2009

Characterizing the Role of Primary Cilia in the Hair Follicle and Skin

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CHARACTERIZING THE ROLE OF PRIMARY CILIA IN THE HAIR FOLLICLE AND SKIN

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2009

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CELL BIOLOGY

ABSTRACT

Primary cilia have been established as a nearly ubiquitous microtubule based signaling organelle, yet their function in many organs, including the hair and skin, is unknown. We hypothesized that the primary cilium would play vital roles in the hair follicle and skin based on the cilium's well characterized interactions with the Shh and Wnt developmental signaling pathways. This dissertation describes our efforts to determine the localization of the primary cilia in the murine hair follicle and assess the function of this organelle in the hair and skin. We used confocal immunofluorescence microscopy to virtually section the entire hair follicle and determined that primary cilia are on most cells of the hair follicle during development and during cycling. We were interested to determine whether the primary cilia had different functions in different cell populations such as the dermal condensate and epidermal placode of the hair follicle. We utilized the Cre-lox system to disrupt primary cilia in the dermal and epidermal portions of the hair and skin, using a dermal Cre construct *Prx1-Cre* and an epidermal construction *K14-Cre* crossed to the floxed *Ift88* and *Kif3a* genes which when disrupted, disrupt the cilia. Using this Cre-lox construct we were able to determine that dermal primary cilia signaling is essential for proper hair follicle formation. In mice with disrupted dermal cilia, the hair follicle arrests at the hair germ stage, which coincides with loss of Shh observed phenotypes. We determined that these dermal cells lost the ability to respond to Shh signals and the hair follicle arrested. The condensate thus requires primary

cilia to receive Shh signal and allow the morphogenesis of the hair follicle. Disrupted cilia in the epidermis resulted in a phenotype of mild alopecia, ectopic hair bud formation, and basal hyperplasia. We determined that primary cilia cause an expansion of the progenitor cell population by changing the axis of division of progenitor cells. They also activate the β-catenin pathway leading to ectopic hair buds. Thus, this dissertation was successful in identifying cilia in the hair and skin and establishing the primary cilia as important for hair follicle morphogenesis and epidermal homeostasis.

ACKNOWLEDGEMENTS

Being a scientist is a funny profession. Once you get beneath the business of it all, the grants and the manuscripts and such, scientists are paid to be professionally curious. This is the dream job of any seven year old. I would like to thank my mentor Bradley K. Yoder for letting me be that seven year old. I was given the opportunity to work on a project which was totally new; new to me, new to the lab, and at least a little new to the world. Brad has been a great mentor and he has been a wonderful guide and teacher in the journey that led to this dissertation.

The Yoder lab has been quite a merry band out in the woods of Cilium. I want to thank all of the current members, Nicholas Berbari, Venus Childress, Mandy Croyle, Erica Hambly, Zak Kosan, Svetlana Masyukova, Amber O'Connor, Neeraj Sharma, Zoe Verney, and Corey Williams for all their help, support, and for being such delightful people. Best wishes for Zoe as her path branches off and continues from this work. I would also like to thank the woodsmen and women who came before, Boglarka Banizs, James Davenport, Jenny Schafer, Marlene Winkelbauer, and particularly Courtney Haycraft who trained me during my rotation and got me started on this particular road.

I also want to thank my committee members, Ed Michaud, Casey Morrow, Trent Schoeb, Rosa Serra, Laura Timares, and Bradley Yoder. They have been instrumental in my growth as a scientist and created a constructive and convivial committee environment. Their comments and advice have improved this work a great deal.

I also want to thank my friends for their support and company in the forest. Thanks for darts and game night and Rock band and Wii bits of fun! If I'm not on call, I'll be there for game night! Also, thanks to Aimee, Christa, Jason, Matt, and Sarah and all the other MD/PhD students for being good classmates, even better friends, and for understanding how it is to be neither fish nor fowl.

Finally, I'd like to thank my family. For once I am at a loss for the appropriate words. I would like to thank my mom, Jane Lehman, and my dad, Joseph W. Lehman III for giving me life and unconditional love and support. I am continually inspired by them and by my wonderful siblings, my sister Juliana Lehman, and my brother, Joseph W. Lehman IV. They are my family. I was born with them and didn't get the option of picking them, but I would have still picked them if I could have. You guys are the best!

Lastly, thank you, the reader. Research isn't meant to stay bound in a dissertation or in a library, but to enlighten and improve the world. Hopefully the work herein will contribute at least some infinitesimal length towards either of those goals.

TABLE OF CONTENTS

Page

TABLE OF CONTENTS (continued)

Page

LIST OF TABLES

Table *Page*

INTRODUCTION

AN ESSENTIAL ROLE FOR DERMAL PRIMARY CILIA IN HAIR FOLLICLE MORPHOGENESIS

LIST OF FIGURES

Figure *Page*

INTRODUCTION

AN ESSENTIAL ROLE FOR DERMAL PRIMARY CILIA IN HAIR FOLLICLE MORPHOGENESIS

LIST OF FIGURES (Continued)

Figures *Page*

EPIDERMAL CILIARY ROLES IN SKIN HOMEOSTASIS AND HAIR FOLLICLE MAINTENANCE

LIST OF FIGURES (Continued)

Figures *Page*

INTRODUCTION

An Introduction to the Dissertation

The goal of this introduction is to review the applicable literature and background on the primary cilia and various associated human diseases referred to as the ciliopathies, as well as an overview of the biology of the hair follicle and skin. The aim is to provide a framework for understanding the studies described and questions asked in this dissertation. This dissertation serves to bridge these two fields and evaluate the roles of primary cilia in hair and skin biology.

An Introduction to Primary Cilia

Primary cilia are nearly ubiquitous (for a partial list of ciliated cell types see http://www.bowserlab.org/primarycilia/cilialist.html) micro-tubule based organelles structurally related to flagella. There are multiple types of cilia characterized by their tubule structure including motile and immotile varieties. Motile cilia, or 9+2 cilia, contain 9 outer microtubules and 2 inner microtubules, with dynein motor arms providing motive force for movement (Pan et al., 2005; Singla and Reiter, 2006). These cilia are

found in locations such as the lung, oviduct, and ependyma and function in mucociliary clearance and fluid movement. In addition to these broad categories of cilia, specialized highly modified cilia such as olfactory or photoreceptor cilia also exist which vary from this simplified nomenclature. For example, nodal cilia are a form of 9+0 cilia that contain 9 outer microtubules without inner microtubules, but are motile and function in early embryogenesis to establish directional fluid flow that regulates left/right axis patterning (Essner et al., 2002; Nonaka, 1998; Okada et al., 1999). Olfactory cilia are a corresponding exception and are specialized (9+2) cilia that are immotile, but sensory (Kerjaschki, 1976). Primary cilia have a 9+0 architecture, but are non-motile and are present on most cells in the mammalian body. They are constructed and maintained via an intricate molecular assembly process known as intraflagellar transport.

Intraflagellar Transport

The process of intraflagellar transport (IFT) was first described in *Chlamydomonas* (Kozminski et al., 1993) and is required for the construction of their flagellum. Proteins destined for the cilia are first localized to the base of the cilia in the basal body/ transition zone. Then, they are transported to the tip of the cilia by a protein raft (IFT particle) powered by a kinesin motor complex comprised of Kif3a, Kif3b, and Kap3. This motor complex moves the raft and cilia "cargo" in an anterograde fashion to the tip of the cilium. Retrograde transport back to the base is mediated by a cytoplasmic dynein complex.

There are many IFT raft proteins, but one of the first IFT proteins identified in mammals was intraflagellar transport protein 88 or IFT88. The establishment of the connection between IFT88, also called Tg737 in mammals, or OSM-5 in the roundworm *Caenorhabiditis elegans,* and cilia resulted from a convergence of research from several laboratories using a variety of model organisms including mouse, *C. elegans*, and *Chlamydomonas reinhardtii* (Haycraft et al., 2001; Murcia et al., 2000; Qin et al., 2001; Taulman et al., 2001). These studies demonstrated that the *Ift88* gene is both highly evolutionarily conserved and that it is required for ciliogenesis. The protein components of the IFT particle form two complexes that can be distinguished both biochemically and by their role in the cilium. Complex A proteins function in retrograde transport and complex B proteins are required for anterograde transport. IFT88 is a complex B protein required for cilia formation and is the focus of this dissertation. Defects in anterograde IFT particle proteins or anterograde motor proteins lead to the expected phenotype of short dystrophic cilia. Defects in retrograde transport lead to improperly constructed, somewhat shorter cilia with accumulated protein in the ciliary axoneme (Pazour et al., 2000; Yoder et al., 2002).

A growing number of signaling molecules have been localized to either cilia or the basal body at the base of cilia, and their activity has been linked to the ability of these signaling proteins to localize to primary cilia. Important cellular signaling molecules now known to localize to cilia include the polycystins, platelet derived growth factor receptor alpha (PDGFRα), smoothened (Smo), patched1, the glioma-associated oncogene homolog (Gli) proteins, and Suppressor of fused (Sufu) (see Fig. 1) (Rohatgi et al., 2007; Singla and Reiter, 2006; Xu et al., 2006). This observation suggests that IFT enables the

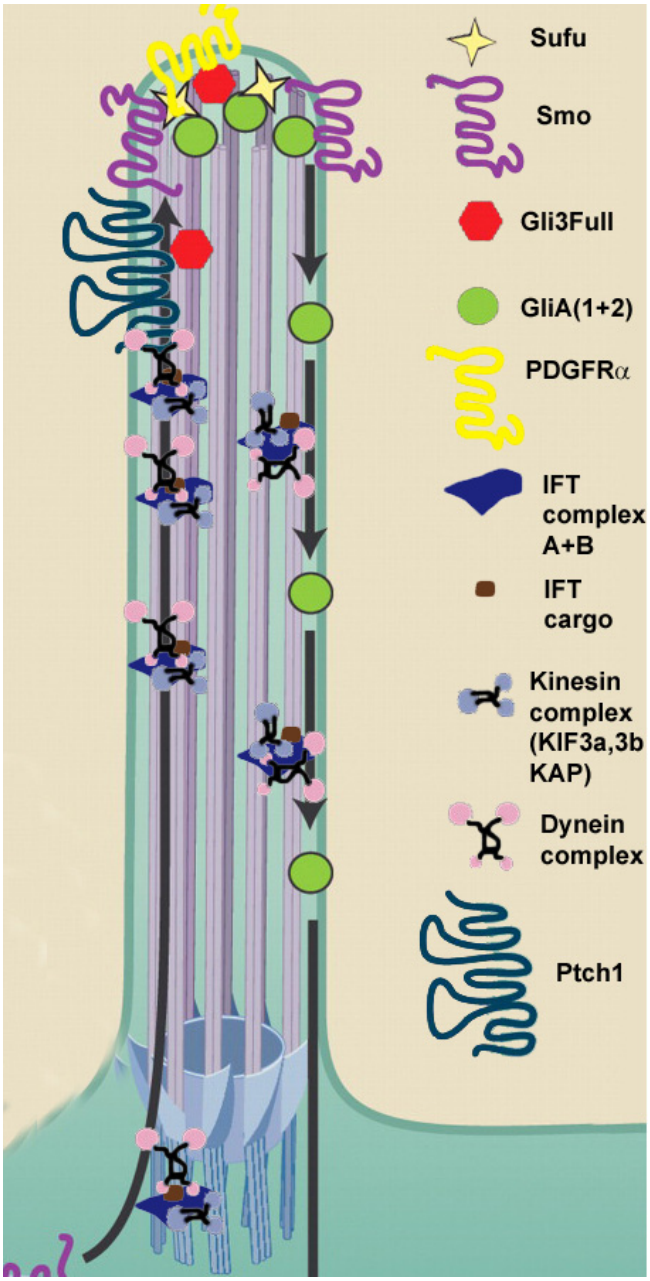

Figure 1 IFT signaling and Mediators
The IFT complex forms at the base of the cilium and is comprised of a kinesin complex, which is responsible for anterograde motion, the IFT complex A+B which forms the protein raft to carry IFT cargo, and a dynein complex which carries the whole assembly back to the basal body of the cilium. The cilium also serves as a site for localization of signaling components such as Ptch1, Gli1-3, Sufu, Smo, and PDGFRα.

Adapted with permission from Singla, V. and Reiter, J. F. (2006). The primary cilium as the cell's antenna: signaling at a sensory organelle. *Science* 313, 629-33. Reprinted with permission from AAAS.

cell to utilize its cilium as both a mechanosensor and an organelle for signaling. The primary cilia and IFT therefore do not simply function as a cellular sensory projection, but also as an organizing center for cellular communications (Singla and Reiter, 2006). Defects in these signaling pathways contribute to the wide range of disease phenotypes associated with ciliary defects.

These diseases are referred to as the ciliopathies and involve genetic defects in proteins that localize to cilia or influence ciliary function. They are noted for their wide variety of possible phenotypes in different organ systems (Badano et al., 2006b). A useful model for the ciliopathies is the Oak Ridge Polycystic Kidney mouse (ORPK), which has a hypomorphic allele of *Ift88*, and is reflective of the hypomorphic alleles responsible for most human ciliopathies.

NOTE:

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Ciliopathy Phenotypes in Humans and the ORPK Model Mouse

Defects in cilia formation and function can affect both motile and/or immotile cilia leading to a surprising variety of phenotypes. The importance of motile cilia in

processes such as mucus clearance and cerebrospinal fluid movement are well known. In contrast, the primary cilium was thought to be of minimal importance for mammalian development and health. In fact, primary cilia are vital for homeostasis and development.

In part, data from the Oak Ridge Polycystic Kidney (ORPK) mouse with its wide spectrum of phenotypes affecting numerous tissues has changed this perception (Fig. 2). The ciliopathies are a growing group of disorders which can be grouped according to several factors based on (1) defects in cilia mediated sensory function (ciliary dysesthesia), (2) defects in ciliary structure or maintenance, (3) defects in ciliary motility (ciliary dyskinesia), and (4) defects in ciliary-mediated signal processing (e.g., the hedgehog pathway). Many of the phenotypes in the ORPK mouse are comparable to several of the ciliopathies observed in humans caused by mutations in cilia proteins that impair cilia function (Table 1). There is remarkable overlap and diversity in the expressivity of phenotypes associated with ciliary defects in humans. Phenotypes associated with ciliary defects in humans include renal cystic disease, polydactyly, situs inversus, obesity, mental retardation, hepatic fibrosis, skeletal defects, cerebellar hypoplasia and many others (Table 1). These phenotypes often have significant differences in expressivity between different disorders and between individuals. Several factors could contribute to the expressivity of phenotypes in humans where cilia function is affected. In part, this may reflect the location where the mutant proteins function in the cilium, whether at the basal body, cilia, or a subdomain within the cilium. Data indicate there is also allelic overlap among the human ciliopathies. This variation is evident in the case of Meckel syndrome (MKS), nephronophthisis (NPHP), and Joubert syndrome (JBT). Despite having multiple distinct phenotypes, mutations in shared genes have been identified as the underlying cause of

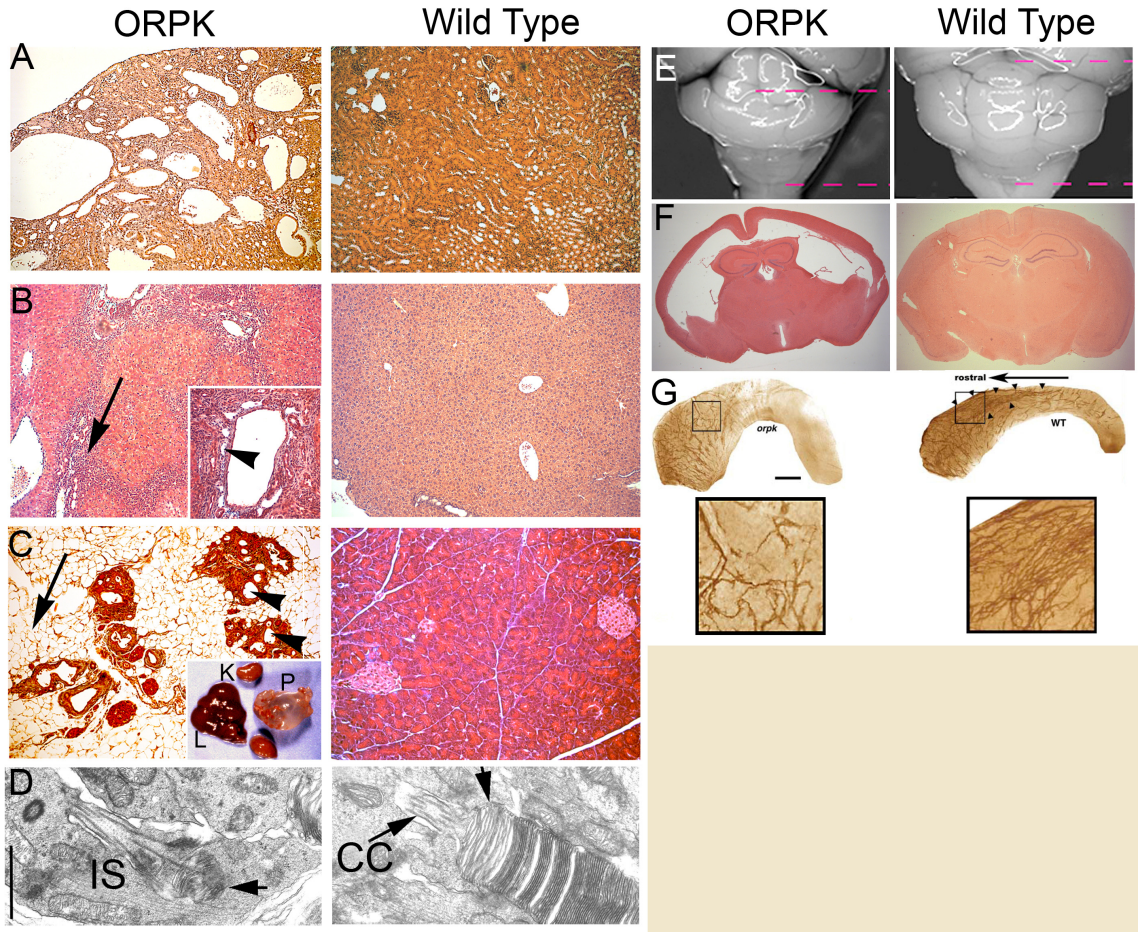

Figure 2 Summary of phenotypes characterized in the Oak Ridge Polycystic Kidney (ORPK) mouse. The ORPK (FVB/N) mouse (left panels) has phenotypes in numerous tissues compared with wild-type controls (right panels). **A:** Cystic lesions are seen in the kidney. **B:** The liver is characterized by biliary (arrow) and bile duct (arrow head) hyperplasia. Inset is a higher magnification view of a central vein showing the multiple dysplastic bile ductules. **C:** In the pancreas, ducts become dilated (arrowhead) and acini atrophy (arrow). Insert shows the typical pancreatic phenotype seen in ORPK-C3H mutants (P is pancreas, L is liver, and K is kidney). **D:** In contrast to wild-type mice, the ORPK mutant has disrupted discs and outer segments that are misshapen and filled with amorphous material that extends into the inner segment of the rod and cone photoreceptors (data are from 10-day-old mice, IS, inner segment; CC, connecting cilium). The images were reprinted with permission from © Pazour et al., 2002. Originally published in *The Journal of Cell Biology*. doi:10.1083/jcb.200107108). **E:** The ORPK mutants are ataxic due to hypoplasia of the cerebellum, which is associated with loss of Shh signaling. Red bars indicate the length of the wild-type and ORPK cerebellum along the anteroposterior axis. (Images reprinted with permission from Chizhikov et al. [2007]; copyright 2007 by the Society for Neuroscience.) **F:** ORPK mutants develop hydrocephalus. **G:** ORPK mutants have defects in neuroblast migration. Shown are neuroblasts in whole mounts of the lateral ventricle stained with an antibody against poly-sialated neural cell adhesion molecule (PSA-NCAM). In wild-type (WT) controls, there are well-organized chains of neuroblasts migrating toward the olfactory bulb. In ORPK mutants, chains of neuroblasts form, but the direction of migration is disorganized. Higher magnification views of the boxed region are shown in the inserts. (Images were reprinted with permission from Sawamoto et al. [2006]; reprinted with permission from AAAS).

TABLE 1. Phenotypes and Associated Clinical Features of the ORPK Mouse and Selected Human Ciliopathies[a]

Ciliopathies	ORPK (*Ift88*^{Tg737Rpw})	ARPKD	ADPKD	SLSN	MKS	JBTS	BBS	NPHP	OFD1	CED^{+}	EVC	USH$^{\wedge}$	PCD	ALS
OMIM*		263200	173900	266900	249000	213300	209900	256100	311200	218330	225500	276900	242650	203800
Retinal degeneration (**M**)	X			X	X	X	X	X		X		X		X
Renal cystic disease (**S**)	X	X	X	X	X	X	X	X	X	X				X
Polydactyly (**P**)	X				X	X	X		X		X			
Situs inversus/isomerism (**D**,**S**)	-			X	X	X	X	X (nph-2)			X	X (USH1+KS)	X (KS)	
Obesity (**?**)	-						X							X
Mental retardation (**?**)	ND			X	X	X	X	X	X			X		
Nociception defects (**S**,**M**)	ND						X							
Cerebellar vermis hypoplasia/aplasia (**P**)	X			X	X	X	X	X			X			
Hypoplasia of the corpus callosum (**?**)	ND				X	X	X		X	X				
Hydrocephalus (**D**,**S**)	X			X	X	X	X	X			X		X	
Respiratory tract infections (**D**)	ND									X		X (USH1)	X	X
Hearing defects (**M**,**S**)	ND			X			X (mild)					X		X
Hepatic fibrosis (**S**)	X	X	X	X	X	X	X	X		X				X
Ectodermal dysplasia (**P**)	X									X	X			
Skeletal defects (**P**)	X			X					X	X	X			X

Legend: ARPKD=Autosomal recessive polycystic kidney disease, ADPKD=Autosomal dominant polycystic kidney disease, SLSN=Senior Loken syndrome, MKS = Meckel syndrome, JBTS=Joubert syndrome, BBS=Bardet-biedl syndrome, NPHP=Nephronophthisis, OFD1=Orofaciodigital syndrome 1, CED=Cranioectodermal dysplasia, EVC=Ellis-van Creveld syndrome, USH=Usher syndrome, PCD= Primary ciliary dyskinesia, KS=Kartagener's syndrome. Contributing Ciliary defects: (**D**)yskinesia, (**S**)ensation, Signal (**P**)rocessing, Ciliary (**M**)aintenance, and (**?**) unknown. (ND)=Not determined. +Cranio ectodermal dysplasia (CED) is a candidate ciliopathy. ^Usher syndrome is a retinal ciliopathy. *Note: Many of the ciliopathies have significant genetic and allelic heterogeneity and resulting variation in phenotype.

[a]ORPK, Oak Ridge Polycystic Kidney.

Table 1 Phenotypes and Associated Clinical Features of the ORPK Mouse and Selected Human Ciliopathies

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several forms of JBT, MKS, and NPHP with the phenotypic outcomes depending on the nature of the mutation (Baala et al., 2007a; Baala et al., 2007b): *NPHP1/JBTS4* (Parisi et al., 2004), *MKS4 /NPHP6/JBTS5* (Baala et al., 2007a; Sayer et al., 2006), and *MKS5/ NPHP8/JBTS7* (Arts et al., 2007; Delous et al., 2007). Additionally, in some ciliopathies such as Bardet Biedl syndrome (BBS) and nephronophthisis, there is oligogenic or triallelic inheritance whereby the resulting phenotype is modified or arises through a combination of mutations in two or more of the BBS or NPHP genes (Badano et al., 2006a; Hoefele et al., 2007; Katsanis, 2004). Modifier gene effects are also evident in the case of the ORPK mouse (Moyer et al., 1994). ORPK mutants on the FVB/N background (ORPK-FVB/N) normally die before weaning with hydrocephalus and severe renal, hepatic, and pancreatic phenotypes (Fig. 2). In contrast, on the C3HeB/FeJLe background (ORPK-C3H), the ORPK mice can survive for longer than a year and have a much milder, slower progressing renal and hepatic phenotype with a significant increase in fibrosis. In contrast to the ORPK-FVB/N mouse, the pancreas in the ORPK-C3H mouse is characterized by a greatly enlarged cyst that can encompass much of the abdominal cavity (Fig. 2C, insert; (Sommardahl et al., 2001). Thus modifier gene effects are seen in the ciliopathies and in corresponding mouse models.

