFP+ cells per touch dome arose per week throughout the imaging period, with no clear correlation with mouse age or hair cycle stage as judged by gross skin appearance (Figure 10A). This increase in new Merkel cell numbers was matched by Merkel cells loss, as average Merkel cell number per

touchdome remained steady throughout the imaging period (Figure 13H). New GFP+ cells had a lower median survival (21 days) relative to original GFP+ cells (84 days), and half of all new GFP+ cells disappeared within two weeks of creation (Figure 13I, Figure 12). The slope of the survival curve for new GFP+ cells within their first two weeks was significantly steeper than that of original GFP+ cells during that time (slope original = -0.83±0.1; slope new = -3.35±0.4; p=0.0005, t-test). However, continued survival of new GFP+ cells remaining 3 weeks after their creation was similar to that of original GFP+ cells (slope original = -0.53±0.1; slope new = -0.45±0.1; p=0.4, t-test; Figure 4I, Figure S3). After 21 weeks of imaging, 93% of new GFP+ cells co-expressed the Merkel cell marker K8 (182/195 GFP+K8+/K8+, n=1 mouse) confirming that they were Merkel cells. These data show that new Merkel cells are at increased risk of death following their creation compared to original Merkel cells.

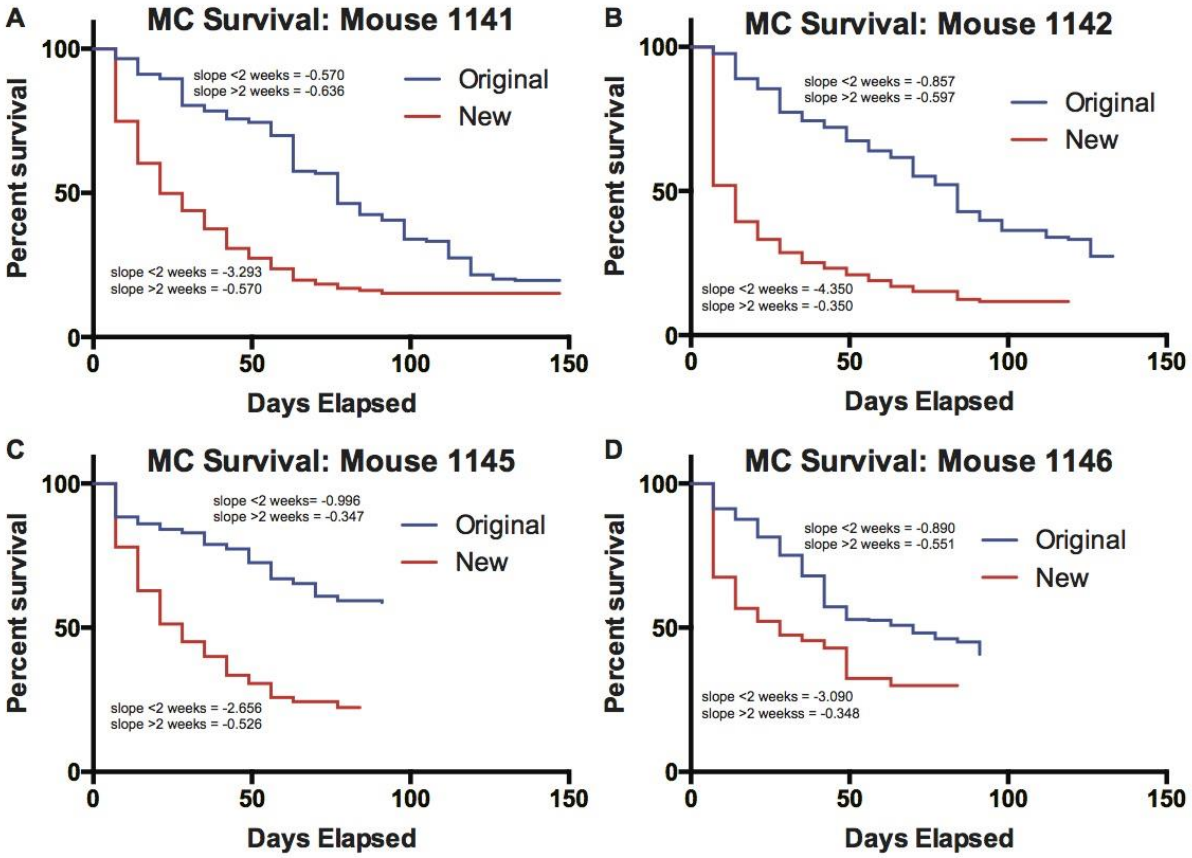

Figure 12- Survival curves for original and new Merkel cells in individual Atoh1GFP mice.

Survival of original (blue) and new (red) GFP+ cells over the imaging periods (A, B - 21 weeks; C, D – 13 weeks). Slopes for survival curves of original and new cells at <2 and >2 weeks for individual mice are shown within each graph.

3.3.4 Survival of new Merkel cells is correlated with innervation

Merkel cell survival depends on innervation (English et al. 1983; Nurse et al. 1984; Xiao et al. 2015), so we hypothesized that survival of new GFP+ cells may be related to innervation by SA1 afferents. To test this, we immunostained skin harvested at 13 weeks for NF200 and quantified the number of GFP+ cells innervated by NF200+ endings. For this analysis, we divided

GFP+ cells into three groups: original, “old-new” (new GFP+ cells surviving >2 weeks) and “new-new” (new GFP+ cells arising ≤2 weeks prior to imaging). We found that fewer new-new (71.1±7.5%) than old-new (84.9±5.2%) or original (91.8±2.5%) GFP+ cells were innervated (one-way ANOVA p=0.023; Tukeys post-hoc p=0.026 new-new vs. original; p=0.5 old-new vs. original; n=255 original and n=323 new cells from n=2 mice; Figure 13J-K’’). These data suggest that prolonged survival of newly-generated Merkel cells is related to innervation status.

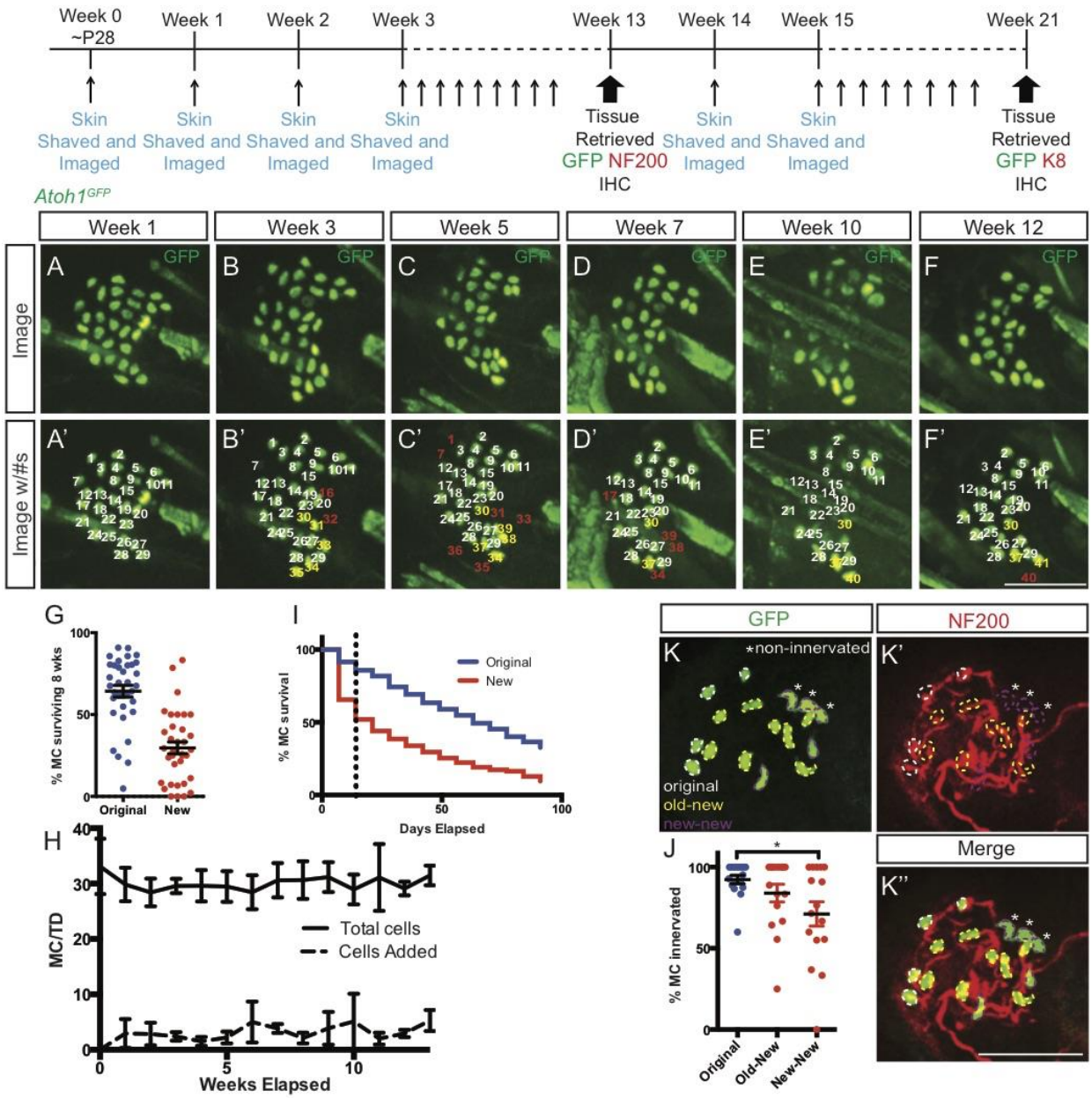

Figure 13- In vivo imaging of touch domes in Atoh1GFP mice

Adolescent *Atoh1*^{GFP} mice were shaved and imaged once weekly to enable tracking of individual touch domes over time. (A-F') *Atoh1*^{GFP}+ cells of the same touch dome at weeks 1 (A, A'), 3 (B, B'), 5 (C, C'), 7 (D, D'), 10 (E, E'), and 12 (F, F') showing endogenous GFP expression. Numbers in (A-F') indicate individual cells that survived (white), died (red) or that were born (yellow) over these weeks. (G) Percentage of original (blue) and new (red) GFP+ cells surviving for the first 8 weeks of imaging (original) or first 8 weeks following creation (new). Each dot represents one touch dome. (H) Average numbers of GFP+ cells (n=4 mice) per touch dome (solid line; p=0.534, one-way

ANOVA) and new cells per touch dome over 13 weeks of imaging (dashed line; p=0.280, one-way ANOVA). (H) Survival curves for original (blue) and new (red) GFP+ cells. In the first two weeks, survival is significantly lower for new cells than for original cells (slope original, -0.828; slope new -3.293; p=0.004) while after two weeks the survival of new and original cells is similar (slope original, -0.532; slope new -0.449; p=0.222) (K-K'') Back skin immunostained for GFP (K, green) and NF200 (K', red) showing innervation of original (white outline), >2 weeks old (yellow outline) and ≤ 2 weeks old (purple outline) cells. Non-innervated cells are indicated by asterisks. (J) Percentage of original (blue) and new (red) GFP+ cells surviving >2 weeks (old-new) and <2 week (new-new) contacted by NF200+ nerve terminals at the end of the imaging period. Scale bars: 50μm.

3.3.5 New Merkel cells arise from touch dome keratinocyte proliferation

We previously reported that Merkel cells in adult mice derive from an *Atoh1*+ lineage (Wright et al. 2015). To determine whether new Merkel cell production after repeated shaving required existing Atoh1+ cells, we ablated these cells by administering tamoxifen (250mg/kg for 3 consecutive days) to $Atoh1^{CreER\text{-}T2/+};ROSA^{DTA}$ mice, a paradigm in which Merkel cell numbers do not recover even six months post-tamoxifen administration (Wright et al. 2015). Consistent with our previous experiments, 28 days after tamoxifen administration $Atoh1^{CreER\text{-}T2/+};\ ROSA^{DTA}$ mice had 98% fewer K8+ cells per touch dome than $Atoh1^{CreER\text{-}T2/+};\ ROSA^{DTA}$ mice that did not receive tamoxifen (0.42±0.1 vs. 16.5±1.5 K8+ cells/TD; p=0.002, t-test; n=2-4 mice/genotype). However, $Atoh1^{CreER\text{-}T2/+};ROSA^{DTA}$ mice (n=3) treated with tamoxifen that had their back and belly skin shaved once per week for four weeks had nearly 8x more Merkel cells than those that were not shaved (3.29±0.5 vs. 0.42±0.1 K8+ cells/TD; p=0.015, t-test; Figure 14A-B''). Furthermore, the density of touch domes containing at least one K8+ cell was 4x higher in shaved vs. unshaved $Atoh1^{CreER\text{-}T2/+};\ ROSA^{DTA}$ mice (50.0±7.1 vs. 13±5 per cm2; p=0.02, t-test). In fact, touch dome density in shaved $Atoh1^{CreER\text{-}T2/+};\ ROSA^{DTA}$ mice was equivalent to that in C57Bl/6J control mice

(58±3 per cm^2, p=0.38, t-test), indicating that essentially all touch domes in tamoxifen-treated, shaved *Atoh1*$^{CreER\text{-}T2/+}$*;ROSA*^{DTA} mice can make new Merkel cells, while touch domes in treated, unshaved mice do not. These data demonstrate that neither the *Atoh1* lineage nor signals from existing Merkel cells are necessary for the production of new Merkel cells induced by shaving.

We next asked whether K14+ epidermal cells found in follicular and interfollicular skin (Vasioukhin et al. 1999) gave rise to new Merkel cells following shaving. To label K14+ cells, we gave tamoxifen (250mg/kg for 3 consecutive days) to P28 *K14*^{CreER}*; ROSA*^{tdTomato} mice, then either shaved them or left them untouched for 5 consecutive weeks (n=3 mice/treatment). Of note, touch dome keratinocytes are not recombined in this paradigm (Wright et al. 2015). No K8+tdTomato+ cells were seen in either shaved or unshaved skin (>400 K8+ cells from 10 back and 10 belly touch domes per mouse; Figure 14C-C''), demonstrating that K14+ progenitors do not give rise to new Merkel cells.

To test the contribution of hair follicle progenitors to Merkel cell generation, we administered EdU in the drinking water to Hairless (*Hr*^{hr}*/Hr*^{hr}) mice, which lack cycling hair follicles secondary to disregulated differentiation of hair follicle progenitors (Zarach et al. 2004; Benavides et al. 2010). After shaving their belly skin once weekly for 4 weeks, we found that 1.7±0.9% of K8+ touch dome cells were also EdU+ in *Hr*^{hr}*/Hr*^{hr} mice, indicating that hair follicle progenitors are not needed for new Merkel cell generation (n>35 K8+ cells/mouse; n=3 mice; Figure 14D-D''').

