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## MERLIN MEDIATED REGULATION OF HAIR FOLLICLE MORPHOGENESIS

Megan K. Fentress

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MERLIN MEDIATED REGULATION OF HAIR FOLLICLE  
MORPHOGENESIS

by

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**MERLIN MEDIATED REGULATION OF HAIR FOLLICLE  
MORPHOGENESIS**

A

Thesis

Presented to the Faculty of  
The University of Texas  
Health Science Center at Houston  
And  
The University of Texas  
MD Anderson Cancer Center  
Graduate School of Biomedical Sciences  
In Partial Fulfillment

Of the Requirement

For the Degree of

MASTER OF SCIENCE

By

Megan Kailani Fentress, B.A.

Houston Texas, USA

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## **DEDICATION**

I would like to dedicate this thesis to my wonderful mom, Debra, whose help and sacrifice has made this possible. She gave me the strength to follow my dreams and taught me the grace to leave space for magic in my life.

I would also like to dedicate this to my grandparents, Marie and Randall Fentress. While they are no longer with us, I know that they would be proud. Their stubbornness and perseverant attitudes were passed down in full.

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# **MERLIN MEDIATED REGULATION OF HAIR FOLLICLE MORPHOGENESIS**

**By Megan Kailani Fentress, B.A.**

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Epidermal homeostasis is paramount for the ongoing function of the skin as the primary barrier between a mammalian organism and the external environment. Homeostasis is achieved through a complex and delicate balance of cell death, cell proliferation and cell differentiation. Critical for regeneration and maintenance of the skin are epidermal stem cells. Within the epidermis two distinct stem cell compartments exist, the bulge and interfollicular/basal stem cell niches, which play a central role in the regeneration of the epidermis through self-renewal and contribution to the differentiated cells of the epidermis. The bulge stem cell niche is established early in epidermal development by the organized expression of transcription factors that regulate stem cell fate. The establishment and proper functioning of this specialized cell compartment is dependent on the stem cells ability to receive and respond to external signals such as Wnt signaling. Disruption in the ability of stem cells to integrate these signals can result in disruption in hair follicle architecture, lack of differentiated cells and disease.

Cells are able to transmit extracellular signals from the cell membrane to the actin cytoskeleton through the adherens junctions (AJ). The AJ is composed of the core proteins E-cadherin,  $\beta$ -catenin and  $\alpha$ -catenin. The transmembrane protein E-cadherin binds the cytoplasmic proteins  $\beta$ -catenin and  $\alpha$ -catenin, the latter tethering the complex to the actin cytoskeleton. In addition to the core AJ

proteins, proper formation and maturation of the AJ is dependent upon a multi-protein complex called the junctional polarity complex (JPC) composed of  $\alpha$ -catenin, the polarity proteins Par3 and aPKC as well as the tumor suppressor Merlin, the protein product of the *Nf2* gene. Merlin plays a pivotal role in the function of the JPC by bridging Par3 to  $\alpha$ -catenin and in the absence of Merlin the interaction between Par3 and  $\alpha$ -catenin is unable to promote formation of functional AJs and tight junctions (TJ). Mice with a deletion of Merlin in the epidermis are unable to form an epidermal barrier due to the loss of TJ in addition to having alterations in basal cell polarity. Post-natal Merlin mutant mice have alterations in differentiation as well as stratification defects of the epidermis. Interestingly Merlin-deficient epidermal basal cells have a loss of basal cell polarity, with disruptions in spindle orientation and subsequent defects in the asymmetric cell divisions necessary for proper epidermal stratification. This study explores a new function of Merlin in the epidermis in regulating hair follicle morphogenesis.

Here we show that in addition to the disruptions in epidermal stratification there is also an alteration in planar polarity of the developing hair follicles combined with defects in hair follicle formation in Merlin-deficient skin.

Disruptions in polarity of the developing hair placodes are observed as early as embryonic day 14.5, as well as defects in cellular orientation and organization of the early placodes as early as E13.5. Interestingly we also see a change in the organization and presence of the early bulge stem cells within the developing follicle. Mutant follicles not only show a decrease in primordial bulge stem cells

but also disorganization and random distribution of these cells throughout the hair germ. Although the requirement of the bulge stem cell niche in maintaining the hair follicle and epidermis is well characterized, the establishment of this niche is not well understood. This study will describe a role for Merlin in the development of the bulge stem cell niche through establishment of polarity. This work will lead to a better understanding of how stem cell niches are initially established and organized through interactions at cellular junctions and the actin cytoskeleton providing new insight into how stem cell niches are regulated in epithelial tissues throughout the body.

## TABLE OF CONTENTS

<b>APPROVAL SIGNATURES</b>	<b>i</b>
<b>DEDICATION</b>	<b>iii</b>
<b>ACKNOWLEDGMENTS</b>	<b>iv</b>
<b>ABSTRACT</b>	<b>vi</b>
<b>TABLE OF CONTENTS</b>	<b>ix</b>
<b>LIST OF ILLUSTRATIONS</b>	<b>xi</b>
<b>ABBREVIATIONS</b>	<b>xii</b>
<b>CHAPTER 1: INTRODUCTION AND BACKGROUND</b>	<b>1</b>
1.1 INTRODUCTION	2
1.2 MERLIN AND THE JUNCTIONAL POLARITY COMPLEX	4
1.3 SKIN DEVELOPMENT DURING EMBRYOGENESIS	7
1.4 HAIR FOLLICLE DEVELOPMENT AND STEM CELLS	9
1.5 THE ROLE OF CELL POLARITY IN HAIR FOLLICLE DEVELOPMENT	13
<b>CHAPTER 2: HYPOTHESIS, SPECIFIC AIMS AND SIGNIFICANCE</b>	<b>17</b>
<b>CHAPTER 3: MATERIALS AND METHODS</b>	<b>20</b>
<b>CHAPTER 4: RESULTS</b>	<b>24</b>
4.1 DELETION OF MERLIN DISRUPTS HAIR FOLLICLE DEVELOPMENT DURING EMBRYOGENESIS	25
4.2 MERLIN IS REQUIRED FOR ESTABLISHMENT OF THE BULGE STEM CELL NICHE	28
4.3 MERLIN-DEFICIENT HAIR FOLLICLES HAVE ALTERED PLANAR POLARITY	32

4.4 MERLIN-DEFICIENT LATE STAGE AND MATURE HAIR FOLLICLES HAVE DECREASED SOX9 EXPRESSION	38
<b>CHAPTER 5: DISCUSSION AND FUTURE DIRECTIONS</b>	<b>45</b>
DISCUSSION	46
FUTURE DIRECTIONS	53
<b>BIBLIOGRAPHY</b>	<b>55</b>
<b>VITA</b>	<b>62</b>

## **LIST OF ILLUSTRATIONS**

### **INTRODUCTION**

FIGURE 1: SCHEMATIC REPRESENTATION OF AN ADHERENS JUNCTION WITH JUNCTIONAL POLARITY COMPLEX	4
FIGURE 2: SCHEMATIC MODEL OF HAIR FOLLICLE DEVELOPMENT IN WILD- TYPE EPIDERMIS	12
FIGURE 3: SCHEMATIC OF SKIN ORGANIZATION AND LOCATION OF BULGE STEM CELL COMPARTMENT	13

### **RESULTS**

FIGURE 4: DELETION OF MERLIN RESULTS IN DEFECTS IN HAIR FOLLICLE DEVELOPMENT	27
FIGURE 5: MERLIN-DEFICIENT EPIDERMIS FAILS TO FORM FUNCTIONAL MATURE HAIR FOLLICLES	30
FIGURE 6: MERLIN IS REQUIRED FOR ESTABLISHMENT OF THE CD34+ ADULT STEM CELL NICHE	31
FIGURE 7: DELETION OF MERLIN RESULTS IN DISRUPTION OF A-P ORIENTATION OF HAIR FOLLICLES	33
FIGURE 8: MERLIN REGULATES POLARITY IN DEVELOPING HAIR FOLLICLES	36
FIGURE 9: MERLIN-DEFICIENT EPIDERMIS HAS DECREASED FOLLICLE NUMBER AND DISRUPTIONS IN SOX9 INITIATION	37
FIGURE 10: MERLIN IS REQUIRED FOR ORGANIZATION OF SOX9+ CELLS AND CELLULAR CONSTRICTION OF THE DEVELOPING FOLLICLES	40
FIGURE 11: MERLIN REGULATES EPIDERMAL STEM CELL INITIATION	42
FIGURE 12: MERLIN IS REQUIRED FOR ORGANIZATION OF SOX9+ CELLS AND CELLULAR CONSTRICTION OF THE EARLY FOLLICLES	44
FIGURE 13: MERLIN REGULATES ESTABLISHMENT OF THE BULGE STEM CELL NICHE	50
FIGURE 14: SCHEMATIC MODEL OF THE ROLE OF MERLIN IN EPIDERMAL DEVELOPMENT	52