Defects in Cilia Mediated Sensory Function

The ciliopathies and the ORPK mouse share several phenotypes associated with primary ciliary dysfunction on epithelial cells. Most notably, this includes cysts in the

renal tubules and ducts of the pancreas and liver and associated pancreatic and biliary ductal hyperplasia and interstitial fibrosis (Fig. 2). In most cases, the pathogenesis of these abnormalities secondary to cilia dysfunction in the ORPK mouse remain poorly understood. In all three of these tissues, the cilium extends into the tubule or duct lumen where data suggest they function as mechanosensors that detect fluid movement (Praetorius and Spring, 2001; Praetorius and Spring, 2003). In the kidney, deflection of the cilium initiates a calcium signaling pathway, which is dependent on the ciliary proteins polycystin 1 (PC1) and polycystin 2 (PC2) (Nauli et al., 2003). Mutations in either PC1 or PC2 cause cyst formation in mice and humans (Mochizuki et al., 1996; Wu et al., 1998). Defects in this flow response were demonstrated in perfused tubules isolated from ORPK mutant mice (Liu et al., 2005). The impaired cilia function in ORPK animals also results in changes in membrane channel organization and activity. In ORPK renal epithelium there are altered Na+/H+ exchanger (NHE) and epithelial sodium channel (ENAC) activities and deregulated apical calcium entry as well as abnormal ciliary distribution of the cation channel polycystin 2 (Olteanu et al., 2006; Pazour et al., 2002b; Siroky et al., 2006). In addition, the choroid plexus of ORPK mice has altered chloride and sodium transport, defects in intracellular pH regulation, and a marked increase in intracellular cAMP levels (Banizs et al., 2007; Banizs et al., 2005). Although the pathogenesis of these defects remains incompletely understood, it is thought they are due to defects in IFT-mediated channel/ receptor transport into or along the cilium and/or transmission of signals from the cilia back into the cytosol.

An important aspect of cilia research is how loss of this organelle leads to cyst expansion. Data obtained recently from three independent rodent models have shown

that there are defects in the orientation of cell divisions in cystic kidney disease, which are thought to cause an expansion of the tubule diameter (cyst formation) rather than tubule elongation (Fischer et al., 2006). Further data from Pazour's group has established that IFT20 is vital for normal kidney tubule elongation as well (Jonassen et al., 2008). These data raise the possibility that the cilium or signals from the cilium have a role in establishing the direction of mitotic spindles. This could occur through cilia-mediated regulation of Wnt signaling (see below) and influence the planar cell polarity pathway or function to position the basal body/centrioles, which are located at the base of the cilium. Furthermore, this could involve the mechanosensory function of the cilium, which would provide spatial cues regarding the axis of the tubule by sensing the movement of fluid through the tubule lumen. In the case of the ORPK mouse, the cilium is stunted and would have defects in establishing this spatial information.

An alternate hypothesis is that ciliogenic proteins such as IFT88 have a direct role in regulating the cell cycle. This role was demonstrated in cell culture using siRNA to knockdown IFT88 expression, which promoted cell-cycle progression to S and G2/M phases (Robert et al., 2007). Furthermore, this study demonstrated a direct interaction between IFT88 and CHE-1, a protein that is known to inhibit retinoblastoma's (RB) growth suppressing function. Intriguingly, proliferative defects due to loss of IFT88 in vivo have given disparate results. For example, analysis of proliferation in the ducts of the pancreas showed that ORPK mutants have a relative increase in proliferation rates compared with other pancreatic cell types, such as the acini, which do not have a cilium or express IFT88 (Cano et al., 2004; Zhang et al., 2005). In contrast, in other tissues, such as the cerebellum, the phenotype is characterized by a failure of progenitor cell ex-

pansion (Chizhikov et al., 2007). Thus, roles for IFT proteins in cell-cycle regulation may be tissue or cell type specific and must be further analyzed. Whether the renal, pancreatic, and hepatic phenotypes in ORPK mutants are due to loss of a mechanosensory role for cilia, changes in cell cycle regulation, or altered transport and localization of channels/transporters in the cilium remains uncertain. However, recent data have raised questions regarding the fluid–flow mechanosensory based model of cyst formation. This comes from several studies indicating that the severity of the cystic phenotypes in *Pkd1* (*Pkd1*$^{tm2.1Ggg}$), *Kif3a* (*Kif3*^{atm2Gsn}, IFT kinesin), and *Ift88* (*Ift88*$^{tm1.1Bky}$) conditional mutant mice is dependent on the time at which cilia function is disrupted. In these studies, cysts were found to develop rapidly if the genes were disrupted before postnatal day (P) 13 but very slowly if disrupted only a few days later (Davenport et al., 2007; Piontek et al., 2007). Furthermore, analysis in the *Pkd1* conditional mutants revealed no significant difference in proliferation between cystic and non cystic age-matched controls. These findings do not fit well with the model that loss of cilia-mediated mechanosensation in itself is sufficient for cyst development or that cysts develop solely from an increase in cell proliferation due to loss of cell-cycle control.

However, the data can be incorporated into the existing theories if cysts result from a defect in cilia-mediated mechanosensation and also require that this occurs in a proliferative environment where defects in mitotic spindle orientation would become evident. Proliferation in the mouse kidney remains high until around P14, drops significantly at P16, and then becomes relatively quiescent in the adult kidney (Piontek et al., 2007). Further support for this hypothesis comes from Igarashi et al. using an ischemia reperfusion model which induces proliferation in the kidney and induces cystogenesis (Patel et

al., 2008). This finding corresponds closely with the time when the rates of cyst formation in the inducible cilia mutants change from a mechanism of rapid to slow progression.

Defects in Specialized Cilia Maintenance

The cilia present on the retinal rods and cones in the eye are a highly modified form of the primary cilium that contain proteins required for photosensation. In the ORPK mouse, the outer segments of the photoreceptors are lost and the cells eventually apoptose, resulting in age-related retinal degeneration (Fig. 2) (Pazour et al., 2002a). These studies indicate that transport of components into the rod and cone outer segments relies on IFT and that this is essential for their maintenance.

Similarly, retinitis pigmentosa and retinal dystrophy are also present in many of the human ciliopathies or retinal ciliopathies, including McKusick-Kaufman syndrome, Senior-Loken syndrome, Usher syndrome, Alstrom syndrome, Meckel syndrome, Joubert syndrome, and Jeune syndrome as well as in some forms of nephronophthisis (Adams et al., 2007; Adato et al., 2005; Overlack et al., 2007; Roepman and Wolfrum, 2007). In general, the genes disrupted in these syndromes do not encode IFT proteins, such as IFT88 in the ORPK mice, but may have a role in regulating IFT movement or cargo entry into the cilium or IFT particle assembly. The one exception to this has been the recent identification of a hypomorphic mutation in IFT80 in a form of Jeune asphyxiating thoracic dystrophy (Beales et al., 2007). In addition to retinal degeneration, there are several other sensory deficits associated with human ciliopathies. They include neu-

rosensory hearing loss, impaired nociception, and anosmia (Kulaga et al., 2004; Liu et al., 2007; Tan et al., 2007; Tosi et al., 2003). The presence of these phenotypes in the ORPK mouse has not yet been evaluated.

Ciliary Motility Defects

IFT88 is required for formation of both immotile and motile cilia. In contrast to the ubiquitous presence of primary cilia throughout the mammalian body, motile (9+2) cilia are relatively restricted and are found on epithelia such as the trachea, efferent duct of the testes, on the oviduct of the female reproductive tract, and ependymal cells lining the ventricles of the brain. In human ciliopathies sterility is evident in males with primary ciliary dyskinesia (PCD) and is associated with immotile sperm flagella or loss of normal ciliary beating on epithelium of the efferent duct. Beating of cilia on the efferent duct in the testes is thought to propel sperm, which at this point have not become motile, in the direction of the epididymis. In human females, fertility is somewhat reduced in PCD and Kartagener syndrome (PCD with *situs inversus*). This finding is thought to be due to impaired movement of cilia, which function in transport of the egg through the oviduct. In keeping with a role for the ORPK mouse as a ciliopathy model, both female and male ORPK mice are sterile when analyzed on the FVB/N or C3H inbred backgrounds (Moyer et al., 1994; Pazour, 2004),Yoder, unpublished observations). Although the cause of the sterility in the ORPK mutants has not been fully evaluated, it is likely due to similar mechanisms seen in human PCD.

Another example of a phenotype associated with defects in motile cilia in ORPK mutants that is shared with human ciliopathies is hydrocephalus. The ependyma of the brain ventricles are heavily ciliated and have a highly coordinated beat that directs the flow of cerebrospinal fluid (CSF). In ORPK mice, the morphology and coordinated beating of the ependymal cilia is impaired, which disturbs normal fluid movement (Banizs et al., 2005). Additionally, small tufts of motile as well as primary cilia are present on epithelial cells of the choroid plexus, which produce CSF. In the ORPK mouse, loss of cilia function in the choroid plexus is associated with abnormal regulation of intracellular pH, increased intracellular cAMP, and altered ion transport that contributes to the excess CSF production exacerbating the hydrocephalus phenotype (Banizs et al., 2007; Banizs et al., 2005). In humans, hydrocephalus has been associated with some cases of PCD, although the phenotype's frequency in patients with PCD is a subject of debate (Afzelius, 2004). Mutations in three dynein chain genes (DNAI1, DNAH11, and DNAH5) have been identified in human PCD (Ibanez-Tallon et al., 2002; McComb et al., 1986; Pedersen and Mygind, 1980; Schwabe et al., 2007; Wessels et al., 2003). As in the ORPK mouse, these mutations can affect the ultrastructure of the cilium on ependymal cells and cause abnormalities in ciliary beating, impairing the ability to direct fluid movement. Immotile cilia also cause an increase in respiratory tract infections in humans with PCD and other ciliopathies; however, this has not been evaluated in the ORPK mouse.

Another function of motile cilia that has been assessed using the ORPK mouse was the role of ependymal cilia in directing neuroblast migration. Analysis suggests that the flow of CSF in the ventricles of the adult brain in mice direct the migration of new-

born neurons forming in the stem cell region of the subventricular zone toward their destination in the olfactory bulb. Sawamoto et al. demonstrated that the neuroblasts fail to align and migrate properly in ORPK mutants, and they attribute this finding to loss of a coordinated ciliary beat on the ependymal cells that disrupts CSF movement. Reciprocal neuroblast transplant data indicate that the defect is not intrinsic to migratory cells, but rather is dependent on the CSF environment and CSF flow. This appears to involve establishing a gradient of a chemorepellent produced by the choroid plexus, which is dispersed by CSF flow generated by ependymal cilia (Sawamoto et al., 2006). Whether similar defects in neuroblast migration are present in human ciliopathies that contribute to phenotypes such as mental retardation or sensory deficits (e.g., nociception or anosmia) is not known.

Cilia-Mediated Signal Reception and Processing

Another exciting class of phenotypes seen in the ciliopathies and the ORPK mouse or cell lines derived from these mutants involves defects associated with cilia-mediated regulation of signaling pathways such as hedgehog (Hh) and platelet-derived growth factor (PDGFR-α/PDGF-A). Regulation of the Hh pathway will be reviewed in depth in a later section of this introduction, though the pathway is described in detail in additional reviews (Oro, 2007; Riobo and Manning, 2007). Very briefly, Hh proteins (Shh, Ihh, and Dhh) are secreted factors that have a complex regulatory network. Hh

regulates the function of both transcriptional activators and repressors of the pathway, in part through posttranslational modifications and proteolytic cleavage steps.

The ORPK mouse has numerous abnormalities expected for defects in the Hh signaling pathway. Here again the hypomorphic nature of the allele in the ORPK mouse allowed analyses at later developmental and postnatal stages than in mice in which cilia function is completely disrupted. ORPK mouse phenotypes associated with Hh signaling defects include cerebellar hypoplasia with ataxia, preaxial polydactyly, stunted long bone growth, supernumerary teeth, and defects in skin and hair follicle development (see Appendix A) (Chizhikov et al., 2007; McGlashan et al., 2007; Zhang et al., 2003). In the cerebellum, cilia were found to play an important role in Shh-mediated regulation of precursor cell proliferation (Chizhikov et al., 2007). This phenotype is also evident in human patients with Joubert syndrome, in which several of the affected proteins localize to the cilium axoneme (Arts et al., 2007; Hildebrandt and Zhou, 2007; Sayer et al., 2006).

The sonic hedgehog (Shh) pathway is also important for patterning of the distal portion of the limb with defects resulting in the formation of either excess (loss of Gli3 repressor, activity) or too few (loss of Shh) digits (Litingtung et al., 2002). Together, Shh and the level of Gli3 repressor function to pattern the anteroposterior axis of the autopod and constrain polydactylous potential. Another phenotype in ORPK mice associated with abnormal regulation of the Shh pathway is preaxial polydactyly, (Table1) which is due in part to defects in the formation of the Hh signaling pathway repressor Gli3. This is supported by genetic studies conducted between the ORPK mouse and the $Gli3^{XT\text{-}J}$ mutant and from the analysis of conditional IFT88 null ($Ift88^{tm1.1Bky}$) mutants in which cilia are lost in the limb bud mesenchyme using the Prx1-Cre deletor (Haycraft et al., 2007; Zhang

et al., 2003). Interestingly, limb bud patterning abnormalities are observed in many of the human ciliopathies, including Bardet-Biedl syndrome, Ellis-van Creveld syndrome, Joubert syndrome, orofaciodigital syndrome type I, and Meckel syndrome (Badano et al., 2006b).

Indian hedgehog (Ihh) also has a role in the developing limb in regulating endochondrial bone formation (Lai and Mitchell, 2005; Long et al., 2004). In ORPK mice, dysfunction of the primary cilium results in stunted linear growth, altered chondrocyte differentiation, and delayed chondrocyte hypertrophy within the growth plate (McGlashan et al., 2007).

Similar bone phenotypes are seen in Ellis-van Creveld syndrome, a rare genetic disorder characterized by short-limb dwarfism, polydactyly, and malformation of the bones of the wrist. One gene (*Evc*) responsible for this disorder has recently been cloned and found to encode a protein that localizes to the base of cilia on chondrocytes. In mice, mutations disrupting the *Evc* gene result in loss of Ihh reception in the growth plate (Ruiz-Perez et al., 2007; Ruiz-Perez et al., 2000). Similarly, disruption of *Ift88* or *Kif3a* using the Col2a-Cre (Tg(Col2a1-cre)1Bhr) was found to cause premature loss of the growth plate, resulting from reduced proliferation and accelerated hypertrophic differentiation of the chondrocytes (Song et al., 2007).

Interestingly, this also altered the columnar orientation of the chondrocytes, which may indicate a role for cilia in regulation of noncanonical Wnt signaling or planar cell polarity (Song et al., 2007). Cilia also have been implicated in platelet-derived growth factor (PDGF) signaling, in particular in the PDGFAA/ PDGFR-αα pathway (Schneider et al., 2005). PDGFR-α and PDGF-A are regulators of cell migration, proliferation, sur-

vival, tissue remodeling, and deposition of extracellular matrix both during development and in adults (Hoch and Soriano, 2003). Mutations disrupting the PDGF-A/PDGFR-α pathway have a wide range of phenotypic consequences and many are embryonic lethal (Hoch and Soriano, 2003). PDGFR-α is located in the cilia membrane, and ligand-dependent activation of PDGFR-α results in activation of Mek1/2 within the cilium and at the basal body (Schneider et al., 2005). Although none of the phenotypes in the ORPK mouse has yet been linked to defects in PDGFR-α signaling, fibroblasts isolated from ORPK mice are unable to respond to PDGF-A and fail to chemotax properly toward PDGF-A (personal communication by Søren Christensen and Biology of the Cilia and Flagella FASEB Conferences, Saxtons River 2007; [Sloboda and Rosenbaum, 2007] meeting review).

Finally, primary cilia also appear to have a role in Wnt signaling by regulating the switch between canonical and the noncanonical or planar cell polarity (PCP) pathways (Benzing and Walz, 2006; Simons et al., 2005). Knockdown of *BBS4* or *Kif3a* expression in cells prevents noncanonical Wnt signals (i.e., Wnt5a) from repressing the canonical Wnt (i.e., Wnt3a) pathway (Corbit et al., 2008; Gerdes et al., 2007). BBS4 is a basal body/cilia localized protein that is disrupted in Bardet-Biedl syndrome patients, whereas Kif3a is the IFT kinesin required for cilia assembly. Thus, defects in the cilium lead to elevated β-catenin levels causing an increase in signaling through the canonical pathway.

Of interest, abnormalities in the Wnt and PCP pathways are further supported from the phenotype in the inner ear when IFT88 is disrupted. The data from this analysis by Jones et al. show that proper positioning of ciliary basal bodies and the formation of polarized cellular structures, such as the stereocilia, require ciliary proteins and, surpris-

ingly, the core PCP proteins were found to partition normally along the polarization axis of the cell (Jones et al., 2007). Thus, IFT88 has a distinct function in basal body positioning and morphological polarization during PCP regulation.

Despite the lack of effect on the distribution of PCP proteins, a role for IFT88 in PCP was confirmed through its genetic interaction with the known core PCP gene *Vangl2* (Jones et al., 2007). IFT88 may be required for a permissive response of core PCP proteins to Wnt signals mediated through the cilium or IFT88/cilia could function independent of the PCP proteins in a parallel pathway regulating polarizing signals that subsequently interact with the core PCP proteins to establish the alignment of stereocilia in hair cells of the organ of Corti. It will be interesting to determine whether there are similar root causes seen with the misaligned cell division in the cystic kidney phenotype as well as in the altered rotation of chondrocytes seen in the limbs of IFT88 mutant mice. In contrast to the conditional IFT88 mutant, only minor defects in the inner ear were observed in the ORPK mutants. Although to our knowledge direct abnormalities in PCP pathways have not yet been reported in human ciliopathies, similar phenotypes have been described in the inner ear of mouse models of BBS and vestibular or sensorineural hearing defects are seen in ciliopathies such as Alstroms syndrome and mildly in BBS (Badano et al., 2006b; Marshall et al., 2007; Ross et al., 2005).

Obesity is associated with several of the human ciliopathies such as BBS and Alstrom syndrome as well as in the corresponding mouse models (Arsov et al., 2006; Collin et al., 2005; Mykytyn et al., 2004; Nishimura et al., 2004). This phenotype is not present in the OPRK mice, which is likely due to an early death and health issues from phenotypes observed in multiple other tissues. However, using a conditional mutation ap-

proach, cilia located on proopiomelanocortin (POMC) neurons in the hypothalamus were identified as being critical for normal satiety responses. Current data suggest that cilia may function as part of the leptin/insulin/melanocortin signaling axis to regulate feeding behavior (Davenport et al., 2007).

Mental retardation is another feature seen in a large number of the human ciliopathies. In ORPK mice, neuronal cilia are largely disrupted; however, possible cognitive deficits in these mice have not been assessed, in part due to their hydrocephalus (Banizs et al., 2007; Fuchs and Schwark, 2004). In light of the data indicating cilia are functionally important for POMC neuronal regulation of satiety responses; it is suggestive that this organelle is involved in reception of signaling peptides that influence neuronal activity. This is further supported by the presence of somatostatin (SSTR3) and serotonin (5HT6) receptors in the cilium of neurons (Fuchs and Schwark, 2004). Overall, the roles of cilia in neuronal patterning and in regulation of neuronal activity remain enigmatic and promise to be an exciting area of research in the future.

Perspectives on the Ciliopathies

Although research on cilial diseases has advanced at an amazing pace over the past decade, there is still a great deal that needs to be learned. The current goals are to uncover how cilia regulate or influence cell behavior and how dysfunction of this organelle alters tissue and cellular physiology. Understanding these key features will initiate pharmacological and molecular approaches to attenuate disease progression associated

with human ciliopathies. Ciliopathy models, such as the ORPK mouse, will continue to be instrumental in realizing these goals, testing therapeutic approaches, and to maintain this rapid progress in cilia-related research.

NOTE: EXCERPTED SECTION ENDS

Primary Cilia and Signaling

Primary cilia have been associated with a growing number of different signaling pathways with implications for human phenotypes ranging from polycystic kidney disease to hair and limb patterning and development. Mutants with disrupted primary cilia due to loss of genes such as *Kif3a* (an anterograde motor protein required for cilia construction) or *Ift88*/*Tg737* (a complex B IFT raft protein) are embryonic lethal during mid-gestation with polydactyly, randomization of left-right organization, and dorsal ventral neural tube patterning defects(Taulman et al., 2001).

Shh signaling. The hedgehog signaling pathway was originally characterized in *Drosophila*(Echelard et al., 1993). There are three mammalian hedgehog genes, desert hedgehog (Dhh), indian hedgehog (Ihh), and the signaling focus of this discussion, sonic hedgehog (Shh). The hedgehog pathway is involved in regulating important developmental events ranging from left-right axis formation and neural tube patterning to organ development. Shh is a secreted protein which interacts with patched1, (Ptch1) a membrane spanning receptor which inhibits Smo (a membrane spanning activator protein).

Binding of Shh to patched represses the inhibition of Smo and leads to the activation of the pathway via the glioma associated oncogene homolog (gli) family of transcription factors. These factors, Gli1, Gli2, and Gli3 are postulated to coexist in a microtubule associated complex along with other factors such as Suppressor of fused (sufu). Gli1 and Gli2 have primarily activator functions, but Gli3 serves both repressor and activator functions and requires proteolytic processing for transcriptional or repressor effects (See Fig. 3).