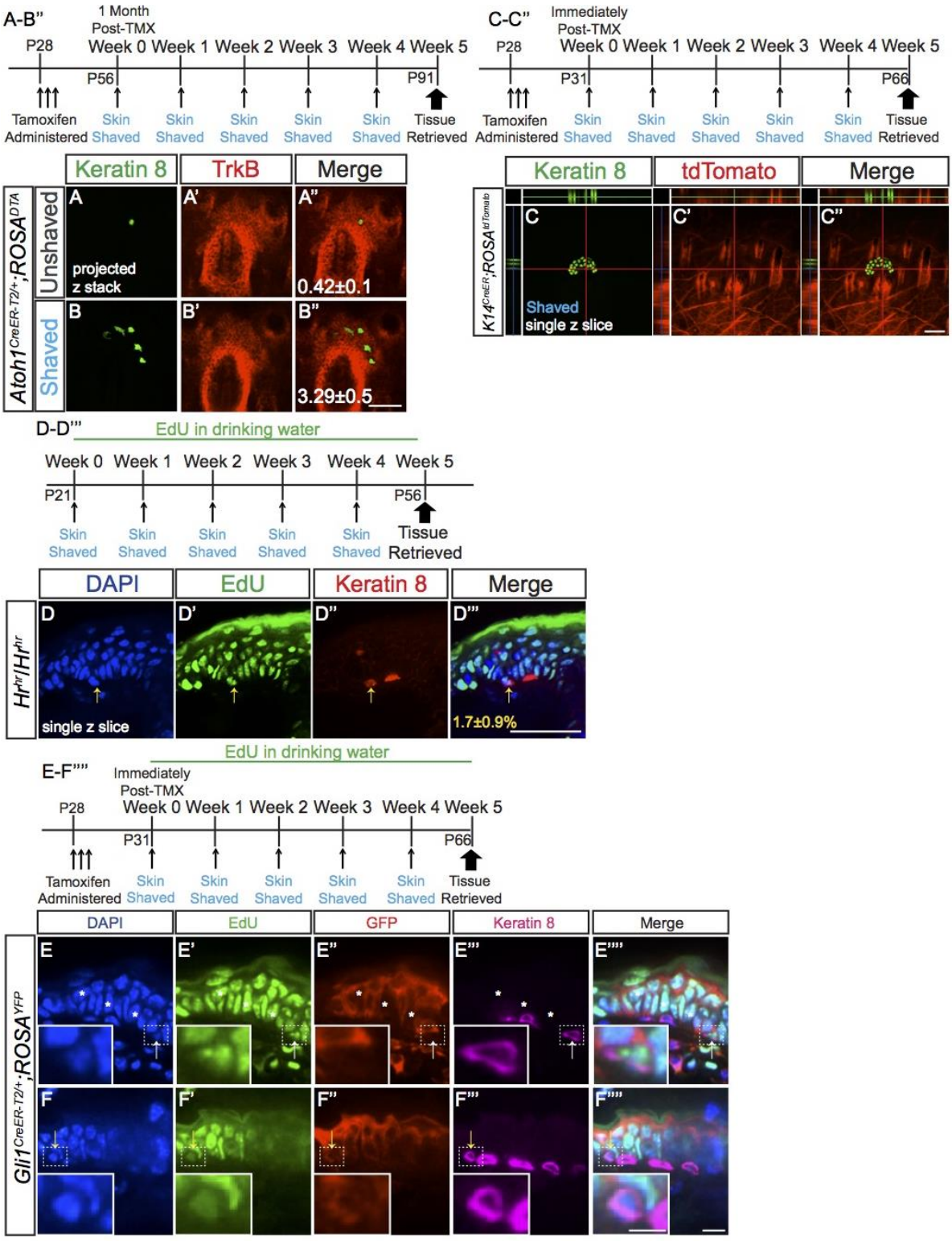

Figure 14- New Merkel cells are derived from *Gli1+* touch dome keratinocytes, not *Atoh1+*, *K14+*, or hair follicle lineages, and production does not require existing Merkel cells

(A-B'') Confocal z-stack projections of wholemount back skin from tamoxifen-treated unshaved (A-A″) or shaved once weekly (B-B'') *Atoh1*$^{CreER\text{-}T2/+}$*; ROSA*^{DTA} mice immunostained for K8 (A, B; green) and TrkB (A′,B′; red). Numbers of K8+ cells (A″,B″) are shown. (C) Average number of K8+ cells per touch dome in shaved and unshaved back and belly skin of mice (n=2–3 mice/condition) (D) Average number of touch domes containing at least 1 K8+ cell per 1 cm2 of back and belly skin (n=2–3 mice/condition). (E-E'') Single z-slice confocal image of wholemount back skin from tamoxifen-treated *K14*^{CreER}*; ROSA*^{tdTomato} mouse shaved once weekly immunostained for K8 (green, E) and imaged for endogenous tdTomato (red, E′). (F-F‴) Single confocal z-slice of sectioned belly skin from a hairless *Hr*^{hr}*/Hr*^{hr} mouse given EdU in drinking water and shaved once weekly for five weeks. Yellow arrow indicates a K8+EdU+ cell. Percentage of K8+EdU+/K8+ cells ±SEM (F‴) are shown (n=3 mice/treatment). (G-H⁗) Single confocal z-slice of sectioned belly skin from tamoxifen-treated *Gli1*^{CreER}*;ROSA*^{YFP} mice shaved once weekly visualized for EdU (green, G',H') and immunostained for YFP (red, G″,H″) and K8 (magenta, G‴,H‴). Insets are of K8+EdU+ cells that are YFP- (G-G⁗, white arrow) and YFP+(H-H⁗, yellow arrow). Asterisks indicate YFP- touch dome keratinocytes. (I) Quantification of the percent of K8+YFP+/K8+ cells in unshaved and shaved *Gli*^{CreER}*;ROSA*^{YFP} mice. Bars on graph are SEMs. Scale bars: A-F''' 50 µm; G-H⁗ 10 µm, insets 5 µm.

Finally, to determine whether touch dome keratinocytes were the source of new Merkel cells, we administered tamoxifen (250mg/kg for 3 consecutive days) to *Gli1*$^{CreER\text{-}T2}$*; ROSA*^{YFP} mice and shaved their belly skin once weekly for 4 weeks or left unshaved (n=3 mice/treatment). Tissue was analyzed one week after the last shave (P66) and K8+ cells analyzed for presence of YFP (Figure 14E-F'''). With this dosage paradigm, a portion of touch dome keratinocytes remained YFP-, indicating incomplete recombination of the population (Fig. 14E''). Consistent with data from others (Xiao et al. 2015), we found that only a small proportion of K8+ cells were YFP+ in unshaved skin (6.5±1.3%). However, this percentage was much higher in shaved skin (23.3±1.6%, p=0.001, t-test), consistent with our prior observed increase in Merkel cell production from shaving. Of note is that this percentage of K8+YFP+/K8+ cells is very close to the average number of new GFP+ cells persisting at 5 weeks of imaging (19.2±2.4%, p=0.24, t-test). Many K8+YFP+ cells were also EdU+, indicating that they arose through proliferation of recombined touch dome keratinocytes (Fig. 14F).

3.4 DISCUSSION

Our data provide several lines of evidence demonstrating that Merkel cells are long-lived, with Merkel cells born during embryogenesis surviving until at least 9 months of age in body skin, whisker follicles and glabrous skin (Figure 6). This evidence distinguishes Merkel cells as by far the longest-lived postmitotic epidermal cell population in mouse hairy and glabrous skin. We propose that long Merkel cell lifespans are critical for maintaining signaling fidelity between Merkel cells and innervating SA1 afferent fibers. Merkel cell turnover rates similar to those of other skin cells would necessitate repeated reestablishment of Merkel cell-neurite contacts. This would represent an unusual arrangement, as neurons typically form relatively stable contacts with cell populations that do not turn over (for example, other neurons or hair cells of the inner ear), and over time this could degrade the mechanosensory apparatus. Secondly, our data illustrate that while very few Merkel cells are generated during normal homeostasis in the first hair cycle, many more are formed after repeated shaving of the skin. This is a novel response that we predict is a product mild superficial wound healing. We confirmed that these new Merkel cells arise from touch dome keratinocytes, as has been previously reported (Doucet et al. 2013; Xiao et al. 2015).

Our data show that touch dome Merkel cell numbers decrease by ~45% between E18.5 and P21 (Figure 9), demonstrating that initial production occurs in excess and is followed by a culling period. We hypothesize that this culling occurs secondary to limiting amounts of trophic factors, likely derived from SA1 afferents. In support of this hypothesis, large scale Merkel cell innervation occurs between E16.5 and birth (Pasche et al. 1990; Vielkind et al. 1995), timing that correlates well with the onset of the decline in Merkel cell numbers. Moreover, skin-derived overexpression of BDNF and NT3 leads to increased numbers of mature and innervated Merkel cells in glabrous and hairy skin, respectively (Albers et al. 1996; Botchkarev et al. 1999; LeMaster et al. 1999).

Merkel cells develop normally in NT3- knockout mice but are lost by P14 along with their innervating afferents, suggesting that NT3 is required for the maintenance of Merkel cell neurite-complexes in hairy skin after birth (Airaksinen et al. 1996). Our data also demonstrate that Merkel cell numbers decrease slightly as mice age (Figure 9). This aging-related decline could result from waning trophic support from the nerve, decreased Merkel cell replacement following injury, or a combination of the two. Further experiments are necessary to test the involvement of BDNF and/or NT3 in adult Merkel cell maintenance during skin homeostasis and injury. Either way, declines in Merkel cell numbers could play an important role in the pathophysiology of aging-related somatosensory deficits. Further studies are needed to directly address this possibility.

Unlike previous studies (Moll, Paus, et al. 1996; Nakafusa et al. 2006), we did not observe changes in touch dome Merkel cell numbers during either natural or induced hair cycles (Figure 9). This conclusion is supported by our live imaging data (Figure 13H) and lack of a significant change in percentage of K8+EdU+ cells in touch domes between P0 and 9 months of age (Figure 6A-B’’’). These data provide strong evidence that Merkel cell numbers do not change during the course of the hair cycle. We believe that the discrepancy between our observations and previously published work is most likely secondary to methodological differences. Our Merkel cell counts at various stages of the natural and induced hair cycles were done in wholemount preparations of skin, thereby insuring that no cells were lost during tissue processing. Analysis of epidermal sheets (Nakafusa et al. 2006) may have led to inaccurate estimates of Merkel cell numbers, as it is our experience that >50% of Merkel cells can adhere to the dermal surface in these preps (unpublished observations). Numbers of Merkel cells that stick to the dermal surface could change during different stages of the hair cycle, leading to the erroneous conclusion that Merkel cell numbers

were changing. Likewise, counts done on small amounts of serially-sectioned mouse skin (Moll, Paus, et al. 1996) could also bias Merkel cell numbers.

Our chronic EdU administration experiments in adult mice showed that 1.8% of touch dome Merkel cells became EdU+ over 5 weeks, but that no new cells were produced in whisker follicles or glabrous skin (Figure 7). Assuming a constant rate of production and survival, approximately 0.36% of touch dome Merkel cells are generated new each week (1.8%/5 weeks = 0.36%). If this is projected out to 9 months of age (the oldest age that we examined), we would expect that 14% of Merkel cells should be new (0.36%*39 weeks = 14%). Following E14.5-E16.5 EdU administration, we found that 45.1±2.5% and 41.9±2.1% of K8+ cells were EdU+ at P0 and 9 months of age, respectively. Therefore, we would predict that 38.8% of 9 month old K8+ cells would retain EdU+ in the embryonic EdU administration experiment (45.1%*(1-0.14) = 38.8%). This number is close to the observed 41.9%, suggesting that our calculations are likely accurate. However, the difference between the percentages of touch dome K8+ cells that were also EdU+ at P0 and 9 months was not statistically significant, likely because the study was underpowered at n=4 mice/age. Regardless, our data support the conclusion that, in touch domes, there is a very low rate of Merkel cell turnover associated with normal skin homeostasis.

Interestingly, we found that Merkel cell homeostasis differs between touch dome Merkel cells and those that reside in whisker follicles and glabrous skin of the forepaw. Greater than 750 and 220 K8+ cells, respectively, were counted in each of these two locations and no Merkel cell was found to have incorporated EdU during 3-8 weeks of age. We predict that this difference in Merkel cell production is due to the proximity of Merkel cells to the epidermal border, and therefore higher potential exposure to environmental insults. Glabrous skin is much thicker than hairy skin, and Merkel cells of the whisker follicle are very deep to the epidermis, likely providing

a protective barrier permitting Merkel cell persistence. The proportion of EdU+ Merkel cells that surround whisker follicles did not decrease between P0 and 9 months of age, consistent with a lack of turnover and replacement. However, Merkel cells of the glabrous skin did decrease between P0 and P21. We have noted that Merkel cells of the paw are generated later in development than Merkel cells of the body skin (Reed-Geaghan et al. 2016), likely explaining this decrease in percentage of cells in early postnatal life. Consistent with a lack of new Merkel cell production in glabrous skin that we noted from chronic EdU exposure from 3-8 weeks of age, the percentage of EdU+ Merkel cells from P21 to 9 months of age is again unchanged.

The existence and identity of the precursor cells that maintain the adult touch dome Merkel cell population has been a source of controversy. Based on fate mapping and conditional deletion studies viewed in light of presumed Merkel cell turnover in adult animals, we recently proposed that Atoh1+ progenitors performed this role (Wright et al. 2015). Our new data force a reconsideration of this interpretation. Because adult Merkel cells express Atoh1 (Lumpkin et al. 2003; Ostrowski et al. 2015), fate mapping in our previous study marked all Merkel cells. Therefore, what we observed previously was undoubtedly the long-term survival of postmitotic Merkel cells in embryonic and adult mice, not replacement of dying cells by an *Atoh1+* progenitor. This explains the very low percentage of Ki67+ Merkel cells that we saw in adult mice (Wright et al. 2015). Of note, our new data do not change the interpretation of our observation that some embryonic *Atoh1+* cells multiply, a conclusion substantiated by EdU incorporation and expansion of lineage-traced cell numbers during embryogenesis (Wright et al. 2015). We also showed previously that tamoxifen administration to adult $Atoh1^{CreER\text{-}T2};ROSA^{DTA}$ mice, where diphtheria toxin A expression is driven in Atoh1-expressing cells, led to Merkel cell death without subsequent replacement (Wright et al. 2015). Again, we interpreted this finding as evidence that *Atoh1+*

progenitors maintain the adult Merkel cell population, and that elimination of those cells prevented creation of new Merkel cells in this paradigm. Rather, we likely deleted only post-mitotic cells, and this action alone was insufficient to induce new Merkel cell formation (see below). Furthermore, our fate-mapping and EdU labeling data in *K14^{CreER}; ROSA^{tdTomato}* and *Hr^{hr}/Hr^{hr}* mice (Figure 14) show that new Merkel cells do not arise from K14+ cells nor hair follicle progenitors as have been previously proposed (Van Keymeulen et al. 2009). Given these data, the presence of columnar K8+EdU+ cells in the touch dome epithelium following repeated shaving (Figure 11D-D'''), and our identification of K8+YFP+EdU+ cells in shaved, tamoxifen-treated *Gli1^{CreERT2}; ROSA^{YFP}* mice (Figure 11), we concur with recent reports (Woo et al. 2010; Doucet et al. 2013; Xiao et al. 2015) suggesting that new Merkel cells arise from K17+/Gli1+ progenitors in touch domes of adult mice. While EdU incorporation has demonstrated that many new K8+ cells arose through cell division, it remains possible that other K8+ cells could form by non-proliferative mechanisms. This is unlikely to occur during normal skin homeostasis, as their production and persistence would be seen through a significant decrease in the percentage of embryonic-born EdU+K8+ cells that persist into adulthood. However, this may have occurred after skin shaving, as some K8+YFP+ cells were present that did not incorporate EdU in tamoxifen-treated and shaved *Gli1$^{CreER-T2}$;ROSA^{YFP}* mice. Whether this illustrates true transdifferentiation of *Gli1*+ cells or a less than 100% EdU incorporation rate for newly-proliferating cells is unclear.

A serendipitous and surprising finding of our study supported by live imaging and chronic EdU administration experiments is that repeated shaving induces Merkel cell death and creation of new Merkel cells in touch domes (Figs. 7, 13). Genetic deletion of adult Merkel cells alone following tamoxifen administration to adult *Atoh1$^{CreER-T2}$; ROSA^{DTA}* mice was insufficient to induce new Merkel cell production (Wright et al. 2015) (Figure 14). This is an important finding

because it shows that no intrinsic counting mechanism exists to determine when Merkel cell production is required. Rather, we conclude that Merkel cell loss coupled to signals induced by repetitive shaving is necessary for Merkel cell production. We hypothesize that these signals arise in the epidermis following skin injury (Hardy et al. 2003; Lai et al. 2012). The identity and cellular origins of these signals, and what types of skin manipulation/injury are capable of inducing them, require further study.