## **ABBREVIATIONS**

**AJ:** Adherens Junction

**AB Polarity:** Apical-Basal Polarity

**A-P axis:** Anterior to Posterior axis

**APC:** adenomatous polyposis coli

**aPKC:** atypical protein kinase C

**BSC:** bulge stem cell

**Cell:Cell:** Cell to cell

**Celsr1:** Cadherin, EGF LAG Seven-Pass G-Type Receptor 1

**E13.4, E14.5, E16.5, E18.5:** Embryonic day 13.5, 14.5, 16.5, 18.5

**ERM:** Ezrin, Radixin, Moesin family of proteins

**FERM:** Four-point-one, Ezrin, Radixin and Moesin domain

**H&E:** Hematoxylin and Eosin tissue histology stain

**HF:** Hair Follicle

**IRS:** Inner Root Sheath of the hair follicle

**JPC:** Junctional Polarity Complex

**K1:** Keratin 1

**K14:** Keratin 14

**KO:** Knock-out referring to knocking out a gene

**Lhx2:** Stem cell transcription factor

**NFATc1:** Nuclear Factor Of Activated T-Cells, Cytoplasmic, Calcineurin  
Dependent 1

**Nf2:** Neurofibromatosis Type 2 gene

**ORS:** Outer Root Sheath

**P0:** Post-natal day 0, day of birth

**Par3:** partitioning-defective protein 3

**PCP:** Planar Cell Polarity

**PDGF:** Platelet Derived Growth Factor

**Sox9:** SRY (Sex Determining Region Y)-Box 9

**Tcf3/4:** Transcription Factors 3/4

**TJ:** Tight Junction

**Zo-1:** zona occludens-1 protein

## **CHAPTER 1: INTRODUCTION AND BACKGROUND**

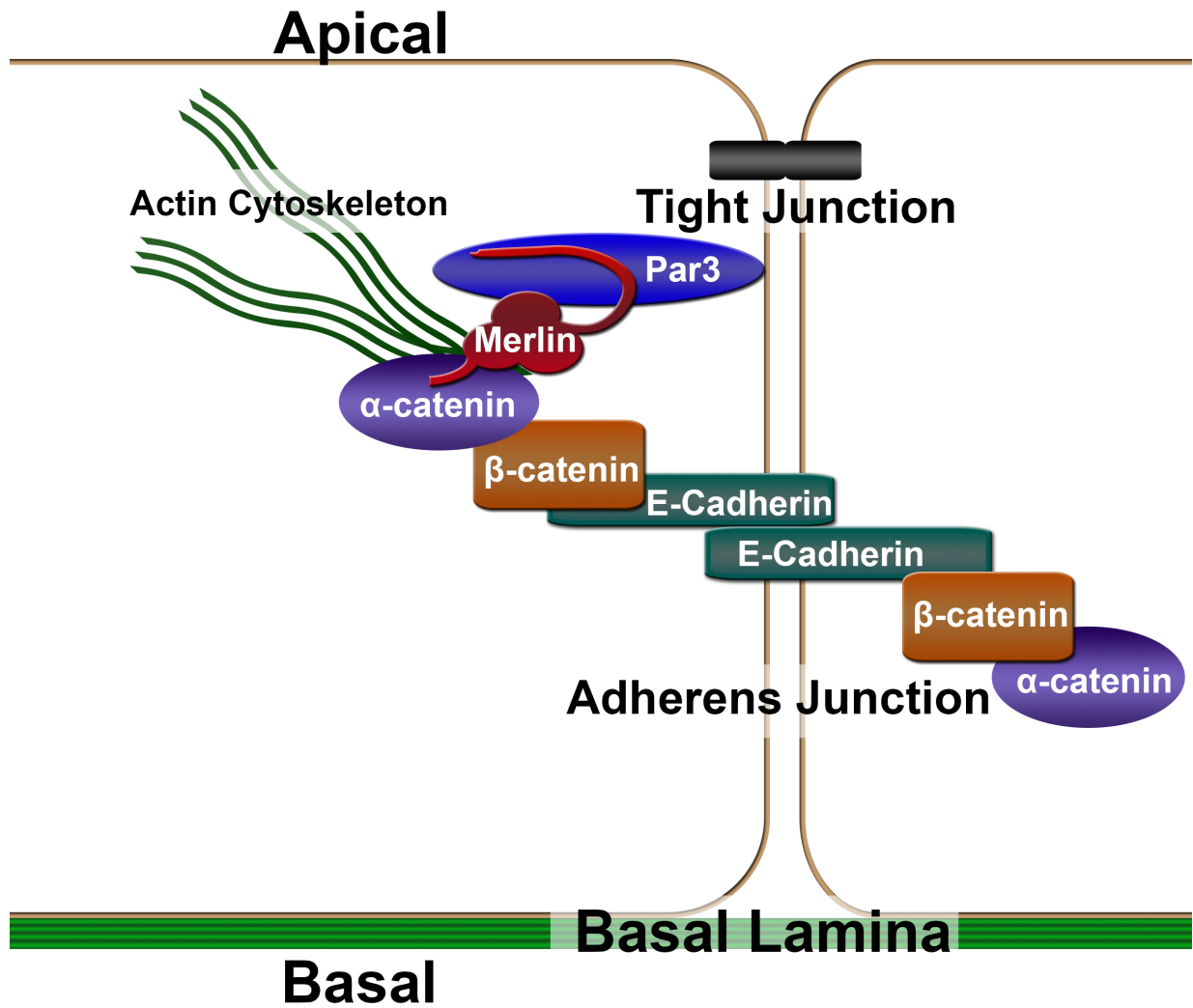
## 1.1 INTRODUCTION

The mammalian epidermis undergoes continual renewal throughout the lifetime of an organism to maintain homeostasis<sup>1</sup>. The ability to maintain homeostasis relies upon two specialized stem cell compartments nestled within the epidermis and hair follicle, the bulge and interfollicular/basal stem cell niches<sup>2-10</sup>. The establishment of the bulge stem cell compartment has been traced back to embryonic day 14.5 when the first stage of hair follicle formation (placode) is observed<sup>10-12</sup>. The stem cell marker Sox9 is expressed by cells in the apical/posterior section of the developing placode and can be followed throughout hair follicle development to the primordial bulge region. These primordial bulge cells express the transcription factor Sox9, which is known to regulate cell fate<sup>9,12,13</sup>. Once established, the maintenance of the stem cell compartments relies on membrane signaling pathways to integrate external cues<sup>2</sup>. One mode of regulating external signals is through the connection of the actin cytoskeleton to the cell membrane through adherens junctions (AJ). Functioning as a link between the cytoskeleton and the surrounding cells in a tissue, the AJ is critical for the regulation of stem cells<sup>4,14</sup>.

Adherens junctions are comprised of the core AJ proteins E-cadherin,  $\beta$ -catenin and  $\alpha$ -catenin<sup>15,16</sup> as well as a recently discovered multi-protein complex called the junctional polarity complex (JPC). The JPC is comprised of  $\alpha$ -catenin, the polarity proteins Par3 and aPKC, and the tumor suppressor Merlin (Figure 1). Merlin is critical for the function of the JPC, disruption of the JPC by deletion of Merlin results in severe epidermal defects by impairing both AJ maturation and

formation of tight junctions as well as alterations to stratification of the epidermis<sup>17</sup>. Working as a link between neighboring cells, integration of external signals through the AJ is an important operation of these dynamic junctions<sup>18–21</sup>.

A critical cell type for the ongoing function of multiple tissues types are stem cells, which work to maintain and replenish tissues, including the epidermis during normal tissue turnover and regeneration following injuries. The stem cells ability to activate during a growth phase or in response to injury depends upon the integration of extracellular signals<sup>14,22</sup>. Disruption in these signaling cascades can result in impaired wound healing, precocious tissue aging, diseases such as psoriasis, alopecia or cancer<sup>9,22–24</sup>. Examining how regulation of the cascades alters stem cell niche development and understanding the mechanisms behind this could lead to specific therapeutics being developed to aid in wound healing and tissue regeneration.



**Figure 1: Schematic Representation of an Adherens Junction with Junctional Polarity Complex.** Merlin mediates the transmission of extrinsic signals from adjacent cells and the extracellular environment via the adherens junction complex.

## 1.2 MERLIN AND THE JUNCTIONAL POLARITY COMPLEX

The ERM family of proteins contain scaffold proteins Ezrin, Radixin and Moesin as well as Merlin, that play roles in a wide array of cellular function from organizing the cell cortex<sup>25,26</sup>, establishment of polarity<sup>25,27,28</sup>, organization of tissues<sup>29</sup> as well as having roles in establishment and maintenance of functional junctions<sup>17,28</sup>. The ERM proteins and Merlin contain a highly conserved FERM (Four-point-one, Ezrin, Radixin and Moesin) domain, located at the N-terminal portion of the proteins<sup>24,26,30,31</sup>. ERM proteins also have a specialized C-terminal tail with an actin binding domain which is important for ERM proteins coordinating membrane signaling proteins with the actin cytoskeleton. While Ezrin, Radixin and Moesin have well defined actin binding domains<sup>25</sup> their close relative Merlin does not, thus how Merlin regulates actin organization is still not completely understood<sup>17,31</sup>. The N and C terminal regions of the ERM proteins are known to interact with each other and numerous studies have shown that regulation of the head to tail (or open and closed) conformation is critical for ERM function and in fact in the case of Merlin it is in the closed conformation that the protein itself is active<sup>32</sup>.

Merlin is a tumor suppressor protein<sup>32,33</sup> that plays an integral role in linking  $\alpha$ -catenin to the polarity protein Par3 and its interacting partner aPKC at the adherens junction (AJ) promoting AJ maturation. In the absence of Merlin Par3 fails to localize to other AJ proteins nor does it recruit tight junction (TJ) proteins, indicating a failure of the AJ to mature thus inhibiting TJ formation<sup>17</sup>. Defects in localization of Par3 and aPKC also lead to disruptions in cell

polarity<sup>34–36</sup>. Thus, deletion of Merlin results in defective cell polarity and asymmetric cell division both *in vitro* and *in vivo*<sup>17,30,32</sup>. Furthermore depletion of Merlin can also disrupt asymmetric cell division by altering Ezrin and subsequently centrosome localization at the cell cortex<sup>17,30</sup>. The association between this group of junctional proteins coupled with polarity defects following depletion of Merlin has lead to the description of a Junctional Polarity Complex (JPC), composed of Merlin, Par3, aPKC and  $\alpha$ -catenin<sup>17</sup> (Figure 1).

Junctional integrity is essential for functional tissues across the entire organism, acting as tethers to other cells as well as a means for communication between neighboring cells and across tissues. As such a basic mode of cell:cell communication seen in virtually all tissue types investigating how junctions develop, mature and maintain their dynamic functions will shed light on how disruptions in the proteins result in a wide array of diseases. With possible implications ranging from the nervous system, where cellular response through junctions is critical in neuronal signaling<sup>37,38</sup>, to the epithelium where the transmission of cellular signals across epidermal tissues is necessary to set up barrier function and epidermal development<sup>17,19,39,40</sup>.

### 1.3 SKIN DEVELOPMENT DURING EMBRYOGENESIS

During gastrulation ectodermal cells are specified to have an epidermal fate, called epidermal commitment, thereby creating the single layer of cells which is the primordial epidermal basal layer<sup>10,41,42</sup>. Stratification of the monolayer of basal cells begins following a switch to polarized cell division. Prior to stratification the monolayer divides parallel to the dermis, at E12.5 a change to asymmetric cell division occurs and the process of stratification begins<sup>36,43</sup>.

Stratification occurs through E18.5 when a fully functional barrier is present. E9.5 when the first monolayer is present is also when the basement membrane, or basal lamina, forms as the initial barrier between the dermis and the epidermis<sup>10,42</sup>. Basal cells attach to the basal lamina through integrins and obtain extracellular matrix proteins as well as growth factors through this interaction<sup>10</sup>. During stratification multiple steps occur to create a fully stratified barrier between the external environment and the organism.

The first layer to result from asymmetric cell divisions of the embryonic basal layer is the embryonic intermediate cell layer<sup>36</sup>. While not sustained past embryogenesis the intermediate cell layer serves as the initial epidermal layer not connected to the basal lamina. By E15.5 the intermediate layer matures into the spinous layer marked by increased Keratin 1 (K1) expression. Further maturation of the spinous layer results in formation of the granular layer by E16.5 and the cornified layer by E18.5<sup>10,42</sup>. The cornified layer acts as the final layer essential in barrier function and mice at this stage are viable and able to survive ex-utero<sup>42</sup>. The ongoing function of the stratified epidermis relies on its ability to

self-renew throughout the organism's lifetime. Within the functionally mature epidermis lies the epidermal stem cells, the workhorses for both epidermal self-renewal as well as regeneration following injury. During stratification the epidermis is also forming appendages called hair follicles that not only function in hair formation but that also house one of the stem compartments necessary for regeneration and homeostasis.

## 1.4 HAIR FOLLICLE DEVELOPMENT AND STEM CELLS

Hair follicle development begins at E14.5 with the formation of placodes. Signals from the dermis initiate placode formation, with Wnt/ $\beta$ -catenin being an essential pathway. In the absence of dermal  $\beta$ -catenin placodes do not form and overexpression of  $\beta$ -catenin initiates increased placode development<sup>44</sup>. Furthermore ablation of epidermal  $\beta$ -catenin prior to hair follicle morphogenesis results in the absence of placodes as well as decreased  $\beta$ -catenin signaling in the dermis. Deletion of  $\beta$ -catenin in the epidermis following the initial morphogenesis of hair follicles results in failure of follicles to form following the first cycle of hair loss<sup>45–47</sup>. Furthermore Wnt/ $\beta$ -catenin signaling is required for differentiation of the stem cells localized to the hair follicle<sup>44,45</sup>, underlying the importance of Wnt/catenin signaling in the development of hair follicles and function of epidermal stem cells. Moreover it is at this stage of incipient hair follicle development when  $\beta$ -catenin/Wnt signaling is important that the early hair follicle stem cells are initially established<sup>9,12</sup>.