Primary cilia have a central role in multiple steps of the Shh pathway. The Shh receptor Ptch1 localizes to the primary cilium and leaves the primary cilium upon interaction with Shh, suggesting that ciliary localization plays a role in the repressor activity of Ptch1 (Rohatgi et al., 2007). Smo requires translocalization to the primary cilia for proper function (May et al., 2005). All three Gli proteins and Sufu also localize to the primary cilia and IFT is required for the proteolytic processing of Gli3. Data from several independent *Ift88* null mutant mice have revealed that cells lacking cilia are unable to respond to Hh ligands (Haycraft et al., 2005; Liu et al., 2005a). These results suggest that primary cilia serve in signal modulation of the Shh pathway at a variety of different steps in Shh signal reception (Haycraft et al., 2005; Singla and Reiter, 2006). This would suggest that cilia knockout mutants may have variable effects on Shh signaling depending on the cell populations affected and the relative influence of the repressor and activator functions of the gli proteins on the tissue. For example, loss of repressor function in a Gli3 repressor mediated environment such as the limb will result in a phenotype resembling a traditional loss of repressor. Activator levels of Gli1 or Gli2 in response to Shh may also be decreased, but they are not the major mediators in the limb environment. This allows

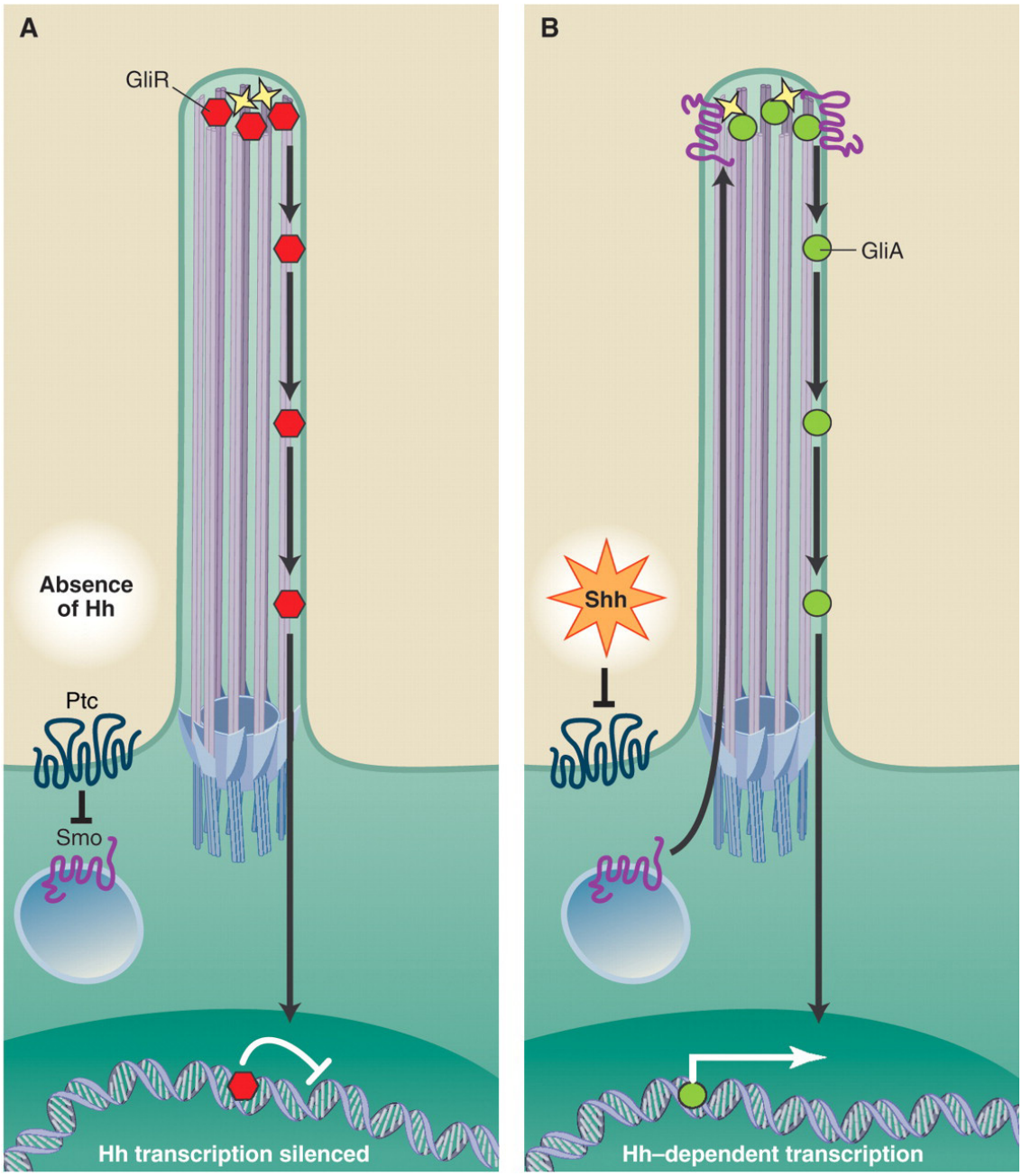

Figure 3 Shh signaling and Primary cilia
(A) Ptc (now localized to the cilium itself) acts constitutively to inhibit the Hh pathway, however, the processing of Gli3 into its GliR repressor form requires the presence of primary cilia and results in a downstream silencing of Hh signaling. (B) Shh relieves this repression and activator Smo localizes to primary cilia in and leads to gliActivator mediated transcription of the Hh pathway.

From Singla, V. and Reiter, J. F. (2006). The primary cilium as the cell's antenna: signaling at a sensory organelle. *Science* 313, 629-33. Reprinted with permission from AAAS.

primary cilia defects to have multiple overall effects on Shh signaling phenotypes in different contexts and reflects a dysregulation of the pathway in response to cilia loss.

Wnt signaling. Wnt signaling serves a variety of roles in embryogenesis from organ and epidermal appendage formation to cellular organization and carcinogenesis. Wnt signaling involves the binding of secreted Wnts to frizzled (Fz) receptor proteins and lipoprotein related receptor proteins (LRPs) and the phosphorylation (activation) of dishevelled (Dsh). These proteins may transduce a signal by either of two main pathways referred to as the canonical and non-canonical Wnt pathway. Canonical Wnt signaling stabilizes β-catenin and prevents its degradation, leading to β-catenin nuclear localization and downstream gene activation (see fig. 4). Non-canonical signaling utilizes other secondary messengers and has a less well characterized and very diverse molecular actor but the pathway generally influences cell fate and tissue organization and positioning. An example is frizzled6, which signals via the non-canonical pathway to control hair patterning and "whorl" formation (Guo et al., 2004; Veeman et al., 2003).

The major non-canonical signaling pathway important in hair follicle development is the planar cell polarity pathway. Inversin, a primary cilia protein mutated in the cystic kidney disease nephronopthisis type II, serves as a molecular switch between the canonical and non-canonical Wnt pathways. It influences the degradation of disheveled (Dsh) and thereby regulates β-catenin canonical signaling (Germino, 2005; Simons et al., 2005). Mutant mice with loss of the inversin gene develop a cystic kidney phenotype similar to that seen with β-catenin overactivation, supporting the above hypothesis.

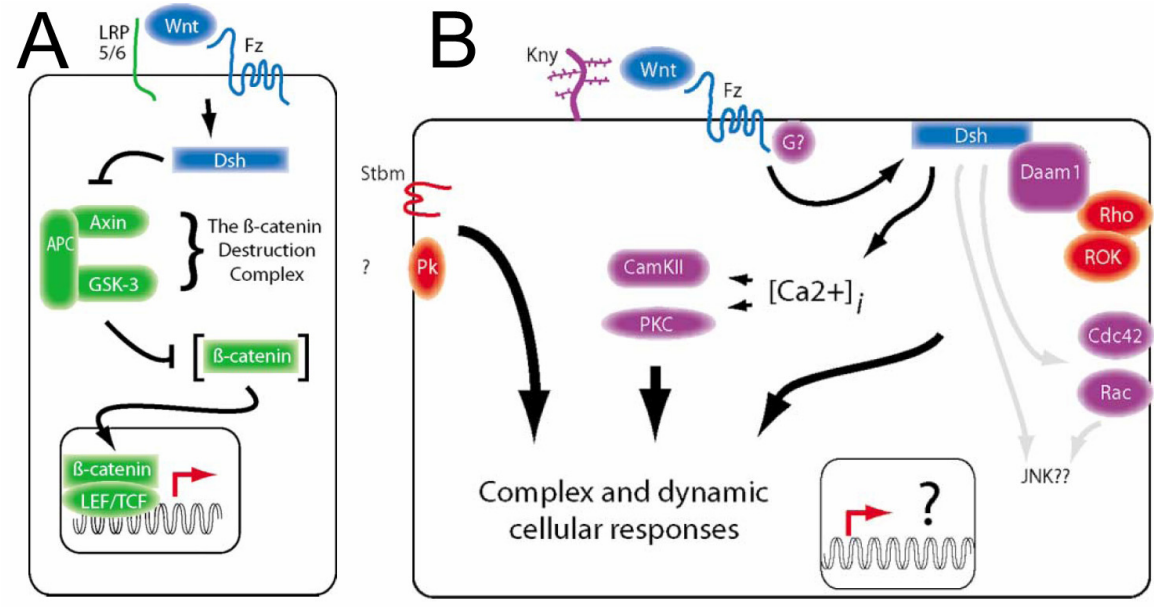

Figure 4 Wnt Canonical + Non-Canonical Pathways
The canonical Wnt pathway (A) involves the interaction of secreted Wnts with lipoprotein receptor like proteins (LRP)s and frizzled receptors leading to the phosphorylation of dishevelled (Dsh) and the inhibition of the destruction of β-catenin. This β-catenin builds up in the cytoplasm and translocates to the nucleus where it interacts with transcription factors LEF/TCF. The general category of Non-Canonical Wnt signaling (B) is a more diverse signaling environment that encompasses Planar cell polarity (PCP) pathways as well as protein kinase C (PKC) and Ca^{2+} mediated cell signaling. These pathways may involve interaction with frizzled receptors but are not dependent upon β-catenin.

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Since inversin is localizes to the cilia, this suggests that primary cilia have a role in the modulation of the Wnt pathway. Intriguingly, these mice also have hair patterning defects reminiscent of Frizzled 6 mutant mice, which have a disordered pattern of hair follicle orientation likely due to loss or disruption of the planar cell polarity pathway. (Guo et al., 2004; Simons et al., 2005). Additionally, there are data suggesting that cilia may play a role in canonical/non-canonical Wnt signaling balance by repressing non-canonical Wnt's ability to repress canonical Wnt signal (Corbit et al., 2008). This suggests a possible link between primary cilia signaling and hair follicle orientation or development.

PDGFRα signaling. Platelet derived growth factor receptor signals as a dimer through tyrosine kinase activation. PDGFRα activity is associated with a variety of developmental cellular activities including organ patterning, specification, and cell proliferation. PDGFA signaling is particularly important in mediating mesenchymal/ epidermal interactions in the developing hair follicle. PDGFA is secreted by epidermal cells in the developing hair placode and PDGFRα is expressed in the corresponding mesenchyme. PDGFA knockout mice also have dystrophic and disorganized hair follicle formation and a small dermal papilla (see review of Hair Follicle Biology).

PDGFRα is localized to the primary cilium in fibroblasts. The importance of this localization is seen in cells isolated from the Oak Ridge Polycystic Kidney (ORPK) mutant mice, which have a hypomorphic mutation in the *Ift88* gene. ORPK MEF cells have a deficient PDGFRα response (Schneider et al., 2005). PDGFRα signaling is thus another member of the growing family of primary cilia signaling associated pathways with implications for hair development and human health.

A Review of Hair Follicle Biology

The hair follicle is a mammalian specific epithelial micro organ that manufactures and maintains hair shaft structures that serve vital roles in insulation, protection, proprioception, communication, and body language. This versatile organ develops in late embryogenesis and features a uniquely complex cycle of growth, apoptosis, and organ regeneration throughout adult life (Botchkarev and Paus, 2003; Paus et al., 1994). These complex cellular behaviors utilize a multitude of signaling pathways including sonic hedgehog (Shh), canonical and non-canonical Wnt signaling, platelet derived growth factor alpha (PDGFα), transforming growth factor beta (TGF-β), bone morphogenic protein (BMP), and others. Many of these pathways crosstalk significantly in the physiological setting (Schmidt-Ullrich and Paus, 2005). Adding to the complexity, there are also several different types of hair follicles, which have overlapping and distinct developmental signaling requirements and timings. These include guard, or primary hairs, and non-tylotrich hairs (secondary) (Fig 5) which serve different functions (Sundberg et al., 2005). These overlapping layers of complexity largely explain why the precise molecular interplay in hair development is relatively uncharacterized.

The Stages of Hair Follicle Morphogenesis

Hair follicle morphogenesis is a highly complex and dynamic process with eight distinct morphogenic stages involving extensive crosstalk between signaling pathways

(See Fig. 6). This process begins about E16.5 in mice for zig-zag hairs, the most common pelage (general murine coat) hair follicle type, and continues to postnatal day 17. The proper initial development of hair follicles requires an intricate ballet of signaling interactions to form an inductive gradient via localized expression of regulatory proteins. The initiating signal for this process is unknown, but is putatively a Wnt based signal. This is supported by studies of Dickkendorf 1(Wnt inhibitor) transgenic mice which fail to support any hair follicle development. The BMP pathway is also important based on studies with BMP antagonists showing that noggin knockout mice fail to develop secondary hair (Botchkarev and Paus, 2003; Schmidt-Ullrich and Paus, 2005). This signal leads to a stage 1 follicle which forms an epidermal placode which acquires a coterie of dermal fibroblasts that interact with the epidermal cells to stimulate their proliferation. This ongoing interplay between mesenchymal and epidermal cells leads to the migration and proliferation of dermal fibroblasts to form a dermal condensate (stage 2).

The dermal condensate transforms into the dermal papilla, a discrete cellular population of differentiated fibroblasts that directs epidermal downgrowth and proliferation. This dermal condensate/ dermal papilla is vital for both hair development and growth during adult cycling. Loss of this dermal papilla or condensate leads to an arrest in hair follicle development or cycling. This is demonstrated in humans with a form of congenital hair loss where defects in the human *hairless* gene cause the dermal papilla to become dissociated from the hair follicle during resulting in later hair loss (Ahmad et al., 1999).

Various signals maintain and support the development of the dermal condensate/dermal papilla. Shh signaling is one of these necessary components. Both Shh and

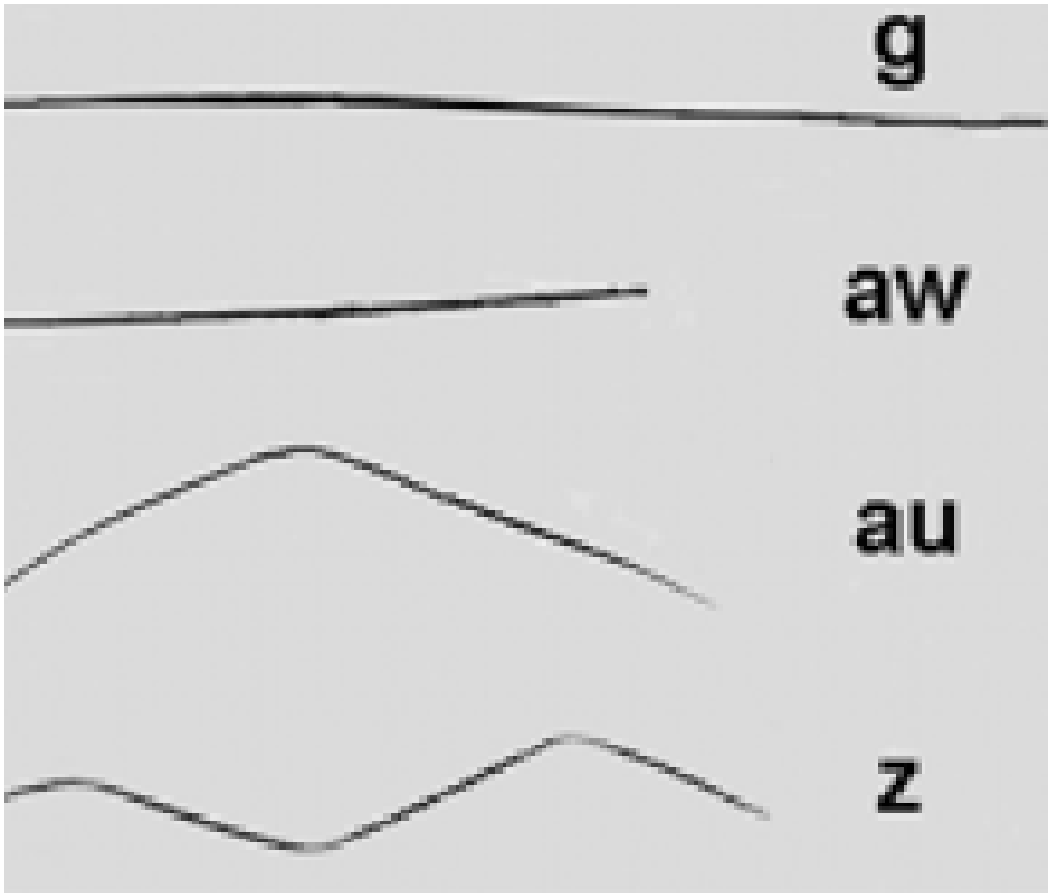

Figure 5 Common types of Murine hair follicles
(g) Guard or Primary hairs, largest pelage hair follicles. (aw)(au)(z) Secondary shorter follicles classified by their number of bends.

Reprinted from Brancaccio, A., Minichiello, A., Grachtchouk, M., Antonini, D., Sheng, H., Parlato, R., Dathan, N., Dlugosz, A. A. and Missero, C. (2004). Requirement of the forkhead gene Foxe1, a target of sonic hedgehog signaling, in hair follicle morphogenesis. *Human Molecular Genetics* 13, 2595-2606 with permission from Oxford University Press.

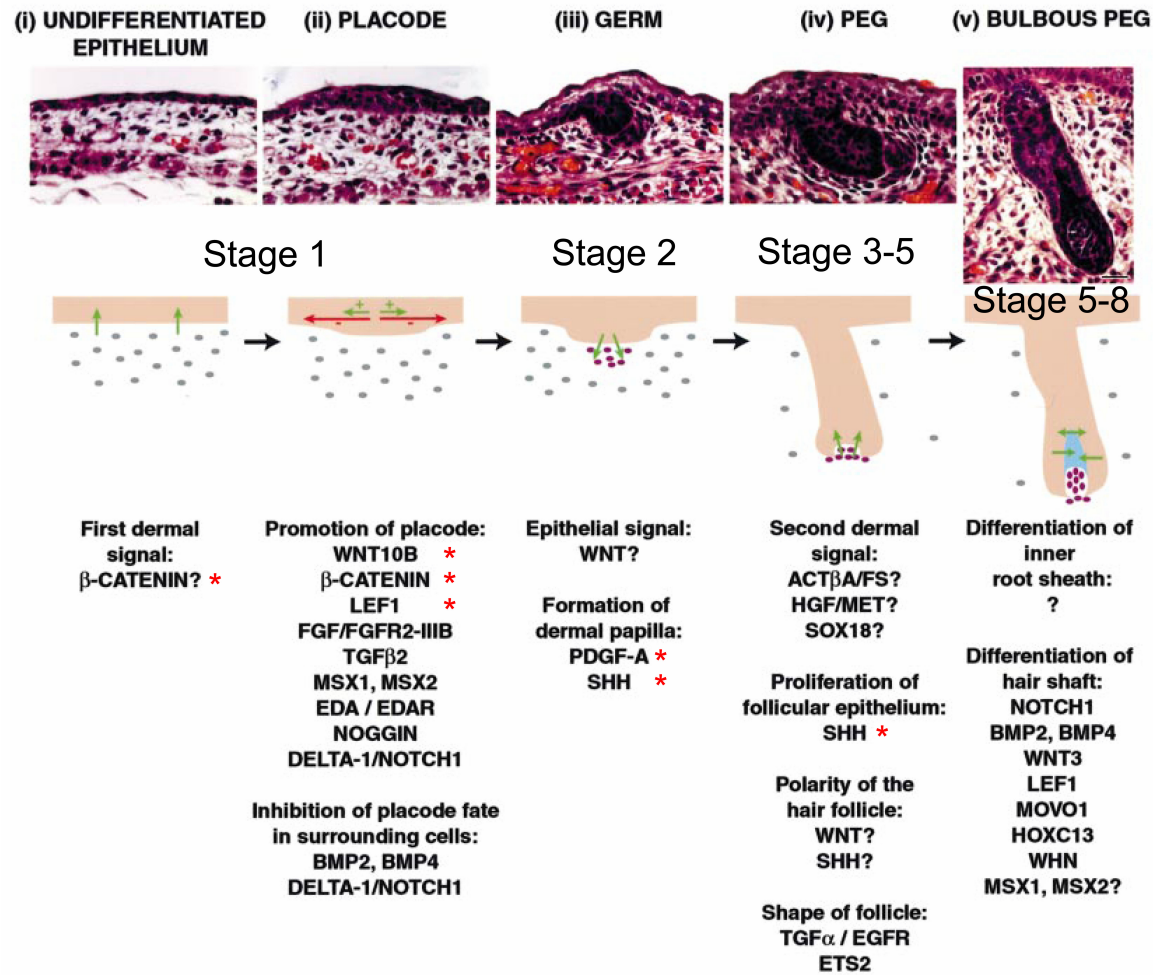

Figure 6 Signaling pathways involved in hair follicle morphogenesis in both the dermal and epidermal cell populations.
Note the importance of Shh and PDGFA in stage 2 hair follicle morphogenesis and the involvement of Shh, PDGFA, and Wnt signaling in hair follicle morphogenesis.

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Gli2 (the primary downstream Shh activator in the skin) knockout mice show follicular arrest at stage 2 of hair follicle morphogenesis and smaller dermal papilla / condensates (Mill et al., 2003; St-Jacques et al., 1998). PDGFα signaling is also important for dermal condensate development and maintenance. PDGFRα knockouts have dystrophic hair follicles and a smaller dermal papilla (Karlsson et al., 1999). Hair follicle morphogenesis continues as the dermal papilla is then enveloped by the developing epidermal cells (stages 3-5). Subsequent lateral epidermal signaling leads to the layers of the adult hair follicle with growth and the formation of a hair shaft with a dermal papilla controlling hair shaft growth (stages 5-8).

Adult Hair Anatomy

The adult hair follicle is a highly organized cellular construction that structurally resembles a series of concentric cylinders. Each of these cylinders correspond to different stages of keratinocyte differentiation (Fig. 7). They are, from interior to exterior, the hair shaft structure itself, which is formed by the replication and differentiation of matrix cells at the bulb of the hair follicle. The matrix cells become keratinized and die in a process similar to normal epidermal development. The hair shaft is followed by two layers of inner root sheath (IRS) which form planes of cells that serve to guide the growing hair shaft. The entire follicle is enclosed by two layers of outer root sheath (ORS) which bind and form a coating over the tough inner root sheath (Paus and Cotsarelis, 1999;

Sperling, 1991). The entire epidermal lineage of the hair follicle is renewed from transiently amplifying progenitor cells generated by the hair follicle bulge stem cells (Blanpain and Fuchs, 2005; Turksen, 2004).

These bulge cells are slowly replicating cells that rest in a protected niche nearby the attachment of the erector pylori muscle and the sebaceous gland, see figure 7B for schematic. These cells are the regenerative engine of the hair follicle and any damage to the bulge cells or loss of the interaction between the bulge cells and the dermal papilla, which stimulates replication of stem cells to form progenitor cells during hair follicle cycling (see below) leads to hair follicle loss. This is evident in such diseases as in lichen planopilaris or lupus where autoimmune attack can destroy the bulge cells leading to permanent hair loss (Wiedemeyer et al., 2004). As long as both the bulge and its surrounding "permanent" follicle region (the upper 1/3 of the follicle) remain intact, the hair follicle can regenerate vigorously despite extreme damage or cell death, as has been shown in cases of chemotherapy that decimate the replicating hair matrix and progenitor cells. This cycling is a vigorous area of research in hair biology.

Hair Follicle Cycling

Hair follicle cycling is the dynamic process of regeneration and destruction responsible for the characteristic hair loss and regrowth found in mammals (Figure 7B). The anagen stage of hair growth is similar to later stages of follicle development where adult hair follicles construct and perpetuate hair shafts via replicating matrix and

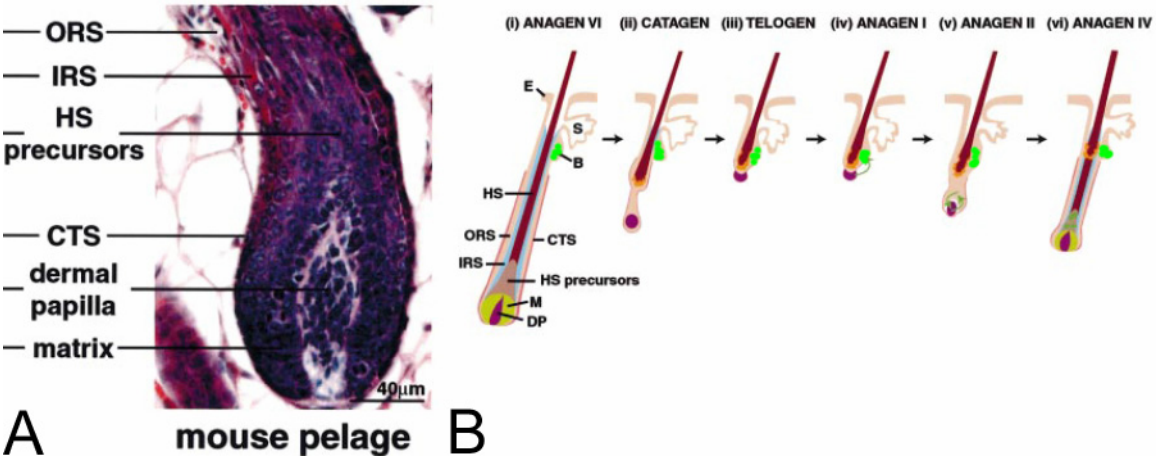

Figure 7 Adult Hair anatomy and cycling
(A) The anatomy of the Anagen (growing) hair follicle with layers of concentric epidermal cells forming the ORS (outer root sheath), CTS(connective tissue sheath) and IRS (Inner root sheath) and the hair shaft and hair shaft precursors (HS) formed by proliferating matrix cells stimulated by dermal papilla cells in the hair bulb. (B) The hair cycle involving the growth (anagen), massive degredation (catagen), and resting interaction of the bulge (B in green) and the dermal papilla (telogen).