These observations may have relevance for understanding genesis of Merkel cell carcinoma (MCC), a rare but aggressive skin cancer. Given similarities in the expression of molecular markers, it is most likely that MCC arises from Merkel cell progenitors (Tang and Toker 1978; Leonard et al. 2002; Eng et al. 2007; Tilling and Moll 2012). Our data suggest that induction of Merkel cell production following even mild skin wounds may, in combination with Merkel cell polyomavirus infection (Feng et al. 2008; Shuda et al. 2015) and/or UV radiation, provide another "hit" that leads to oncogenesis. Identifying the signaling pathways responsible for Merkel cell progenitor activation could therefore provide insight into the molecular pathways responsible for initiating this devastating cancer.

4.0 THE ROLE OF SAI NEURONS DURING MERKEL CELL PRODUCTION

The data presented in this chapter has not been published. All experiments were done by myself. Adam Kubicki helped with live animal imaging.

4.1 INTRODUCTION

As discussed in Chapter 1 of this dissertation, Merkel cell survival is dependent on signals from the neuron. Merkel cells do not survive in chronically denervated skin (Krimm et al. 2004), and neural-derived Shh is one of the factors that promotes Merkel cell survival (Xiao et al. 2015). These studies focus largely on the effects of chronic denervation, which brings up the question of whether SAI neurites reorganize during the lifespan, and if so how do Merkel cells respond to transient disruptions in innervation. One way to study this question would be through live imaging techniques.

In section 3.3.3, we used a live animal imaging tool to assess the lifespan of touch dome Merkel cells. While original cells lived for relatively long periods of time, newly-produced cells were shorter lived. Postmortem NF200 immunostaining, showed that new cells were less likely to be innervated. This suggested that newly formed Merkel cells have poor survival because they are not immediately innervated. It also suggests that innervation is not required for Merkel cell production.

Unfortunately, postmortem staining could only give a snapshot of how many touch dome Merkel cells are innervated. Furthermore, innervation can not be reliably detected by postmortem

staining because NF200 does not completely label nerve terminals. To determine if Merkel cells can be produced prior to innervation and to understand more about the dynamics of the Merkel cell-neuron interaction, we needed a live animal imaging tool that allowed us to visualize both Merkel cells and neurons.

In this chapter, I will describe the steps we took to generate a mouse model that can be used to image Merkel cells and neurites simultaneously. First we determined the efficacy of using *Thy1*$^{CreER\text{-}EYFP}$ mice to label complete SAI terminals. Next we show how the *Advillin*^{Cre} mouse can be used to label Merkel cells. Finally, we describe a successful mouse model that can be used to simultaneously image Merkel cells and SAI neurons. This model can be used to study Merkel cell and neurite interaction in both normal and injury conditions.

4.2 MATERIALS AND METHODS

4.2.1 Mice

Female and male *Atoh1*^{GFP} (JAX 013593;(Lumpkin et al. 2003)), *ROSA*^{tdTomato} (JAX 007914; (Madisen et al. 2009)), *Advillin*^{Cre} (Zurborg et al. 2011), and *Thy1*$^{CreER\text{-}EYFP}$ (Young et al. 2008) mice were maintained in accordance with International Animal Care and Use Committee guidelines at the Children's Hospital of Pittsburgh of the University of Pittsburgh Medical Center. Mice were anesthetized with 100mg/kg ketamine, 10mg/kg xylazine mixture. Mice in section 4.3.1 were given tamoxifen by oral gavage. We created a solution of 5% Tamoxifen in 9:1 corn oil: ethanol solution for oral gavage. Under isofluorane anesthesia, tamoxifen was administered at a dose of 250mg/kg once daily for three consecutive days.

4.2.2 Live Imaging

After anaesthesia, mice abdomens or backs were shaved using a straight razor, and an India ink marker was used to outline the imaging area. Mice were placed on a specialized platform with their skin pressed to a coverslip, ensuring that no skin wrinkles were present. Mice were moved to a 37°C temperature-controlled chamber for imaging. Confocal images for live imaging were acquired with spinning-disc confocal imaging system (UltraVIEW VoX; PerkinElmer) utilizing a sensitive EM-CCD camera (C9100-13; Hamamatsu Photonics) allowing for minimal light exposure and photoxicity. The system was coupled to an inverted microscope (Axio Observer; Carl Zeiss). Images were obtained minimizing light exposure and resulting photoxicity and analyzed with the Volocity (Perkin Elmer) Acquisition and Analysis software. A 10X objective with 1.6x optivar was used to capture Z-stacks of 120μm thickness with single images taken every 3μm. Presented images are extended focus projections of the entire Z-stack. Mice were repeatedly shaved and imaged once a week for 6 weeks, at which time they were sacrificed and tissue retrieved. Touch domes were identified from week to week based on their location to the square drawn on their skin and their proximity to other touch domes. Cells were classified as original or new based on positioning relative to the hair follicle and other cells from week to week. Images were cropped and brightness and contrast enhanced with Adobe Photoshop and/or Illustrator.

4.2.3 Histology

Sectioned tissues were stained on glass slides. Tissues were rehydrated in 1xPBS for 2 minutes at room temperature. Slides were then blocked in PBS with 0.3% Triton-X and 5% normal donkey serum (Millipore) for 30 minutes. Primary antibodies were diluted in blocking buffer and tissue sections were incubated for one hour at room temperature with the following antibodies: rat anti-keratin 8 (1:20; TROMA-1; Developmental Studies Hybridization Bank), chicken anti-GFP (1:000; Aves), and rabbit anti-NF200 (1:500; Sigma). After primary incubation, slides were washed 3x5 minutes at room temperature and incubated for 30 minutes in secondary antibodies: Cy3 or alexa fluor 647 conjugated anti-rat (1:250), alexa fluor 488 conjugated anti-chicken (1:250) or cy3 conjugated anti-rabbit (1:250). Nuclei were stained with DAPI (1:1000; Thermo Fisher Scientific). For wholemount, skin was dissected to remove subcutaneous fat and tissue was washed for 4-6 hours in PBS with 0.3% Triton-X. A four day primary and two day secondary incubation was done with the same concentrations and antibodies as above with 5% normal goat serum and 20% DMSO. Tissues were washed for 4-6 hours in PBS with 0.3% Triton-X between the primary and secondary incubations. All tissues were mounted in Prolong Gold (Invitrogen) on glass coverslips.

4.2.4 Data analysis

For every touch dome, each Merkel cell was tracked to determine location, lifespan, and innervation status. Each Merkel cell was assigned a number and categorized as “original” if they were detected the first week of imaging or “new” if they appeared at any subsequent week during the imaging period. Merkel cell innervation were considered innervated if the calyx of the neuron

contacted the Merkel cell. Graphs were created and statistics were preformed using Prism software (Graphpad).

4.3 RESULTS

4.3.1 *Thy1*$^{CreER\text{-}EYFP}$ mice effectively label SAI nerve terminals

To simultaneously image Merkel cells and neurons, we required a mouse model that expressed an endogenous fluorophore specifically in SAI neurons. *Thy1* is expressed by large diameter peripheral neurons including SAI neurons (Taylor-Clark et al. 2015); however, a common and publically available *Thy1*^{YFP} mouse does not label touch dome neurons (Taylor-Clark et al. 2015). Instead we acquired a similar transgenic mouse, *Thy1*$^{CreER\text{-}EYFP}$ (Young et al. 2008). These Single-neuron Labeling with Inducible Cre-mediated Knockout mice (SLICK), simultaneously express an inducible Cre, and a fluorescent EYFP reporter.

To characterize the efficacy of the *Thy1*$^{CreER\text{-}EYFP}$ mouse in labeling SAI neurons, skin from adult (P28) *Thy1*$^{CreER\text{-}EYFP}$ mice was collected and immunostained for K8 and GFP (which is sensitive to EYFP) by wholemount and tissue sections for both K8 and GFP. Wholemount immunostaining showed that Merkel cells were innervated by GFP+ SAI neurons (Figure15A). By cross section, a calyx afferent can be seen innervating the basal side of Merkel cells (Figure 15B). Next, we stained *Thy1*$^{CreER\text{-}EYFP}$ mice for GFP, NF200, and K8 and measured innervation to determine which technique was more effective in determining innervation. Significantly more Merkel cells were innervated by GFP staining than by NF200 immunostaining (97% ± 4 vs. 86% ± 14, $p<0.0001$).

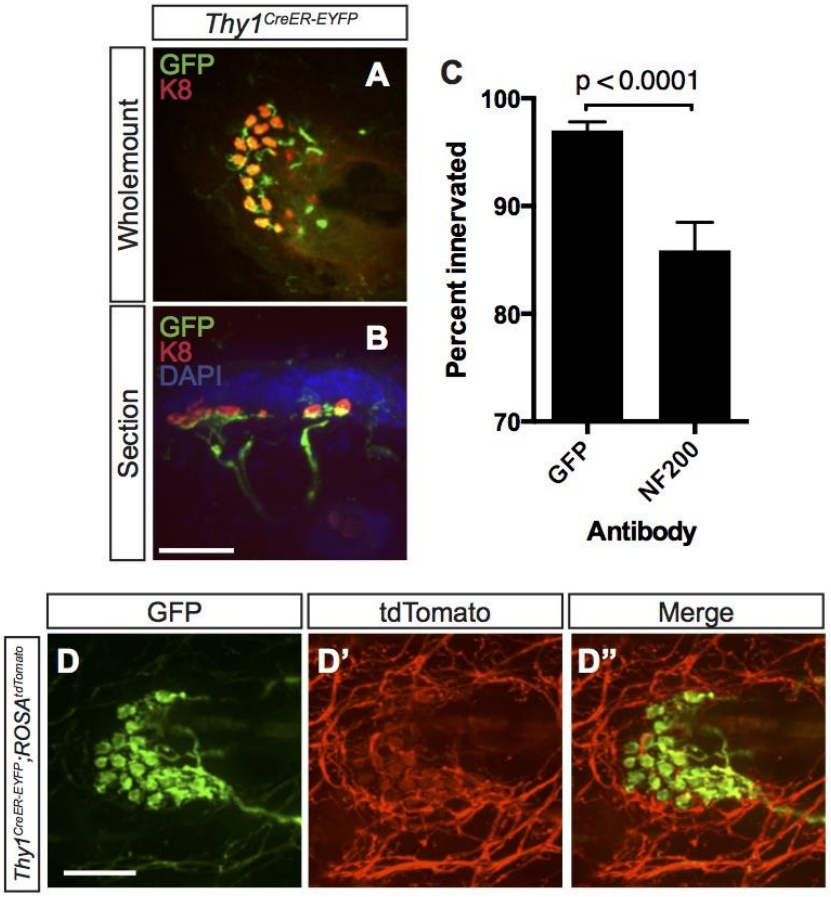

Figure 15- Complete SAI neuron labeling in *Thy1*$^{CreER\text{-}EYFP}$ transgenic mice

(A) Wholemount immunostain of GFP (green) and K8 (red) from fixed skin from *Thy1*$^{CreER\text{-}EYFP}$ mouse shows innervation at all Merkel cells. (B) Tissue sections from *Thy1*$^{CreER\text{-}EYFP}$ mice immunostained for GFP (green) and K8 (red) and DAPI (blue) shows that neurites form bouton calyxes which contact Merkel cells. (C) Percent of Merkel cells innervated using two staining techniques, immunostaining *Thy1*$^{CreER\text{-}EYFP}$ mice with a GFP antibody or immunostaining for NF200 (n=30 touch domes from 2 mice). (D-D'') Live imaging of *Thy1*$^{CreER\text{-}EYFP}$*;ROSA*^{tdTomato} mice one week after receiving three doses of tamoxifen. (D) Complete SAI neurons can be detected. (D') tdTomato does not efficiently label nerve terminals in *Thy1*$^{CreER\text{-}EYFP}$*;ROSA*^{tdTomato} mice after one week. Scale bar = 50μm.

We considered the possibility of administering tamoxifen to *Thy1*$^{CreER\text{-}EYFP}$*;ROSA*^{tdTomato}*;Atoh1*^{GFP} mice. In this model, Merkel cells will express endogenous GFP, and SAI neurons will express tdTomato as well as EYFP. We hypothesized that Merkel cells could be

distinguished from SAI neurons because of the lack of tdTomato expression. To determine the feasibility of this model *Thy1*$^{CreER\text{-}EYFP}$*;ROSA*^{tdTomato} mice were treated with tamoxifen and live imaged one week later (Figure 15D-D''). Although SAI neurons were clearly visible in the green channel, tdTomato expression was weak, and localized to many other cutaneous nerves. tdTomato expression was detected in the dorsal root ganglion, suggesting that recombination was occurring (data not shown). We live imaged the same mice two weeks later and observed increased tdTomato signal in the SAI neuron but also other cutaneous nerves (data not shown). This suggests that detection of tdTomato in the SAI is limited by diffusion from the dorsal root ganglion. Regardless, this model would not be effective for live imaging. Instead, we needed to find a transgenic mouse which expresses a red fluorophore in Merkel cells. This mouse could be crossed to *Thy1*$^{CreER\text{-}EYFP}$ mice to visualize both Merkel cells and SAI neurons.

4.3.2 *Advillin*^{Cre}*;ROSA*^{tdTomato} mice express tdTomato in Merkel cells

Advillin is an actin regulatory protein that regulates peripheral neuron outgrowth and is expressed at high levels in SAI neurons (Ravenall et al. 2002). Originally, we planned to use *Advillin*^{Cre} mice (Zurborg et al. 2011) to drive expression of a fluorescent reporter in SAI neurons. Interestingly, when we generated *Advillin*^{Cre}*;ROSA*^{tdTomato} mice, we were surprised to see tdTomato expression in Merkel cells far exceeded tdTomato expression in neurons (Figure 16), making *Advillin*^{Cre}*;ROSA*^{tdTomato} mice an effective model for visualizing Merkel cells in live mice.

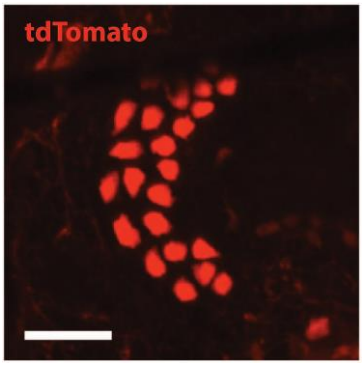

Figure 16- *Advillin^{Cre};ROSA^{tdTomato}* mice express tdTomato in Merkel cells

Live animal imaging of *Advillin^{Cre};ROSA^{tdTomato}* mouse. tdTomato expression is detected in Merkel cells. Scale bar = 50μm

4.3.3 *Advillin^{Cre};ROSA^{tdTomato};Thy1$^{CreER-EYFP}$* mice are effective for live imaging Merkel cells and their SAI neuron.

We generated *Advillin^{Cre};ROSA^{tdTomato}Thy1$^{CreER-GFP}$* mice which effectively label Merkel cells and the SAI afferent nerve in live mice (Figure 17). We wanted to be able to denervate mice while imaging, and since denervation is frequently performed by transecting the dorsal cutaneous nerve, we decided to image back skin for this experiment. Three mice were shaved and imaged weekly for 6 weeks to observe the dynamics between the neuron and the Merkel cells. Only touch domes that were detected for at least 3 consecutive weeks were analyzed. Between 4 and 10 touch domes were tracked for each mouse for a total of 19 touch domes. Merkel cells were given a number and categorized as either original or new based on if they had been detected during the first week of imaging. Each cell was designated innervated or non-innervated. For more information on how touch domes were tracked, see figure 10 and section 3.3.3 of this dissertation.