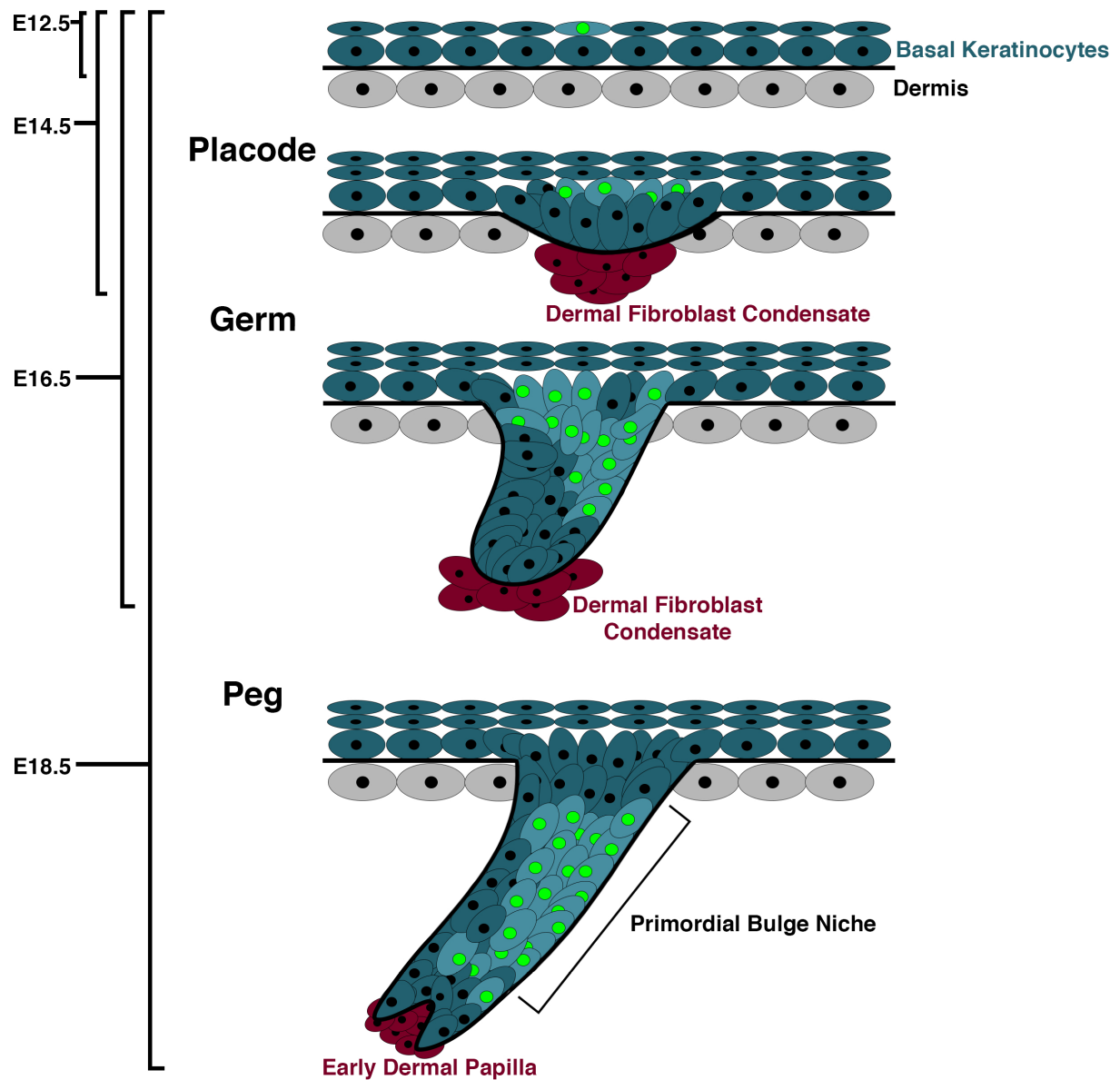
The adult bulge stem cells are specified at E14.5 in the developing epidermis when the placode forms and can be traced by the transcription factor Sox9<sup>12</sup>. At the placode stage Sox9 expressing cells are found in the apical portion of the placode in cells not touching the basal lamina. In the absence of Sox9 in the epidermis placodes form, however the adult bulge stem cell niche fails to form<sup>9</sup>, suggesting the formation of the placode and the BSC are not dependent on one another and that additional signals may be needed to initiate BSC formation. In addition to the absence of the BSC niche the outer root sheath

does not differentiate, sebaceous gland morphogenesis is disrupted and wound repair is impaired<sup>9,12,13</sup>.

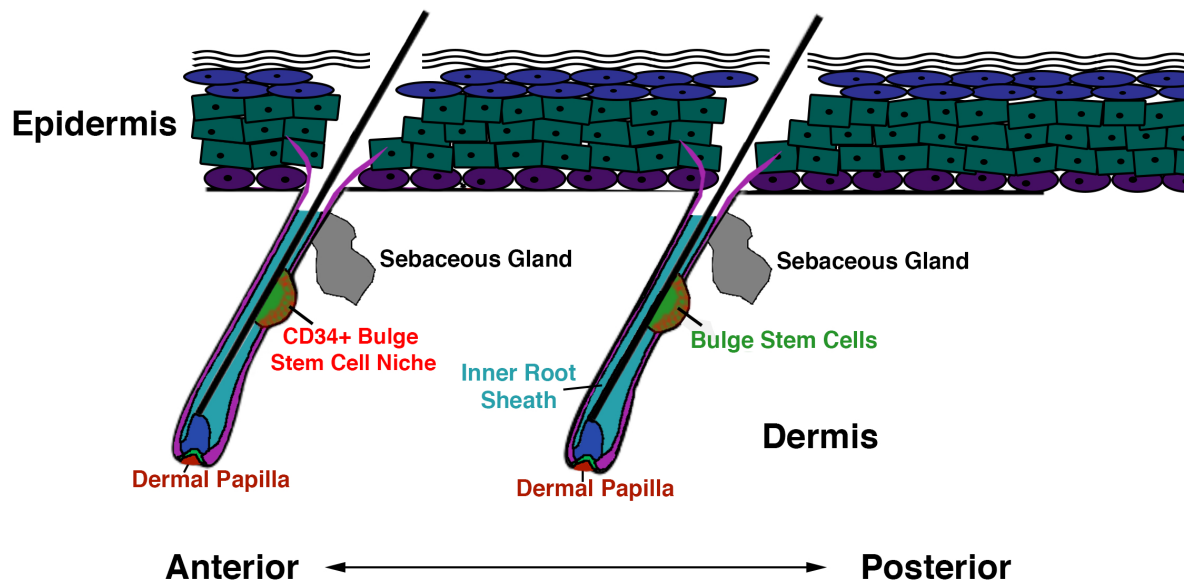
Following initial placode development fibroblasts in the dermis immediately basal of the placode respond to sonic-hedgehog (SHH) signaling from the epidermis to condense and form the dermal condensate. This aggregation of dermal cells signals to the developing follicle to guide its downgrowth. Signaling pathways from the dermal condensate such as Wnt and platelet derived growth factor (PDGF) results in the hair follicle extending down to form the hair germ. At this stage in hair follicle development Sox9 expressing cells are located in the posterior portion of the developing follicle and begin to be observed further down toward the middle of the hair germ. Down-growth continues to occur eventually forming the hair peg, the most mature form of hair follicles in the embryo. At the peg stage Sox9+ cells cluster in the middle-shaft of the follicle<sup>9,10,12</sup>. Also at this stage the dermal condensate is engulfed by the hair peg, thus forming the dermal papilla. The dermal papilla is responsible for the signaling required for hair cycling (Figure 2).

Full maturation of the hair follicle occurs at post-natal day 21. The hair follicle is composed of layered epithelial tissue including the outer root sheath (ORS) and inner root sheath (IRS) as well as the sebaceous gland (Figure 3). At this stage follicles contain the fully formed bulge stem cell niche located below the sebaceous glands which can be marked by the presence of CD34, a surface protein of bulge stem cells<sup>2,8-10</sup>. The stem cells in the hair follicle BSC niche are responsible for replenishing the epidermis following injury as well as the hair

follicle during normal homeostasis<sup>2,3,6,48</sup>. Cells within the BSC niche express multiple stem cell markers such as Sox9, Lhx2, NFATc1 and Tcf3/4<sup>2,3,6,10,12,49,50</sup>. While it is evident that the bulge stem cell niche is important for proper epidermal function and hair follicle morphogenesis, but how the niche itself is established during embryogenesis remains to be fully understood.



**Figure 2: Schematic Model of Hair Follicle Development in Wild-Type Epidermis.** Sox9+ cells are indicated by a green nucleus. Primordial bulge stem cells can be tracked during hair follicle morphogenesis through expression of Sox9.



**Figure 3: Schematic of Skin Organization and Location of Bulge Stem Cell Compartment.** Multiple layers make up the epidermis and hair follicle. Within the hair follicle the inner most layer is the Inner Root Sheath (light blue) which is surrounded by the outer root sheath (purple). Sebaceous glands are indicated in grey. Bulge stem cells (green) reside in the CD34+ bulge stem cell niche (red) directly below the sebaceous gland. The Dermal Papilla (red) lies at the base of the follicle. Hair follicles orient along the Anterior-Posterior axis.

## 1.5 THE ROLE OF CELL POLARITY IN HAIR FOLLICLE DEVELOPMENT

The ability of cells to sense polarity across a field of cells (planar cell polarity (PCP)) is critical in establishment of functional organs and tissues<sup>36,39,51–53</sup>. In the murine epidermis, cells must be able to identify top from bottom (apical-basal (AB) polarity) and anterior to posterior (PCP). Cell polarity relies on the cell's ability to communicate with its neighbors through cell:cell interactions. This is accomplished both by direct association with neighboring cells through junctions as well as extracellular ligand association. Multiple proteins have been shown to be critical in the establishment of polarity, with several of them such as *Frizzled* (*Fzd3*, *Fzd6*)<sup>11,20,34,54</sup>, *Flamingo* (*Celsr1*)<sup>11,20,52</sup>, *Disheveled* (*Dvl1*, *Dvl2*)<sup>55</sup>, *Bazooka* (*Par3*)<sup>17,34,36,51</sup>, *aPKC*<sup>34,35</sup>, Wnt/ $\beta$ -catenin<sup>45,46,56–59</sup>,  $\alpha$ -catenin<sup>56,59</sup> and the ERM proteins<sup>17,25–30</sup>, having high conservation throughout diverse taxa. The high incidence of functional conservation across such a wide and diverse group of taxa underlies the importance of polarity in morphogenesis and development.

Disruption in the ability of cells to establish polarity results in several phenotypic consequences. In the mouse epidermis polarity proteins play a role in hair follicle development as well as stratification during embryogenesis. Apical-basal polarity is apparent at E12.5 when the epidermis begins stratification<sup>36,42</sup>. Failure to achieve correct apical-basal polarity during stratification of the epidermis results in the disruption of spindle orientation resulting in defects in asymmetric cell division. The breakdown of asymmetric cell division of the dividing basal layer leads to alterations in stratification and decreased barrier function<sup>19,36,42,60</sup>. This phenotype is exemplified by Merlin-deficient epidermis,

which exhibits basal layer polarity defects, random spindle orientation, expansion of the basal cell layer and severe barrier defects<sup>17,30</sup>. Planar cell polarity in the developing epidermis is initially established at E13.5 and can be visualized through polar localization of *Celsr1*. By E14.5, when the first stage of hair follicles formation occurs the placodes are polarized along the anterior-posterior axis<sup>11</sup>.

PCP knockout mice have multiple phenotypes, ranging from disruption in neurulation<sup>55</sup>, to defects in epidermal development<sup>11</sup>, cochlear formation abnormalities<sup>54</sup> as well as ablation of organized cellular migration<sup>51</sup>, indicating that planar polarity has a role in many developmental contexts. Mouse models deficient for *Celsr1* or *Frizzled* in the epidermis have shown disruptions in hair follicle orientation, alluding to the importance of polarity in establishment of functional tissues and organs. Wnt/  $\beta$ -catenin signaling is involved in polarity as well as adhesion<sup>46,61</sup> with striking phenotypes associated with  $\beta$ -catenin knockout in the epidermis. In the murine epidermis  $\beta$ -catenin KO results in complete failure of hair follicles to form, as well as defects in hair cycling<sup>45</sup>. Disruption of both interacting partners of  $\beta$ -catenin as well as downstream proteins in the Wnt pathway results in multiple types of disruptions, from lack of hair follicle formation in *Wntless (Wls)* KO mouse epidermis<sup>46</sup> to defects in hair follicle polarity visualized as incorrect orientation of hair follicle angles in KO *Frizzled*<sup>62</sup>. From this data it is clear that proteins involved in establishment of polarity are important in formation of the hair follicle itself as well as for establishment of epidermal stem cell niches and subsequent epidermal differentiation both during embryogenesis as well as during hair cycling<sup>11,56,63</sup>.

Due to defects in both hair follicle development and establishment of epidermal stem cell niches following disruption in polarity pathways it is clear that polarity plays significant role in formation of the bulge stem cell niche, though the direct link between this pathway and the bulge niche has not been established. In this study we aimed to explore the role polarity and polarity proteins have in establishment of hair follicle morphology through disruption in adherens and tight junction maturation by epidermal ablation of Merlin.

## **CHAPTER 2: HYPOTHESIS, SPECIFIC AIMS AND SIGNIFICANCE**

## Hypothesis

We hypothesize that Merlin is required for hair follicle morphogenesis through establishment of polarity. To discover the role of Merlin in the organization of the hair follicle the following aims were explored:

**Aim 1:** To determine the role of Merlin mediated regulation of the early hair follicle morphogenesis we will a) examine the role of Merlin in organizing the Sox9+ progenitor cells through analysis of control and K14cre;*Nf2*<sup>L/L</sup> embryonic epidermis; b) ascertain if Merlin is necessary for development of the adult bulge stem cell niche with an epidermal transplantation assay.

**Aim 2:** To ascertain the function of Merlin-regulated polarity in hair follicle development we will define the role of Merlin in establishment of planar polarity in the developing follicles of control and K14cre;*Nf2*<sup>L/L</sup> embryos.

## Significance

Stem cells function to maintain and replenish epithelial tissues during normal tissue turnover and to regenerate tissue following injuries. The stem cell's ability to activate during a growth phase or in response to injury depends upon the integration of extracellular signals. Disruption in these signaling cascades can result in impaired wound healing, precocious tissue aging, diseases such as psoriasis, alopecia or cancer. Examining how regulation through adherens junction proteins alters stem cell niche development and understanding the

mechanisms behind this could lead to specific therapeutics being developed to aid in wound healing and tissue regeneration.