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progenitor cells. This is followed by massive apoptosis and break down of the hair follicle structure and associated matrix cells (catagen). The follicle then undergoes a "rest" cycle, which likely involves the replacement of these cells with new progenitor cells from a protected stem cell population (bulge cells in telogen) (Fig. 7). It is worth noting that this complete cycling process is a significant organ event involving digestion of extracellular matrix components and even vascular remodeling. The major known signaling mediators of this process have many similarities to those found in hair morphogenesis during development and include Shh, BMP, and PDGFRα signaling playing conserved roles in this signaling orchestra.

Murine hair follicles are very similar to human hair follicles, but there are important differences. The mouse anagen cycle is much shorter at a few weeks compared to human anagen of up to six years (Krause and Foitzik, 2006). Mice also have multiple hair shafts in a single follicle, though one shaft is growing at any given time, whereas humans have a single shaft per follicle. Each of these stages also has several substages; a discussion of which is beyond the scope of these studies but they are reviewed by Muller et al (Muller-Rover et al., 2001). Despite the differences, murine hair follicles still serve as a valuable model for hair follicle development and cycling and have provided a wealth of information on the pathogenesis of human disease.

The first hair cycle begins approximately at P17 in catagen in the mouse dorsal neck and perpetuates in a wave-like fashion from head to tail. Murine hair follicles are synchronized for the first two hair cycles and then desynchronize. Resynchronization can be induced by depilation treatment with wax or depilatory agent (Nair) which "resets" the hair cycle clock of neighboring hair follicles allowing them to begin synchronized anagen

growth. The precise signaling mediators and the actual physical determinants of the hair cycle clock are incompletely understood. An understanding of these interactions remains a "holy grail" in hair follicle research, as the most common alopecias in humans result from progressive defects in hair follicle cycling and understanding of this cycling process could lead to more efficacious treatments for baldness and alopecias.

Interfollicular Epidermis and Stratification

In addition to serving as the scaffold for follicle formation, the mammalian skin also serves a variety of important roles as a waterproof and protective layer, with functions in thermal regulation, UV protection, and chemical and microbiological barrier roles that together shield the body from the hostile external environment. In order to serve these functions the epidermis must be able to repair from injury and form a waterproof seal. It does this by forming a squamous stratified epithelium with multiple distinct layers that have different functional roles in epidermal homeostasis.

The epidermis is composed of three living layers and one dead. From the exterior to interior these layers are the stratum corneum, composed of tightly interlocked, waterproof dead squames, the granular layer, with dying cells filling with waterproofing components, the spinous layer, where cells are generating a keratin structure that will provide support for the future squames, and most important for this project, the basal layer which replicates to replenish the continually dying epidermal cells. The replicative potential of the basal keratinocytes and their exposure to a toxic environment (UV) renders them par-

ticularly susceptible to malignant transformation. Thus, it is not surprising that skin cancers arising from the basal keratinocytes are the most common human malignancies (Athar et al., 2006).

Basal keratinocytes are identified by expression of the paired keratins K14/K5 (Fuchs, 1990; Nelson and Sun, 1983). This basal layer of keratinocytes is the sole mitotic layer of the adult epidermis and continually regenerates throughout life to replace cells being lost (Fuchs, 1990). This long term regenerative ability is conferred by a combination of interfollicular stem cells that are dispersed in the basal layer between hair follicles and follicular stem cells that are present in the bulge area of follicles. Multiple pathways are involved in the regulation of these stem cells and in the fate of their progeny. Defects in the Shh, Wnt, Myc, and Notched pathways have effects on the differentiation of these progenitor cells or on the maintenance or size of the stem cell pool (Estrach et al., 2008; Fuchs, 2008; Merrill et al., 2001; Zanet et al., 2005).

As indicated previously, defects in these same pathways are also associated with skin tumors including squamous cell carcinoma (SCC), basal cell carcinoma (BCC) and follicular hamartomas (Hoseong Yang et al., 2008; Nicolas et al., 2003; Saldanha et al., 2004). There is debate in the skin literature between two competing paradigms of skin homeostasis/stratification. In one model, interfollicular stem cells produce transiently amplifying cells that replicate to form basal keratinocytes. In a second model, asymmetric divisions from a single stem cell produce a cell that delaminates from the basement membrane, but does not replicate (See Fig 8) (Fuchs, 2008; Lechler and Fuchs, 2005). In either case, these basal keratinocytes push upwards and differentiate to form the spinous layer and eventually all layers of the epidermis. This collection of cells from a single

stem cell form a structure called an epidermal proliferative unit (EPU) with the upper most cell being a fully mature squame cell. The EPU is built by replication of the a single interfollicular stem cell at the base that produces cells that then undergo differentiation (Fuchs, 2008). The mechanism of this stratification is poorly understood. However, it is intriguing, based on a putative role of cilia or IFT in the kidney in controlling the axis of cell division, that stratification of the epidermis may involve a shift in the frequency of cell divisions from perpendicular to the basement membrane to parallel in order to drive the stratification process (Lechler and Fuchs, 2005). The consequences of defects in maintenance of this basal layer stem cell population and the control of their differentiation have important implications for skin carcinoma.

The skin is an excellent example of the convergence of developmental pathways and cancer. BCC, the most common skin cancer in humans, involves deregulation of the Shh pathway, and mutations affecting several components of this pathway have been found in *de novo* and genetic cases of BCC (Grachtchouk et al., 2003; Hutchin et al., 2005). Ectopic activation of the pathway is also sufficient for carcinogenesis, as basal cell tumors develop in mice overexpressing Gli1 or Gli2 and trichoepitheliomas develop in mice with Smo overactivation in the skin (Athar et al., 2006). There are direct connections between additional signaling pathways, such as Wnt, PDGF, and Myc that are required for normal hair follicle development and whose deregulation contributes to the formation of skin tumors (Hoseong Yang et al., 2008; Roop and Toftgard, 2008). Importantly, the activity of these pathways is also influenced by primary cilium function/IFT, suggesting there may be links between these processes with implications for cancer treatment, diagnosis, and management.

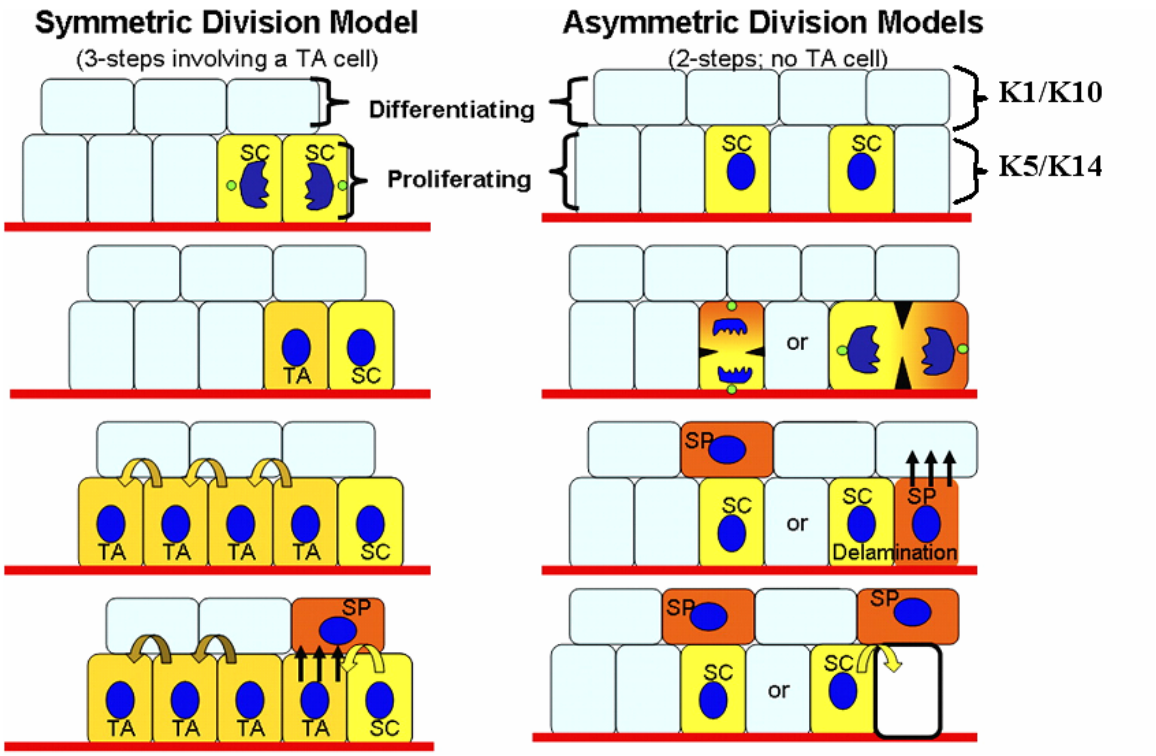

Figure 8 The Symmetric and Asymmetric Division models for stratification of the epidermis

The symmetric model postulates parallel division of the stem cells (SC) to form transiently amplifying cells which delaminate (orange) and stratify the epidermis. The asymmetric model postulates a single interfollicular progenitor population which divides perpendicularly to stratify, or in parallel fashion, to replenish stem cells, and generate cells which may immediately delaminate, and the ratio of perpendicular to parallel divisions influences stem cell population and stratification. K5/K14 are basal keratinocyte keratins which label the progenitor cell populations. K1/K10 keratins label differentiating cells.

Purpose of the Research

The goal of this research was to evaluate the role of primary cilia in hair and skin development and physiology. At the outset of this work, there was a great deal of excitement concerning signaling roles of primary cilia in a variety of organ systems and their involvement in the Shh pathway with its intimate connection to skin cancer was of great interest. We hypothesized, based on this literature, that primary cilia would be essential in the development of the hair and skin due to vital roles in signal transduction. However, there was very little known about the potential localization of cilia in the hair follicle and even less known about their functional roles. This meant that the initial question of interest was in determining whether primary cilia localize to cells in the murine skin and hair follicle. Primary cilia localization in skin has been described in anecdotal and often contradictory fashion for many years (Haust, 1995; Strugnell et al., 1996; Wandel et al., 1984), but an in-depth study of primary cilia and the murine hair follicle had not previously been conducted due to a lack of specific antibodies and the difficulty of visualizing the primary cilia in the context of large, diverse 3-d structures such as hair follicles via EM. The discovery of specific cilia antibodies such as acetylated tubulin and anti-IFT88 combined with confocal microscopy and 3-d reconstructive imaging allowed this work to go forward and produced the first ciliary localization for the stages of the murine hair follicle. These results, in combination with β-galactosidase staining of an *Ift88* lacZ knock-in mouse model, enabled a more comprehensive study of cilia and re-

lated protein expression in the developing and adult hair follicle and are described in the following section.

After establishing and evaluating the localization of primary cilia, the next goal was to use a conditional allele of an IFT gene and Cre-lox systems to specifically disrupt cilia maintenance in either the dermal or epidermal populations. This allowed analysis of cilia function in the context of the intricate dermal/epidermal molecular conversations that are vital to hair follicle development and maintenance and may play roles in skin cancer and epidermal homeostasis. Additionally, the long term goal of this research was to better understand what role primary cilia play in the pathways and extensive crosstalk in the murine hair and skin, including pathways important to human disease and carcinogenesis. These initial results aimed at bridging the hair/skin phenotypes observed with ciliary signaling pathways involved in disease and are detailed in the following two chapters. Implications of these data are further discussed in the summary and future directions section. Thus, this project set out to begin the long process of answering interesting questions and characterizing the vital roles of primary cilia in the hair follicle and skin.

AN ESSENTIAL ROLE FOR DERMAL PRIMARY CILIA IN HAIR FOLLICLE MORPHOGENESIS

by

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Journal of Investigative Dermatology Advance Online Publication 6 November 2008

ABSTRACT

The primary cilium is a microtubule-based organelle implicated as an essential component of a number of signaling pathways. It is present on cells throughout the mammalian body; however, its functions in most tissues remain largely unknown. Herein we demonstrate that primary cilia are present on cells in murine skin and hair follicles throughout morphogenesis and during hair follicle cycling in postnatal life. Using the Cre-lox system, we disrupted cilia assembly in the ventral dermis and evaluated the effects on hair follicle development. Mice with disrupted dermal cilia have severe hypotrichosis (lack of hair) in affected areas. Histological analyses reveal that most follicles in the mutants arrest at stage 2 of hair development and have small or absent dermal condensates. This phenotype is reminiscent of that seen in the skin of mice lacking *Shh* or *Gli2*. *In situ* hybridization and quantitative RT-PCR analysis indicates that the hedgehog pathway is downregulated in the dermis of the cilia mutant hair follicles. Thus, these data establish cilia as a critical signaling component required for normal hair morphogenesis and suggest that this organelle is needed on cells in the dermis for reception of signals such as sonic hedgehog.

Abbreviations:

DC, dermal condensate; DP, dermal papilla; IFT, intraflagellar transport; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; Shh, sonic hedgehog

INTRODUCTION

The development and patterning of many tissues in the mammalian body involves conserved inductive signaling events between the epithelium and underlying mesenchyme. The hair follicle is a prototypic example of an organ formed through such reciprocal inductive interactions. Although in most tissues these signaling events occur only during embryogenesis, the hair follicle is exceptional in that it continually regenerates itself throughout life, utilizing many of the same signaling pathways that are essential for hair follicle morphogenesis. These properties, along with the abundance and accessibility of hair follicles, and the existence of many murine mutations affecting follicle morphogenesis, have made it an attractive system to analyze reciprocal signaling events between the epithelium and the mesenchyme.

In the mouse, primary hair follicle morphogenesis begins at approximately embryonic day 14.5 (Paus *et al.*, 1999). The signal initiating hair follicle formation (stage 0) is thought to be an unidentified Wnt that is sent from the dermal mesenchyme to the epithelium. This "first dermal signal" results in a clustering of cells in the epidermis to form an epithelial placode (stage 1). In the germ hair stage (stage 2), the epithelial placode sends the "first epithelial signal" back to the mesenchyme promoting clustering of mesenchymal cells into the dermal condensate/dermal papilla (DP) (Hardy, 1992). The dermal condensate serves as an organizing center for the developing hair follicle. In response to signals from the dermal condensate "second dermal signal", the epidermal cells undergo a period of intense proliferation and down growth to establish the hair peg (stages 3–5); (Paus *et al.*, 1999; Schmidt-Ullrich and Paus, 2005). Sonic hedgehog (Shh) signaling is vital for this epidermal proliferation. It is thought that Shh produced by the

epidermal placode helps maintain the dermal condensate and that Shh responsiveness is necessary for epidermal follicular down growth (St-Jacques *et al.*, 1998; Nanba *et al.*, 2003). In keeping with this hypothesis, mice lacking *Shh* or *Gli2*, the primary Shh pathway transcriptional activator in the skin, have stage 2 arrested follicles (St-Jacques *et al.*, 1998; Botchkarev and Paus, 2003; Mill *et al.*, 2003). Transgenic expression of wild-type Gli2 specifically in epidermal cells was sufficient to rescue hair follicle arrest in $Gli2^{-/-}$ animals, indicating that the Shh pathway in the epidermis is essential for follicular morphogenesis and down growth. A constitutively active form of Gli2 (but not wild-type Gli2) expressed in the epidermis of $shh^{-/-}$ mutants was able to promote epidermal proliferation and induce Shh targets; however, this only partially rescued the follicular phenotype. These data suggest there is a Shh-dependent function required to activate Gli2 in the epidermis and a Shh-dependent signal from the dermis back to the epidermis that is needed to complete the down growth necessary for follicle development (Mill *et al.*, 2003).

In the stage 6–8 follicle, the invading follicular epithelium continues to envelop the dermal condensate forming the DP, which is important in further hair follicle development and cycling in postnatal periods. The number of cells recruited to form the DP is directly related to the size of the follicle that will form and the thickness of the resulting hair (Paus and Foitzik, 2004). Surrounding the DP at the base of the follicle are the epithelial matrix cells that differentiate into multiple concentric epithelial cell layers that constitute the mature hair follicle including the hair shaft and the inner root sheath surrounding the hair shaft (Blanpain and Fuchs, 2006). Mutations in several genes have been generated that affect the number of cells in the DP, including *Shh* and platelet-

derived growth factor A (*Pdgfa*), both of which result in significantly fewer DP cells and severely impaired hair follicle formation (Karlsson *et al.*, 1999; Mill *et al.*, 2003).

Intriguingly, primary cilia, which are small microtubule-based appendages extending from the surface of most cells in the body, are required for normal signaling activity in several pathways, including Shh and PDGFRαα, as well as for regulating the balance between canonical and noncanonical Wnt signaling (Schneider *et al.*, 2005; Christensen *et al.*, 2007; Gerdes *et al.*, 2007). In the case of PDGF-mediated signaling, data indicate that PDGFRαα homodimers localize to, and are activated in, the ciliary membrane in response to PDGF-A (Schneider *et al.*, 2005). In the absence of cilia, PDGF-AA is unable to activate PDGFR-αα. Similarly, multiple components of the hedgehog signaling pathway, including the Shh receptor Ptch1, the pathway transducer Smoothened, the negative regulator suppressor of fused, and all three of the Gli transcription factors are present in cilia (Corbit *et al.*, 2005; Haycraft *et al.*, 2005; May *et al.*, 2005; Rohatgi *et al.*, 2007a). Cells with mutations in genes required to build cilia are unable to respond to Shh and have defects in both Gli2 activator and Gli3 repressor functions (Haycraft *et al.*, 2005; Huangfu and Anderson, 2005; Rohatgi *et al.*, 2007b). In addition, cilia have a role in regulating Wnt signaling, as mutations affecting cilia can prevent noncanonical Wnts (such as Wnt5a) from suppressing the canonical Wnt (Wnt3a) pathway (Simons *et al.*, 2005). Furthermore, using fibroblasts with a mutation in the ciliogenic gene Kif3a, Corbit *et al*. (2008) recently demonstrated that loss of cilia leads to an increased signaling response to canonical Wnts such as Wnt3a. Together these data raise the possibility that primary cilia have essential functions in skin and hair follicle morphogenesis through a role in coordinating signaling activities of Shh, PDGF-AA, and

the activity ratio of canonical versus noncanonical Wnt signaling (Christensen *et al.*, 2007).

The construction and maintenance of the primary cilium is dependent on intraflagellar transport (IFT). IFT mediates the bidirectional movement of proteins between the tip and base of the cilia axoneme using numerous proteins (for example, IFT88) involved in the formation of the IFT particle and the molecular motors kinesin II (Kif3a, Kif3b, and Kap3) and cytosolic dynein motor proteins. Disruption of IFT in mice prevents cilia formation and causes early to mid-gestational lethality (Murcia *et al.*, 2000; Huangfu and Anderson, 2005; Liu *et al.*, 2005; Houde *et al.*, 2006) due to defects in left–right axis specification and in neural tube closure and patterning. Ciliary dysfunction is also associated with the formation of cystic kidneys, hepatic and pancreatic abnormalities, skeletal malformations, and obesity (Davenport and Yoder, 2005; Huangfu and Anderson, 2005; Liu *et al.*, 2005; Singla and Reiter, 2006; Haycraft *et al.*, 2007). Thus, primary cilia have essential functions in development and homeostasis in multiple tissues and many of the phenotypes observed in cilia mutant mice have been linked with impaired regulation of hedgehog and Wnt signaling (Cano *et al.*, 2004; Zhang *et al.*, 2005; Chizhikov *et al.*, 2007; Haycraft *et al.*, 2007).

Despite the prevalence of primary cilia in the mammalian body, their functional importance in most tissues, such as the skin, remains unknown. In part, this is due to the early embryonic lethality caused by loss of cilia function. Thus, we utilized conditional alleles of two ciliogenic genes *Kif3a* (*Kif3a*^{tm2Gsn}) and *Ift88* (*Ift88*^{tm1Bky}) to disrupt cilia assembly specifically in the dermis of the skin to explore ciliary function during skin and hair follicle morphogenesis. These dermal cilia mutants have defects in hair development

similar to that seen in *Gli2*$^{-/-}$ or *Shh*$^{-/-}$ mutant mice (St-Jacques *et al.*, 1998; Mill *et al.*, 2003). These data demonstrate an unappreciated role for dermal cilia during hair follicle morphogenesis where they appear to be involved in reception of hedgehog signals.

RESULTS

Primary cilia in hair follicle development

Primary cilia are solitary, small organelles (normally 2–5 μm long) that are often overlooked or are difficult to detect using standard epifluorescence microscopy on a single section through tissue. Thus, our initial objective was to thoroughly evaluate whether cilia are present on cells in the skin and hair follicle during morphogenesis and during follicular cycling in postnatal stages. Expression of the ciliogenic gene *Ift88* (previously known as *Tg737*) was assessed using the β-galactosidase reporter gene incorporated into the *Ift88*^{tm1Rpw}(*Ift*$^{\Delta 2\text{-}3\text{-}\beta\text{-}gal}$) targeting construct used to generate the null mutant mouse (Murcia *et al.*, 2000). *Ift88*^{tm1Rpw} heterozygous skin samples had β-galactosidase activity in both dermal and epidermal cell populations in the developing and cycling follicle. Dermal cells include the dermal condensate, the DP and interfollicular fibroblasts. Epidermal cells include the matrix, inner and outer root sheaths, as well as cells in the bulge region (Figure 1 a and e).