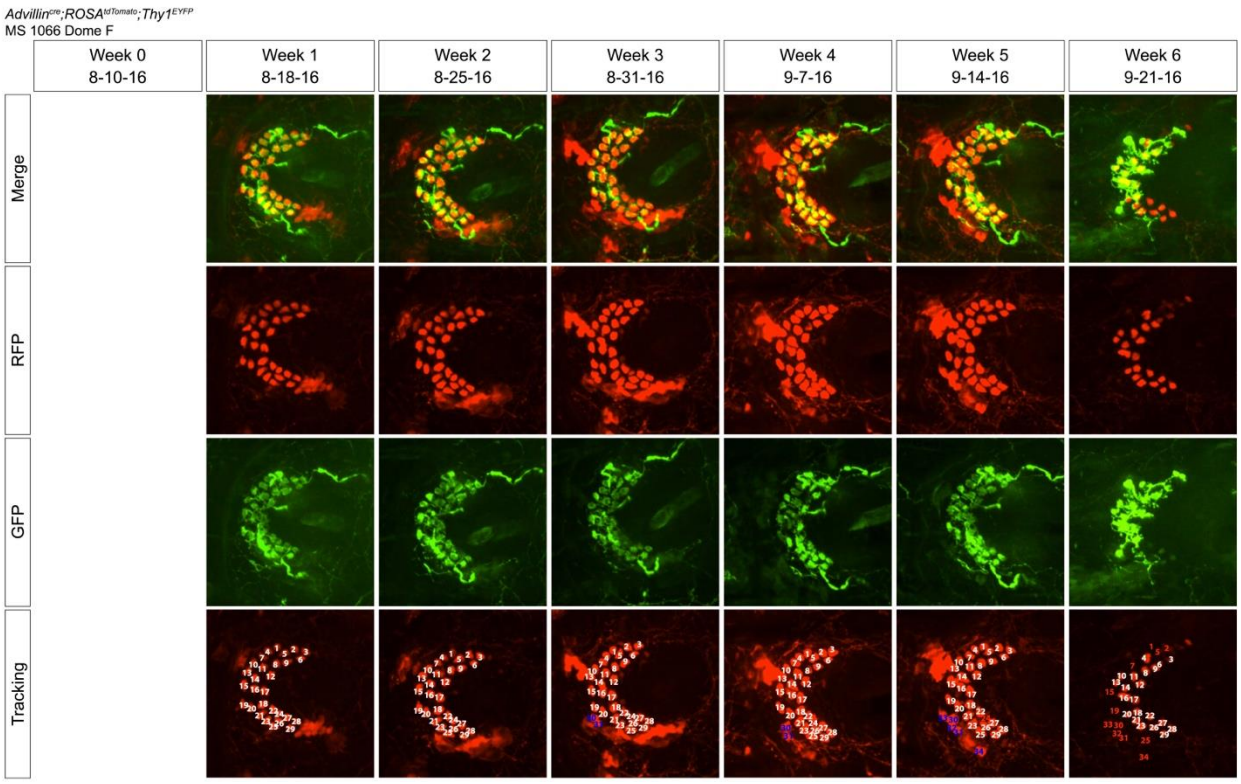

Figure 17- *Advillin^{Cre};ROSA^{tdTomato};Thy1$^{CreER-GFP}$* mice can be live imaged to show Merkel cell Neuron dynamics.

An example of one touch dome imaged for 5 consecutive weeks (this touch dome was not detected at week 0). Each column represents an image of the same touch dome from a single week. Top row is a merged image of the second and third row. In the second row from the top, red cells mark tdTomato positive Merkel cells. The third row from the top is the GFP positive SAI afferent neuron. The bottom row has an overlay of numbers representing each Merkel cell. White Merkel cells are original cells that were detected since the first week of imaging. Blue Merkel cells are new Merkel cells that appear at a later week. Red Merkel cells are cells that have disappeared during the imaging period.

4.3.4 Newly formed Merkel cells are less likely to be innervated and have poor survival than original cells

In section 3.3.3, we determined the percent of innervated Merkel cells based on post-mortem NF200 staining. This only gave a snapshot of innervation status during the imaging period. Using this dataset, we could determine if new and original Merkel cells were ever innervated. The percent

of innervated Merkel cells was significantly less than the percent of innervated original cells (73% ± 5 vs 98% ± 2, p=0.0053, Figure 18A). These findings are consistent with what we observed from postmortem staining in section 3.3.3.

To determine if survival is affected by innervation, we created a survival curve for original Merkel cells, new and innervated Merkel cells, and non-innervated Merkel cells (Figure 18B). Only 4 original Merkel cells of the 336 cells were non-innervated, so these cells were grouped with new non-innervated cells. The median survival for original Merkel cells is undefined because it is greater than the imaging period (there was no point at which 50% of original Merkel cells remained. New-innervated Merkel cells had significantly worse median survival than original Merkel cells (2 weeks vs. undefined, $p<0.0001$, Mantel-Cox test). Non-innervated Merkel cells also had a significantly lower median survival than original Merkel cells (1 week vs. undefined, $p<0.0001$, Mantel-Cox test). There was no significant difference in survival between new innervated and non-innervated Merkel cells (2 weeks vs. 1 week, $p=0.125$, Mantel-Cox test). In Chapter 3 we hypothesized that innervated new Merkel cells would have higher survival than non-innervated Merkel cells. Instead, all new Merkel cells have a lower survival than original Merkel cells, regardless of innervation status. These results could be explained in three ways: 1- survival of new Merkel cells is not dependent on innervation 2- New Merkel cells that come in close contact with neurons must form mature synapses in order to survive, or 3- The low survival of new Merkel cells is an artifact of relatively low sample size (48 out of 336 Merkel cells tracked).

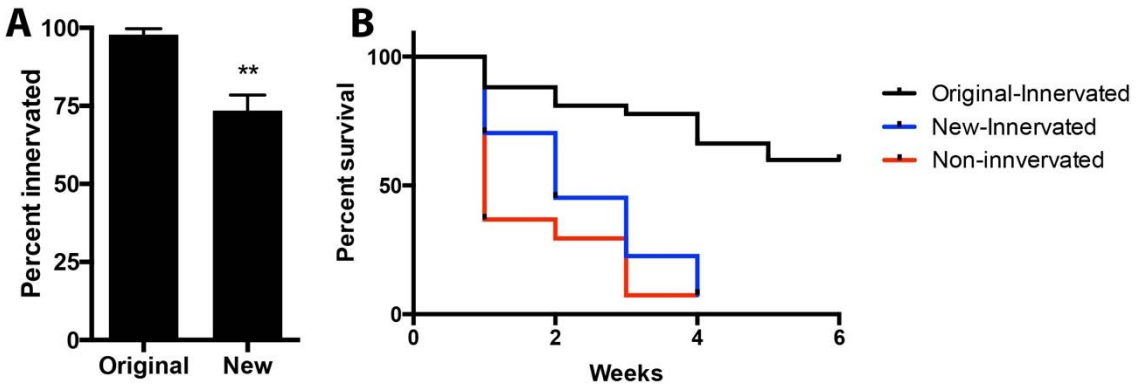

Figure 18- Original Merkel cells are innervated more often and survive longer than new Merkel cells

Quantifications on data collected from live imaging neurons and Merkel cells (see figure 17). (A) Percent of original and new cells innervated (p=0.0053, n=3 mice). (B) Survival curve with original-innervated Merkel cells (black line, 284 Merkel cells), new-innervated Merkel cells (blue, 33 Merkel cells), and all non-innervated Merkel cells (red, 19 Merkel cells) (n=3 mice; 4-10 TDs/mouse)

The model highlighted in this section could make a valuable tool for studying the dynamic interactions between the Merkel cell and the neuron. This could be used to study how neurons re-innervate after nerve injury or how Merkel cells and neurons respond to abrasive skin injury.

4.4 DISCUSSION

In this chapter, I described the steps we took to develop a model to visualize Merkel cells and SAI neurons in live mice. We discovered that *Thy1*$^{CreER\text{-}EYFP}$ mice expressed high levels of EYFP in nerve terminals, and *Advillin*^{Cre}*;ROSA*^{tdTomato} mice expressed high levels of tdTomato in Merkel cells. *Advillin*^{Cre}*;ROSA*^{tdTomato}*;Thy1*$^{CreER\text{-}EYFP}$ mice can be live imaged to detect neurons and innervating SAI neurons. We used this model to track touch domes for 6 weeks and found that new Merkel cells were innervated less often than original cells, and new Merkel cells had a lower survival than original Merkel cells.

The strong EYFP expression in SAI neurons of *Thy1*$^{CreER\text{-}EYFP}$ mice was a serendipitous discovery. Currently immunostaining with antibodies to NF200 (NFH), is the most commonly used method for detecting peripheral neurons; however, this staining does not completely label nerve terminals. Conversely, *Thy1*$^{CreER\text{-}EYFP}$ mice express EYFP in SAI terminals labeling the entire afferent calyx. This mouse can be used broadly for staining for SAI neurons. One area that would benefit from a better method of detecting peripheral neurons is determining the date of Merkel cell innervation during embryogenesis. Past reports have placed innervation broadly between E15.5 and postnatal (Pasche et al. 1990; Vielkind et al. 1995). *Thy1*$^{CreER\text{-}EYFP}$ mice can be used to determine when innervation occurs (Appendix A).

The live imaging model for imaging Merkel cells and SAI neurons can be used to answer questions about how Merkel cells interact with neurons. Thus, there are many future directions for using this model to answer questions about how the neuron promotes Merkel cell survival.

In this Chapter and in Chapter 3, we show that new Merkel cells are formed without being innervated. However, the neuron is still intact and in close proximity newly formed Merkel cells. Neural-derived growth factors could still promote Merkel cell survival from a distance. To determine if Merkel cells can be produced in complete absence of the neuron, the dorsal cutaneous nerve can be transected during the imaging period. If new Merkel cells are produced in complete absence of a neuron, it would imply that signals from the neuron are not required for Merkel cell production. If this is true it could imply that the touch dome compartment contains all the necessary factors to produce Merkel cells after injury.

This mouse model can be used to study the effects of wounding on Merkel cell number and innervation. *Advillin*^{Cre}*;ROSA*^{tdTomato}*;Thy1*$^{CreER\text{-}EYFP}$ mice can be bred to hairless *Hr*^{hr}*/Hr*^{hr} mice to produce hairless mice with fluorescent Merkel cells and SAI neurons. This model would allow for

imaging without inducing an abrasive wound by shaving. Thus mice could be left untreated or subjected to minor skin injuries.

Though this mouse model is useful; it is still imperfect. First, the tdTomato channel has non-specific staining in the epidermis, making it difficult to ensure that tdTomato signal is derived from a Merkel cell. Further, hair needs to be removed prior to imaging. Since hair removal induces Merkel cell production (Wright et al. 2016), this model does not represent non-wounded Merkel cell homeostasis. The main problem with the method is that it assumes that complete innervation occurs when a neurite encounters a Merkel cell. This is a false assumption; just because a nerve contacts a Merkel cell does not necessarily mean that a mature synapse is formed. Still, this model can be used to analyze the interactions between Merkel cells and neurites in live animals, and it is currently the only model that has this function.

5.0 HAIR FOLLICLES PROMOTE MERKEL CELL DEVELOPMENT AFTER INJURY

The data from this chapter is unpublished. Some of the data presented are preliminary, but they represent interesting findings with substantial implications to the future directions of this work. All experiments in this Chapter were done by myself. Adam Kubicki helped with measurements for Figure 20

5.1 INTRODUCTION

Shaving skin can induce production of new Merkel cells in the epidermis (Wright et al. 2016); Shaving affects the skin in several ways: removing hair, causing a mild abrasive wound (Hardy et al. 2003), and inducing mechanical stimulation (Johnson et al. 2000). Presumably, any combination of these effects could induce Merkel cell production. If we can understand which effects from shaving induce Merkel cell production, it could give insight for which signals promote Merkel cell production in adult mice.

As a preliminary experiment, we subjected C57Bl6 and $Hr^{Hrr};Hr^{Hr}$ (hairless) mice to a variety of skin conditions designed to replicate hair removal (shaving), abrasive wounding (tape stripping), and mechanical stimulation (massage). We measured the number of Merkel cells per touch dome and found that Merkel cell number decreased in shaved and tape-stripped hairless mice but not C57Bl6 mice. This suggests that the hair follicle promotes Merkel cell recovery after injury. To determine if Merkel cells are being produced near the hair follicle compartment, we generated

a heatmap to visually display the location of newly produced Merkel cells with respect to the hair follicle. We found that newly formed Merkel cells are produced most frequently near the hair follicle. These results suggest that the hair follicle promotes restoration of Merkel cell number after injury.

5.2 MATERIALS AND METHODS

5.2.1 Mice

Female C57BL/6J (JAX 000664), *Atoh1*^{GFP} (JAX 013593;(Lumpkin et al. 2003)), and Hairless (Charles River Crl:SKH1-*Hr*^{hr}) mice were maintained in accordance with International Animal Care and Use Committee guidelines at the Children's Hospital of Pittsburgh of the University of Pittsburgh Medical Center.

5.2.2 Skin wounding conditions

For all skin conditions, including control, mice were anesthetized with a 100mg/kg ketamine, 10mg/kg zylasine mixture. For shaved mice, abdomens were damped with water and covered with generic shaving cream. Hairy and hairless mice were shaved with a straight razor for two minutes. For tape stripping, a piece of adhesive tape was pressed to the abdomen and rapidly removed. This was repeated 20 times with a fresh piece of tape for each application as described previously (Holzmann et al. 2004). For massaged mice, the abdomen was stroked with the index and middle

fingers for 2 minutes as previously descrived (Major et al. 2015). After treatments, mice were allowed to recover under close observation for an hour. Each treatment was done weekly for 6 consecutive weeks. Tissue was collected at week 6.

5.2.3 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 dissected from pregnant dams and decapitated before tissue dissection. Skin processed for immunohistochemistry was fixed in 4% paraformaldehyde for 30-60 minutes and washed in PBS. Skin was dissected, underlying adipose tissue removed, and washed for 5-8 hours in 0.3% PBS-T. Tissue was incubated with rat anti-keratin 8 (1:20; TROMA-1; Developmental Studies Hybridization Bank) for 4 days, washed for 5-8 hours in 0.3% PBS-T, and then incubated with Cy3 conjugated anti-rat and cy3 conjugated anti-rabbit (1:250) for 2 days at room temperature. Skin samples were mounted in Prolong Gold (Invitrogen) on glass coverslips.

5.2.4 Heatmap generation

In section 3.2.3 of this dissertation, we described a method of live animal imaging, where we could track the location and lifespan of Merkel cells. Using ImageJ coordinates were measured for each Merkel cell in 14 touch domes as well as a coordinate for the hair follicle. ACK performed these measurements. The coordinate of the hair follicle was subtracted from the coordinates of each Merkel cell to normalize each touch dome to the location of the hair follicle. In other words, the hair follicle was assigned a coordinate of 0,0 and all Merkel cells were distributed with respect to

that point. Heatmaps were created using SciPy, a python-based open source programming tool for generating scientific figures package.

5.3 RESULTS

5.3.1 Wounding hairless mice decreases Merkel cells number

We previously show that skin shaving induces Merkel cell production (Wright et al. 2016). But we didn't know if this was due to hair removal, abrasive wounding, or mechanical stimulation. It is not possible to directly wound C57Bl6 mice without shaving the skin. Instead, we used hairless (Hr^{hr}/Hr^{hr}) mice, which have a defect in hair follicle stem cells (Benavides et al. 2010). Hairless mice develop hair follicles normally, but the hair follicle loses its capacity to regenerate after the first hair cycle. Hairless produce normal-appearing Merkel cells (Xiao et al. 2015). In these experiments, Hairless mice were subjected to one of four skin treatments: shaved, tape stripped, massaged, or untreated.