## **CHAPTER 3: MATERIALS AND METHODS**

## Mouse Strains

K14Cre;*Nf2<sup>L/L</sup>* mouse line was previously generated<sup>17</sup>. K14cre mice<sup>64</sup> were crossed with *Nf2<sup>L/L</sup>*<sup>65</sup> mice were genotyped as described<sup>17</sup>. All animal procedures were approved by the MD Anderson Cancer Center Institutional Animal Care and Use Committee (IACUC).

## Embryo preparation and Immunofluorescence

Embryonic mice were collected at the indicated developmental time-points and for sagittal sectioning embryos were fixed in 4% PFA for 1hr followed by sucrose dehydration (10% sucrose in PBS for 24hrs, 20% sucrose in PBS for 24hrs, 30% sucrose in PBS 24hrs) and then embedded into Optimized Cutting Tissue media (OCT), frozen and cryosectioned (7µm). Sections for immunofluorescence were then permeabilized for 10min in PBS-0.1% Triton, rinsed in PBS once, blocked for 1hr in 10% HISS/1% BSA in PBS, and then incubated in indicated primary antibodies overnight at 4°C using the following dilutions: Sox9 (1:250, H-90, Santa Cruz), K14 (1:250, AF 64, Covance), CD34 (1:200, gp105-120, Pierce), E-Cadherin (1:200, ECCD-2, Invitrogen), Zo-1 (1:250, 61-7300, Invitrogen), Vinculin (1:200, MAB3574, Millipore), Tcf4 (1:200, C481411, Cell Signaling). Sections were then washed 5min/3X in PBS and incubated in secondary antibodies as indicated. Filamentous actin was used to label phalloidin (1:200, Alexa Fluor 488 and 568, Invitrogen) and nuclei were labeled with DAPI. For whole mount microscopy embryo backskin was dissected from embryos following overnight fixation, permeabilized for 10min in PBS-0.5% Triton and incubated in

indicated antibodies as above except secondary antibody incubation was performed overnight at 4°C. For histological analysis sagittal sections were stained for Hematoxylin and Eosin and then imaged.

## **Microscopy**

Embryonic epidermis and adult epidermis for immunofluorescence was imaged using Nikon A1 laser scanning confocal microscope at 40X magnification and processed with Nikon-Elements software (Nikon). For whole mount imaging 15-30 z-stacks at 0.5-0.6µm were obtained. Representative single planes are shown in 2D or compiled planes are shown in 3D movies. All images were collected at 1024 X 1024 square pixels resolution. Images were assembled and labeled in Adobe Photoshop CS6. 3D movies were created using z-stacks described above with Bitplane Imaris Data Visualization software.

Images of adult epidermis for histological analysis and P0 epidermis for angle measurements were acquired using a Nikon 80i upright microscope at 20X (for angle measurement) or 40X magnification. Images were assembled and labeled in Adobe Photoshop CS6.

## **Measurements and Quantification**

Following acquisition described above angles were measured using Fiji (ImageJ) angle tool. The angle of the follicle was captured at the intersection of the follicle with the adjacent basal epidermis. The angle of the basal epidermis surrounding the hair follicle was used as the horizontal control. Measurement data was

exported and plotted with Microsoft Excel. T-test of whole mount Sox9 expression and hair follicle number was performed using GraphPad Prism6.

### **Grafting Transplantation Assay**

Full thickness epidermis was dissected from K14Cre;Nf2<sup>L/L</sup> mutant mice and wild-type littermates at P0. Epidermis was then washed thoroughly in PBS and placed onto recipient nude mouse graft bed and secured using surgical staples. The graft bed was created by removing backskin of comparable size to graft from recipient nude mouse. Epidermis was then allowed to heal for 21 days and then harvested. Nude recipients were sacrificed and graft was dissected from backs.

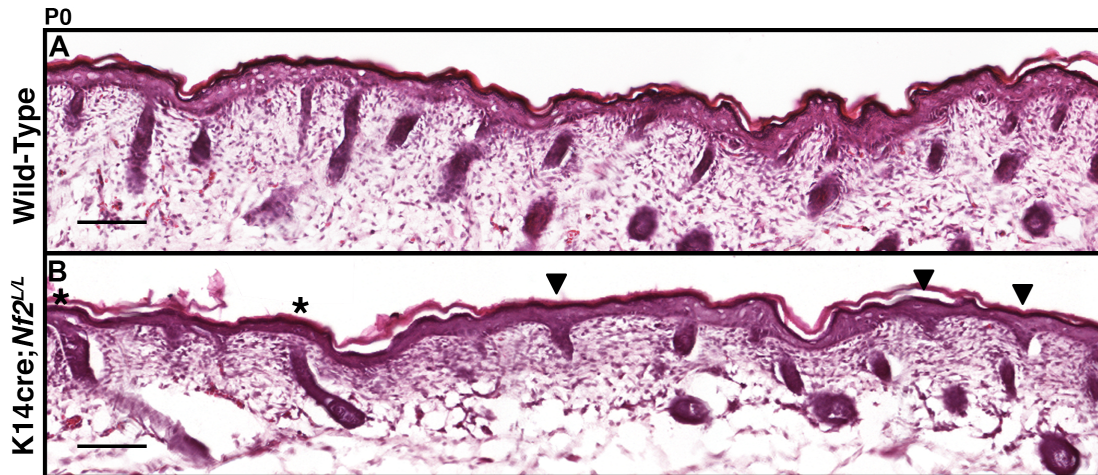
## **CHAPTER 4: RESULTS**

#### **4.1 DELETION OF MERLIN DISRUPTS HAIR FOLLICLE DEVELOPMENT DURING EMBRYOGENESIS**

Epidermal development and hair follicle morphogenesis are linked during development. Signals from the epidermis and dermis work in concert with each other to create a functional barrier to the outside world, failure in one or the other results in defect in epidermal development as well as hair follicle morphogenesis. Following initial embryogenesis the ongoing homeostasis of the epidermis relies heavily on stem cells located in the bulge stem cell niche of the hair follicle. Bulge stem cells contribute to all epidermal lineages during normal homeostasis as well as following injury, underlying the importance of communication between the epidermis and its hair follicle appendages. Thus proper formation of the hair follicle is important for the continued function of the epidermis. Defects in hair follicle development results in several diseases and deficiencies in the epidermis itself, from alopecia<sup>9,66</sup> to impaired wound healing<sup>12,67-71</sup> to tumor formation<sup>66,72</sup>. Clearly the ability to communicate between the epidermis and the hair follicle is imperative for development and maintenance of the main barrier to the external world.

Merlin-deficient mice have defects in AJ maturation, a key player in cell:cell communication. Mice lacking Merlin in the epidermis die shortly after birth due to loss of barrier development. Further examination of the P0 skin of mutant mouse found that hair follicles appeared disorganized and some appeared to be developmentally delayed due to the lack of penetration into the dermis (Figure 4). Due to the loss of barrier function long-term studies of

epidermal development and hair follicle morphogenesis have not been previously performed using K14cre;*Nf2*<sup>L/L</sup> mice. To examine whether Merlin-deficient follicles are able to mature and function correctly we performed an epidermal transplantation assay.



**Figure 4: Deletion of Merlin Results in Defects in Hair Follicle Development:**  
**(A,B)** Hematoxylin and Eosin of sagittal sections of skin from **(A)** wild-type and **(B)** K14cre;*Nf2<sup>L/L</sup>* imaged at 20X magnification. Scale bar (SB) = 100μm. Asterisks indicate hair follicles oriented in the wrong direction. Arrows indicate malformed hair follicles.

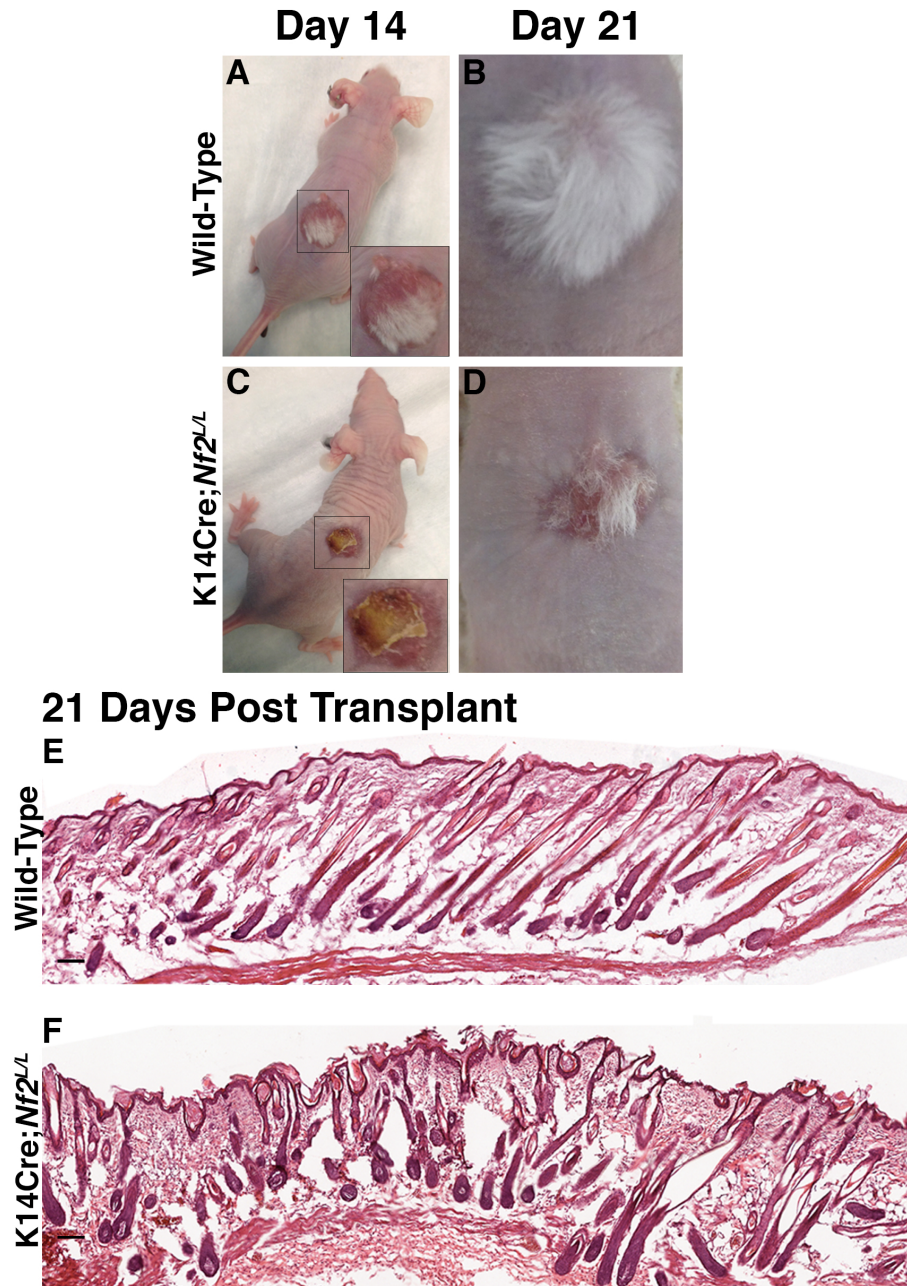
## 4.2 MERLIN IS REQUIRED FOR ESTABLISHMENT OF THE BULGE STEM CELL NICHE

Hair follicles form during early epidermal development and bulge stem cell precursors form during epidermal development. Complete formation of the hair follicle and development of the bulge stem cell niche does not occur until 21 days of age. To investigate if mutant epidermis had the ability to develop functional mature follicles we performed grafting of mutant and wild-type backskins onto nude mice. Full-thickness skin from newborn mutant and control littermates was isolated at birth (P0). Skin was grafted onto the backs of immune compromised Nude mice and allowed to attach and grow for 21 days.