To determine if cilia are present on skin cells, we immunoprobed 40–50 μm skin cryosections with antisera against IFT88 (previously called polaris) and the cilia marker

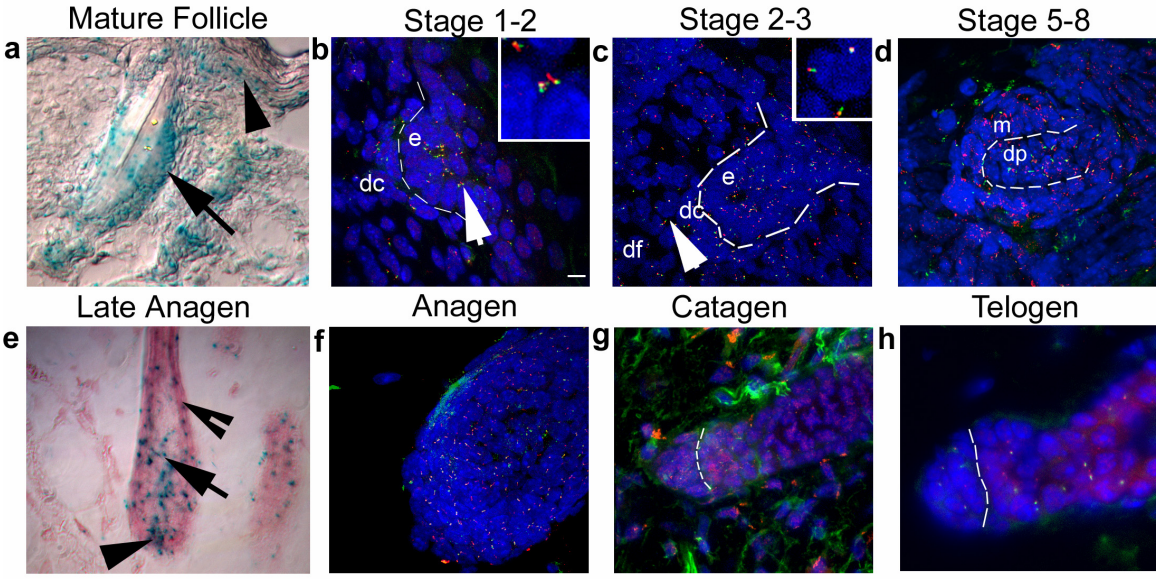

Figure 1. *Ift88* expression and primary cilia are found in most cells of the developing follicle and skin and most stages of the mature hair follicle.
Temporal and spatial analysis of an *Ift88/Tg737* reporter gene (a, e) and protein (b–d, f–h) reveals the presence of a primary cilium on epithelial and mesenchymal cells of the skin and hair follicle in embryos and adults. (a, e) *Ift88* expression was analyzed in heterozygous *Ift88tm1Rpw* (*Ift88*$^{\Delta 2\text{-}3\beta\text{-}gal}$) mice using a β-galactosidase reporter gene by X-Gal staining of skin sections. In the mature follicle (a) and late anagen follicle (e) and in the developing skin and hair, *Ift88* expression (blue) is detected in most cell types. Expression is prominent in the (arrow) cortex and (indented arrow) ORS (epithelium), (arrowhead) dermal papilla (mesenchyme) (e), and in cells near the (arrow) bulge region (stem cells) of the hair follicle as well as the (arrowhead) interfollicular epidermis (a). (b–d, f–h) IFT88 (red) localizes to primary cilia as shown by immunofluorescence colocalization obtained using antibodies against the ciliary axoneme marker, acetylated a-tubulin (green). The nuclei (blue) were labeled with Hoechst. (b) Analysis in a stage 1–2 developing hair follicle shows primary cilia on cells of both the forming dermal condensate (DC) and epidermal placode (e). The dashed line indicates the region of the basement membrane and the intersection between the epidermis and dermis. The insert shows a magnified image of the region indicated by the arrow. (c) In a stage 2–3 developing hair follicle, cilia were present on the epidermal and dermal portion of the hair germ. The epidermal (e), DC, and dermal fibroblast (DF) cells are indicated. The insert shows a magnification of the region corresponding to the arrow in the dermal fibroblasts. (d) Analysis in a later stage (5–8) follicle shows primary cilia present on cells of both the matrix (m) and dermal papilla (DP). (f–h) Immunofluorescence colocalization of acetylated α-tubulin (green) and IFT88 (red) during hair follicle cycling shows that a single primary cilium is present on most cells at (f) anagen, (g) most dermal cells in catagen with reduced epidermal cilia, and (h) most cells in telogen. Scalebar is 10 mm. Three-dimensional confocal images of cilia in the developing (1) or mature anagen (2) follicle can be seen in Supplementary Movies 1–2.

acetylated α-tubulin. Studies in multiple tissues have shown that both proteins localize to the cilia axoneme and that IFT88 is also present in the basal body at the base of cilia (Piperno and Fuller, 1985; Piperno *et al.*, 1987; Taulman *et al.*, 2001). Confocal microscopy was used to reconstruct the entire three-dimensional architecture of the developing and mature hair follicle (see Supplementary Movies 1–2). Using this approach, primary cilia were evident on most if not all dermal and epidermal cell populations of the hair follicle and skin (Figure 1). Cilia were present on cells throughout the development of the follicle, including the placode (stage 1), to advanced hair bulbs (stages 5–8). Cilia were also found in the dermal fibroblasts, cells of the dermal condensate and DP, and in the epidermis in keratinocytes, as well as the epidermal matrix cells, and cells of the inner root sheath near the follicle bulb and throughout the outer root sheath. Fewer cilia, with the exception of the outer root sheath, were found in the keratinizing cells of the upper anagen follicle near the hair shaft. Fewer epidermal cilia were also observed in catagen follicles, possibly due to intense epidermal apoptosis during this period. Primary cilia in the epidermis were most prominent on basal keratinocytes in the interfollicular regions. Progressively fewer cilia were observed on differentiated cells in the spinous layer and cilia were lost in the upper layers of the epidermis much as reported by Elofsson *et al.* (1984) in human skin. Most epidermal cilia had an apical orientation that was conserved in outer root sheath cells as the follicle matured, but was lost in the matrix cells. Cilia on dermal cells did not have such an obvious orientation.

Disruption of primary cilia in the dermis

Dermal cells, notably the dermal condensate/DP, have a major influence on hair follicle development and cycling as both an inductive and responding mesenchymal tissue. Thus, to begin assessing the importance of the primary cilium in the skin and hair follicle, we utilized two floxed alleles (*Kif3a*^{tm2Gsn} and *Ift88*^{tm1Bky} hereafter referred to as *Kif3a*^{fl} and *Ift88*^{fl}, respectively) of ciliogenic genes (*Kif3a* or *Ift88*) to disrupt cilia assembly specifically in cells of the dermis using the *Prx1-cre* (*Tg*(*Prrx1-cre*)*1Cjt*) line. This transgene drives Cre recombinase activity in the dermal mesenchyme of the skin on the ventrum, limbs, and laterally between the limbs starting at E9.5 (Logan *et al.*, 2002). In contrast to *Ift88* null mutant mice (*Ift88*^{tm1Rpw}), *Prx1-cre;Ift88*$^{fl/n}$ (*n* refers to the deleted allele *Ift88*$^{tm1.1Bky}$ arising from *Ift88*^{fl}) conditional mutants are viable.

To determine the temporal and spatial locations of Cre activity in the skin and hair follicles, we crossed *Prx1-cre*-positive mice with mice carrying the Cre reporter, *R26R* (Zambrowicz *et al.*, 1997). Analysis of β-galactosidase staining indicated that Cre activity was tightly restricted to cells in the dermis including dermal condensates/DP and was not evident in epidermal cells (Figure 2a and b, inset). Conditional cilia mutant mice (*Prx1-cre;Ift88*$^{fl/n}$) were then analyzed using thick sections of ventral skin for loss of cilia by immunofluorescence microscopy (Figure 2). In agreement with the spatial distribution of Cre activity, very few cilia were present on dermal mesenchyme cells of *Ift88* conditional mutants. The few cilia remaining on dermal cells likely reflect incomplete activity of the Cre recombinase. In contrast, epidermal cells near the hair follicles in wild-type mice and *Prx1-cre* conditional mutant mice possessed a primary cilium.

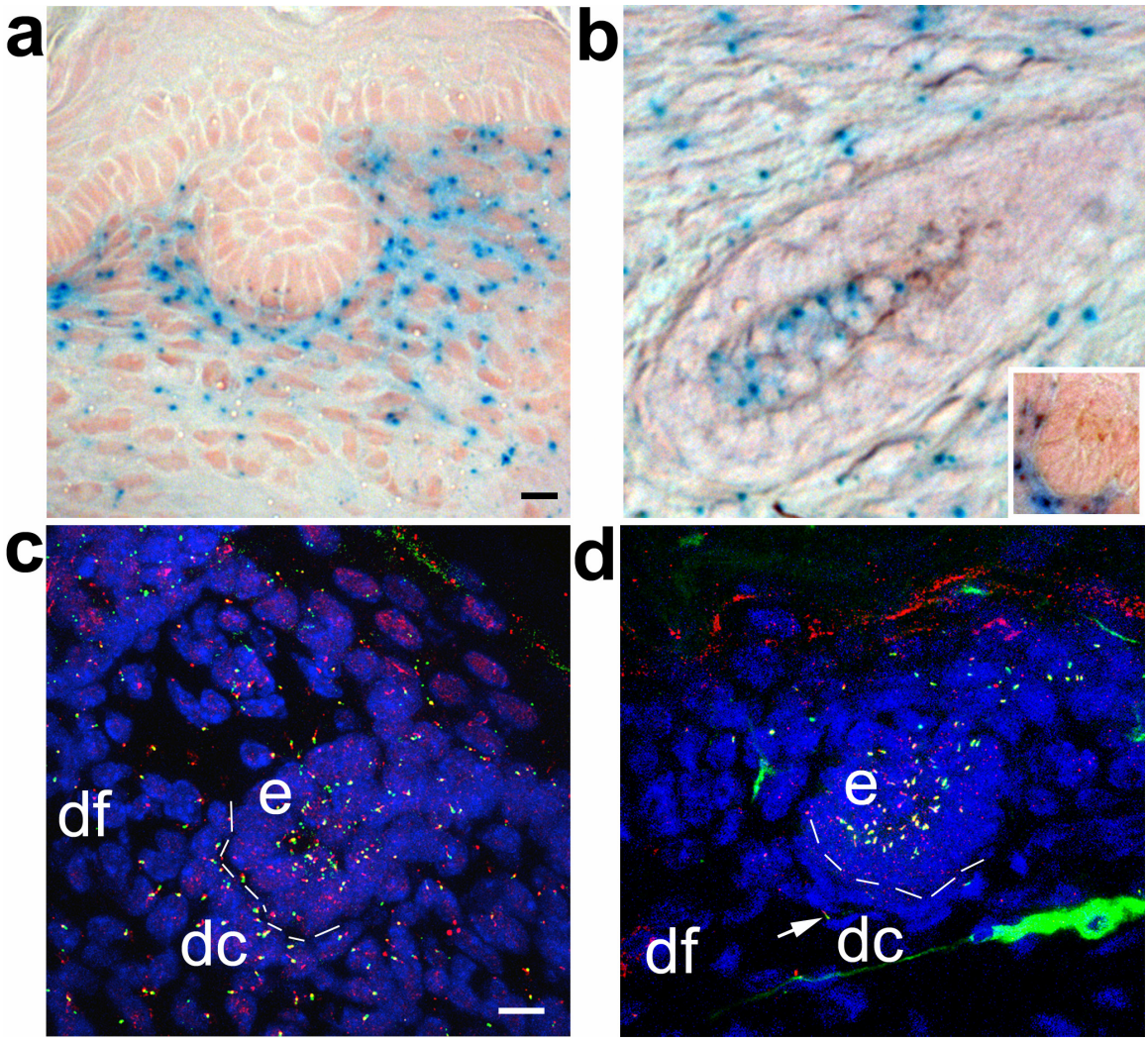

Figure 2. Cre activity and disruption of cilia in *Prx1-cre* mice is restricted to the dermal compartment of the skin and hair follicles covering the limbs, flanks, and ventrum.
(a) Cre activity in the skin and stage 2 hair follicle from the ventrum of a *Prx1-cre;R26R* mouse is present only in dermal cells, as revealed by the *lacZ* reporter gene (blue). (b) Cre activity is also seen in dermal components of advanced follicles including the dermal papilla of an advanced follicle and a stage 2 follicle (inset) from a P5 *Prx1-cre;R26R* mouse. (c) In wild-type control mice, primary cilia are present in the epidermal (e) and dermal (DC, dermal condensate; DF, dermal fibroblast) compartments of the skin and stage 2 hair follicle, as shown by colocalization of antibodies raised against acetylated α-tubulin (green) and IFT88 (red). (d) In *Prx1-cre;Ift88*$^{fl/n}$ conditional mutant mice, primary cilia are ablated from most DC and DF cells, but cilia are unaffected on epidermal (e) cells. Rare dermal cilia are unaffected, likely due to incomplete cre activity (arrow). Scalebars are 10 μm.

Loss of cilia in the dermis of the skin results in hypotrichosis

Loss of cilia on dermal cells in the conditional mutants resulted in persistent and severe hypotrichosis (Figure 3). The sparse hair phenotype in both the *Prx1-cre;Ift88* and *Prx1-cre;Kif3a* conditional mutants was identical and was observed specifically in the regions where *Prx1-cre* is expressed, including the upper ventrum, limbs, and a small domain on top of the head (Logan *et al.*, 2002). Hair follicle number and morphology appeared normal on regions of the body where Cre is not expressed. There were some hair follicles that formed normally on the ventrum in the *Prx1-cre* conditional cilia mutants (Figure 3e). The mechanism by which these follicles escape developmental arrest is unknown, but a similar observation was made in *Gli2*$^{-/-}$ mutant skin (Mill *et al.*, 2003).

The hypotrichosis in the cilia mutants is caused by an arrest in follicle morphogenesis

To further assess the follicular phenotype, we conducted histological analysis of the ventral skin from *Prx1-cre;Ift88* conditional mutant mice at P1 and P5. The data indicate there is a significant delay in follicle development in the conditional mutants (Figure 4b, c and e), with most arresting at stage 2 (Figure 4f and g). This is in contrast to the wild-type controls (Figure 4a and d) where most follicles advanced to stages 5–8 by P5 (Figure 4f and g). Interfollicular skin appeared normal as determined by staining for different cell populations using antibodies for K5 (basal layer), K10 (suprabasal layers), and loricrin (granular layer; Figure 4h–m). Mild fibrosis was seen in the *Prx1-cre;Ift88* mutant animals via trichrome staining (Figure S1).

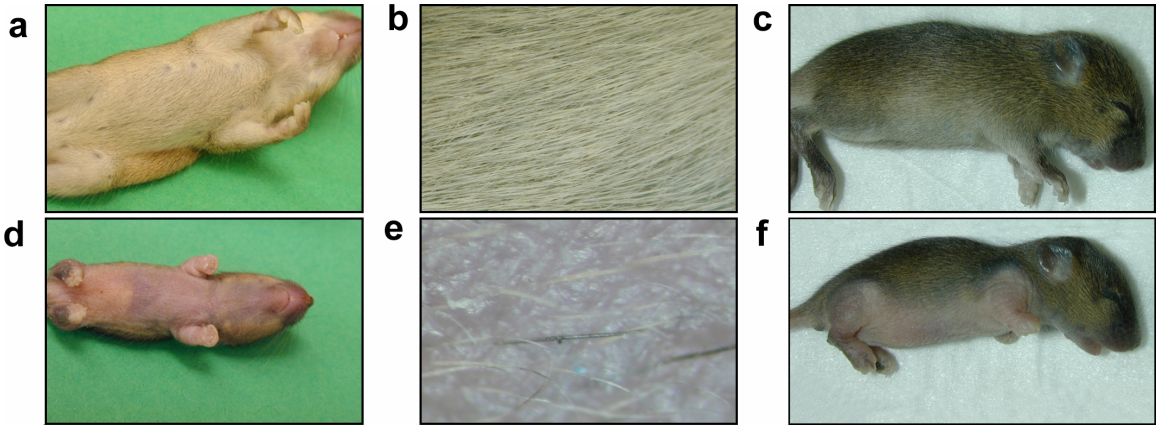

Figure 3. Conditional disruption of primary cilia in the dermis of the ventral skin in *Prx1-cre;Ift88*$^{fl/n}$ and *Prx1-cre;Kif3a*$^{fl/n}$ mice results in hypotrichosis.

The pelages of (a, b, c) wild-type littermate control mice, (d, e) a *Prx1-cre;Ift88*$^{fl/n}$, and (f) *Prx1-cre; Kif3a*$^{fl/n}$ conditional mutant mouse are shown at P14. (b, e) Higher magnification images of the ventrum of the mice in (a, d) show that the mutant skin has an extremely sparse coat and a few follicles that appear normal. The conditional cilia mutant mice also have deformed limbs that were described previously (Haycraft et al., 2007).

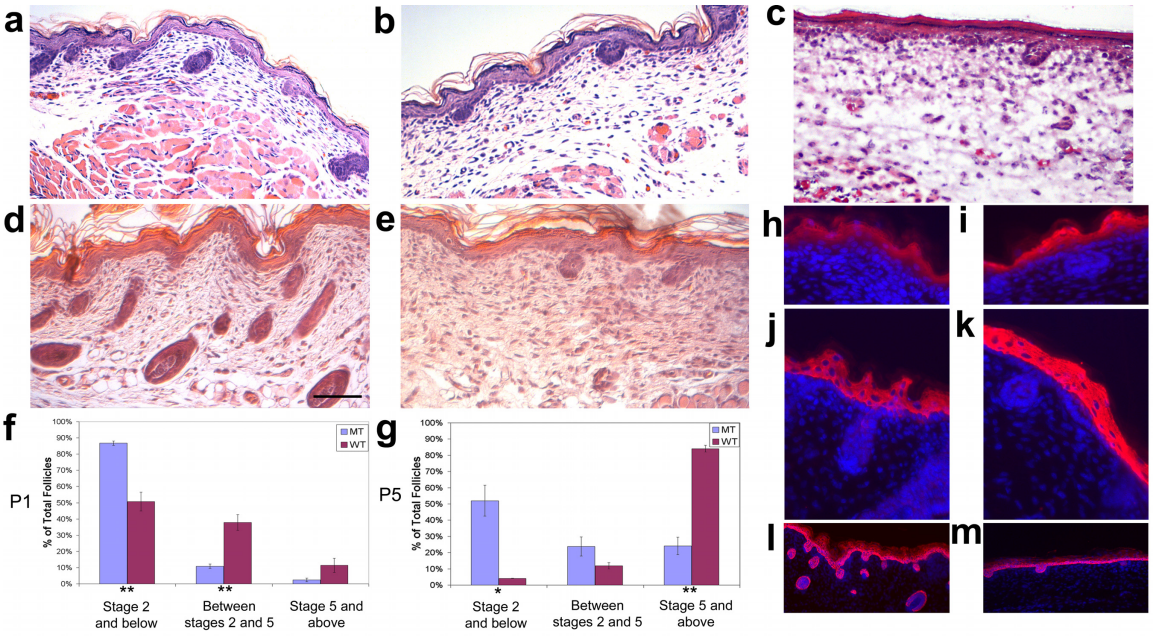

Figure 4. Hair follicle morphogenesis is arrested at stage 2 (hair germ) in mice with primary cilia ablated from dermal cells of the skin and hair follicles.

Histological sections of skin from (a) P1 wild-type, (b) mutant P1 *Prx1-cre;Ift88*$^{fl/n}$, and (c) P1 *Prx1-cre; Kif3a*$^{fl/n}$ mice show fewer and less advanced follicles in the mutant mice. This phenotype worsens at P5 in (e) mutant animals when compared to age-matched (d) wild-type controls. Histomorphometric analyses of the hair follicle phenotypes in skins harvested from mutant and control mice at (f) P1 and (g) P5 indicate that most follicles arrest at stage 2 of morphogenesis in *Prx1-cre;Ift88*$^{fl/n}$ mice. The analyses were performed using a minimum of 40 longitudinal follicles in each group from (f) P1 (n=5 pairs) and (g) P5 (n=3 pairs) mice. Error bars represent SEM. Statistical comparisons were conducted using the two-tailed independent Student's t-test. *P<0.05; **P<0.01. (h–m) Defects in differentiation were analyzed by immunofluorescence using antibodies against (h, i) loricrin (granular layer), (j, k) K1 (stratum spinosum), and (l, m) K5 (basal layer) of the epidermis reveal no overt differences in staining between (h, j, l) WT and (i, k, m) MT interfollicular epidermis. Scale bar (a–e) is 50 μm.

Comparison between the conditional cilia mutant and wild-type skin revealed that there were also defects in the dermal condensate in cilia mutants. Using endogenous alkaline phosphatase as a marker for dermal condensate cells as well as by histological and immunofluorescent (K5 stain) approaches, it is evident that in most follicles of the mutant mice, the dermal condensate was not detected or had fewer cells than in wild-type controls (Figure 5, Table 1 , and Figure S2). This dermal condensate hypoplasia was less pronounced at P1 than at P5. Additionally, wild-type stage 2 dermal condensates at P5 appeared similar to those at P1, though there were too few present for statistical analysis (Table 1). At P21, the arrested hair germs in *Ift88* and *Kif3a* mutants are no longer visible (data not shown) indicating that they likely degenerate.

Loss of dermal cilia impairs hedgehog signaling activity

Together, the phenotypes observed in the *Prx1-cre;Ift88* and *Prx1-cre;Kif3a* conditional mutant mice reveal that ciliary function in the dermal mesenchyme is essential for normal hair follicle morphogenesis. Interestingly, the skin and hair follicle phenotype seen in these mutant mice recapitulates that seen in $Gli2^{-/-}$ and $Shh^{-/-}$ mutants. Thus, we analyzed the activity of the Shh pathway in the dermal cilia mutants by *in situ* hybridization and real-time quantitative RT-PCR using RNA isolated from laser microdissected stage 2 dermal condensates (Figure S3). At P1, hedgehog pathway activity was impaired as shown by quantitative RT-PCR where Gli1 expression was reduced by 4.7 ($P<0.01$ ($P=0.0073$)) fold. Similarly, *in situ* hybridization analysis

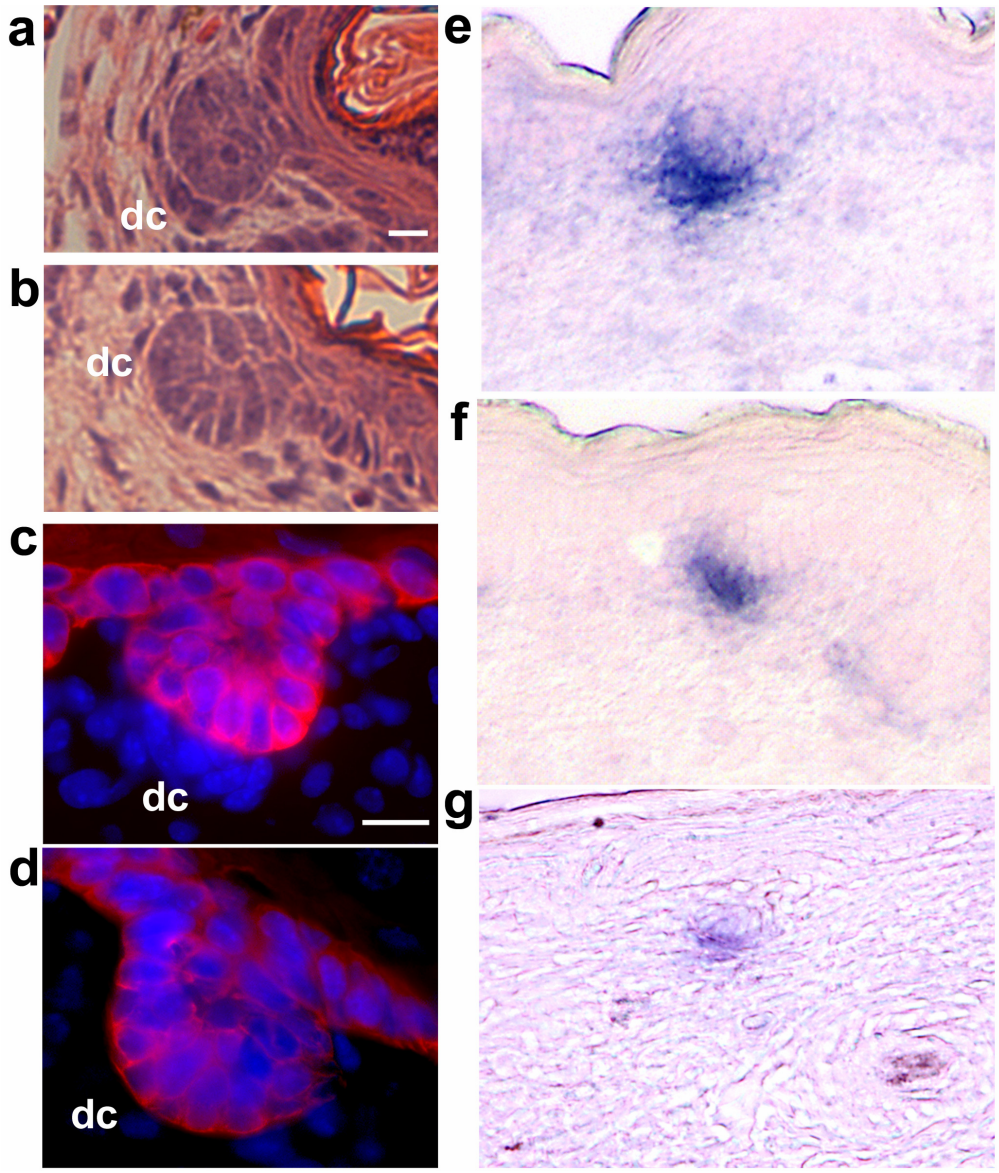

Figure 5. Disruption of cilia in the dermis of *Prx1-cre;Ift88*$^{fl/n}$ conditional mutant mice results in a marked reduction or absence of cells in the dermal condensate of stage 2 hair follicles.
Histological analysis of (a, b) hematoxylin and eosin stained sections and (c, d) thick section immunofluorescence analysis using epidermal marker Keratin 5 (red) antibodies shows a marked reduction in the number of cells in the dermal condensate (DC) of (b, d) *Prx1-cre;Ift88*$^{fl/n}$ conditional mutants compared to (a, c) controls. The reduced number of DC cells was also evident in (f, g) *Prx1-cre;Ift88*$^{fl/n}$ conditional mutants when compared to (e) controls as determined by staining for endogenous alkaline phosphatase activity. (e, f) are stage 2 follicles from P1 mice and (g) is a stage 2 follicle at P5. Scalebars are 10 mm.