First, we were interested to see how hairless mice responded to shaving compared to hairy mice. Mice were left untreated or shaved weekly for 6 consecutive weeks, skin was collected, and wholemount immunostained for K8. We counted the number of Merkel cells per touch dome for each treatment group (Figure 20A). Hairless mice had 10% fewer Merkel cells per touch dome than hairy mice, though this was not significant (hairless, 26 ± 4; hairy, 29 ± 4; p=0.88; Tukey's post-hoc comparison). Interestingly, while shaving had no effect on the total number of Merkel cells per touch dome in hairy mice (untreated, 29 ± 4; shaved, 30 ± 2; p = 0.99), shaving hairless mice resulted in a significant decrease in the number of Merkel cells (untreated, 26 ± 4; shaved,

15 ± 1, p = 0.047). This could suggest that shaving skin decreases Merkel cell number, and hair follicle regeneration is required for restoring Merkel cell number.

Next we determined the effect of different skin treatments on total Merkel cell number hairless mice (Figure 20B). As discussed above, shaving causes a decrease in Merkel cell number in hairless mice. Similarly, tape stripped hairless mice had fewer Merkel cells than untreated hairless mice (untreated, 26 ± 4; tape stripped, 14 ± 1; p = 0.02). Massaging hairless mice had no effect on the number of Merkel cells per touch dome (untreated, 26 ± 4; massaged, 23 ± 2; p = 0.7). These results suggest that harsher skin treatments, such as shaving and tape stripping, causes in a decrease in Merkel cell number. This effect is most likely a result of an induced abrasive wound because physical stimulation alone by massaging is not enough to cause an effect.

The results, though preliminary, are interesting. They suggest two things: 1- abrasive wounding causes a decrease in the number of Merkel cells per touch dome and 2- hair follicle regeneration is required to induce Merkel cell production after wounding.

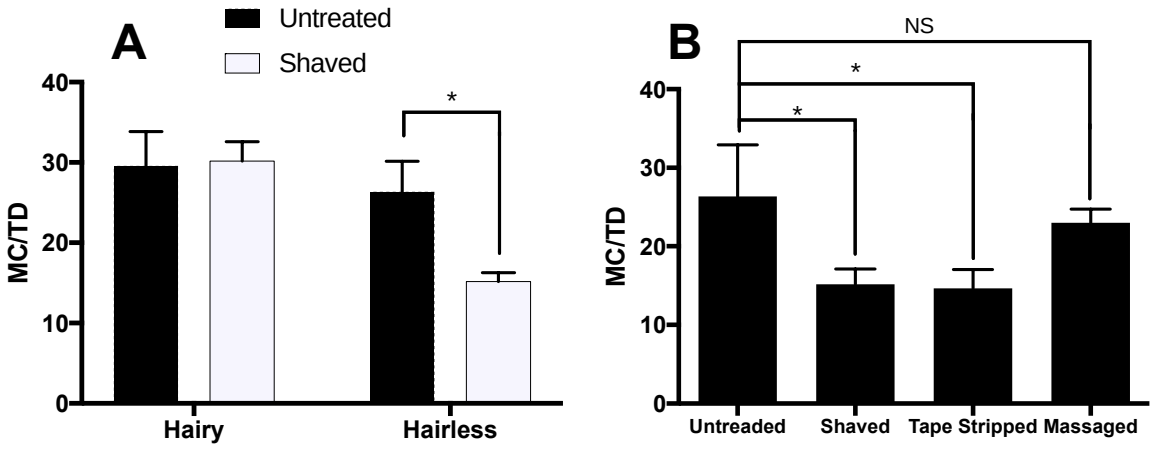

Figure 19- Abrasive wounding decreases Merkel cell number in hairless mice

(A) Average number of Merkel cells per touch dome (MC/TD ± SEM) for hairy and hairless mice left untreated or shaved for 6 consecutive weeks. Shaved hairless mice had significantly fewer Merkel cells than untreated hairless mice (p = 0.047). (B) Average

number of Merkel cells per touch dome (±SEM). In addition to shaving, tape stripped mice also had significantly fewer Merkel cells per touch dome ($p = 0.02$). Massaging mice had no effect on Merkel cell number compared to untreated mice ($p=0.7$). These results suggest that abrasive wounds cause a decrease in Merkel cell number, and hair follicle regeneration contributes, in part, to restoration of Merkel cell number. ANOVA with Tukey's post-hoc comparison was done for all statistics. NS=not significant, *$p<0.05$, n=3 mice/treatment

5.3.2 Heatmap reveals newly produced Merkel cells near the hair follicle

Results from shaving hairy and hairless skin suggest that hair follicle generation promotes Merkel cell recovery after injury. If this is true, then we would expect newly formed touch dome Merkel cells to appear near the hair follicle. In section 3.3.3 of this dissertation, we described a method whereby the location and lifespan of Merkel cells was measured during 13-21 weeks of shaving. We used information on Merkel cell location from this dataset to make conclusions on where newly produced Merkel cells appear in relation to the hair follicle.

The heatmap of original Merkel cells reveals an even distribution of cells in the characteristic crescent shape around the hair follicle (Figure 20). Interestingly, new Merkel cells appeared in the highest density closer to the hair follicle. This could be explained in three ways: 1- Merkel cells produced after injury are derived from the hair follicle, 2- Merkel cell progenitors localize near the hair follicle, or 3- the hair follicle provides signals to promote Merkel cell differentiation after injury.

The dataset from section 3.3.3 also included information about survival. We were interested in determining if cells survived for longer based on their location. To visually demonstrate where Merkel cells live the longest, we generated a map showing the location of Merkel cells with different colored points to represent lifespan of Merkel cells. The distribution of Merkel cell lifespan showed no obvious pattern; however, long-lived new Merkel cells seem to

cluster closer to the center of the touch dome, where innervation would be more consistent. This suggests that the hair follicle does not promote Merkel cell survival.

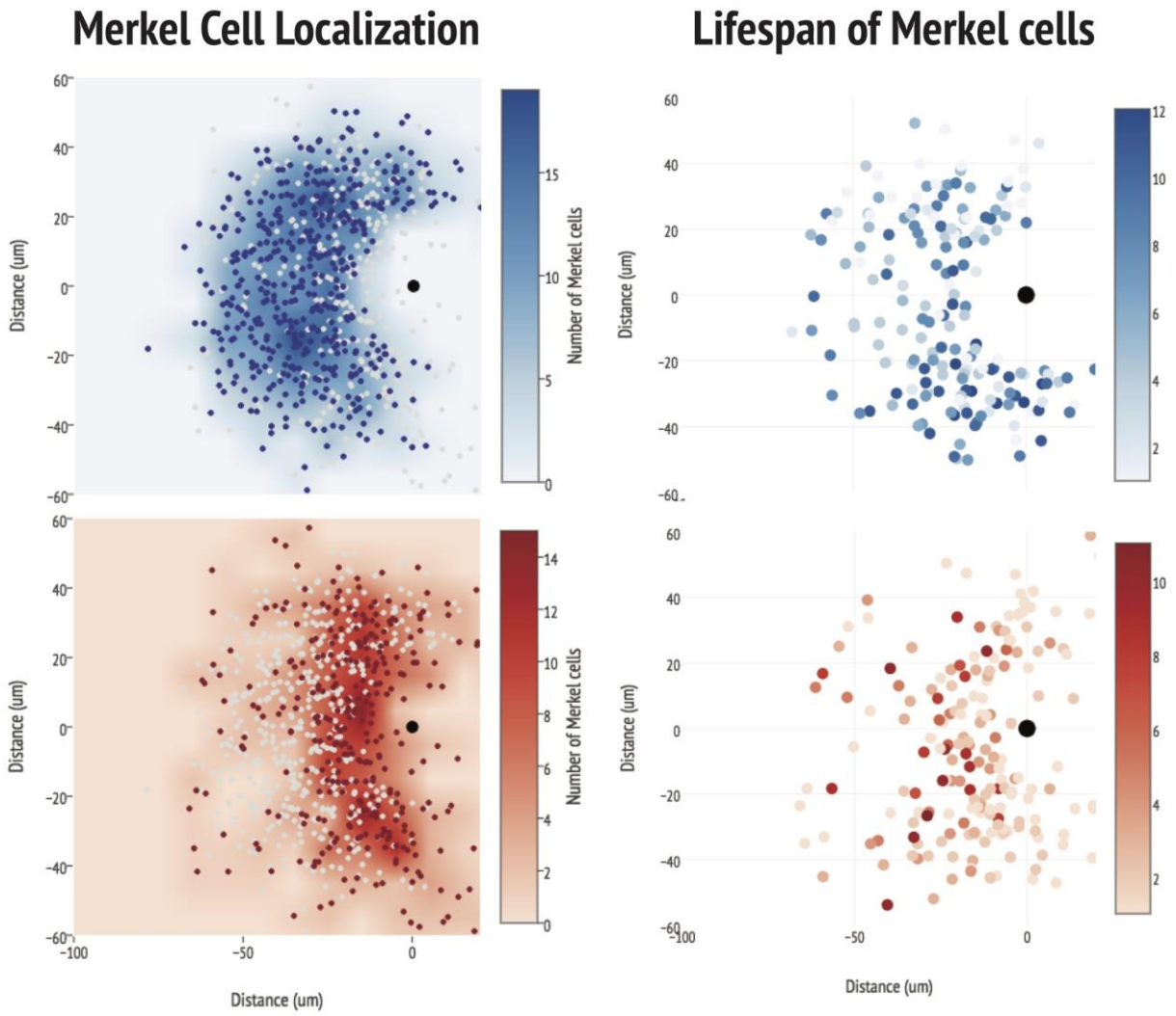

Figure 20- New Merkel cells appear more frequently near the hair follicle

The figures on the left are heatmaps demonstrating the localization of Merkel cells. Darker hues represent areas where high consentrations of Merkel cells can be detected. All graphs show the location of Merkel cells with respect to the hair follicle (black). The top left graph is a heatmap showing the location of original Merkel cells (blue), new Merkel cells (white), and the hair follicle (black). The bottom right graph is a heatmap showing the location of new Merkel cells (red), original Merkel cells (white),

and the hair follicle. The figures on the right demonstrate the lifespan of Merkel cells based on location. Darker hues represent longer-lived Merkel cells. The top right figure represents the lifespan of original cells based on location, and the bottom right figure represents the lifespan of new Merkel cells based on location.

Though these heatmaps are qualitative, the visual display gives insight to the location of newly formed Merkel cells and survival. These results suggest that newly formed Merkel cells appear most often near the hair follicle after injury, and the Merkel cells near the center of the touch dome are more likely to survive for longer periods of time.

5.4 DISCUSSION

Though the data from this chapter are preliminary, they provide interesting information about how the skin responds to wounding, and how the hair follicle regulates regeneration. First, we show that shaving causes a decrease in the number of Merkel cells per touch dome in hairless mice, but not hairy mice. Next we show that tape stripping the skin of hairless mice causes a decrease in Merkel cell number similar to the effect shaving. Furthermore, mechanical stimulation by massaging does not affect the number of Merkel cells. Finally, we show that when hairy mice are shaved weekly, they form new Merkel cells near the hair follicle.

There are a few experiments that must be done to fully understand what is occurring in this model. The first step would be to provide evidence that injury causes cell death. We would use induce abrasive wounds in hairless and hairy mice by shaving or tape stripping. Tissue can be collected and stained with TUNEL or cleaved caspase 3 to measure cell death. We hypothesize that both hairy and hairless mice will have increased number of TUNEL and caspase 3 positive Merkel cells.

The next step is to determine if new Merkel cells are produced in hairy mice but not hairless mice. Fortunately, this experiment has been done previously (Wright et al. 2016). In Wright et al. hairless and hairy mice are shaved weekly for 5 weeks while receiving EdU water. At the end of 5 weeks, Wright observed that 7.8 ± 2.7% of Merkel cells were EdU positive in hairy mice, while only 1.7 ± 0.9% of Merkel cells were EdU positive in hairless mice. These results suggest that the presence of a fully functional hair follicle is important for Merkel cell production after injury.

The live imaging tool described in Chapter 4 of this dissertation can also be used to strengthen the conclusions from these experiments. $Advillin^{Cre};ROSA^{tdTomato};Thy1^{CreER\text{-}EYFP}$ can be bred to hairless ($Hr^{hr};Hr^{hr}$) mice to create a hairless mouse with fluorescent Merkel cells and neurons. These mice can be shaved or left untreated while imaging weekly to produce a timecourse that shows how Merkel cell number changes as a result of certain skin conditions. We predict that there will be a steady decline of Merkel cells during the imaging period. This will stand in contrast to hairy mice which maintain a constant number of Merkel cells per touch dome during the imaging period (Figure 13H).

The next question would be to determine how the hair follicle promotes Merkel cell production after injury. One possibility is that Merkel cells are derived from hair follicle progenitors. A previous report suggests that Merkel cells are derived from hair follicle bulge stem cells (Van Keymeulen et al. 2009). Van Keymeulen administered RU846 daily to induce recombination in $K15^{CrePR};ROSA^{YFP}$ mice. This method was used to label K15 positive bulge stem cells and their progeny (Morris et al. 2004). He observed that 4.5% of K8+ Merkel cells were YFP positive in whisker follicles after 5 days while no YFP positive Merkel cells were observed in the paws. These results suggest that whisker follicle Merkel cells are derived from K15+ bulge stem cells. Van Keymeulin did not look at Merkel cells of hairy skin; therefore, an interesting

experiment would be to administer RU846 to *K15^{CrePR};ROSA^{YFP}* mice and leave untreated or shave to determine if touch dome Merkel cells are derived from hair follicle derived stem cells after shaving.

If Merkel cells are not derived from hair follicle progenitors, then perhaps they provide signals which promote Merkel cell production after injury. During the resting phase of the hair follicle BMP signaling represses activation of hair follicle stem cells (Zhang et al. 2006). During regeneration, BMP mediated repression is reversed by TGF-β, which induces the expansion and differentiation of bulge stem cells (Oshimori and Fuchs 2012). Hair follicle growth is then promoted by subsequent release of growth factors such as IGF, FGF, EGF, and PDGF (Stenn and Paus 2001). These signals, derived from the hair follicle niche, could promote Merkel cell production after injury. Further experiments would be necessary to determine the roles of TGF-β, IGF, FGF, EGF, and PDGF in Merkel cell production.

Though the experiments described in this Chapter are preliminary, they underscore a concept that has been left out during Merkel cell research: How do Merkel cells recover after minor injury? Here we present data that suggests that abrasions cause decrease in Merkel cells and functional hair follicles are required for restoration of Merkel cell number.