Grafts of wild-type epidermis were able to form functional hair follicles by day 14 post-graft (Figure 5A) and by day 21 had full hair growth within the grafted skin (Figure 5B). In contrast grafts of Merlin-mutant skin failed to grow hair by day 14 (Figure 5C). By day 21 patches of small hairs were present on the grafted mutant epidermis however most of the epidermis was hairless (Figure 5D). H&E staining shows fully mature hair follicles across the entirety of wild-type donor skin (Figure 5E). Mutant epidermis displayed malformed follicles with disorganization of the epidermis (Figure 5F). The bulge stem cells are required for formation of hair<sup>9</sup>. Due to the defects in hair formation of the Merlin-deficient epidermis we next sought to determine if the adult bulge stem cell compartment had developed I performed fluorescence immunohistochemistry with antibodies directed against Sox9 and the adult bulge niche marker CD34<sup>2,12</sup> (Figure 6A). I observed cells positive for both Sox9 and CD34 in the mid-region of the wild-type hair follicle indicative of the adult bulge stem cell niche. Contrary to wild-type

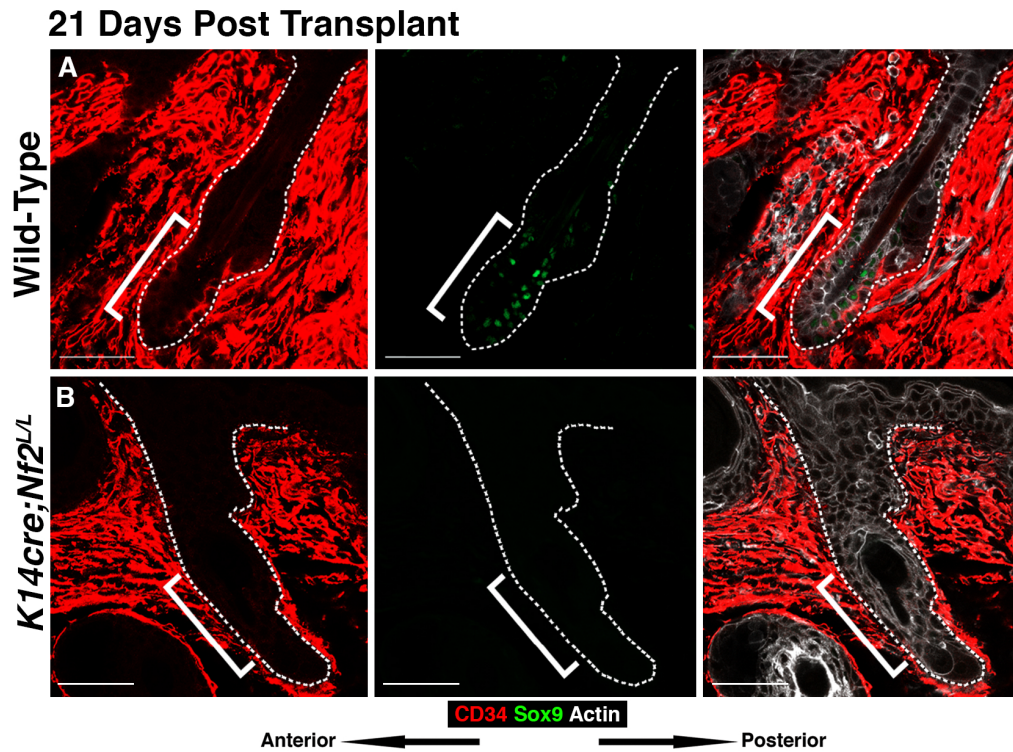
expression mutant follicles displayed decreased and random Sox9 expression with no apparent CD34 expression within the follicle (Figure 6B).

The lack of CD34 expression within mutant hair follicles coupled with a disruption in stem cell marker expression suggests that in the absence of Merlin developing hair follicles are unable to form the bulge stem cell niche during morphogenesis. The lack of the bulge niche coupled with alterations in hair follicle organization and lack of hair growth led us to question whether hair follicle polarity is disrupted early in development, which could lead to these late stage defects.



**Figure 5: Merlin-deficient Epidermis Fails to Form Functional Mature Hair Follicles.**

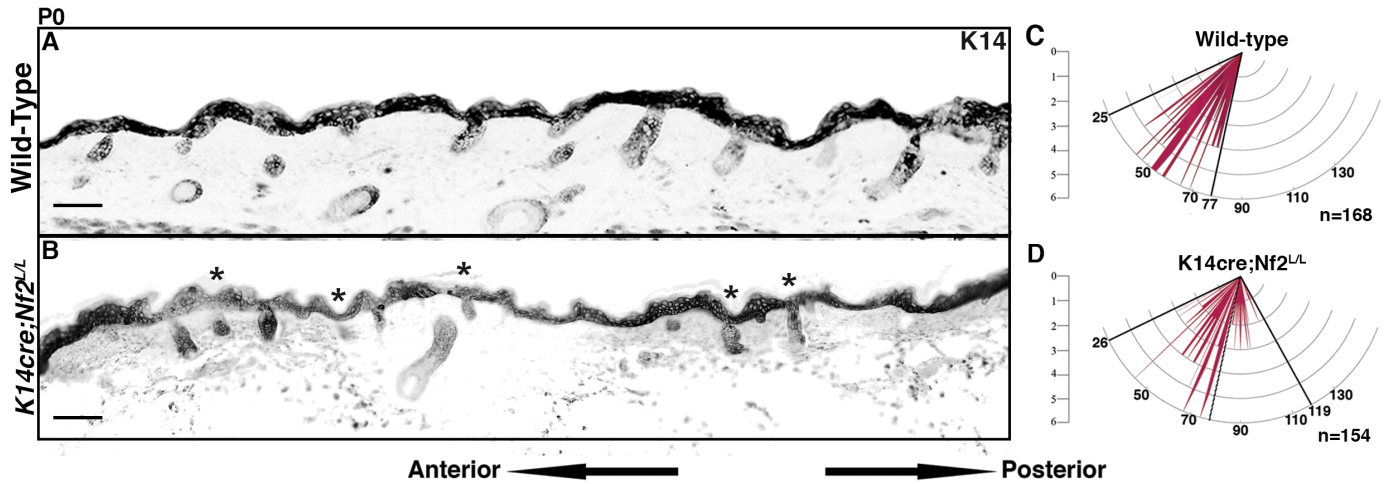
(A-D) Images of mice from transplantation assay. (A-B) Wild-type transplants had partial hair growth by (A) Day 14 and full growth at (B) Day 21. (C-D) K14cre;*Nf<sup>L/L</sup>* transplants had no hair growth at (C) Day 14 and only partial patchy growth at (D) Day 21. P0 Wild-type or K14cre;*Nf2<sup>L/L</sup>* epidermis was transplanted onto the backs of nude mice. Epidermis was collected 21 days post-transplantation. (E-F) Hematoxylin and Eosin stain of (E) wild-type (F) and K14cre;*Nf2<sup>L/L</sup>* epidermis. SB=100μm.



**Figure 6: Merlin is Required for Establishment of the CD34+ Adult Stem Cell Niche:** 21 days post transplantation of **(A)** Wild-type or **(B)** *K14cre;Nf2<sup>L/L</sup>* epidermis graft sites were stained for CD34 (Red) and Sox9 (Green) to label the adult bulge stem cell niche. Sox9 expression in **(A)** wild-type follows previously described pattern with clear CD34+ niche. **(B)** mutant epidermis has no CD34+ niche and no Sox9 expression. Dotted boundaries indicate edges of the hair follicle. Brackets indicate CD34+ bulge stem cell region in wild-type and corresponding presumptive niche area of the mutant. SB=50µm.

### **4.3 MERLIN DEFICIENT HAIR FOLLICLES HAVE ALTERED PLANAR POLARITY**

Hair follicle morphogenesis begins with the formation of the placode and continues with organization and downgrowth of the developing follicle. During morphogenesis proper hair follicles formation is dependent upon polarization of cells within the epidermis. Merlin depleted epidermis has altered basal cell polarity<sup>17,30</sup>, resulting in changes in epidermal differentiation. Mice deficient of Merlin in the epidermis have marked defects in hair follicle orientation at the P0 stage when compared to wild-type littermates (Figure 7A,B). In order for hair to have a characteristic pattern the follicles develop at specific angles during embryogenesis and following wounding. Failure to orient in the correct angle results in disruptions in hair growth and texture<sup>11,58</sup>. Wild-type follicles exhibited orientation along the A-P axis as previously described<sup>11,73</sup> and angles were within a range of 25°-77°(Figure 7C). The majority of hair follicles measured between 50°-53° anteriorly. While hair follicles were present in mutant epidermis (Figure 7B) their orientation along the anterior posterior axis was severely disrupted. In contrast to wild-type follicles the Merlin-deficient epidermal hair follicles had a larger range of orientation of 26°-119° (Figure 7D) with the majority measuring between 70°-75°. Disruptions in angles of the Merlin-deficient follicles indicated defects in polarity of the developing epidermis. To discover if developing Merlin-deficient epidermis had alterations in polarity we went back in developmental time to embryogenesis.



**Figure 7: Deletion of Merlin Results in Disruption of A-P Orientation of Hair Follicles:**

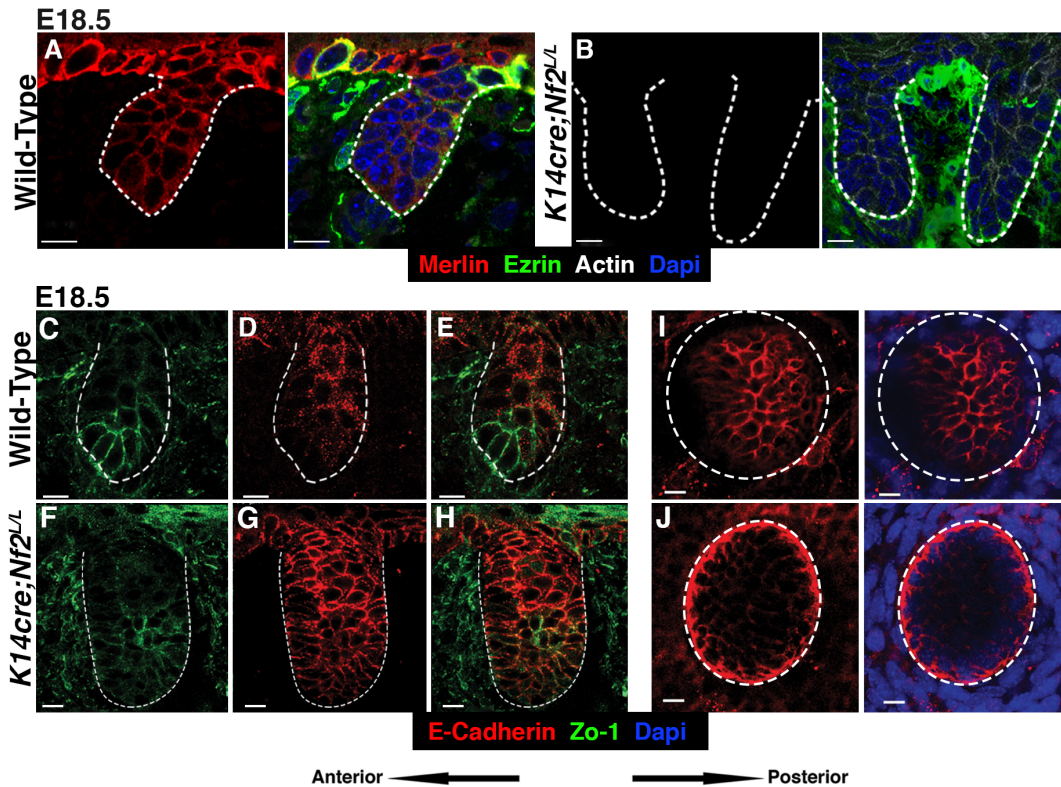
(A,B) Anti-Keratin 14 labeling of sagittal sections of skin from (A) wild-type and (B) K14cre;Nf2<sup>L/L</sup> imaged at 20X magnification. Scale bar (SB) = 100μm. (C,D) Schematic of hair follicle angle quantification. X axis, measured angle between anterior end of follicle and epidermis. Y axis, number of follicles measuring angle X. (C) Wild type angles measured between 25-77° with a mode of 51°. (D) Mutant angles measured between 26-119° with 32% of follicles measuring outside of wild-type range and a mode of 67°. Asterisks indicate hair follicles that measure outside of wild-type angle range.