Genotype	Timepoint	No. of DC cells	Standard error	*n*
Wild-type 1	P1	7.18	0.48	11
Wild-type 2	P1	6	0.65	10
Wild-type 3	P1	5.75	0.31	12
Avg. wild-type	*P1*	***6.31***	*0.44*	*3 animals*
Mutant 1	P1	4	0.42	10
Mutant 2	P1	4.43	0.59	14
Mutant 3	P1	5.1	0.66	10
Avg. mutant	*P1*	***4.51***	*0.32*	*3 animals*
Avg. wild type	*P5*	*Only 5 stage 2 follicles*		*3 animals*
Mutant 1	P5	2.8	0.55	10
Mutant 2	P5	2.18	0.44	10
Mutant 3	P5	1.8	0.42	10
Avg. mutant	*P5*	***2.26****	*0.29*	*3 animals*

Abbreviations: Avg., average; DC, dermal condensate.
Bold connotes significant *t*-test comparing wild-type condensates at P1 to mutant condensates at P1 ($P<0.05$) or P5 ($^*P<0.01$).

Table 1. Statistical representation of dermal condensate cell number in wild-type and dermal cilia disrupted mutant follicles at P1 and P5.

indicated that expression of the hedgehog responsive genes *Ptch1* and *Gli1* were reduced in the dermal region of the follicle at P5. However, this was difficult to assess due to the reduced size of the dermal condensates in most of the mutant follicles (Figure 6). Unexpectedly, levels of expression of *Ptch1* and *Gli1* in epidermal cells, which retain their cilia, appear to be elevated compared to the controls.

Disruption of dermal cilia and the canonical Wnt pathway

In several tissues, ciliary dysfunction has been shown to cause an increase in nuclear β-catenin levels and over activation of the canonical Wnt pathway. To determine whether this is also the case in the skin and hair follicle, we analyzed β-catenin expression and localization by immunofluorescent confocal microscopy in the dermal cilia mutants (Figure 7). There were no overt changes evident in the subcellular localization of β-catenin in the mutants when compared to similarly staged control follicles at P1.

DISCUSSION

There have been sporadic reports of primary cilia in mammalian skin; often these were in association with disease states such as basal cell carcinoma (Wilson and Mc, 1963; Daroczy and Feldmann, 1974). In addition, *in vivo* cilia have been found in basal keratinocytes and a minority of primary keratinocytes in culture also express a cilium under certain growth conditions; however, the functional importance of this organelle in the skin is unexplored (Elofsson *et al.*, 1984; Strugnell *et al.*, 1996). Discrepancies in the literature regarding the extent of ciliation in the skin may reflect the technical difficulties

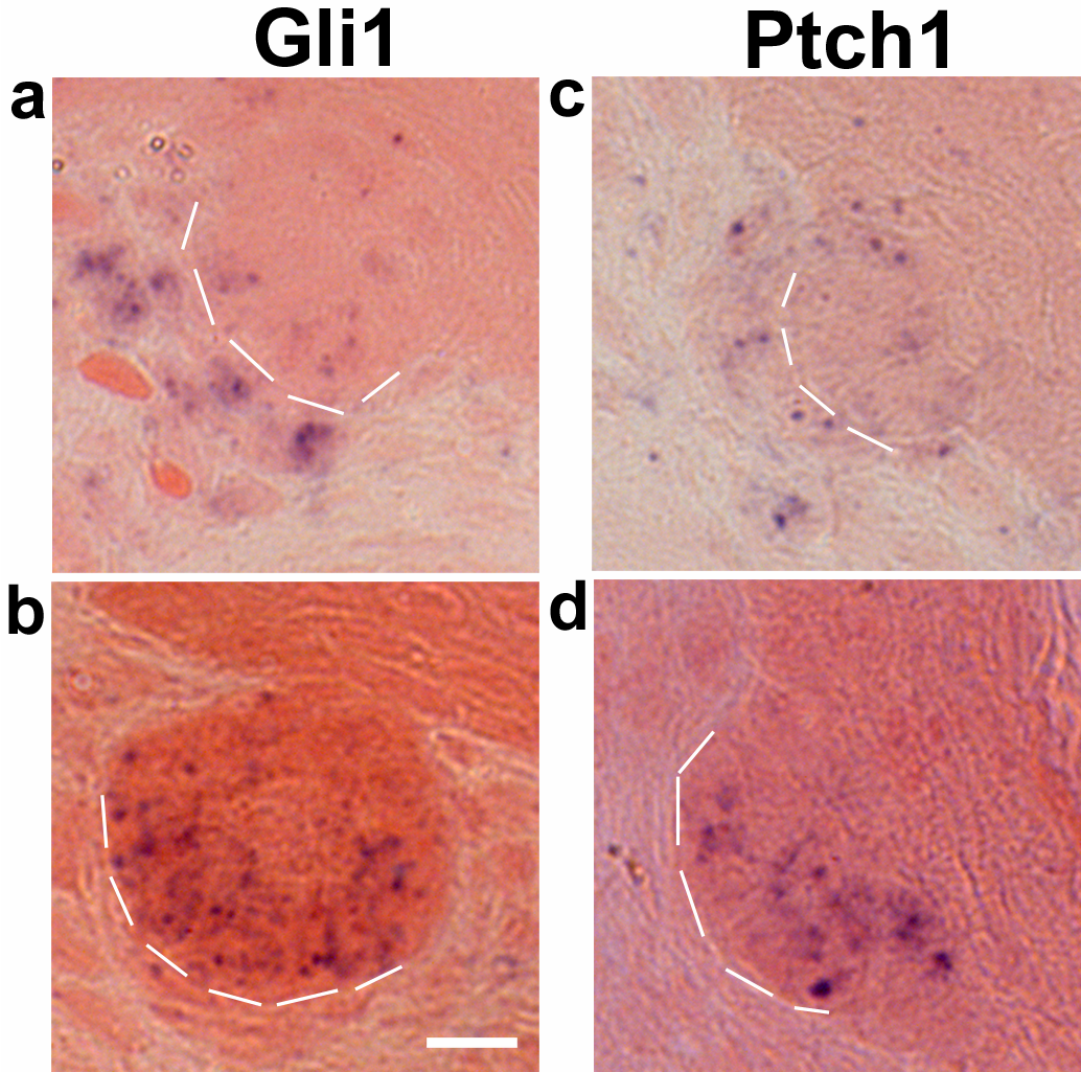

Figure 6. Ablation of dermal cilia in the skin of *Prx1-cre;Ift88*$^{fl/n}$ conditional mutant mice results in downregulation of the hedgehog responsive genes, Gli1, and Patched1, during hair follicle morphogenesis.
Analysis of the hedgehog signaling pathway was performed in sections of P5 skin by in situ hybridization to detect expression of the hedgehog responsive genes (a, b) Gli1 and (c, d) Patched1 (Ptch1) in (a, c) control and (b, d) *Prx1-cre;Ift88*$^{fl/n}$ conditional mutant mice. The mutants show reduced or absent expression for both genes in the dermal condensates and an increase in the epidermal cells. Dashed lines separate the epidermal components of the hair follicle from the underlying dermal condensate. Scalebar is 10 mm.

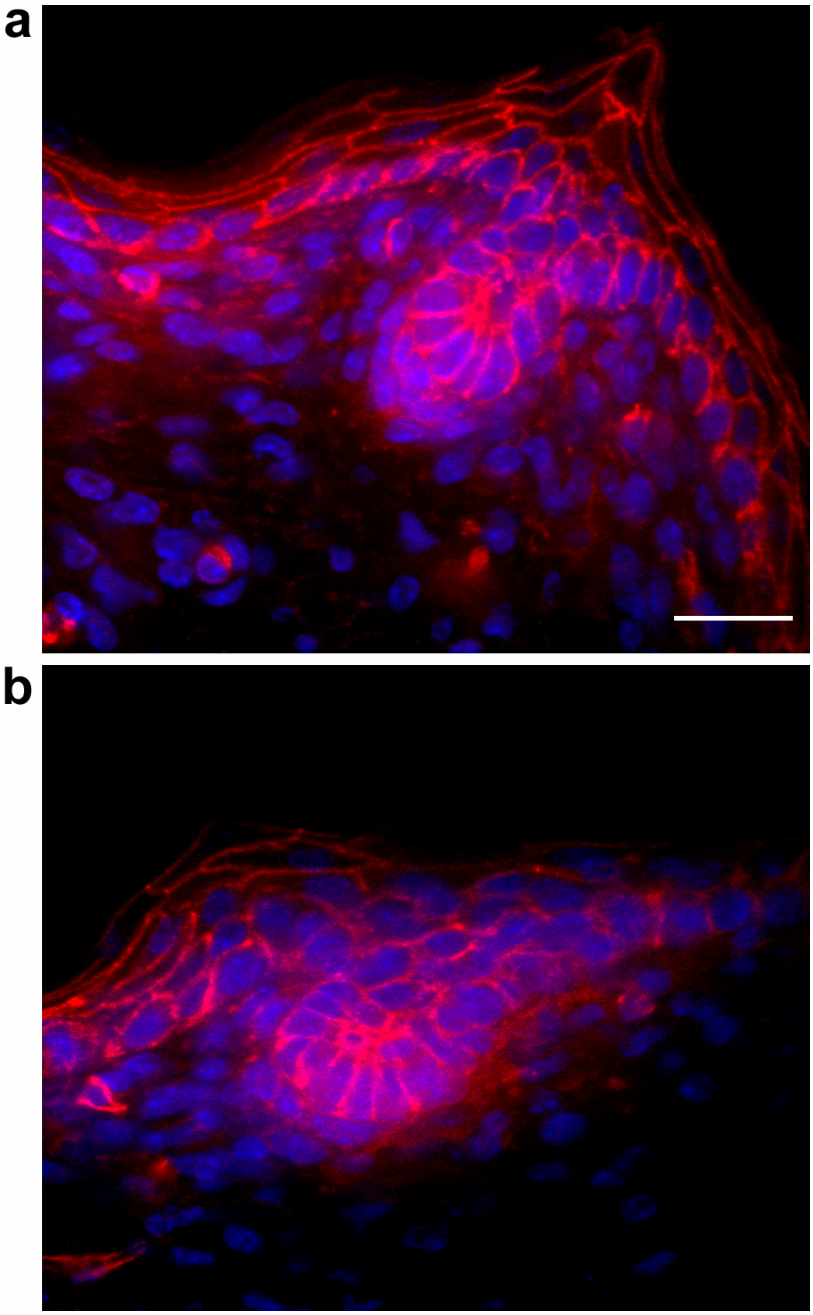

Figure 7. No difference in subcellular b-catenin localization was observed in hair germs of *Prx1-cre; Ift88*$^{fl/n}$ mice and wildtype littermates at P1.
There are no overt changes in Canonical Wnt signaling in dermal cilia conditional mutants as determined by thick section immunofluorescent confocal analysis of β-catenin (red) localization between (a) wild-type and (b) *Prx1-cre;Ift88*$^{fl/n}$ mutant mice at P1. Nuclei are stained with Hoescht (blue). Scalebar is 20 mm.

related to fixation (such as Karnovsky's solution) or that serial section electron microscopic analysis was required to observe this small organelle (Elofsson *et al.*, 1981, 1984; Wandel *et al.*, 1984; Roberto *et al.*, 1999). Hair follicle cilia, by contrast, have remained unexamined, with the exception of the mature anagen wool follicle where cilia were found on a few epidermal cells of the bulb and the DP (only 41 epidermal cilia and 6 dermal cilia in a total of 9 follicles were found; Orwin and Woods, 1982).

Using immunofluorescent confocal microscopy, we were able to analyze cells in the complex three-dimensional structure of the epidermis and dermis of the skin and hair follicle and demonstrate conclusively that primary cilia are found on most if not all cells throughout development of the skin. The presence of cilia on epidermal and dermal cells in the skin and particularly in the dermal condensate/DP, a cell population that functions to regulate cycling and development of the follicle, supports the idea that primary cilia are important for normal skin and hair follicle morphogenesis.

This study was initiated to directly address the question of whether cilia are functionally important in skin. Due to the mid-gestation lethality associated with the loss of cilia in mice, we utilized conditional alleles of two genes required for ciliogenesis, *Kif3a* and *Ift88*, to disrupt cilia in the dermal mesenchyme by crossing them with the *Prx1-cre* mice. Analysis of the phenotype in either line (*Prx1-cre;Ift88* and *Prx1-cre;Kif3a*) revealed that the disruption of dermal cilia results in stage 2 follicular arrest and severe hypotrichosis. Therefore, ciliary function in the dermis is essential for hair follicle development. Histological analysis indicates that cilia on cells of the dermal condensate/DP are particularly important, as the dermal condensate is often absent or severely hypocellular compared to controls. Mice with mutations in one of several

components of the Shh and PDGF signaling pathways exhibit defects in DP maintenance or formation (St-Jacques *et al.*, 1998; Karlsson *et al.*, 1999; Mill *et al.*, 2003). The fact that loss of dermal cilia, with intact epidermal cilia, leads to follicular arrest suggests that cilia are required for reception of a signal, possibly originating from the epidermis, needed for their maintenance, proliferation, or recruitment of cells into the dermal condensate.

The *Prx1-cre;Ift88* and *Prx1-cre;Kif3a* conditional cilia mutants are the first dermal-specific knockouts with a follicular arrest phenotype at stage 2 of development. Previous work has implicated signaling in the epidermis as critical for progression past this stage of follicle morphogenesis (Mill *et al.*, 2003, 2005). Our data with the conditional cilia mutant mice indicate that reception of signals in the dermal cells is also critically important for follicular morphogenesis. As identical phenotypes are obtained with both the *Kif3a* and the *Ift88* conditional mutant mice, the data suggest that their phenotypic sequelae stem from a loss of ciliary function as opposed to other unknown cellular activities of these proteins. Although we have not fully evaluated the pathway(s) affected in the *Kif3a* and *Ift88* conditional cilia mice, as indicated below, defects in hedgehog signaling likely contribute to this phenotype.

Recently, cilia dysfunction has been associated with impaired hedgehog signaling activity leading to abnormalities in the patterning of the limb bud and neural tube, and to defects in the expansion of cerebellar progenitor cells (Haycraft *et al.*, 2005; Huangfu and Anderson, 2005; Chizhikov *et al.*, 2007). Similarly, both our quantitative RT-PCR analysis using laser capture enriched dermal condensates and *in situ* hybridization data show that there is reduced expression of *Shh* target genes in the dermal condensate region

in ciliary conditional mutant mice; although this could also be associated with the hypocellularity of the dermal condensates in older mutants. Moreover, the observed arrest of hair follicle morphogenesis at stage 2 and the defects in dermal condensate/DP maintenance are similar to the phenotypes observed in mice lacking *Shh*, *Gli2*, or other likely mediators of Shh, such as the *Lama5*$^{-/-}$ mice (St-Jacques *et al.*, 1998; Li *et al.*, 2003; Mill *et al.*, 2003). These results are also supported by the previously characterized limb patterning defects observed in these mutants that we have shown involve defects in hh signaling (Haycraft *et al.*, 2007). Together these data support a model in which the loss of dermal cilia results in arrested follicle development and hypotrichosis due to dysregulated Shh signaling activity.

Our expression data also suggest there is a feedback effect resulting in an increase in *Gli1* and *Ptch1* expression in the epidermal hairgerm of the mutant stage 2 follicles. A possible explanation for this phenomenon is that the reduction in Ptch1 expression in the dermis leads to a local increase in the availability of Shh in the neighboring epidermal hairgerm upregulating Ptch1 and Gli1. A similar observation was made when smoothened was disrupted in the epidermis. In these mice, dermal Shh signaling activity is elevated due to an increase in Shh availability caused by the reduction in Ptch1 expression in the epidermis (Gritli-Linde *et al.*, 2007).

In the dermal cilia mutants, we also observed that there were a number of follicles that escaped developmental arrest. In part, this could reflect an inefficiency of Cre activity in the *Prx1-cre* line. An alternate explanation is that loss of cilia in the dermis also disrupts proteolytic processing of Gli3 to its repressor form. In the absence of a Shh signal (that is *Shh* mutants or in regions where Shh signal is low), Gli3 is normally

cleaved into a repressor; however, in mesenchymal cells lacking cilia this processing is impaired despite the inability of these cells to respond to Shh signals. Thus, some of the follicles may escape the arrest expected for cells unable to respond to Shh due to the additional loss of Gli3 repressor activity. This was shown previously in *Shh*$^{-/-}$ mutants where subsequent loss of *Gli3* (*Shh*$^{-/-}$;*Gli3*$^{-/-}$ double mutants) was able to rescue many aspects of the *Shh*$^{-/-}$ mutant phenotype, including a partial rescue of the dermal condensate in hair follicle morphogenesis (Mill *et al.*, 2005).

Although our analyses indicate that cilia on cells in the dermis of the skin are essential for normal hair follicle morphogenesis and that they likely function in Shh reception, our findings are perplexing in light of data from Mill *et al.* (2003). These authors demonstrated that the arrested hair follicle phenotype in *Gli2*$^{-/-}$ mutants can be rescued by expression of *Gli2* specifically in the epidermis. In contrast to our results, their data suggest that Shh reception in the epidermis, rather than dermis, is critical for morphogenesis of the hair follicle past stage 2. However, it is interesting that Mill *et al.* were unable to completely rescue the follicular phenotypes in *Shh*$^{-/-}$ mutants by exogenous expression of an activated form of *Gli2* in the epidermis. Thus, the understanding of how cilia mediate hedgehog signaling during hair follicle morphogenesis is incomplete, and it is likely that loss of cilia from the dermal condensate (DC)/dermal papilla (DP) affects both the activator and repressor functions of the Gli transcription factors. To fully address this issue will require further analyses using cre lines that that disrupt ciliary function specifically in the epidermis or in more defined cell populations in the follicle, such as the dermal condensate.

The hair follicle phenotypes displayed by the dermal cilia mutants phenocopy those seen in mice lacking Shh or Gli2. However, in addition to its role in hedgehog signaling, the primary cilium has been implicated in several other pathways that are known to be important for hair follicle and skin development including Wnt and PDGFR-αα signaling. Cilia influence the Wnt pathway by regulating noncanonical Wnt (e.g. Wnt5a) repression of the canonical Wnt signals (e.g. Wnt3a) and by restricting the strength of a cell's response to canonical Wnt signals. In the absence of cilia, the canonical pathway is no longer repressed by noncanonical Wnts leading to an increase in β-catenin and canonical Wnt signaling (Simons *et al.*, 2005; Benzing and Walz, 2006; Corbit *et al.*, 2008). However, in our dermal cilia knockouts the epidermal cilia are intact and β-catenin appears to be properly regulated. This may be due to the fact that in later hair follicle development the canonical Wnts are primarily expressed in the epidermis (Reddy *et al.*, 2001), though early loss of dermal canonical function leads to defects in ventral dermal specification (Ohtola *et al.*, 2008). The lack of an observed change in β-catenin localization may indicate that canonical Wnt activity is more important in the epidermis, or that disruption of cilia that theoretically leads to a mild to moderate increase in activation of the canonical pathway would, unlike loss of canonical Wnt signaling, not impact dermal development significantly. Thus, future studies using epidermal-specific cilia knockout lines may be more informative concerning the effects of ciliary disruption on canonical versus noncanonical signaling in the skin and hair follicle, particularly considering the recent data indicating that β-catenin signaling in the epidermis is important for specification of hair follicle fates (Zhang *et al.*, 2008). Unfortunately, the importance of the noncanonical Wnt pathway is less well

characterized in the hair follicle and the role of this pathway in hair follicle morphogenesis as opposed to polarity is not understood.

Cilia have also been implicated as important regulators of PDGF signaling. The receptor (PDGF receptor (PDGFR)-α) localizes to the ciliary membrane and cells with defects in ciliogenesis are unable to respond to the PDGF-AA ligand (Schneider *et al.*, 2005). In the skin, PDGF-A is expressed in the developing epithelial portions of the epidermis and hair follicle, whereas the dermal condensates cells express PDGFR-α. Furthermore, *Pdgf-a*$^{-/-}$ mutant mice develop skin and hair phenotypes characterized by a constellation of dermal phenotypes, including a hypoplastic dermis, small DP, abnormal dermal sheaths, and thin hair with misshapen follicles. The expression patterns and mutant phenotypes suggest that epidermal PDGF-A stimulates proliferation of dermal mesenchymal cells that contribute to the DP, mesenchymal sheaths, and dermal fibroblasts (Karlsson *et al.*, 1999). Thus, disruption of PDGFR-αα signaling in the mesenchyme of the dermal cilia mutants may be a contributing factor, in addition to loss of Shh responsiveness, to the reduced size of the dermal condensate. However, these conditional mutants do not display the general dermal atrophy phenotype normally associated with loss of PDGF-A signaling in the skin; in fact, trichrome staining suggests mild fibrosis or thickening of the dermis (Figure S1). This argues against a predominant role for PDGFR-αα signaling in these mutant phenotypes. This may reflect compensation by PDGF-AB and PDGF-BB signaling, which function normally in cells lacking cilia (Schneider *et al.*, 2005). Indeed, both PDGF-BB and PDGF-AA have shown anagen inducing effects upon injection into skin, and a study in lung fibroblast suggests that

PDGF-AB is stronger at driving fibroblast proliferation than PDGF-AA (Bonner *et al.*, 1991; Tomita *et al.*, 2006).

The cross talk between the Wnt, PDGF, and Shh pathways in hair follicle development remains incompletely understood, but may contribute to the phenotypes observed. Previous analysis of shh knockout mice has ruled out a direct epistatic interaction, but shh knockout mice were missing positive PDGFRα mesenchymal cells neighboring $Shh^{-/-}$ follicles suggesting a role for Shh in the clustering of these dermal condensate cells (Karlsson *et al.*, 1999). It has also been suggested that the Shh pathway initiates stage-specific activation of Wnt signaling components β-catenin and Lef1, but this was evaluated in the context of epidermal Shh pathway activation (Mill *et al.*, 2005) and changes in β-catenin expression or localization were not evident in our dermal conditional mutants. Expression of noncanonical Wnt5a in the dermal condensate does require the sonic hedgehog pathway, suggesting Wnt5a may be a downstream Shh target in the dermis (Reddy *et al.*, 2001). Although data indicate that cilia are essential for hair follicle development, the role of this organelle in regulating the cross talk between the Shh, PDGF-AA, and canonical versus noncanonical signaling pathways remains to be fully explored.

The primary cilium is an organelle with a previously unappreciated and wide distribution in the developing hair follicle and skin. Evolving research on cilia in the past decade has changed the perspective on this organelle from a functionless remnant to a signal transduction center in multiple tissues. In this study, we have localized primary cilia to both epidermal and dermal populations of the skin and developing follicle. We further show that dermal cilia are essential for normal hair follicle morphogenesis with

disruption of this organelle leading to hair follicle arrest that is associated with abnormal Shh signaling activity. Furthermore, the involvement of cilia in multiple signaling pathways such as canonical and noncanonical Wnt, PDGFR-αα, and hedgehog has exciting implications for understanding human skin biology and disease processes where these signaling pathways become dysregulated and contribute to hair loss or carcinogenesis.