6.0 GENERAL DISCUSSION

6.1 SUMMARY AND INTERPRETATION

Perceiving the sense of touch is critical for experiencing the world around us. The skin must be sensitive enough to detect even the lightest physical stimuli, yet it must be thick enough to protect us from the environment. This conundrum is most apparent in the study of Merkel cell biology. These cells are most sensitive to light touch, and being localized to the epidermis helps with this function; however, the superficial location of Merkel cells also makes them prone to damage. Presumably, there must be a quick and efficient mechanism of differentiation to make sure that Merkel cell function is quickly recovered after routine abrasions; however, these mechanisms are poorly understood. Our work seeks to understand the signals the promote Merkel cell development by analyzing Merkel cell production during development and wound repair. In this dissertation, we have tested three main hypotheses:

Hypothesis 1: Merkel cell development is regulated by Notch signaling

Hypothesis 2: Merkel cells are long-lived and replaced after skin injury

Hypothesis 3: The nerve is required for Merkel cell survival, but not Merkel cell production

Hypothesis 4: The hair follicle promotes Merkel cell production after injury

In chapter 2, we tested hypothesis 1 by manipulating elements of the Notch signaling pathway in transgenic mice. Three conclusions came from these experiments:

1- Transgenic mice overexpressing NICD had lower numbers of touch dome and whisker follicle-associated Merkel cells.
2- Mice lacking an important element of Notch signaling (RBPj) in the epidermis had more Merkel cells in whisker follicles at E15.5. Although the number of Merkel cells per touch dome did not change, there was a greater number of ectopic Merkel cells in the interfollicular epidermis of *RBPj* conditional knockout mice.
3- Mice lacking a downstream target of Notch signaling (Hes1) had a greater number of Merkel cells in E15.5 whisker follicles.

Together these experiments suggest that Notch signaling inhibits Merkel cell specification in the developing mouse embryo. As discussed in chapter 1 of this dissertation, Merkel cell development and patterning is controlled by a precise cascade of signals from the surrounding tissue. These results identify Notch as one of those signals.

With the help of MCW, we tested hypothesis 2 in Chapter 3 and made the following conclusions:

1- MCW did an EdU pulse-chase experiment on mouse embryos and observed that Merkel cells born during embryogenesis retain EdU up to 9 months later.
2- MCW did an EdU proliferation assay for 5 weeks to detect dividing Merkel cells, and she detected no Edu positive Merkel cells in whisker follicles or paws. She detected EdU in only 1.8% of touch dome Merkel cells.
3- MCW counted Merkel cells at specific stages during the natural and induced hair cycles, and showed that the number of Merkel cells does not change at any point during the hair cycle.

4- We used live animal imaging techniques to show that 64.3% of Merkel cells detected on the first day of imaging persisted for 8 weeks. Furthermore, when new Merkel cells were produced, they were less likely to be innervated and less likely to survive for 8 weeks.

5- MCW gave EdU water to shaved mice and observed that shaving induces Merkel cell production. She observed that 7.8% of Merkel cells were EdU positive after 5 weeks of shaving, compared to 1.8% of EdU positive Merkel cells in non-shaved mice.

6- MCW used lineage tracing experiments to determine which progenitors differentiate into Merkel cells after shaving. These results showed that Merkel cells do not likely come from *Atoh1+*, *K14+*, or hair follicle progenitors. Instead, Merkel cells appear to be derived from a *Gli1+* progenitor.

Together these results suggest that Merkel cells are long-lived cells, whose production is stimulated by skin shaving. This is an interesting observation that is contradictory to other reports in the literature, which hypothesize that Merkel cells are replaced consistently through the lifespan of the mouse (Doucet et al. 2013; Xiao et al. 2015; Marshall et al. 2016). A detailed discussion of the contradictions between this work and the literature will be discussed in section 6.2 of this dissertation.

To test hypothesis 3, we generated a mouse model that allows for simultaneous imaging of Merkel cells and neurons in live mice. In the process of generating this model, we found the following:

1- *Thy1*$^{CreER\text{-}EYFP}$ mice express EYFP in the neurites of SAI neurons. These mice can be used to visualize the entire nerve terminal, and the signal is strong enough to be visualized in live mice.

2- *Advillin*^{Cre}*;ROSA*^{tdTomato} mice express tdTomato in Merkel cells. The signal intensity is strong enough to be visualized in live mice.

3- *Advillin*^{Cre}*;ROSA*^{tdTomato}*;Thy1*$^{CreER=EYFP}$ mice express EYFP in SAI nerve terminals and tdTomato in Merkel cells. These mice can be used to detect the interaction between Merkel cells and neurons.

4- Mice shaved and imaged for 6 weeks create new Merkel cells, fewer of which are innervated than original Merkel cells. This suggests that new Merkel cells are produced prior to innervation.

5- New Merkel cells have poorer survival than original cells regardless of innervation status. This suggests either that SAI neurons do not promote survival of new Merkel cells or that Merkel cells are not always completely innervated, even though the neuron physically contacts the Merkel cell.

Some of these results are preliminary and warrant further investigation. They suggest that the SAI neurons are required for Merkel cell survival, but are not required for Merkel cell production. We propose a mouse model for studying the interaction of nerves and Merkel cells that can be used to continue studying this subject. More future directions for this research will be discussed in Chapter 7.

To test hypothesis 4, we subjected hairy and hairless mice to various skin conditions to determine how these conditions affect Merkel cell number. We observed the following:

1- While shaving hairy mice has no effect on the number of Merkel cells per touch dome, shaving hairless mice decreases Merkel cell number. This suggests that hair growth promotes Merkel cell recovery after skin injury
2- An abrasive wound by tape stripping causes Merkel cell loss that resembles shaving-induced Merkel cell loss. Mechanical stimulation has no effect on Merkel cell number. This suggest that abrasive wounding causes Merkel cell death.
3- When the location of newly formed Merkel cells is visually displayed with a heatmap, it reveals that newly formed Merkel cells cluster closer to the hair follicle than longer-lived Merkel cells.

These results are preliminary, but interesting. They suggest that shaving skin induces an abrasive wound which decreases Merkel cell number, and hair growth promotes production of new Merkel cells after injury.

The experiments in Chapters 2-5 provide novel insight into genetic and environmental signals that promote Merkel cell production during embryogenesis and homeostasis. Notch signaling prevents Merkel cell development during embryogenesis; injury promotes production of long-lived Merkel cells; the SAI neuron promotes Merkel cell survival but is not required for Merkel cell production; and the hair follicle promotes Merkel cell production after injury. These results broaden our understanding of how Merkel cells are produced in normal and injured states.

The sense of touch is important for perceiving our surroundings. Touch is impaired in certain individuals, including aging adults and patients with skin grafts after burns (Ward et al. 1989; Wickremaratchi 2006). These individuals could benefit from therapies that restore their sense of touch. Further research on how the sensory system repairs after injury could result in

potential therapies. Furthermore, understanding the basic biology of how Merkel cells develop and differentiate could have implications for Merkel cell carcinoma, a rare but deadly cancer, whose cells share characteristics of Merkel cells (R Moll et al. 1984; Sidhu et al. 2009). This is an interesting area of study with many future directions, which I will discuss in Chapter 7.

6.2 CONTROVERSY OVER MERKEL CELL LIFESPAN

The findings in chapter 3 were published in Developmental Biology in the Spring of 2017 (Wright et al. 2016). Shortly before publication a paper in Cell Reports was published documenting results that contradict our findings (Marshall et al. 2016). Marshall et al. concluded that touch domes undergo rapid remodeling during anagen of the natural and induced hair cycle. This sharp contrast to our findings warrants further discussion.

6.2.1 Summary of Marshall et al. 2016

Previous studies have shown that Merkel cell number changes through the hair cycle (Moll, Paus, et al. 1996; Nakafusa et al. 2006). Marshall et al. 2016 hypothesized that both Merkel cell number and frequency of SAI responses decreases during specific parts of the hair cycle.

Marshall et al. determined the number of Merkel cells during stages of the natural and induced hair cycle using a strikingly similar method to one our lab performed in Wright et al. 2016 (discussed in chapter 3, Figure 9). To assess the changes during the natural hair cycle, hindlimb and back skin was collected from C57Bl6 mice between the ages of P23 and P70. Hair cycle stage was determined in sectioned tissue as described previously (Müller-Röver et al. 2001). Tissue was

wholemount stained for K8, NFH (NF200), and MBP. They observed fewer Merkel cells in anagen of the hair cycle (ANOVA $P<0.0001$). They also observed fewer neurite branches in anagen of the natural hair cycle.

Next, they measured change in Merkel cell number in induced hair cycle. They induced by plucking hind limb hair and collected tissue 4, 6, 10, 14, 18, 22, and 26 days later. They found fewer Merkel cells at 10, 14, and 18 days after hair plucking. They also detected fewer neurite branches 14, 18, and 22 days after hair plucking.

For the rest of the report they assess how SAI response corresponds to changes in Merkel cell number during the induced and spontaneous hair cycles. First, they generated a model for how SAI neurons remodel during the hair cycle. Then they measured SAI responses in different stages of the induced and natural hair cycles. They found that fewer SAI responses were detected in anagen compared to telogen; however, they found no difference in SAI responses during stages of the natural hair cycle. Finally, they perform a behavioral experiment to show that perceived touch changes during the induced hair cycle. They found that mice had a slower reaction time to a stimulous during induced anagen than in telogen.

Marshall et al. concludes the following 1- Merkel cell number decreases in anagen of the natural and induced hair cycle 2- Nerve branching decreases in anagen of the natural and induced hair cycle, 3- SAI responses are less frequent during anagen of the induced hair cycle but are not different during anagen of the natural hair cycle, and 4- mouse reaction to light touch is delayed during anagen of the induced hair cycle.

6.2.2 Marshall et al. 2016 vs. Wright et al. 2016

Marshall et al. and Wright et al. both measure Merkel cell numbers during the induced and natural hair cycles, but we found contradicting results which led us to opposing conclusions. It is important to note the differences in technique between the two reports. These differences are summarized in table 3. Many of these discrepancies are minor and are not sufficient to explain the contradicting conclusions. However, a combination of these technical differences could account for the discrepancies.

In Wright et al. 2016, we performed all cell counts in back skin from C57Bl6 mice. Marshall et al. first did counts in hind limbs of C57Bl6 mice. Later they perform counts on back skin of *Atoh1*^{GFP} mice. The average number of Merkel cells per touch dome can vary between different parts of the mouse (unpublished observations). This could explain why Marshall et al reports low counts of Merkel cells per touch dome (<20MC/TD) than we report in Wright et al.; however, body location is not likely to explain why Marshal et al. observed a decrease in Merkel cell number during anagen, while we did not.

The difference in statistics could account for major differences between Marshall et al. and Wright et al. The sample size was strikingly different between the two. In Wright et al. we counted over 20 touch domes from N=3-5 mice per age. In total we counted 420-700 touch domes for the natural hair cycle and 300-500 touch domes for the induced hair cycle. Marshall counted N=8-10 touch domes from 2-3 mice per age. In total, Marshall counted 64-120 touch domes for the natural hair cycle and 128-240 touch domes in the induced hair cycle. Wright et al. counted over 5 times as many touch domes as Marshall et al. for the natural hair cycle and double the number of touch domes for the induced hair cycle. Furthermore, Wright et al. reported standard error of the mean from the 3-5 mice per age, a more robust measurement than reporting counts from individual touch

domes, as they did in Marshall et al. Counting individual touch domes artificially increases the sample size, increasing the likelihood of achieving statistical significance. Most egregious of all, to statistically test if hair cycle stage influences Merkel cell number, Marshall et al. reports an ANOVA of $P<0.0001$, but does not perform a post-hoc comparison to determine if Merkel cell number in anagen is any different from other stages of the hair cycle. The authors cannot conclude that Merkel cell number decreases during anagen without this statistical test.

Table 3- Wright et al. 2016 vs. Marshall et al. 2016

	Wright et al. 2016	*Marshall et al. 2016*
Tissue collected	Back skin from C57Bl6 mice	Hind limb and back skin from C57Bl6 and *Atoh1*^{GFP} mice
Sample size	3-5 mice/age; >20 touch domes/mouse	2-3 mice/age; 8-10 touch domes/mouse
Values reported	Merkel cell per touch dome (Average per mouse)	Merkel cell per touch dome (Median per touch dome)
Ages for natural hair cycle	P21, 28, 35, 42, 51, 85, 140	P23, 35, 44, 66
Ages for induced hair cycle (D0=day of depilation)	D0, 3, 5, 12, 18 (Induced with surgicream)	D0, 4, 6, 10, 14, 18, 22, 26 (Induced by plucking)
Immunostaining	Wholemount immunostained for K8	Wholemount immunostained for K8, NF200, and MBP
Results	No difference in Merkel cell number detected at any age	Fewer Merkel cells detected at P35 and D10, 14, 18 (corresponding to anagen)
Additional supporting experiments	1- EdU uptake during embryogenesis persists for up to 9 months 2- Only 1.8 % of Merkel cell incorporate EdU in adult mice 3- Live imaging of touch dome reveals Merkel cells that can last for up to 21 weeks 4- Wright et al. 2015	1- SAI responses decrease during anagen of induced hair cycle 2- Behavioral responses to light touch is delayed during induced anagen 3- Moll et al. 1996 Nakafusa et al. 2006

Marshall et al. and Wright et al. collected tissues at different ages (see table 3). It could be possible that Wright et al. did not collect tissue during anagen, missing the window of rapid touch dome turnover. This is not likely the case, as we verified hair cycle stage at each age with H&E staining. Marshall observes a decrease around P35. Wright also collected tissue at P35 corresponding to anagen. During the induced hair cycle, Marshall et al. observed decreased Merkel cells at D10, 14, and 18. Wright observed no changes in Merkel cell number at D12 and 18. The differing results can not be explained by the difference in collection days.

Perhaps the biggest difference between the two reports is additional experimentation that supports the conclusions. Marshall et al. shows that there are few Merkel cells during anagen of the natural hair cycle, but fails to show that SAI response changes during anagen. Conversely, Wright et al. shows that Merkel cell number does not change during the hair cycle, and supports this observation with pulse-chase and proliferation assays.

Wright et al. isn't the first publication to suggest that Merkel cells live for a long time. First, Wright et al. 2015 shows that most Merkel cells lived for up to 9 months (Wright et al. 2015). Doucet also shows that Merkel cells live for over 8 weeks (Doucet et al. 2013), which is longer than a typical hair cycle (Müller-Röver et al. 2001). However, there are several reports that show Merkel cell number changes with the hair cycle, and it is currently the more accepted dogma in the field (Moll, Paus, et al. 1996; Nakafusa et al. 2006; Xiao et al. 2015).

Our findings in Wright et a. 2017 are novel and contradictory to some previous reports. We believe that our data is substantial and strong enough to call into question the dogma that Merkel cells are consistently replaced. Further exploration from third party labs is necessary in order for this hypothesis to become accepted by the field.

6.2.3 Hypotheses for the discrepancy

Although there can be many explanations for the discrepancies between these two reports, there is one hypothesis that could unite the differing conclusions:

Hypothesis: The SAI neuron retracts during anagen of the hair cycle, but Merkel cell number only changes with skin injury.

Cutaneous nerves, including SAI neurons, are intimately associated with hair follicles, which regenerate every 3-5 weeks (Müller-Röver et al. 2001). It seems unlikely that neurons do not reorganize during the hair cycle. Previous studies show that nerve density changes with the natural hair cycle (Johansson et al. 1997; Peters et al. 2001). Inducing the hair cycle by cutting hair can also induce neuron regeneration (Cheng et al. 2010). Similarly, Marshall et al. shows that SAI neurons retract during anagen of the hair cycle (Marshall et al. 2016). It is unlikely that the structure of the SAI neuron remains consistent through the hair cycle, but this does not necessarily mean that Merkel cell number changes as well.

Marshall et al. also show that Merkel cell number decreases during anagen of the natural hair cycle. This observation could be attributed to low sample size and statistical errors as described above, or it could be due to technical errors. Marshall shows that Merkel cell number is lowest during anagen, when the hair follicle is the largest. Large hair follicles can block touch domes, making them difficult to detect and count with accuracy. Furthermore, it is possible that expression of Merkel cell markers could change over time (Marshall et al. 2016), making them difficult to detect during specific stages of the hair cycle.