Developing hair follicles show clear planar polarization (PCP) during embryogenesis that can be visualized by staining tissue with adhesion markers that are planar polarized<sup>11</sup>. PCP is initially established at E13.5 and can be visualized using E-cadherin by E15.5<sup>11</sup>. To visualize planar cell polarity within the developing hair follicle sagittal sections of E18.5 backskin were analyzed using E-cadherin and ZO-1<sup>11</sup>. Figure 8 A,B demonstrates complete loss of Merlin in the epidermis. Wild-type follicles show clear anterior expression of ZO-1 and posterior expression of E-cadherin (Figure 8C-E). Mutant follicle germs exhibit E-cadherin expression across the entire germ with minimal ZO-1 expression in the middle of the follicle (Figure 8F-H). We further analyzed early polarity defects by utilizing whole mount backskin from E16.5 embryos (Figure 8I-J). Wild-type follicles displayed polarized E-cadherin expression at the posterior portion of the developing follicles (Figure 8I). In contrast, Merlin mutant follicles exhibit a complete lack of polarization with E-cadherin at all edges of developing follicles (Figure 8J).

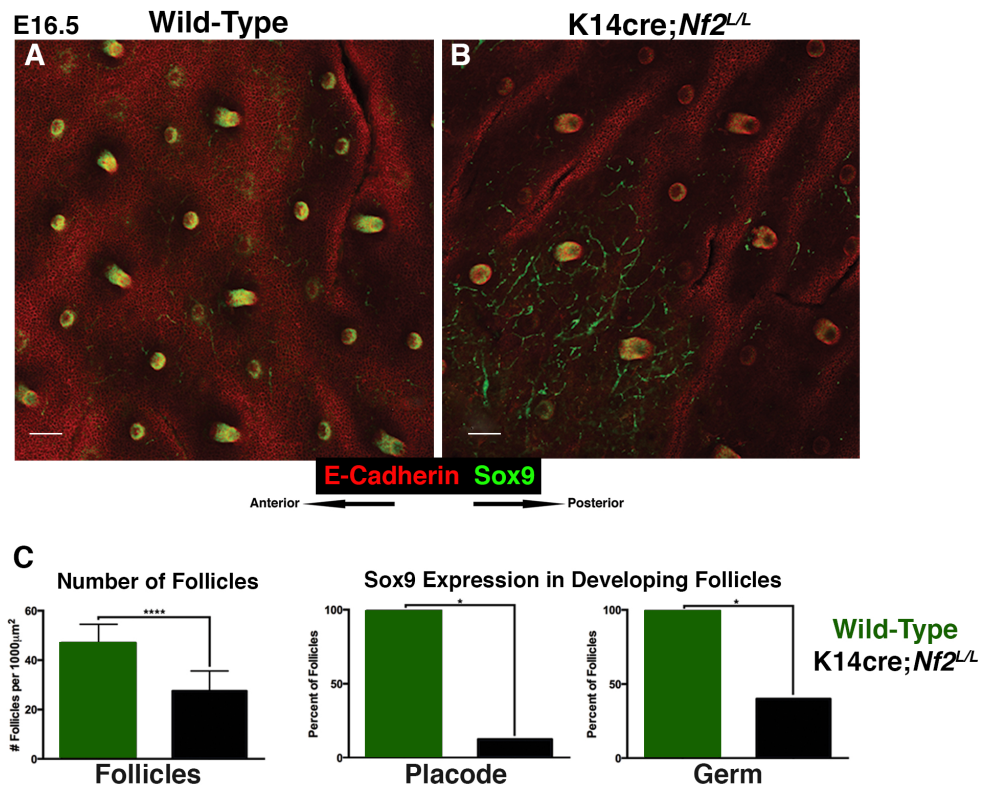
The defect in E-cadherin polarization is visible in early and later stage developing follicles hereafter referred to as “placodes” for early stage and “germ” for late stage. Through visualization of localization of E-cadherin and Sox9, a known marker for developing hair follicles<sup>9,12,13</sup>, obvious disruptions in both polarization and in follicle orientation are evident in hair germs of the mutant epidermis when compared to wild-type (Figure 9A,B). It is interesting to note that the orientation defects observed in P0 and grafted mutant epidermis (Figure 7) is evident in the E16.5 mutant epidermis. Whole mount staining clearly shows

mutant hair germs developing at random angles (Figure 9B) in contrast to the uniform orientation of developing wild-type germs (Figure 9A). While an obvious decrease in hair follicle number is observed in the mutant epidermis (Figure 9B,C:  $p < 0.0005$ ) it was also observed that mutant follicles exhibit decreased Sox9 expression both in hair placodes and hair germs. Quantification of this showed that of the follicles present there was a marked decrease in Sox9 expression when compared to wild-type. 100% of wild-type follicles contained Sox9+ cells while only 12.5% of mutant placodes and 40% of mutant germs contained Sox9+ cells (Figure 9C:  $p < 0.05$ ).

These data suggest that Merlin is required for the polarization of hair follicles during embryogenesis and that depletion of Merlin disrupts early hair follicle polarity in turn causing defects in the orientation of follicles.



**Figure 8. Merlin Regulates Planar Polarity in Developing Hair Follicles: (A-H)** Sagittal section from E18.5 **(A)** wild-type and **(B)** K14cre;Nf2<sup>L/L</sup>. Staining for Merlin indicates knock-down was successful in the epidermis. **(C-H)** Sagittal sections with polarity marker expression for **(C-D)** wild-type and **(F-H)** K14cre;Nf2<sup>L/L</sup>. Note the failure to properly polarize. SB= 10μm. **(I,J)** E16.5 epidermis whole-mount staining. Planar view of **(I)** wild-type epidermis shows polarized expression of E-cadherin to the posterior portion of the follicle while **(J)** K14cre;Nf2<sup>L/L</sup> epidermis exhibits lack of planar polarity. (A-D) SB= 10μm.



**Figure 9: Merlin Deficient Epidermis has Decreased Follicle Number and Disruptions in Sox9 Initiation. (A,B)** Planar view E16.5 epidermis of **(A)** wild-type and **(B)** K14cre;Nf2<sup>L/L</sup>. Decreased follicle number as quantified by **(C)**.  $p < 0.0005$ . **(C)** Quantification of Sox9+ follicles K14cre;Nf2<sup>L/L</sup> epidermis has decreased Sox9 expression compared to wild-type.  $P < 0.05$ . (A,B) SB= 100μm.

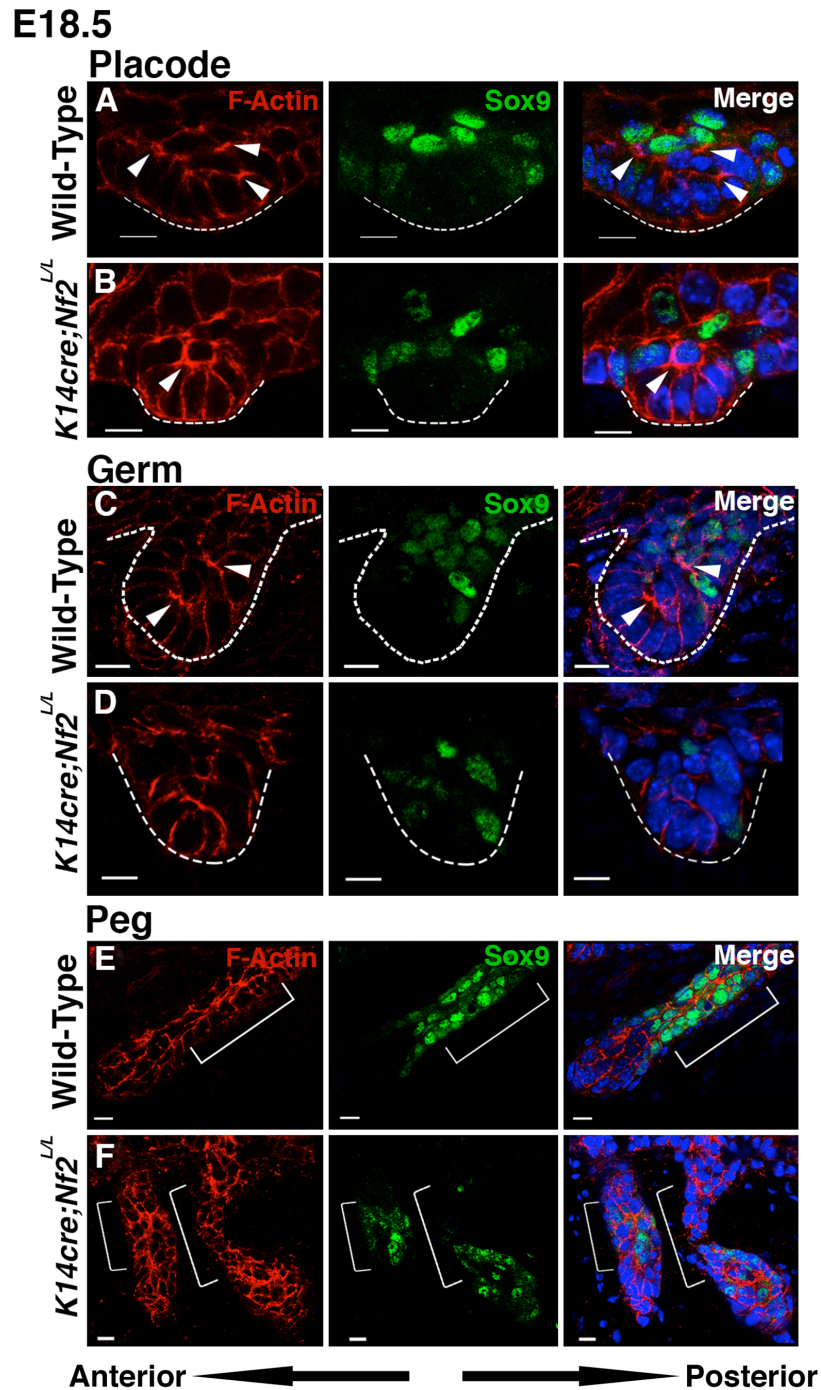
#### **4.4 MERLIN-DEFICIENT LATE STAGE AND MATURE HAIR FOLLICLES HAVE DECREASED Sox9 EXPRESSION**

Sox9 is a well established marker of primordial bulge stem cells destined to establish the bulge stem cell niche<sup>9,12</sup>. The decrease in Sox9 expression within grafted Merlin mutant epidermal follicles compared to wild-type along with the decrease in Sox9 expression within E16.5 epidermis led us to explore whether timing of Sox9 initiation is disrupted during embryological morphogenesis resulting in the inability to correctly form the bulge stem cell niche. To answer this question we examined both Sox9 expression and actin organization during multiple stages of hair follicle development during embryogenesis.

We examined Sox9 expression and actin organization in E18.5 backskin, when all early stages (placode, germ and peg) of hair follicles are present (Figure 2). In wild-type placodes Sox9<sup>+</sup> cells cluster near the center of the suprabasal portion of the placode (Figure 10A). As follicle development continues these Sox9<sup>+</sup> cells are visible in the apical and posterior side of the germ (Figure 10C). By the peg stage of development Sox9 is expressed in the middle portion of the follicle in the primordial bulge region (Figure 10E). In contrast mutant follicles display altered expression of Sox9 throughout follicle development. Mutant placodes have decreased apical Sox9 expression with the few Sox9<sup>+</sup> cells randomly located throughout the placode. (Figure 10B). Germ stage follicles (Figure 10D) of mutant epidermis continue to exhibit a random Sox9 expression pattern across the developing follicle, moreover some Sox9<sup>+</sup> cells are located at the basal edge of the follicle. The random and sometimes basal pattern of

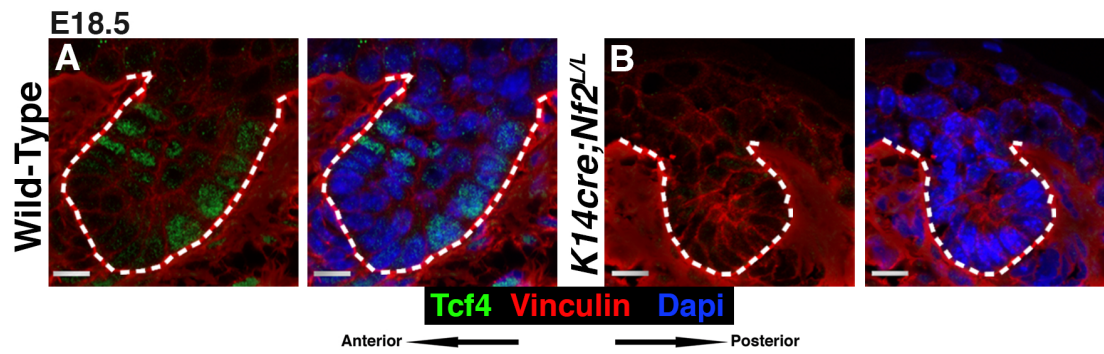
expression is stark contrast to the obvious apical expression pattern in wild-type germ follicles. By the peg stage of hair follicle development (Figure 10F) Sox9+ cells have a completely random pattern throughout the entire follicle in the mutant follicles with Sox9+ cells located in the primordial bulge region of the wild-type peg.