METHODS

Mice

Ift88^{tm1Rpw} (previously referred to as *Tg737*$^{\Delta 2\text{-}3\text{-}\beta\text{-}gal}$) mice on the FVB/N genetic background that carry a knockout allele of the *Ift88* (previously called *Tg737*) gene were generated at the Oak Ridge National Laboratory and described previously (Murcia *et al.*, 2000). The *Ift88* conditional allele, *Ift88*^{fl} (*Ift88*^{tm1Bky}), was generated at the University of Alabama at Birmingham and was described previously (Haycraft *et al.*, 2007). The conditional *Kif3a* allele, *Kif3a*^{fl} (*Kif3a*^{tm2Gsn}), was obtained from Dr. Goldstein (UCSD) (Marszalek *et al.*, 2000). The Prx1-cre mice (*Tg*(*Prx1-cre*)^{1Cjt}) were generated by Logan *et al.* (2002) and obtained from Jackson Laboratory, Bar Harbor, ME. Conditional cilia mutant mice were analyzed on a mixed C57BL/6J ×129P2/OlaHsd genetic background. The experimental mice were generated using male *Prx1-cre* mice due to the germline activity of *Prx1-cre* in females. PCR analysis of DNA obtained from tail biopsies was used to genotype mice as described previously (Yoder *et al.*, 1997; Murcia *et al.*, 2000). All animals in this study were maintained in AAALAC accredited mouse facilities at

UAB and in accordance with IACUC regulations and protocols at the University of Alabama at Birmingham (UAB).

Immunofluorescence microscopy

Hair and skin from appropriate regions as indicated in the figures was adhered to nitrocellulose filter paper in a phosphate-buffered saline (PBS) bath then longitudinally trimmed. The samples were embedded in Optimal Cutting Temperature compound (Sakura, Torrance, CA) and flash frozen in a 2-methyl butane bath cooled in liquid nitrogen. To evaluate the presence of cilia on cells of the skin, 40–50 μm thick skin sections were cut using a Leica CM1900 cryostat and fixed either for 2 hours in 4% paraformaldehyde or for 30 minutes in ice-cold methanol. The sections were permeabilized by incubation for 30 minutes in either 0.02% SDS in PBS or in ice-cold methanol. Sections were washed in PBS, blocked with 1% BSA for 30 minutes, and incubated with primary antibodies and sequentially with secondary antibodies overnight at 4 °C in PBS with 1% BSA and 0.01% Triton. Antibodies used in this analysis include anti-polaris/IFT88 (B1700, 1:1,000, (Haycraft *et al.*, 2007)), anti-acetylated α-tubulin (Sigma, St. Louis, Missouri, catalog no. T6793, 1:1,000), anti-β-catenin (Sigma catalog no. C2206, 1:1000), anti-K5 (Covance, Berkeley, CA, catalog no. PRB-160P, 1:1000), anti-K1 (Covance catalog no. PRB-165P, 1:500), and anti-loricrin (Covance, catalog no. PRB-145P, 1:500). Nuclei were stained with Hoechst 33258 (Sigma) diluted 1:1,000 in PBS. After extensive washing in PBS, the sections were covered in mounting medium consisting of 1 mg ml^{-1} *p*-phenylenediamine in 90% glycerol and coverslips were attached with nail polish. Images were captured using either an inverted Nikon TE200

epifluorescence microscope with a CoolSnap HQ/FX (Roper Scientific) CCD camera operated through MetaMorph (Molecular Devices, Downington PA) imaging software, a Leica Confocal Imaging Spectrophotometer TCS SP unit (UAB High Resolution Imaging Core), or a PerkinElmer Spinning Disc confocal microscope. The resulting images were viewed and analyzed using Adobe Photoshop (Adobe). For confocal microscopy, optical sections were captured at ~0.4–0.5 μm intervals and the three-dimensional structure of skin and hair follicles was rendered from the Z-stacks using Voxx2 imaging software (available from the Indiana Center for Biological Microscopy, Indiana University http://nephrology.iupui.edu/imaging/voxx/index.htm; Clendenon *et al.*, 2002).

β-galactosidase assays

Analysis of *Ift88/Tg737* expression was performed as described (Taulman *et al.*, 2001) using 8–10 μm sections of skin biopsies obtained from *Ift88*^{tm1Rpw} (*Tg737*$^{\Delta 2\text{-}3\text{-}\beta\text{-}gal}$) heterozygous mice. The location of Cre recombinase activity was analyzed in sections of mice doubly transgenic for *Prx1-cre* and the ROSA26 Cre-reporter, *R26R* (Zambrowicz *et al.*, 1997), using X-gal as a substrate.

Histomorphometry, endogenous alkaline phosphatase staining, and trichrome staining

Histomorphological analysis was performed on hematoxylin and eosin stained sections (5 μm) obtained from ventral skin over the sternum and analyzed using the staging guidelines described by Paus *et al.* (1999). Longitudinal sections were separated by at least 40 μm to prevent double counting of hair follicles. Statistical analysis was

performed using a two-tailed independent Student's *t*-test ($P<0.05$) on at least three littermate controls or five age-matched controls with at least 40 staged follicles per mouse. Endogenous alkaline phosphatase staining was performed on 8 μm cryosections postfixed for 8 minutes in 4% paraformaldehyde/PBS, washed with NTMT, (100 mM NaCl, Tris-HCl (pH 8.0), 50 mM $MgCl_2$, 1% Tween-20) and incubated in BM Purple substrate (Roche Applied Science, Indianapolis, IN) for 2 hours at 4 °C. Trichrome staining was performed using Lillie's trichrome on 5 μm paraffin embedded sections.

In situ hybridization

In situ hybridization for *Ptch1* and *Gli1* expression was performed as described in Sheng *et al.* (2002) using *Ptch1* and *Gli1* probes as previously described in Haycraft *et al.* (2007).

Laser cutting microdissection and quantitative real-time PCR

Laser microdissection was performed using a Zeiss P.A.L.M. microdissection instrument. Captures were enriched for dermal condensate using the dissection and capture regimen indicated in Figure S3. Briefly, 8 μm cryosections were cut, stained with hematoxylin and eosin, and then laser dissected and catapulted into collecting tubes. A minimum of 80 mutant or 40 wild-type stage 2 follicles were microdissected per sample. RNA was extracted using RNAqueous (Ambion, Austin, TX) and converted to cDNA. Quantitative PCR analysis was performed using a Roche Lightcycler 480, and resulting fold differences and statistical significance was assessed using the Relative Expression Software Tool program (Pfaffl *et al.*, 2002) using a pairwise fixed reallocation

randomization test with 50,000 iterations. Gene expression was referenced to 18S rRNA (to compensate for cell size/loading) and normalized to *Ift88* levels using an amplicon in the deleted region (to compensate for variable contamination of samples with neighboring epidermal placodes in the laser microdissection process and Cre efficiency). TaqMan probes for Gli1 (Mm00494645_m1), 18S rRNA(Hs99999901_s1), and *Ift88* (Mm0133466_m1) were obtained from Applied Biosystems, Foster City, CA.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Acknowledgments

We gratefully acknowledge Dr Trenton Schoeb for guidance in pathology as well as the fellow members of the Yoder laboratory for critical reading and comments on the paper. We acknowledge the UAB Comparative Pathology laboratory for histology services and the Indiana Center for Biological Microscopy for developing the program Voxx2. We also thank the UAB laser microdissection facility and Natalya Frolova for assistance, and acknowledge the UAB Heflin Genomics Core and Dr Michael Crowley for providing facilities and assistance with qRT-PCR analysis. This work was supported in part by a Pilot and Feasibility award (BKY) from the UAB Skin Diseases Research Center (SDRC, P30 AR050948, to Dr Craig Elmets), as well as an RO1 award from NIAMS (AR052792, BKY), and by the Laboratory Directed Research and Development Program of Oak

Ridge National Laboratory, managed by UT-Battelle, LLC for the US Department of Energy under contract no. DE-AC05-00OR22725 (EJM).

SUPPLEMENTARY MATERIAL

Movie S1. Immunofluorescence analysis of a stage 2 hair germ showing that cilia are present on most epidermal and dermal cells including the interfollicular epidermis, epidermal hair germ, dermal fibroblasts, and forming dermal papilla as shown by cilia *axoneme* markers acetylated α-tubulin (green) IFT88 (red), and Hoescht nuclear staining(blue).

Movie S2. Immunofluorescent analysis of a P25 anagen follicle with cilia present on most epidermal and dermal cells including the matrix and dermal papilla (center) as shown by ciliary axoneme markers acetylated α-tubulin (green), IFT88 (red), and Hoescht nuclear staining (blue).

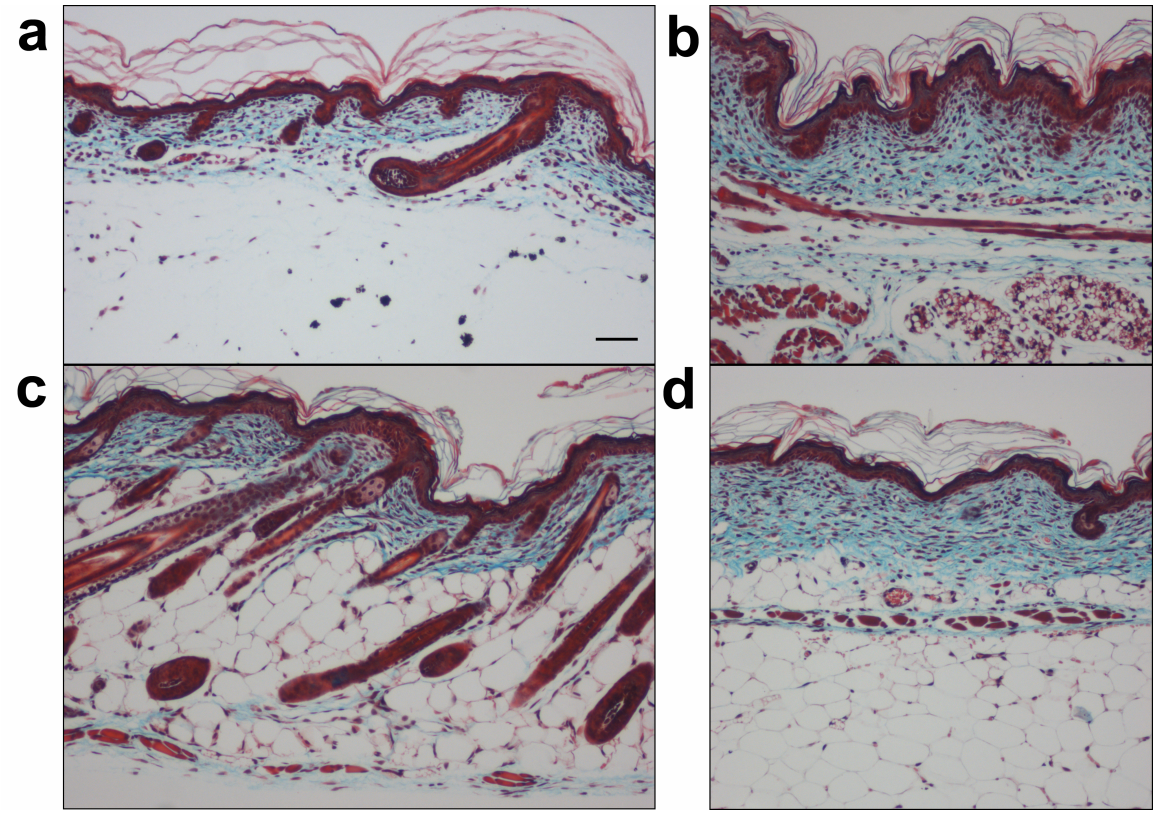
Figure S1. Mild fibrosis and possible dermal expansion was present in (B, D) *Prx1cre;Tg737*$^{fl/fl}$ animals compared to (A, C) wild-type littermates at (A,B) P1 and (C, D) P5 as determined by trichrome staining (green).

Figure S2. Representative 8 μm images before laser microdissection of stage 2 follicles in P1 (A) wild-type and (B) conditional cilia knockout mice showing the reduced number of dermal condensate cells in mutants compared to control samples.

Figure S3. Representation of the Laser Capture Microdissection strategy used to analyze expression in the dermal condensates.

Supplemental material available online at

http://www.nature.com/jid/journal/vaop/ncurrent/suppinfo/jid2008279s1.html?url=/jid/journal/vaop/ncurrent/abs/jid2008279a.html



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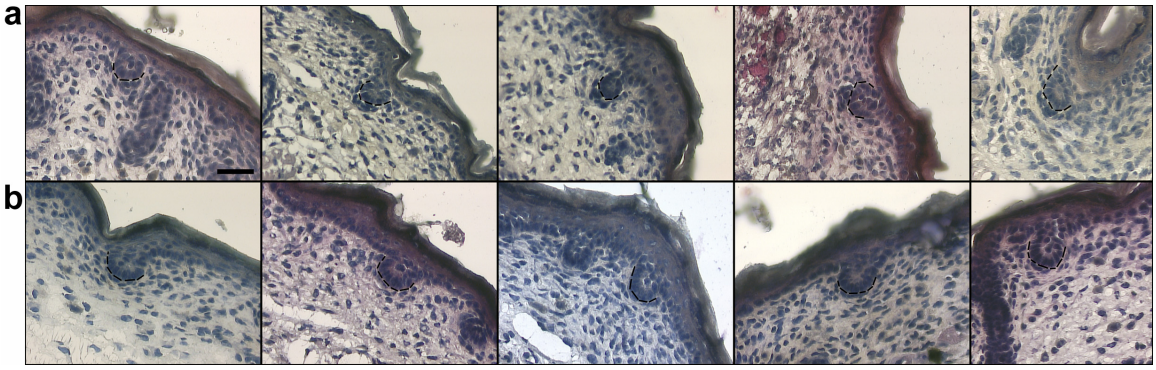

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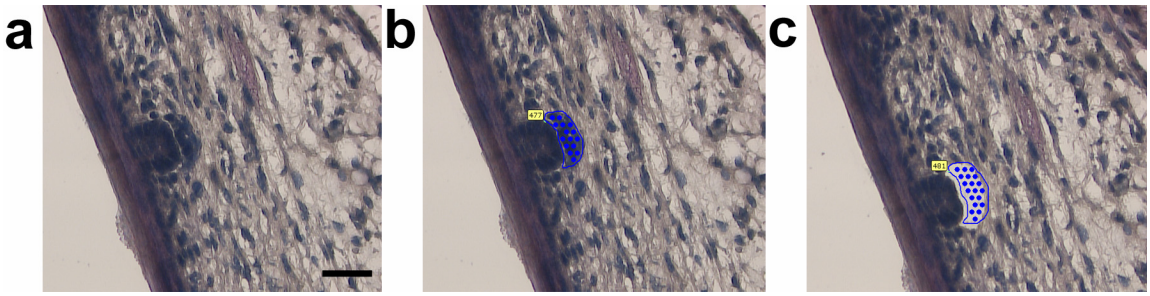

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Representation of the Laser Capture Microdissection strategy used to analyze expression in the dermal condensates.

EPIDERMAL CILIARY ROLES IN SKIN HOMEOSTASIS AND HAIR FOLLICLE DEVELOPMENT

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In preparation for *Development*

Format adapted for dissertation

Summary

The skin is a stratified squamous epithelium functioning as a thermal, chemical, and microbiological barrier. Its maintenance relies on follicular and interfollicular stem cells and regulated differentiation of progenitor cells from the basal layer into the upper layers of the skin. The process regulating stratification, maintenance, and differentiation of these cells is incompletely understood, but involves asymmetric versus symmetric cell divisions and the hedgehog, canonical, and noncanonical Wnt signaling pathways. Primary cilia function has recently been implicated in all of these processes. The cilium is present on most mammalian cells, including the skin, cilia are reported in the dermis, interfollicular epidermis, and hair follicle as well as the stem cell compartments. Previous studies have shown that disruption of the cilium in dermis results in hair follicle arrest; however, the function of epidermal cilia has not been explored. We address this issue using the Cre-lox system to disrupt ciliogenesis in the basal layer of the epidermis. Compared to established dermal cilia mutants, epidermal cilia mutants appear normal at birth and form normal pelage hair. However, these mice develop abnormal ventral hair patterning, epidermal basaloid hyperplasia, and have ectopic hair bud like growths. Marker analysis reveals this phenotype is associated with an expanded basal progenitor layer and altered differentiation. This ectopic hair bud and hyperplasia phenotype along with altered marker expression resembles mice with epidermal activation of the Shh pathway. Furthermore, there is an accumulation of nuclear β-catenin suggesting altered canonical Wnt signaling. Finally, analysis of the spindle pole marker NumA reveals that the pattern of orientation of cell divisions is abnormal in the epidermal cilia mutants.

These data indicate that cilia may have a role in regulating expansion or homeostasis of the interfollicular stem cell population and in regulating progenitor differentiation.

INTRODUCTION

Mammalian skin and hair serves as a waterproof and protective layer with functions in temperature control, UV protection, and essential thermal, chemical, and microbiological barrier roles, shielding the body from the hostile external environment. The epidermis meets these protective functions and achieves a durable, repairable, waterproof barrier by forming a stratitified squamous epithelium.

Briefly, the mouse epidermis is composed of 3 living layers and one dead; from exterior to interior, the stratum corneum, composed of tightly interlocked, waterproofed dead squames, the granular layer, with dying cells filling with waterproofing components, the spinous layer, where cells are constructing a keratin structure which will house and provide support for the future squames, and the basal layer which replicates to replace the shed squames and replenish this dynamic system.

Basal keratinocytes are identified by expression of the paired keratins K14/K5 (Nelson and Sun, 1983; Fuchs, 1990). This basal layer of keratinocytes is the sole mitotic layer of the adult epidermis and regenerates throughout life (Fuchs, 1990). The power source of this replicative engine is postulated to be a combination of an interfollicular stem cell population and contributions from neighboring follicular stem cells, particularly in the context of wound healing. These basal keratinocytes push upwards and differentiate to form

the spinous layer and eventually all layers of the epidermis. The basal layer of the skin contains and is renewed by an interfollicular stem cell population. The mechanism of this stratification and renewal is incompletely understood, but there is increasing support for the existence of an interfollicular stem cell population which divides symmetrically (or parallel to the basement membrane) to replenish the stem cell population, as well as asymmetrically (perpendicular) to initiate the differentiation program.

Recent data suggests that the orientation of these divisions, whether parallel or perpendicular to the basement membrane, along with asymmetric distribution of Notched regulates the stratification of the epidermis (Lechler and Fuchs, 2005; Clayton et al., 2007; Fuchs, 2008). This use of parallel and perpendicular divisions and associated planar cell polarity is not confined to stratified squamous epithelium. Other organ systems such as the kidney utilize a similar process of perpendicular and parallel divisions to regulate kidney tubule length and size. Interestingly, abnormalities in the axis of cell division are thought to contribute to the formation of kidney cysts. Although the mechanism responsible for the altered orientation of cell divisions is unknown, it is intriguing that most proteins involved in cystic kidney diseases localize to the primary cilium or at the base of the cilium.

Primary cilia are microtubule based singular organelles present on most cells of the mammalian body. They are maintained by a highly evolutionarily conserved process referred to as intraflagellar transport (IFT) which mediates the bidirectional movement of large protein complexes between the base and tip of the cilium. Mutations disrupting the IFT process result in ciliary structural or functional defects and cause a wide variety of developmental abnormalities. These range from mid-gestational lethality due to random left-right body axis specification, to neural tube closure and organ patterning defects (Murcia et

al., 2000; Pazour et al., 2000; Taulman et al., 2001). More subtle mutations affecting ciliary function result in postnatal phenotypes in multiple organ systems from polycystic kidney and liver disease, to hydrocephalus, to central obesity, to ectodermal dysplasia and growth defects reviewed in (Badano et al., 2006; Lehman et al., 2008b). Although not fully understood, the primary cilium is now known to be a key regulator of a variety of signaling pathways such as the Shh and canonical and noncanonical Wnt signaling pathways. In the case of the Shh pathway, data suggest the cilium is required for cells to respond to the ligand which binds to its receptor (patched) at the cilia membrane (Rohatgi et al., 2007).

Surprisingly, despite their ubiquity, the primary cilium has only recently been investigated for functional involvement in formation and maintenance of the hair follicle and skin. The fact that the Shh and Wnt pathways are closely associated with skin diseases, such as basal cell carcinoma, and hair follicle morphogenesis and cycling, raised the possibility that cilia may play important roles in the development and maintenance of this organ. To address this issue, we have been using the conditional loxP model system to disrupt primary cilia assembly in different tissues and cell populations to bypass the early developmental requirement for cilia function. Previously, we established that loss of cilia in the dermis results in a phenotype similar to that seen in mice lacking Shh or Gli2 in the skin, with an arrest of hair follicle development at stage 2 (Lehman et al., 2008a). This finding suggested that there is an essential role for cilia on cells in the dermis in the skin for Shh signaling and is similar to that demonstrated in the limb and neural tube. However, these data were somewhat surprising considering work by Mill et al (Mill et al., 2003). They demonstrated that the hair follicle morphogenesis defects of the Gli2 mutants could be *rescued* by expression of an active Gli2 in the K5 basal layer of the epidermis. Thus, their data indicate

that Shh signaling in the epidermis was of great importance for this phenotype. Together these data raised the question about what role the cilium plays in the epidermis.

To evaluate this we used conditional floxed alleles of *Ift88*$^{tm1Bky/tm1.1Bky}$ (hereafter referred to as *Ift88*^{fl}) and *Kif3a*^{tm2Gsn} (hereafter referred to as *Kif3a*^{fl}) which encode proteins required for IFT raft assembly and IFT raft movement, respectively, and are needed for cilia assembly. To disrupt cilia, we crossed the conditional alleles with the K14-Cre transgenic line (*FVB-Tg(K14-Cre)8Brn*) which expresses Cre recombinase in the basal cells of the epidermis. Somewhat surprisingly, the mice lacking IFT in the epidermis appear overtly normal at birth. However, with age these mutants develop a constellation of phenotypes, which are not typical of mice lacking Shh signaling, including mild ventral alopecia, ectopic hair bud formation in the interfollicular epidermis, and epidermal hyperplasia. In the mutants, the population of p63/K5 positive cells was increased and mitotic figures were observed in suprabasal layers, which are normally not proliferative in wild type skin. This is consistent with an expansion of the interfollicular/progenitor cell population.

Similar to what is seen in the cystic kidneys of the cilia mutant mice, this progenitor hyperplasia was associated with abnormalities in the orientations of the mitotic spindle in the basal keratinocytes. Together these data indicate there are distinct roles for primary cilia in the epidermal and dermal compartments of the skin with regards to hair follicle formation and skin development, and support a novel role for primary cilia in maintaining homeostasis of the interfollicular epidermis.

RESULTS

In our previous studies, we described the presence of cilia on the interfollicular epidermis and throughout the developing and cycling hair follicle (Lehman et al., 2008a; Lehman et al., 2008b). Most if not all basal keratinocytes are ciliated. The frequency of cilia decreased on cells in the spinous layer, and there were no observed cilia present on granular cells (Elofsson *et al.*, 1984). We determined that cilia are also present on cells in the dermis and that they are essential for normal hair follicle morphogenesis, likely due to the requirement of ciliary function in regulation of the hedgehog signaling pathway (Lehman et al., 2008a). However, the importance of the cilium in the epidermis remains unexplored. In order to address this question, we used conditional alleles of two ciliogenic genes and a transgenic line expressing Cre recombinase from a K14 promoter to disrupt cilia in the basal keratinocytes.