Contrary to this hypothesis are experiments that show the dependence of Merkel cells on the nerve (Krimm et al. 2004; Xiao et al. 2015). These studies show that Merkel cell numbers decrease after chronic denervation, but the effect of transient denervation has not been assessed. There is currently data to suggest that Merkel cells can survive without the neuron for a short period of time. Merkel cells develop prior to being innervated (Pasche et al., 1990; E. Peters et al., 2002; Vielkind et al., 1995, Appendix A) and even when nerve development is attenuated in $NT3^{-/-}$ mice, Merkel cells can still be detected two weeks after birth (Airaksinen et al. 1996). Furthermore, in Chapter 4, we show that Merkel cells are produced without being innervated. These reports show it is plausible that Merkel cells can survive shortly without the neuron.

Injury could explain why Marshall et al. observes a stark drop in Merkel cell number during the induced hair cycle. They induce the hair cycle with hair plucking, which could induce a minor wounding response. The subsequent decrease in Merkel cell number could be a response to injury instead of hair follicle growth. Wright et al. used surgicream to remove hair, a less physically aggressive procedure than hair plucking. Lastly, as we discuss in Chapter 5, injury decreases Merkel cell number in hairless mice, but the presence of hair follicle promotes restoration of touch dome Merkel cells.

This hypothesis can explain the discrepancy between Marshall et al. 2016 and Wright et al. 2016. Furthermore, it is supported by our findings in Chapters 4 and 5. This hypothesis could be tested with experiments discussed in Chapter 7 of this dissertation.

7.0 FUTURE DIRECTIONS

Despite the progress described in this dissertation, our understanding of the genetic and environmental factors that promote Merkel cell development remains incomplete. There are many directions for this research. Here, I will discuss future directions to continue the research described in this dissertation as well as new directions that can broaden our understanding of how Merkel cells are produced.

7.1 CONTINUING PROJECTS

7.1.1 Notch signaling antagonizes development of Merkel cells

In Chapter 2 of this dissertation, we show that Notch signaling antagonizes Merkel cell development. These findings bring up several questions that can be addressed in the future. Below are a few questions that we think are important future directions for this work.

1- Which Notch receptors and ligands are expressed in touch domes and whisker follicles, and how do they contribute to Merkel cell development? Several Notch receptors and ligands such as Notch 1-3, Jagged 1-2, and Delta-like 1 are expressed in the epidermis and are involved in regulating differentiation of basal epidermal stem cells, but their expression in touch domes and whisker follicles has not been described (Watt et al. 2008). The differentiation of other *Atoh1*-positive progenitors into secretory cells of the gut or hair cells of the inner ear requires the expression of specific Notch ligands.

Gut secretory cell differentiation is promoted by Jagged-1 (Kim and Shivdasani 2011; Gomi et al. 2016), and inner ear hair cell differentiation is promoted by Delta-like1 and Jagged-2 (Kelley 2006; Kiernan 2013). We predict that Merkel cell differentiation requires expression of Notch ligand(s); further experiments are necessary to understand which ligands regulate this process.

2- How does Notch signaling interact with other pathways that are important for Merkel cell development? It is unlikely that Notch pathway signaling receives no crosstalk from pathways that are known to regulate Merkel cell development. There are two specific pathways that have recently been shown to be important for Merkel cell development. First, a cascade of Wnt, Eda, and Shh signaling was shown to be essential for touch dome formation (Xiao et al. 2016). Second, the PRC2 has been suggested to restrict Merkel cell generation to first-wave primary hair follicles by preventing Merkel cell creation in secondary hair follicles that develop later in embryogenesis (Bardot et al. 2013; Dauber et al. 2016; Perdigoto et al. 2016). How these pathways interact with Notch has not been studied during Merkel cell development.

3- How does notch signaling influence production of Merkel cells during wounding? We show that Merkel cell production is inhibited by Notch signaling during development, and we have previously shown that it can inhibit the production of *Atoh1*-induced ectopic Merkel cells in adult mice (Ostrowski et al. 2015). In Chapters 3-5, we show how skin abrasions caused by shaving can induce the production of Merkel cells. Interestingly, we only observe the production of Merkel cells near the touch dome. We hypothesize that Notch signaling inhibits production of new Merkel cells. $K14^{CreER};RBPj^{flox/flox}$ mice can be treated with tamoxifen and shaved weekly to

determine see if Merkel cells are produced in the interfollicular epidermis. This would be an interesting observation because it would suggest that shaving somehow promotes expression of *Atoh1* in interfollicular epidermal cells.

7.1.2 Merkel cells are long-lived cells whose production is stimulated by skin injury

In Chapter 3, we show that Merkel cells are long-lived cells that are produced during embryogenesis and have the capacity to persist for the lifespan of the mouse. We also show that Merkel cell production can be induced by shaving. The following are questions that should be addressed in the future:

1- What progenitors produce Merkel cells after injury? In Wright et al., $Gli1^{CreER};ROSA^{YFP}$ were administered with tamoxifen and shaved weekly for 5 weeks. We showed that around 20% of Merkel cells were YFP positive. This low percentage could be due to low recombination, but it could also imply that some Merkel cells are derived from *Gli1*- progenitors. If this is true, what are the progenitors? It is likely that shavin-induced Merkel cells are derived from *K17*+ progenitors, as they are thought to be one of the primary progenitors for Merkel cell maintanence (Doucet et al. 2013). As I discuss in chapter 5 of this dissertation, we hypothesize that hair follicles promote Merkel cell production after injury. Therefore, we would be interested in testing if a portion of shaving induced Merkel cells are produced by K15+ hair follicle progenitors. Wright et al. showed that 1.7% of Merkel cells were EdU positive in hairless mice given EdU water while shaving for 5. She concluded from this that Merkel cells produced after shaving were not derived from hair follicles, as hairless mice have defects in their hair follicle stem cells. However, hairless mice have functioning hair

follicles that grow and progress as normal until 3-4 weeks of age (Benavides et al. 2010); therefore, it is still possible that residual hair follicle progenitors remained in these mice during this experiment.

2- Does Merkel cell number change during phases of the induced and natural hair cycles. As discussed in detail in Section 6.2 of this dissertation, there is a discrepancy in the literature between whether or not Merkel cell number changes with the hair cycle. To understand if this is true, the experiment should be repeated by a third party.

7.1.3 The role of SAI neurons during Merkel cell production

In Chapter 4 of this dissertation, we show that $Thy1^{CreER\text{-}EYFP}$ mice effectively label SAI nerve terminals. We then show that $Advillin^{Cre};ROSA^{tdTomato};Thy1^{CreER\text{-}EYFP}$ mice can be live images to show the interaction between Merkel cells and SAI nerve terminals. We use this model to show that after shaving mice, new Merkel cells are formed. These new Merkel cells are less likely to be innervated and survive for a shorter period of time. The following are the next questions that we think should be addressed:

1- Do Merkel cells require SAI neurons for production? Results from our experiments show that Merkel cells form without being directly contacted by the SAI nerve terminal. However, the SAI neuron is still intact and near the newly formed Merkel cell. Survival factors could still promote Merkel cell from a distance. To determine if Merkel cells can be produced in complete absence of the neuron, the dorsal cutaneous nerve can be transected during the imaging period. If new Merkel cells are produced after injury and in absence of a neuron, it would imply that signals from the neuron are

not required for Merkel cell production. If this is true it could imply that the touch dome compartment contains all the necessary factors to produce Merkel cells after injury.

2- How do nerves and Merkel cells change after abrasive skin injuries? The mouse model proposed in Chapter 4 can be used to study the effects of wounding on Merkel cell number and innervation. *Advillin^{Cre};ROSA^{tdTomato};Thy1$^{CreER-EYFP}$* mice can be bred to hairless *Hr^{hr}/Hr^{hr}* mice to produce hairless mice with fluorescent Merkel cells and SAI neurons. This model would allow for imaging without inducing a wound by shaving. Thus, mice could be left untreated or subjected to minor skin injuries. In Chapter 5, we show that skin injury decreases the number of Merkel cells. It will be interesting to see how the nerve reacts to Merkel cell loss. Merkel cells release factors that are important for SAI function (Reed-Geaghan et al. 2016), yet the SAI neuron does not require Merkel cells to innervate the touch dome (Maricich et al. 2009). If the neuron retracts after Merkel cell loss, than this would suggest that Merkel cells release neurotrophic factors that are necessary for maintenance of mature afferent terminals.

3- When are Merkel cells innervated during embryogenesis? The strong EYFP expression in SAI neurons of *Thy1$^{CreER-EYFP}$* mice was a serendipitous discovery. Currently immunostaining with antibodies to NF200 (NFH), is the most commonly used method for detecting peripheral neurons; however, this staining does not completely label nerve terminals. Conversely, *Thy1$^{CreER-EYFP}$* mice express EYFP in SAI terminals labeling the entire afferent calyx. This mouse can be used broadly for staining for SAI neurons. One area that would benefit from a better method of detecting peripheral neurons is determining the date of Merkel cell innervation during embryogenesis. Past reports have placed innervation broadly between E15.5 and postnatal (Pasche et al. 1990;

Vielkind et al. 1995). *Thy1*$^{CreER\text{-}EYFP}$ mice can be used to determine when innervation occurs (Appendix A).

7.1.4 Hair follicles promote Merkel cell development after injury

In Chapter 5 of this dissertation, we interrogate how the hair follicle promotes Merkel cell growth after injury. First, we show that shaved and tape stripped hairless mice have fewer Merkel cells than untreated mice, suggesting that a functioning hair follicle is required for the maintenance of the touch dome after abrasive skin injury. Next, we produced a heatmap that visually showed that newly produced Merkel cells localize near the hair follicle. This work is preliminary and has many interesting future directions. The main questions that I think should be addressed are as follows:

1- Do skin abrasions cause Merkel cell death? We show that shaving and tape stripping mice results in decreased Merkel cell number; however, We would need to immunostain for apoptosis markers to determine if abrasive wounds cause cell death. Tissue from shaved and tape-stripped mice can be collected and stained with TUNEL or cleaved caspase 3 to measure cell death. We hypothesize that both hairy and hairless mice will have increased number of TUNEL positive Merkel cells.

2- How quickly do Merkel cells disappear after skin injury? The live imaging tool described in Section 3.3.3 can be used to test this question. *Atoh1*^{GFP} mice can be bred to hairless (*Hr*^{hr}/*Hr*^{hr}) mice to create a hairless mouse with fluorescent Merkel cells. These mice can be shaved or left untreated while imaging weekly to produce a timecourse that shows how Merkel cell number changes as a result of certain skin conditions. We predict that there will be a steady decline of Merkel cells during the

imaging period. This will stand in contrast to hairy mice which maintain a constant number of Merkel cells per touch dome during the imaging period (Figure 13H).

3- Are newly produced Merkel cell derived from hair follicle progenitors? We show that new Merkel cells are produced near the hair follicle, but it is not clear whether Merkel cells are derived from the hair follicle, or if the hair follicle simply releases factors that promote Merkel cell production. A previous report suggests that Merkel cells are derived from hair follicle bulge stem cells (Van Keymeulen et al. 2009). Van Keymeulen administered RU846 daily to induce recombination in $K15^{CrePR};ROSA^{YFP}$ mice. This method was used to label K15 positive bulge stem cells and their progeny (Morris et al. 2004). He observed that 4.5% of K8+ Merkel cells were YFP positive in whisker follicles while no YFP positive Merkel cells were observed in the paws. These results suggest that whisker follicle Merkel cells are derived from K15+ bulge stem cells. Van Keymeulin did not look at Merkel cells of hairy skin; therefore, an interesting experiment would be to administer RU846 to $K15^{CrePR};ROSA^{YFP}$ mice and leave untreated or shave to determine if touch dome Merkel cells are derived from hair follicle derived stem cells after shaving.

4- What signals from the hair follicle promote Merkel cell production? Several growth factors are released from the hair follicle during the hair cycle. Some of these factors could promote production of Merkel cells after injury. During the resting phase of the hair follicle BMP signaling represses activation of hair follicle stem cells (Zhang et al. 2006). During regeneration, BMP mediated repression is reversed by TGF-β, which induces the expansion and differentiation of bulge stem cells (Oshimori and Fuchs 2012). Hair follicle growth is then promoted by subsequent release of growth factors

such as IGF, FGF, EGF, and PDGF (Stenn and Paus 2001). These signals, derived from the hair follicle niche, could promote Merkel cell production after injury. Further experiments would be necessary to determine the roles of TGF-β, IGF, FGF, EGF, and PDGF in Merkel cell production.

7.2 OTHER QUESTIONS ABOUT MERKEL CELLS

7.2.1 How can we replicate Merkel cell development *ex vivo*?

As I described in chapter 1 of this dissertation, Merkel cells develop because of signals between the epidermis, the dermis, the developing hair follicle, and the nerve. This presents a challenge for studying Merkel cell development because conditional deletion of a gene for Merkel cell development can disrupt proper development of the entire epidermis. Consequently, it is difficult to make specific conclusions about how Merkel cell development is regulated.

The field of Merkel cell research could benefit greatly from an *ex vivo* model of Merkel cell development. With an *ex vivo* approach, we could target genes and pathways at more specific timepoints, which may decrease off target affects that we observe in global and conditional knockout mouse models.

For example, in chapter 2, we show that $K14^{Cre};ROSA^{NICD}$ mice, which overexpress the Notch Intracellular Domain (NICD), had fewer Merkel cells. $K14^{Cre};ROSA^{NICD}$ mice also show significant defects to their epidermal structure. Mice overexpressing NICD, die shortly after birth due with poorly developed limbs and blistered skin. It is difficult to conclude from this experiment alone if NICD is directly affecting Merkel cells of if the effect is secondary to significant

alterations in epidermal morphology. An *ex vivo* model of Merkel cell development would be a valuable tool for testing this hypothesis. We could treat *ex vivo* embryonic tissue with a Notch agonist at E14.5 and E15.5, when Merkel cell production is at its maximum. Narrowing the window of treatment would decrease the off-target effects of the treatment, thus epidermal morphology should be less affected.

An *ex vivo* approach would also allow for more high-throughput experiments with larger sample sizes. Multiple genes and pathways could be targeted pharmacologically without the cost, time, and challenge of generating multi-transgenic mice. *Ex vivo* tissue can also be live-imaged, providing more information about short-term effects of experimental treatments and conditions.

Currently no such model exists, but there have been models of *in vitro* Merkel cell culture (Boulais et al. 2009) and *ex vivo* embryonic skin culture (Mort et al. 2014). Aspects of these techniques could be used to generate an effective *ex vivo* system for Merkel cell development.

Since Merkel cells are post-mitotic, they do not proliferate in normal culture medium, and most will die in two weeks of culture (Vos et al. 1991). To increase survival, Merkel cells can be grown in a monolayer with keratinocytes (Shimohira-Yamasaki et al. 2006). Supplementing media with bFGF can increase Merkel cell survival and can even promote Merkel cell proliferation (Boulais et al. 2009). These results suggest that Merkel cells may not survive in an *ex vivo* system without supplementing media with growth factors (such as bFGF).

An *Ex vivo* embryonic skin culture system has been used to track migrating melanocytes during development (Mort et al. 2010; Mort et al. 2014). In this method, embryonic skin is dissected from embryos at E14.5 and skin is grown in special inserts that allow tissue to grow in a liquid-air interface. This method allows for live imaging in culture for up to 24 hours. Since Merkel

cell production is at its maximum between E14.5 and E15.5, this should be sufficient time to monitor Merkel cell development.

If an *ex vivo* model for embryonic Merkel cell development is designed, a similar method may be produced to study adult tissue, which could be helpful for understanding how mature Merkel cells regenerate.