In conjunction with alterations in Sox9 expression within the developing follicles of Merlin-deficient epidermis I have also observed aberrant actin organization. During hair follicle morphogenesis areas of actin concentration correlate with the apical constriction of the cells below the actin and is associated with hair follicle downgrowth directionality<sup>11</sup>. Wild-type follicles exhibit concentrated actin at the posterior and apical sides of the developing placode (Figure 10a arrows). Areas of actin concentration can be seen in the germ of wild-type follicles near the central apical and anterior portion of the follicles (Figure 10C arrows). In contrast to the organized constriction seen in wild-type placodes mutant placodes have high actin concentration at the central suprabasal layer of the placode (Figure 10B arrow). By the germ stage actin is extremely disorganized with no apparent constriction (Figure 10D) and obvious disruption to the cellular architecture are apparent. Rather than concentrating around cell borders such as what I observed in wild-type germs (Figure 10C) actin retains a punctate staining pattern in mutant follicles (Figure 10D). The disruption in actin organization culminates in the peg stage of Merlin-deficient hair follicles with defects in gross overall morphology of the follicles (Figure 10F).



**Figure 10: Merlin is Required for Organization of Sox-9+ Cells and Cellular Constriction of the Developing Follicles: (A-F)** Immunofluorescence microscopy with indicated antibodies of sagittal wild-type and K14cre;Nf2<sup>L/L</sup> skin at embryonic day 18.5. **(A)** Wild-type placode, **(C)** germ and **(E)** peg follicle stages show appropriate Sox9 localization. **(B)** Mutant placode, **(D)** germ and **(H)** peg follicles show decreased and random Sox9 expression. SB=10μm

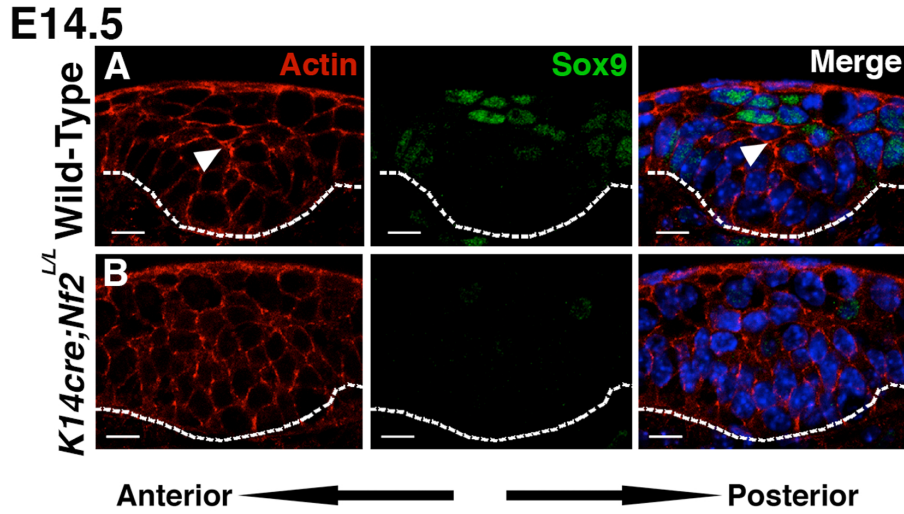
To identify if Merlin depletion was specifically acting on Sox9 expression or on stem cell marker expression as a whole we next looked at expression of another stem cell marker, Tcf4<sup>49,50,70,74</sup>. Tcf4 is a member of a family of DNA binding proteins that bind  $\beta$ -catenin and transactivates Wnt target genes. In the epidermis Tcf4 is initially expressed in the primordial bulge stem cells of early stages of hair follicles similar to the expression pattern of Sox9. Functionally redundant to its sister protein Tcf3, deletion of both Tcf3/4 in the developing epidermis leads to lack of hair follicle morphogenesis as well as defects in stem cell activation which results in deficient wound healing<sup>5,49,70,75</sup>. Tcf4 localization in wild-type epidermis is present in the shaft of the developing hair follicles (Figure 11A) while mutant epidermis had complete depletion of Tcf4 expression within the follicle (Figure 11B). These results indicate that Merlin is regulating the initial formation of the primordial bulge stem cell niche during epidermal development.



**Figure 11: Merlin Regulates Epidermal Stem Cell Initiation.** (A,B) Sagittal section of (A) wild-type and (B) K14cre;Nf2<sup>L/L</sup>. Note failure to express stem cell marker Tcf4 in K14cre;Nf2<sup>L/L</sup>. epidermis (A,B) SB=10μm.

We strove to determine how early in development Sox9 and actin disruptions begin so we moved further backwards in developmental time to E14.5 when placodes are just beginning to form. While wild-type placodes have Sox9+ cells as early as E14.5 (Figure 12A), mutant placodes at this early embryonic stage lack Sox9 in the placode entirely (Figure 12B). Disruptions in the actin cytoskeleton of the Merlin mutant placode are also very obvious at this stage. Wild-type follicles have concentrated actin at the apical and anterior portion of the follicle (Figure 12A arrows) while mutant placodes have actin concentration at random areas of the follicle (Figure 12B). These results indicate that in Merlin-deficient epidermis both the spatial and temporal initiation of Sox9 is disrupted, this disruption is seen at the same time as defects in actin cytoskeletal architecture.

Actin acts as the link between cellular junctions and the intracellular environment through mechanical signaling<sup>23,76</sup>. Mechanotransduction is a known force in cellular signaling and even stem cell fate<sup>76-78</sup>. This is achieved through the integration of mechanical signals from neighboring cells through junctions to the cytoskeleton<sup>17,19</sup>. Arrangement of the actin cytoskeleton is dependent on the correct polarization of the cell, asymmetry of the actomyosin network is believed to be responsible for the cells ability to integrate spatial cues and promote correct contractility of the cell<sup>30,60,79</sup>. Further work to identify the role of mechanotransduction in establishment of polarity and subsequent development of the bulge stem cell niche will greatly increase our understanding of this vital epidermal stem cell compartment.



**Figure 12: Merlin is Required for Organization of Sox-9+ Cells and Cellular Constriction of the Early Follicles: (A-B)** Immunofluorescence microscopy with indicated antibodies of sagittal wild-type and K14cre;*Nf2*<sup>L/L</sup> skin at embryonic day 14.5. **(A)** Wild-type placode shows appropriate Sox9 localization. **(B)** Mutant placode has completely diminished Sox9 expression. Arrowheads indicate sites of cellular constriction within developing hair follicle. SB=10μm

## **CHAPTER 5: DISCUSSION AND FUTURE DIRECTIONS**

## DISCUSSION

This study aimed at expanding our knowledge of the link between establishment of the epidermal bulge stem cell niche and polarity. During embryogenesis polarity plays a pivotal role in the organization of tissues, organs and structures throughout the animal and plant kingdoms<sup>10,11,15,39,42,51–55,80–83</sup>.

This conservation across such a vast array of organisms underlies the necessity of polarity in organismal biology and development. Polarity relies upon an individual cells ability to communicate with its neighbors and orient itself within a developing tissue<sup>4</sup>. When cell contacts are unable to function properly, defects in polarity are inherent in the overall phenotype<sup>17,39,40,54,82,84,85</sup>.

Hair follicle morphogenesis is a tightly synchronized process that involves both AB polarity and planar cell polarity, however until this work it was not known how alterations in polarity result in defects in follicle formation. Not only does the inherent angle of the follicle need to be established, but also the multiple cell types of the follicle need to be organized correctly. One important sequestered cell type within the follicle is the adult hair follicle stem cell. Epidermal stem cells reside in a specific area of the follicle called the bulge stem cell niche. Failure to properly localize results in defects in differentiation, lack of hair growth and disruptions in wound healing<sup>6,9,12,13,49,86</sup>. Much work has been done using lineage tracing and functional studies of the bulge stem cells, revealing them as multipotent<sup>3,5</sup> cells that are specified in the early stages of hair follicle development<sup>12</sup>. While the function and importance of the bulge stem cells and their niche is apparent how they localize to the bulge niche area remains to be fully understood.

This study is unique in that it bridges polarity with establishment of the hair follicle niche during development through cell contact function. This is accomplished through characterization of epidermal knockout of the protein Merlin. Merlin-deficient epidermis was previously characterized as having severe barrier defects and loss of apical-basal polarity resulting in disruptions in the stratification of the mutant epidermis and defects in the actin cytoskeleton<sup>17</sup>. Through this study we have begun to characterize the phenotypic role of Merlin in establishing the bulge stem cell niche of hair follicles.

To begin studying the role of Merlin in establishment of the adult bulge stem cell niche we performed full thickness transplantation assays of wild-type and Merlin-deficient epidermis onto the backs of nude mice. Following transplantation of epidermal backskin grafts the epidermis was allowed to heal and grow for 21 days. By day 14 the wild-type epidermis was beginning to grow hair in patches, however the Merlin-mutant epidermis failed to have hair growth. On day 21 the mice were sacrificed and grafts were harvested for analysis. At this stage in the healing process all wild-type epidermis had full hair growth while Merlin-deficient epidermis has only patchy, small hairs if any.

Immunofluorescence microscopy uncovered that while wild-type hair follicles possessed a CD34+ bulge stem cell niche with cells positive for the stem cell marker Sox9, Merlin-deficient epidermis failed to form a CD34+ niche and stem cell marker expression was decreased and random throughout the follicle.

From the transplantation assays I concluded that Merlin is required for the development of the CD34+ bulge stem cell niche and localization of stem cells

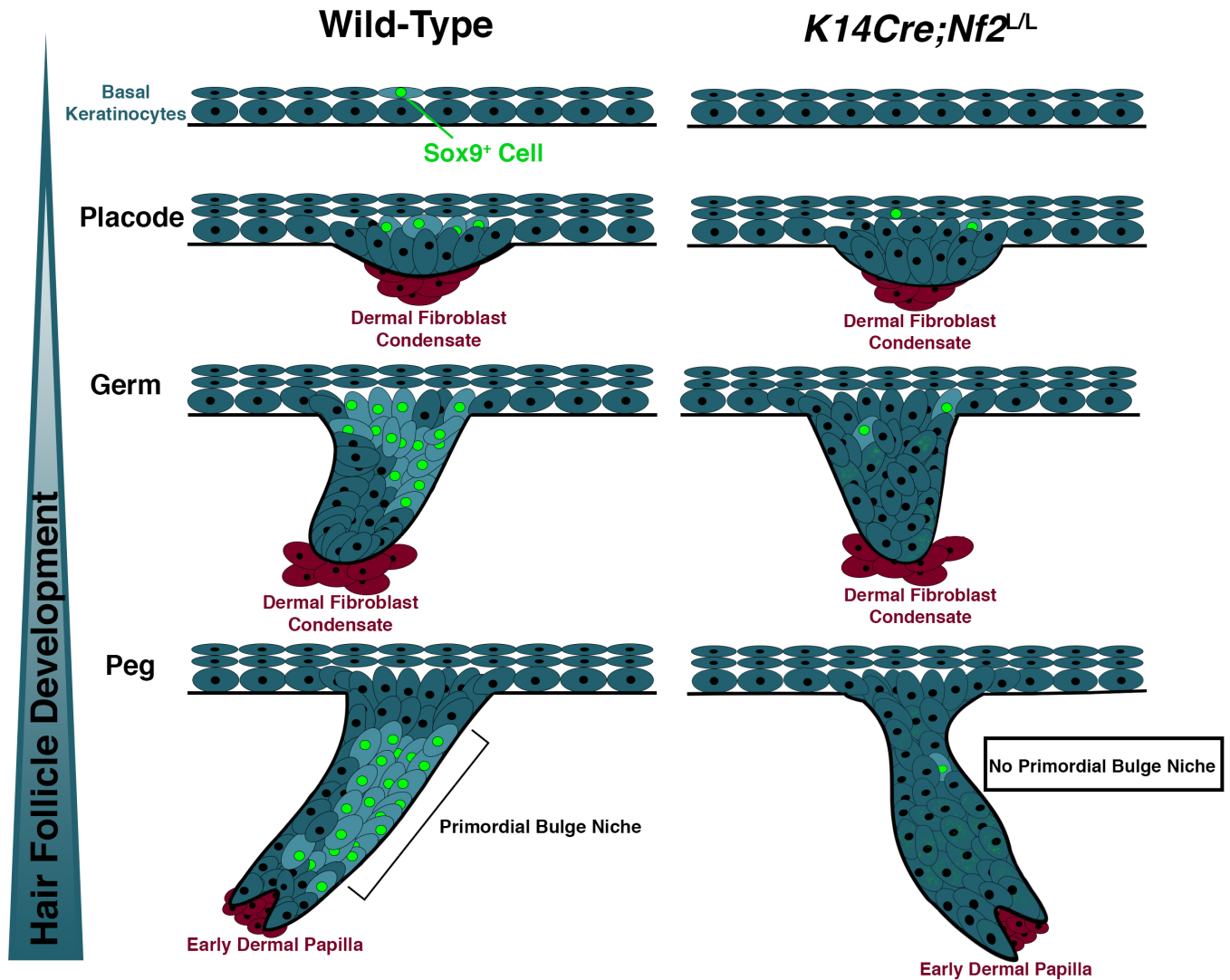
within the follicle is impaired. The grafted mutant skin has severe disorganization and malformation of hair follicles. To understand the biology behind the defects seen in hair follicle morphogenesis it was pertinent to examine hair follicle morphology in newborn mice. While past work has focused on how Merlin regulates the epidermis itself<sup>17</sup> how and if hair follicle development was effected by loss of Merlin in epidermal tissue remained to be fully explored.

Close examination of P0 hair follicles revealed a striking phenotype with randomized orientation of follicles along the A-P axis in Merlin-deficient epidermis. Following quantification of hair follicle angles between Merlin-deficient and wild-type follicle angles further brought to light the stark difference between the two phenotypes. Wild-type embryos exhibited angles measuring between 25°-77° along the A-P axis while Merlin-deficient follicles were found between 26°-119°. Taken together, defects in both with the defects in hair follicle orientation observed in Merlin-deficient P0 mice and previous knowledge of Merlin's role in establishment of AB polarity in the epidermis we began to explore the possibility that Merlin is needed for establishment of hair follicle polarity during embryonic epidermal development. To answer this question I began examining earlier time-points in development to analyze both the state of polarity in the developing epidermis and possible defects in hair follicle morphogenesis.

Throughout the entirety of embryonic hair follicle development, Sox9 expression is disrupted in Merlin-deficient epidermis (Figure 13). Rather than following the known expression pattern across development Sox9+ cells are found randomly throughout the follicles and at decreased numbers. Interestingly

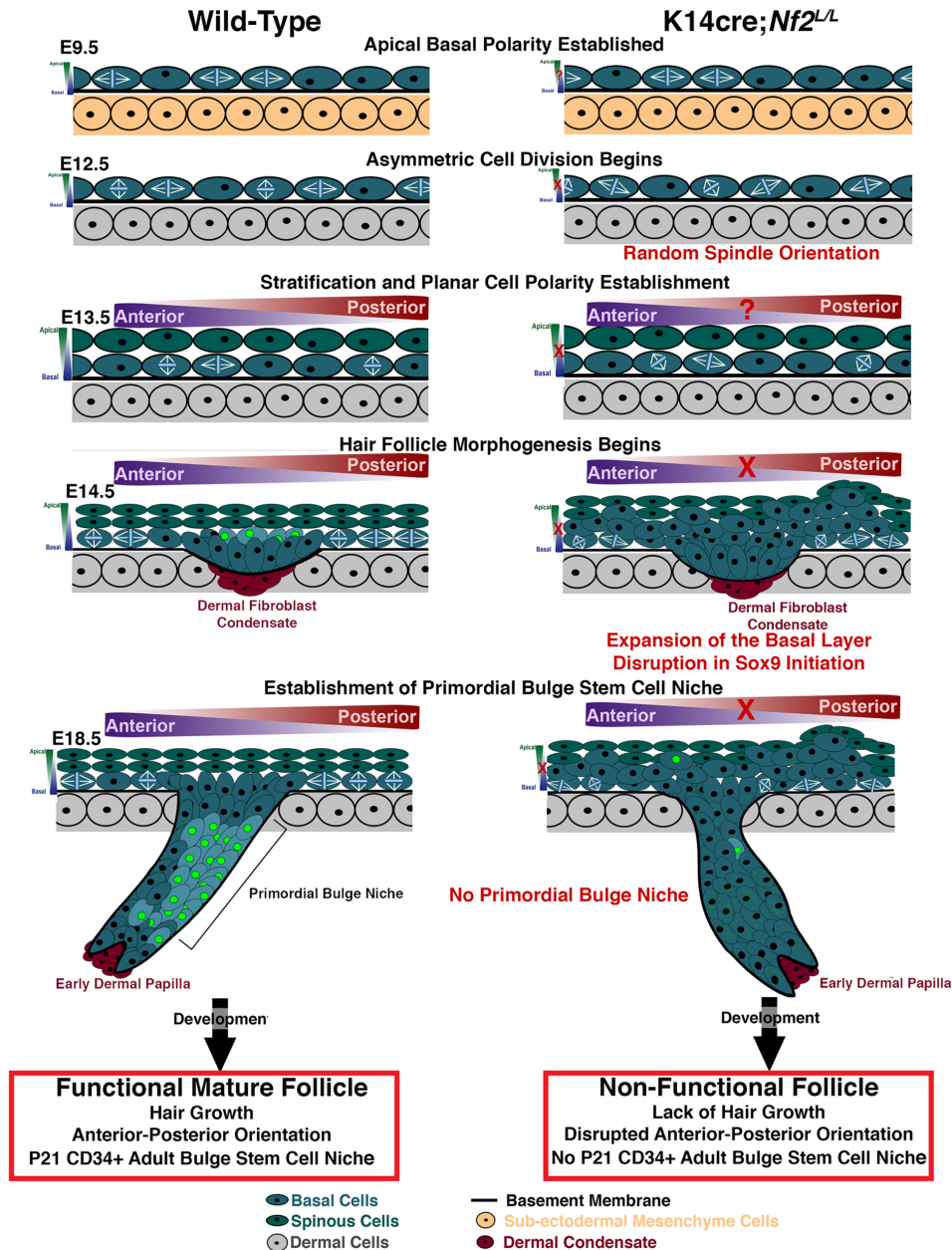
at E14.5 when the placode is first forming and Sox9 marks the apical cell of wild-type follicles, Merlin-deficient epidermis has no Sox9 expression. However by E18.5 Sox9 does mark a few cells in an apparently random fashion, indicating not only a disruption in spatial Sox9 expression but in temporal expression as well. Disruption in the establishment of hair follicle stem cells through depletion of Sox9 in the epidermis results in blocked progression through hair follicle cycling and the CD34 niche does not form<sup>9,12</sup>. Thus Merlin's role in establishment of the bulge stem cell niche is supported through both a decrease in Sox9+ cells during development and failure to form CD34 bulge niche following transplantation when Merlin is ablated in the epidermis.

During hair follicle development the final angle of the hair follicle is set up early in morphogenesis. By the early germ stage a clear angle is visible and is characterized through actin constriction in specific areas of the developing follicle, physically forcing the hair follicle angle<sup>10,11,73</sup>. I have found that during this critical time in hair follicle development the actin cytoskeleton of the follicles in Merlin-deficient epidermis lacks proper areas of constriction. Thus explaining the morphological consequence of Merlin depletion and subsequent alteration in hair follicle angles. Without proper actin constriction the hair follicles are unable to form in the organized pattern along the A-P axis. Disruption in actin constriction is often seen in ablation of other polarity proteins such as *Celsr1* and *Vangl2* in the epidermis<sup>11</sup>, further emphasizing the role of Merlin in establishment of polarity and hair follicle morphogenesis.



**Figure 13. Merlin Regulates Establishment of the Bulge Stem Cell Niche:** Schematic model of hair follicle development in wild-type and *K14cre;Nf2<sup>L/L</sup>* epidermis. Sox9 expression begins early in follicle formation when planar cell polarity is initiated and follows a polarized movement down the posterior portion of the follicle during development eventually forming the primordial bulge niche. Mutant follicles fail to follow polarized expression of Sox9 in any stage of follicle development and the primordial bulge niche does not form. In addition the overall morphology of the follicle is disrupted.

The data presented in this work demonstrates the importance of Merlin in establishment of the bulge stem cell niche during development through regulation of polarity. One proposed model (Figure 14) is that Merlin regulates AB polarity in the earliest stage of epidermal stratification as well as regulating subsequent establishment of planar cell polarity in the stratified epidermis. In the absence of Merlin, cells are unable to form mature junctions and AB polarity is disrupted, followed by a disruption in PCP. In the absence of PCP and with decreased functional cell junctions the cells within the follicle are unable to develop the bulge stem cell niche because they cannot communicate with their neighbors and so cannot establish polarity within the tissue. Also during this time as cell contacts function as the link between cells and the actin cytoskeleton the disruption in both polarity and junctions results in defective actin cytoskeletal arrangement and follicles are unable to form with the correct morphology.



**Figure 14. Schematic Model of the Role of Merlin in Epidermal Development:** In wild-type (left) E9.5 marks the first epidermal commitment step and the basement membrane is formed. At this point establishment of apical-basal polarity it established. At E12.5 assymetric cell division begins and is followed by initiation of stratification. Hair follicle development begins at E14.5 with the initiation of placodes with the primordial bulge stem cell marker Sox9 (green) localized to the apical portion of the placode. It is also here that anterior-posterior polarity is evident. Hair follicles develop and organize in and anterior to posterior orientation and at E18.5 the peg stage contains the primordial bulge stem cell niche. Further maturation of the hair follicle results in hair growth at P4 and the CD34+ bulge stem cell niche at P21. In the Merlin-deficient epidermis (right) it is unclear if apical-basal polarity is established at E9.5 however at E12.5 spindle orientation is random and stratification is altered. Initiation of primordial bulge stem cells is delayed and organization of Sox9+ cells is disrupted. Thus the primordial bulge stem cell niche is not formed at E18.5, hair growth is aberrant in transplanted epidermis and the adult CD34+ bulge stem cell niche is absent.

## FUTURE DIRECTIONS

While a possible model has been outlined the molecular mechanism behind it remains unknown. One possibility is that in the absence of Merlin cells are unable to integrate mechanosensory cues from the external environment. Integration of signals from the external environment to the inside of cells requires multiple pathways acting in concert with one another, one such being mechanosensory transduction. Cellular junctions play a critical role in integration of external signals resulting in intracellular changes and initiation of signaling cascades<sup>79,87-91</sup>. The role of mechanical stress in initiating such intracellular changes is an important piece in understanding how tissue development and cell-type regulation occurs. Mechanosensory transduction has a role in establishment of planar polarity<sup>19,84</sup>, cell sorting<sup>79,92</sup> and stem cell activation<sup>90,93,94</sup>, although the bridge between these cellular responses and tension remains to be fully understood. Arrangement of the actin cytoskeleton is dependent on the correct polarization of the cell, asymmetry of the actomyosin network is believed to be responsible for the cells ability to integrate spatial cues and promote correct contractility of the cell<sup>79</sup>. Merlin is a known interacting partner of actin binding proteins at the adherens junctions thus this model could explain how deletion of Merlin could effect the cells ability to integrate mechanosensory cues in the developing follicle in turn effecting initiation of Sox9. Discovery into this field will yield exciting possibilities for the creation of therapeutics based on mechanical tension for treatment of wounds and disorders characterized as having disrupted stem cell signaling.

Further molecular studies are needed to identify how Merlin may be regulating stem cell fate within the epidermis as indicated by the decrease in Sox9 expression in the hair follicle both through development as well as following epidermal transplantation. Bulge stem cells are multipotent stem cells that can contribute to multiple cell types within the epidermis<sup>2,9,13</sup>. There are several pathways involved in the activation of bulge stem cells, with  $\beta$ -catenin playing a pivotal role<sup>45,72</sup>. Overexpression of  $\beta$ -catenin within the niche promotes precocious stem cell activation and proliferation, while down regulation of  $\beta$ -catenin causes a decrease in bulge stem cell marker expression<sup>72,95</sup>. A recent study showed that  $\alpha$ -catenin is required for the degradation of  $\beta$ -catenin by stabilizing  $\beta$ -catenin to the APC destruction complex<sup>96</sup>. As Merlin interacts with  $\alpha$ -catenin at the adherens junction it would be interesting to ascertain if Merlin also interacts with  $\alpha$ -catenin at the APC destruction complex. A detailed exploration of the molecular mechanisms driving Merlin's role in regulating epidermal stem cells would shed light on the intricate world of stem cell biology.

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## **VITA**

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