7.2.2 Role of FGF during Merkel cell development

In Chapter 2 of this dissertation we describe the role of Notch pathway signaling as an agonist of Merkel cell production during embryonic. Several other pathways are important for Merkel cell development including Wnt, Eda, Shh, and PRC2 (Perdigoto et al. 2016; Xiao et al. 2016). It is unlikely that these are the only pathways that regulate Merkel cell development, so further exploration of Merkel cell regulatory pathways are necessary. As mentioned above (section 7.2.1), bFGF is important for survival of Merkel cells in culture, but the role of FGF in regulating Merkel cell development has not been explored in detail.

FGF pathway signaling regulates several aspects of skin and hair follicle development (Turner and Grose 2010). FGF signaling also plays an important role in the specification of *Atoh1*+ hair cell progenitors in the inner ear (Pirvola et al. 2002; Hayashi et al. 2008; Sweet et al. 2011). In particular, FGF20 is important for both skin and inner ear development. In the skin, FGF20 expressed by hair placodes and the surrounding epidermis is required for formation of dermal condensates (Huh et al. 2013), while in the ear FGF20 is required for development of sensory epithelium (Hayashi et al. 2008). We hypothesize that epidermal FGF20 promotes Merkel cell specification.

Interestingly, this question has been briefly addressed in a recent report. Xiao et al 2016 counted the number of Merkel cells per touch domes in P0 *FGF20*$^{LacZ/Lacz}$ mice and observed a 17% decrease compared to the control, which they concluded was not significant (Xiao et al. 2016, Figure 6D). However, in this experiment they use *FGF20*$^{LacZ/+}$ mice as a control. The control mice had an average of around 17 Merkel cells per touch dome. Our lab normally detects between 20 and 25 Merkel cells per touch dome at this age (Figure 4). This suggests that losing one allele of FGF20 in *FGF20*$^{LacZ/+}$ mice could result in a partial loss of Merkel cell function. Xiao et al. should have compared *FGF20*$^{LacZ/LacZ}$ mice to *FGF20*$^{+/+}$ mice to determine the effect of FGF on Merkel cell development.

FGF has many isoforms which have redundant functions (Ornitz and Itoh 2015). Xiao et al. concluded from their experiments that FGF20 is not required for Merkel cell specification. This may be due to redundancy from other FGF molecules in the developing epidermis. More experiments would be needed to determine the role of FGF in Merkel cell development.

8.0 CONCLUSIONS

Merkel cells serve an important role for the sense of touch; therefore, it is important for these cells to remain in the epidermis, undisturbed while they carry out this vital function. Yet, as residents of the epidermis, Merkel cells are often exposed to injuries and abrasions; therefore, a repair mechanism is necessary for the replacement of damaged Merkel cells. This dissertation focuses on the genes and signals that regulate the production of these cells during embryogenesis and after injury.

Merkel cells are born in late embryogenesis and their production is regulated by many signaling pathways. In this dissertation, we show that one of these signaling pathways is Notch, which prevents Merkel cell production in certain compartments of the skin. Postnatally, the number of Merkel cells were thought to oscillate during certain phases of the hair cycle. We show that this is not the case; rather, Merkel cells born during embryogenesis have the capacity to persist for the lifespan of the mouse or until injury or abrasion occurs. After skin abrasion, the number of Merkel cells decreases, but it is restored through a repair mechanism. Our preliminary data suggests that the hair follicle promotes Merkel cell regeneration. After Merkel cells regenerate, they must be innervated by SAI neurons to ensure their survival.

The findings from this dissertation characterize the roles of genetic and environmental factors on Merkel cell development, lifespan, and regeneration after injury. Broadly, this research increases our understanding of how the sensory system develops and is maintained in adulthood and could have implications for ageing individuals and patients with skin injuries who suffer from partial loss of their sense of touch.

APPENDIX A

USE OF *THY1*$^{CREER\text{-}EYFP}$ MICE TO DETERMINE DATE OF INNERVATION

The data presented in Chapter 4 of this dissertation suggests that innervation is not required for Merkel cell production during skin homeostasis in adult mice. This can be further supported by observing innervation in during embryogenesis, when Merkel cell production is at its maximum (Wright et al. 2015). As discussed in Chapter 1 of this dissertation, the age during embryonic development when innervation occurs is not clearly defined. Some reports show Merkel cells are innervated as early as E15.5 (Pasche et al. 1990), while others say innervation occurs postnatally (Vielkind et al. 1995). The discrepancy is due in part to limitations in immunostaining during embryonic timepoints.

In Chapter 4, we introduced the *Thy1*$^{CreER\text{-}EYFP}$ mouse, which expresses high levels of EYFP in SAI neurons. Here we use this transgenic mouse to determine innervation at birth. P0 *Thy1*$^{CreER\text{-}EYFP}$ were collected and drop fixed in 4% PFA. Skin from four quadrants of the mouse were collected to determine when innervation occurs in different parts of the mouse. Skin was sectioned and immunostained with Rat anti K8 (1:20, TROMA) and chicken anti-GFP (1:1000, Aves) using our standard immunofluorescence protocol (see section 2.2.3 for example). Innervation was determined for >100 Merkel cells per quadrant (n=3 mice). Results are displayed in Figure 21.

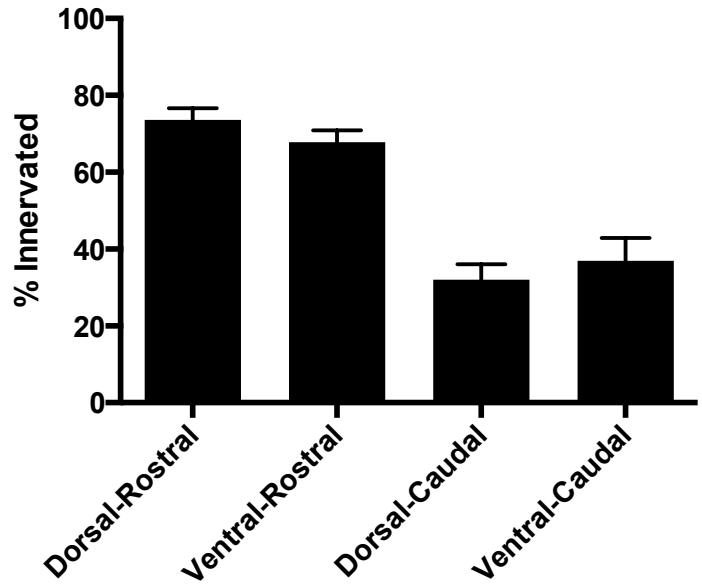

Figure 21- Not all Merkel cells are innervated at P0

Percent of Merkel cells innervated in *Thy1*$^{CreER=EYFP}$ mice from four regions of the skin (n=3 mice)

At P0 more Merkel cells are innervated at the rostral end of the mouse than the caudal end (Dorsal-Rostral, 74 ± 3; Ventral-Rostral, 68 ± 3; Dorsal-Caudal, 32 ± 4; Ventral-Caudal, 37 ± 6). There was no obvious difference between innervation in the dorsal side of the mose and the ventral side. These data show that depending on location in the body, some Merkel cells remain non-innervated for days after they are produced.

These data are preliminary. To gain a full understanding of when Merkel cell innervation occurs more timepoints would be needed. It is also possible that the *Thy1*$^{CreER=EYFP}$ allele does not turn on in all cells until later in development. Therefore, some Merkel cells may falsely appear non-innervated using this detection mechanism. This data should be taken in context with all other reports on Merkel cell innervation, which place innervation between E15.5 and postnatal (Pasche et al. 1990; Vielkind et al. 1995). Both extremes may, in part, be correct with innervation beginning at E15.5, but remaining incomplete until after birth.

APPENDIX B

USING TCGA TO STUDY MERKEL CELL CARCINOMA

The Cancer Genoma Atlas (TCGA) is a publically available database that contains genomic information, including expression profiles and mutations, from real patient tumors. It can be helpful for identifying genes and pathways involved in specific cancer subtypes. Unfortunately, there are no Merkel cell carcinoma (MCC) tumors available in the database; however, we thought that some MCC patients could have been misdiagnosed as cutaneous melanoma patients. Presumably, MCC patients could be separated from cutaneous melanoma patients based on their expression of MCC biomarkers.

To analyze this dataset, we imported gene expression arrays (RNAseq) from 473 cutaneous melanoma patients. We used python programming language to perform a hierarchal clustering analysis on these patient samples (Figure 22A). We used the Ward's method to minimize variance, and cluster tumors based on expression of 5 MCC biomarkers: KRT20, BDNF, ENO2, S100A1, and TTF1. Patient tumors clustered in 6 groups. A group of 16 patient tumors had elevated levels of the 5 biomarkers of MCC (Figure 22B). These patient tumors represent MCC-like tumors.

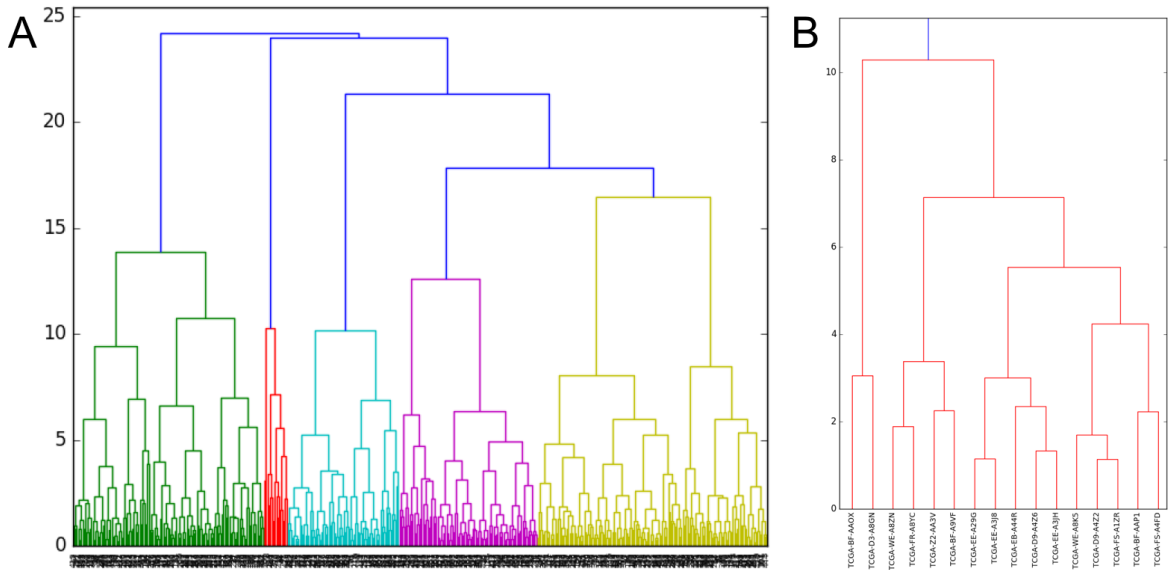

Figure 22- Phylogenetic tree of cutaneous melanoma patients

(A) Hierarchal clustering analysis (Wards method) of 473 cutaneous melanoma patient tumors resulted in 6 groups of patient tumors. One group (red), expressed high levels of 5 MCC biomarkers: KRT20, BDNF, ENO2, S100A, and TTF1. (B) enlarged image of MCC-like tumors.

Next, we wanted to determine which genes were differentially expressed in the MCC-like tumors. We used python programming language to run a t-test for each gene, comparing gene expression in MCC-like tumors, to cutaneous melanoma tumors (CMT). 188 genes were differentially regulated in MCC-like tumors ($p<0.001$). The top 50 differentially regulated genes are listed in Table 4.

As expected, KRT20 was at the top of the list. This represents a proof of principle for the method. Of interest, ATOH1 (number 34) was upregulated in MCC tumors. This dataset could be useful in identifying genes and pathways that are specifically expressed in Merkel cell carcinoma.

Table 4- Top 50 differentially regulated genes in MCC-like tumors

Number	Gene	Expression_MCC	Expression_CMT	ratio MCC/CMT	p value
1	KRT20	2.21002	0.108880983	20.29757577	2.81989E-34
2	MLN	0.23514	0.003609188	65.15038778	2.96962E-23
3	CCDC105	0.78396	0.014989957	52.29901501	6.54323E-19
4	RBP2	1.52614	0.096334188	15.84214318	1.99562E-18
5	S100G	0.12958	0.000872436	148.5266716	3.34915E-17
6	OR10A5	0.30816	0.011611111	26.54009569	9.23822E-16
7	RPL3L	2.25596	0.219992735	10.25470227	1.90772E-13
8	FAM48B1	1.33202	0.126886752	10.49770742	6.55341E-13
9	PRR23C	1.50576	0.059492735	25.30998111	3.62734E-12
10	PRR23B	1.42092	0.075507692	18.81821516	3.91587E-12
11	ZG16	0.56812	0.036499359	15.56520487	2.63954E-11
12	LOC340074	0.65956	0.027565385	23.92711037	2.65141E-11
13	C10orf71	1.3756	0.090324573	15.22952127	8.29388E-11
14	NKX1-2	0.93214	0.049971154	18.65356167	9.36379E-11
15	GLRA4	1.59834	0.109436325	14.60520538	1.30584E-10
16	CHAT	0.6463	0.024038034	26.88655798	2.41475E-10
17	CYP2W1	3.81354	0.887185256	4.298470891	4.98869E-10
18	LOC388428	0.935	0.109767521	8.518002305	7.26898E-10
19	TMEM105	2.47788	0.453991667	5.457985646	1.01615E-09
20	OR10G7	0.10556	0.001961325	53.82076479	1.14409E-09
21	TMEM88B	1.48	0.123764957	11.95815062	5.73485E-09
22	CLEC3A	1.56606	0.092913462	16.85503881	9.13054E-09
23	HCRTR1	1.9505	0.288587607	6.758779496	1.58507E-08
24	LY6G6E	0.4931	0.035234188	13.99493014	1.77933E-08
25	LGALS4	3.6042	1.044437821	3.450851673	2.01727E-08
26	TMEM8C	0.58616	0.02178312	26.90890971	2.68357E-08
27	C6orf221	0.82326	0.053611111	15.35614508	3.18604E-08
28	HPYR1	0.36082	0.027074359	13.32700066	3.7077E-08
29	SPAG11A	0.10556	0.002309829	45.70035153	4.62126E-08
30	FLJ39609	0.63026	0.050504487	12.47928719	6.62852E-08
31	H2BFM	0.68438	0.050956624	13.43063859	6.68698E-08
32	CLDN19	3.65466	0.582521795	6.273859677	1.28607E-07
33	CAPZA3	1.12892	0.061791453	18.26984066	1.44262E-07
34	ATOH1	0.36914	0.025959402	14.21989629	1.48776E-07
35	OR5V1	1.04756	0.080316026	13.04297606	2.23291E-07
36	SPDYC	1.9777	0.263788889	7.497283181	2.30209E-07
37	OR8G2	0.2026	0.012708333	15.94229508	2.3701E-07
38	PHGR1	0.55328	0.035206838	15.71512915	2.6924E-07
39	VIL1	1.5677	0.270795726	5.789234639	2.93833E-07
40	FOXI3	1.98558	0.199844017	9.935648957	4.3684E-07
41	TULP1	3.3138	0.809776068	4.092242448	4.67085E-07
42	UGT3A1	2.06798	0.220007479	9.399589563	4.73412E-07
43	C6orf222	1.36814	0.246812607	5.543233863	4.83793E-07
44	MSGN1	0.4038	0.037798932	10.68284162	6.19729E-07
45	SCARNA3	0.07302	0.001814957	40.23235225	6.78414E-07
46	UGT2A1	2.16704	0.342264103	6.331484908	7.1997E-07
47	IL9	0.10556	0.00366688	28.78741332	7.7666E-07
48	OR52K2	0.46596	0.052323504	8.905366842	7.93089E-07
49	KRTAP17-1	1.3862	0.060951068	22.7428335	8.56798E-07
50	HNF4A	2.87256	0.656117094	4.378120958	1.47301E-06

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