We evaluated the expression pattern of Cre activity from the K14 promoter (*FVB-Tg(K14-Cre)8Brn*, hereafter referred to as K14-cre) by crossing the Cre mice to the *R26R* Cre reporter line and evaluating the extent of β-galactosidase staining (Figure 1 a-b). We detect Cre activity at low levels in the early stage hair follicle such as hair germs or placodes (Figure 1a). This activity increased dramatically in postnatal stages and in the epidermal portions of advanced follicles (Figure 1b) and in the interfollicular epidermis. Due to the late onset of Cre activity in this line, we also crossed our conditional alleles with an additional K14-Cre (*Tg(KRT14-cre)1Amc/J*) generated by Andy McMahon whose characterized Cre expression is evident early in follicular morphogenesis ((St-Jacques et al., 1998; Gritli-Linde et al., 2007). We then analyzed sections of skin from *K14cre; Ift88*$^{fl/n}$ and control mice at

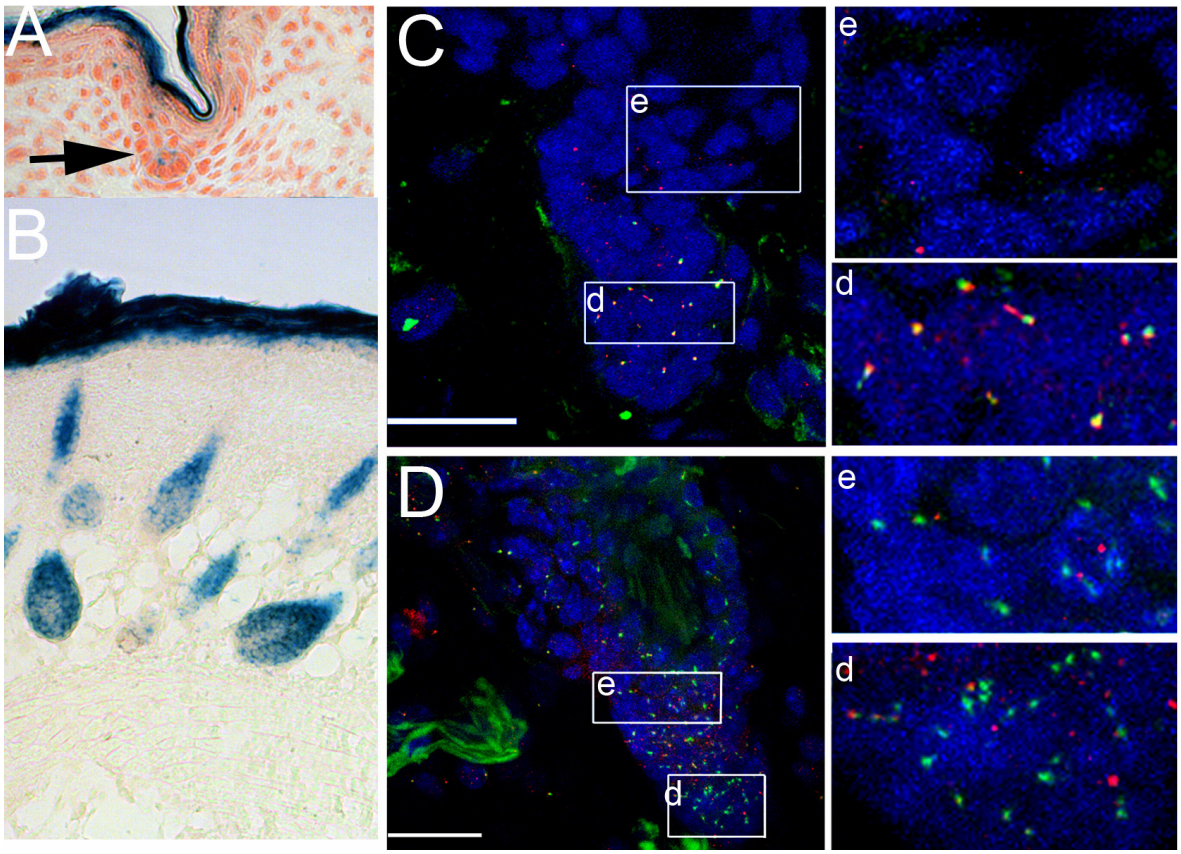


Figure 1 *K14-cre* is highly expressed in later stages of epidermal development and induces epidermal specific cilial disruption in *K14-cre;Ift88*$^{fl/n}$ animals.
K14-cre; R26R animals have sparse early β-galactosidase staining at P2 (A), but develop extensive staining in the interfollicular epidermis and advanced hair follicles by P6 (B). Immunofluorescence analysis of *K14-cre;Ift88*$^{fl/n}$ and wildtype littermate animals with anti-α-acetylated tubulin (green) and anti-IFT88 (red) antibodies and Hoescht nuclear stain (blue) show disrupted epidermal hair follicle cilia in P50 animals with cilia loss in epidermal mutant animals (C), but normal cilia in wildtypes (D).

P50 for cilia by immunofluorescence microscopy. There was a significant loss of cilia on cells in the epidermal portions of the hair follicle (Figure 1 c-d), though occasional epidermal cilia were still visible. Cilia on cells of the dermal papilla and in neighboring dermal fibroblasts were maintained.

Observed Phenotypes in Epidermal Cilia Conditional Mutants

K14-cre;Ift88$^{fl/n}$ and *K14-cre;KIF3a*$^{fl/n}$ are born healthy and have no external visible phenotypes until weaning age. At about 21 days of age the mutants begin to develop a "ridged" pattern in their hair with areas of progressive alopecia (Figure 2 e,g). This ridging is largely visible in the ventrum, although mild hair loss is also present in portions of the dorsum. These phenotypes continue to worsen with age. This can be seen in figure 2 by comparing P159 (Figure 2 a,e) animals to P730 animals (Figure 2 c,g). Similar macroscopic phenotypes are observed in the *Tg(KRT14-cre)1Amc/J; Ift88*$^{fl/n}$ mice which have similar "ridging" and progressive mild alopecia (data not shown). The ridging pattern in the hair is accompanied by histological evidence of epidermal hyperplasia. This hyperplasia is confined to the lower layers of the epidermis. There are no visible signs of hyperkeratosis or flaky skin suggesting no defects in stratum corneum. At more advanced stages, the basal epidermis has signs of palisading morphology changes (inset), which are also seen in models and disorders with increased Shh signaling activity or truncations in Patched1 (Nieuwenhuis et al., 2006; Nieuwenhuis et al., 2007). The progressive alopecia is mirrored in the histology with a qualitative reduction in hair follicle adnexae in older animals. Additionally, epidermal

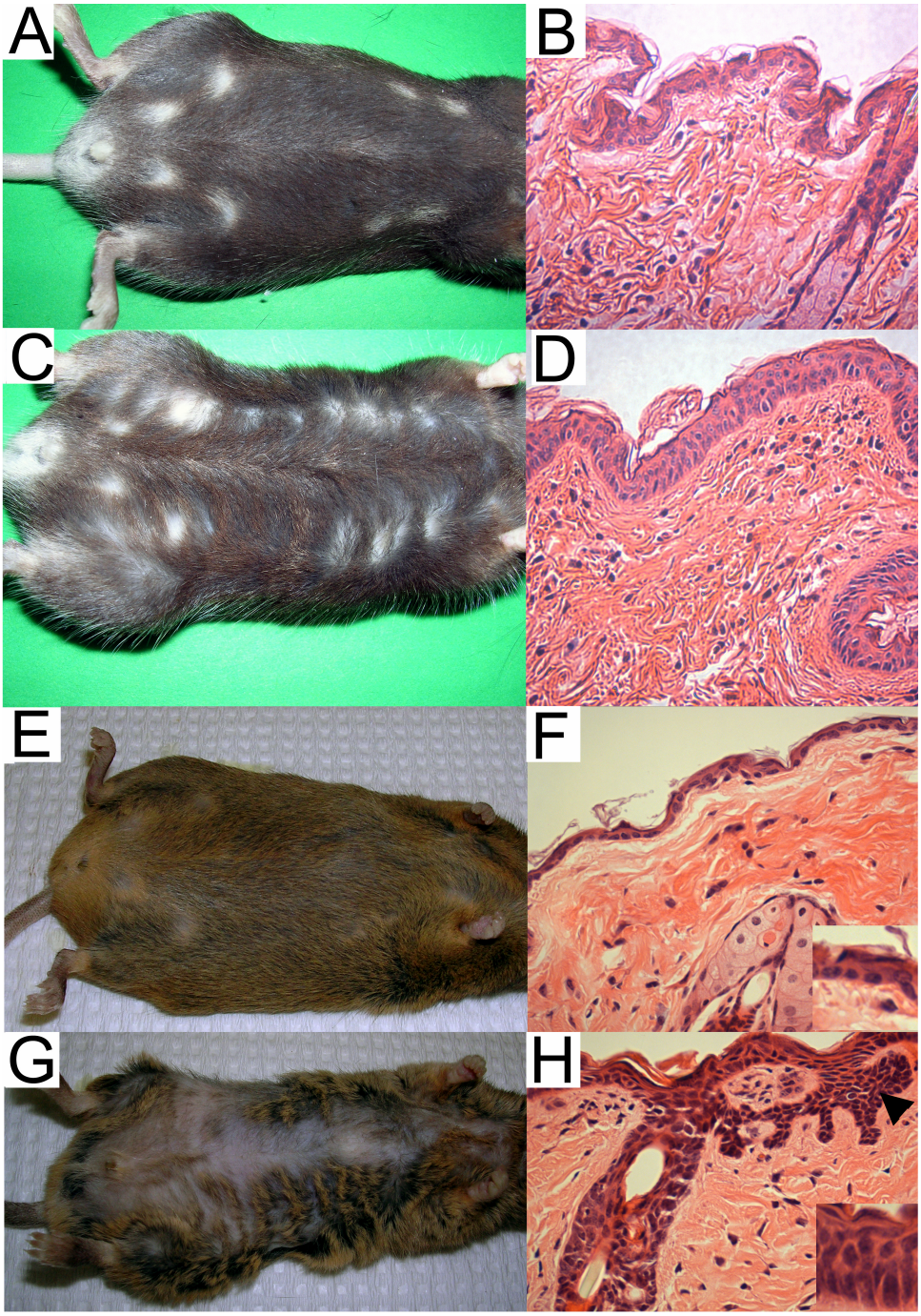


Figure 2 *K14-cre; Ift88*$^{fl/n}$ and *K14-cre;Kif3a*$^{fl/n}$ animals display progressive ventral alopecia with basaloid hyperplasia and epidermal ingrowths.
Both *Kif3a* (C) and *Ift88* (G) epidermally disrupted animals have a "ridged" disorganized hair appearance with progressive ventral alopecia from weaning onward compared to wildtype littermates (A, E) (shown are P159 (A-D) to P730 (E-H). H&E staining shows basaloid hyperplasia with palisading morphology changes (H,inset) compared to thin normal mouse epidermis (F, inset) accompanied by epidermal ingrowths resembling hair germs (H, arrowhead).

ingrowths resembling ectopic hair buds are seen, particularly in the ventrum, both interfollicularly and arising from nearby hair follicles (Figure 2 h).

Epidermal Marker Analysis Confirms the Histological Analysis and Suggests Pathways Involved in the Hyperplasia and Ingrowths.

Markers for the different layers of the epidermis were used to further characterize the source of the hyperplasia and epidermal ingrowth phenotypes (Figure 3). Analysis of Loricrin and K1 expression indicates that the granular and spinous layers of the epidermis are not markedly hyperplastic; however, K1 staining is discontinuous in some areas of the conditional mutants. In contrast, staining the skin with antibody against the basal marker K5 revealed that this region of the epidermis is significantly expanded 3-4 fold that seen in wildtype mouse epidermis. Normally, the basal layer consists of a monolayer of cells. Together these data indicate that the primary source of the hyperplasia is the basal keratinoctye population. The lack of expansion of the spinous layer in the cilia mutant mice is in contrast with many other hyperplasia models and suggests that the primary source of the hyperplasia is from the basal keratinocytes /interfollicular stem cell population. These data are similar to the basaloid hyperplasia and interfollicular stem cell expansion observed in mice with C-terminal truncation mutations in the Shh receptor patched1 (Nieuwenhuis et al., 2007).

Our analysis revealed several markers that are ectopically expressed. This was observed for K6, which was expressed in patches in the interfollicular epidermis, and K17,

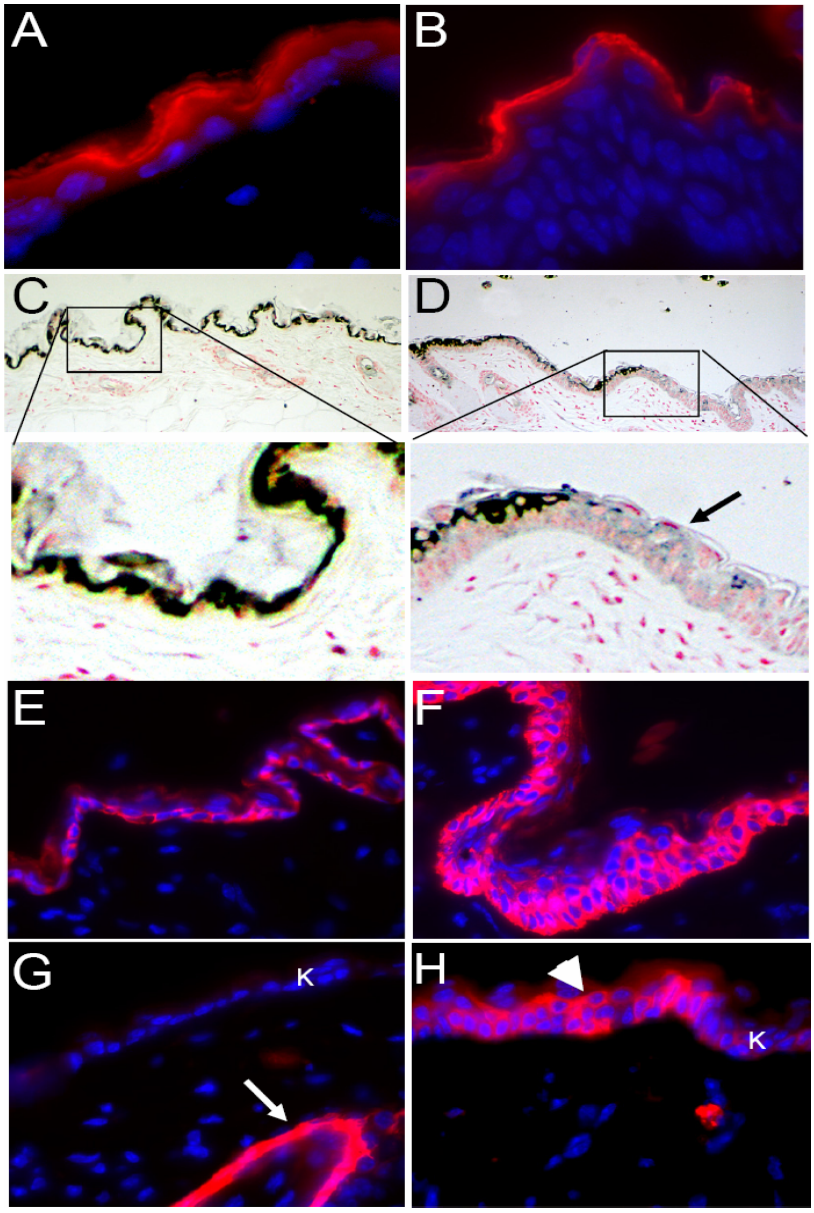


Figure 3 Marker analysis of epidermal hyperplasia showing expanded K5 staining, a lack of corresponding spinous layer expansion, regions of sparse K1 staining, and ectopic K6 cell populations.

Marker analysis of Wt (A,C,E,G) and *K14cre; Ift88*$^{fl/n}$ (B,D,F,H) shows normal granular/stratum corneum layer staining in Wt and Mt animals (A,B) as seen by immunofluorescence imaging with anti-loricrin antibody (red) and Hoescht nuclear staining (blue). Wildtype (C) mice have a single continuous layer of K1 positive, spinous layer epidermal cells as detected by anti-K1 antibody and ABC immunohistochemistry with nuclear fast red counterstain. Mutant mice (D) with epidermally disrupted cilia have a single discontinuous layer of K1 expression suggesting a lack of corresponding expansion in this cell layer and possible differentiation defects. K5 staining (E,F red) is expanded, and K6 is ectopically located in the interfollicular epidermis in the Mt (H, arrowhead) as opposed to the normal localization in the inner root sheath of the hair follicle (G, arrow). K= interfollicular keratinocytes.

which was expressed throughout the interfollicular epidermis of the conditional mutants, (Figures 3 and 4). In normal mice, K6 expression is restricted to the companion layer and K17 to the outer root sheath of the hair follicle. Ectopic expression of these keratins is often associated with an abnormal proliferative or "activated" keratinocyte response.

Furthermore, K17 is also expressed in basal cell carcinoma and basal follicular harmartomas. The expansion of K17 in our mutants is similar to that seen in mouse models with upregulation of Shh signaling such as those where an activated form of smoothened (Δ*K5 M2SMO*) is expressed in the basal cell layer of the epidermis (Grachtchouk et al., 2003). K17 is also observed in wildtype hair germs and the ectopic hair buds observed in conditional mouse mutants with an epidermally activated form of smoothened. These data support the conclusion that K17 may be a target for the Shh and Wnt signaling (via Lef1) pathways (McGowan and Coulombe, 1998).

Axis of Division in Cilia Disrupted Epidermis

The mechanism by which stratification and keratinocyte differentiation is regulated in the skin remains a matter of controversy. In one model, division of a stem cell leads to a transiently amplifying progenitor cell that rapidly divides and subsequently delaminates from the basal membrane and then initiates the differentiation program. In the other model, an asymmetric division of a stem cell in the basal layer results in a mother cell that remains in the basal layer and a daughter that losses contact with the basement membrane resulting in stratification of the epidermis and also initiates terminal differentiation. This asymmetric

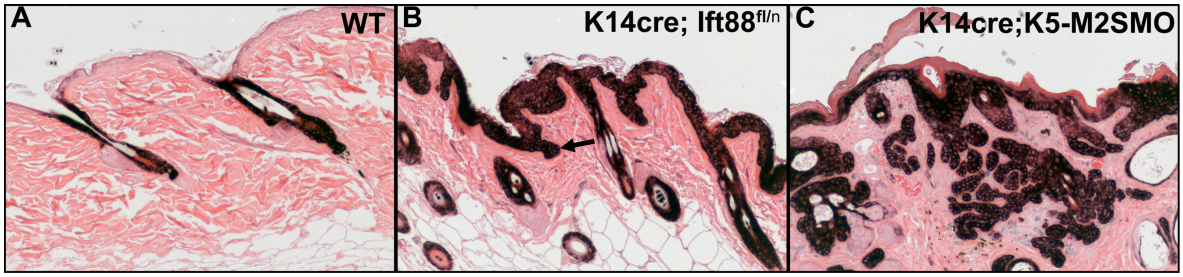


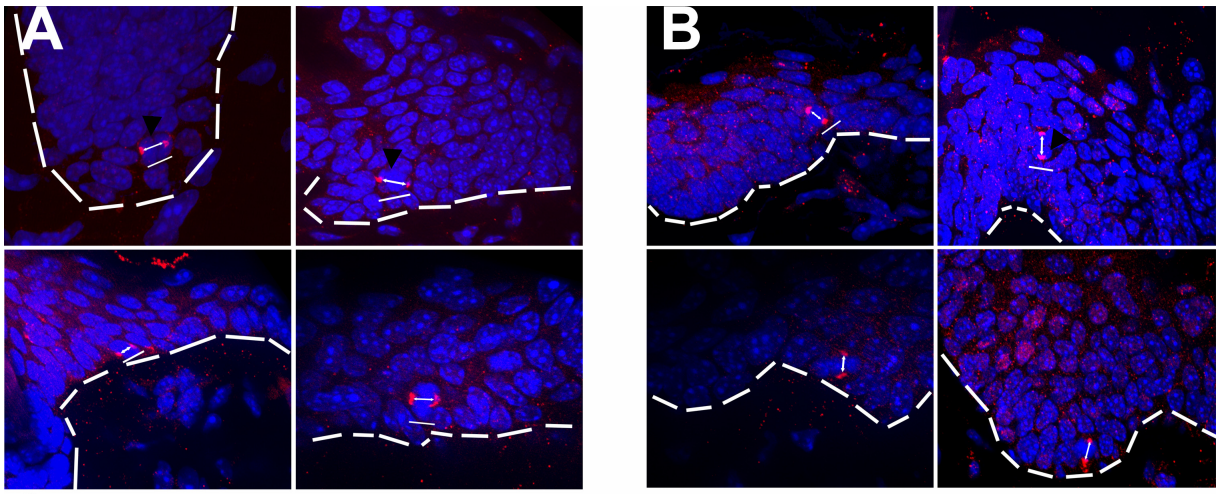
Figure 4 Immunohistochemistry staining for K17 showing an expanded localization of K17 staining throughout all the layers of the follicular and interfollicular epidermis.

Adult wildtype mice (A) have a normal pattern of K17 staining in the outer root sheath of the hair follicle. No staining is observed in the interfollicular WT epidermis. *K14 cre;Ift88*$^{fl/n}$ mice (B) with epidermally disrupted IFT have ectopic expression of K17 throughout all layers of the epidermis and in ectopic hair buds. This K17 expression is comparable to that observed in *K14cre; K5-M2SMO* mice (C) with activated Smo in the K5/K14 distribution.

division in the skin was noted as early as 1970 (Smart, 1970). According to studies by Lechler and Fuchs, the orientation of these divisions is temporally regulated. During midgestation (~E12.5), while the epidermis is single-layered, most cell divisions occur parallel to the membrane resulting in two equal cells. However, when stratification is initiated, the mitotic spindles orient such that the cleavage results in an apical to basal or perpendicular division relative to the basement membrane, releasing the newly formed daughter cell into the suprabasal layer. For a short period of time during gestation, these suprabasal cells are able to proliferate and expand the epidermis. These divisions in the suprabasal layer do not occur in the postnatal skin, with only cells in the basal layer being proliferative.

Studies by Lechler et al. demonstrate that spindle orientation is associated with asymmetric distribution of nuclear mitotic apparatus antigen (NuMA), a component of the PAR3/PAR6/aPKC complex, and that localization of NuMA can be used to assess perpendicular or parallel (to the basement membrane) division of basal keratinocytes. Since a similar mechanism has recently been proposed for cyst formation in the kidney of the cilia mutants, we analyzed NuMA localization in adult epidermis to evaluate whether an alteration in orientation of cell divisions could be responsible for the expansion of the K5 basal layer in the adult cilia mutants. Based on data from Fuchs' laboratory, a large majority (85%) of the cell divisions in the epidermis of adult mice occur perpendicularly (Lechler and Fuchs, 2005; Fuchs, 2008). These adult wildtype mitotic events are quite rare and technically daunting to evaluate.

By contrast, in adult epidermal cilia mutant mice the orientation of the spindle, based on NuMA localization, was random (ratio of ~55%, Figure 5a-c). We also noticed a number



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	Cell counts		Ratios		Total Number of NUMA+ Figures	References
	Perpendicular divisions	Parallel divisions	Perpendicular divisions	Parallel divisions		
K14cre;Ift88$^{fl/n}$	10	8	56%	44%	18	
Wild type			85%	15%	20	Lechler T, Fuchs E. 2005.*Nature* 437:275-280.

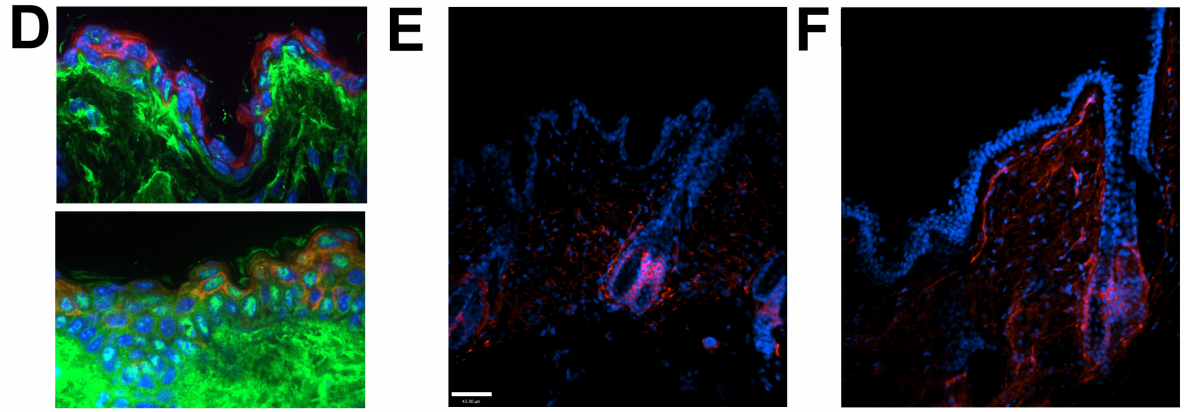

Figure 5 Alterations in the progenitor/stem cell population in Wt and ciliary disrupted Mt epidermis and follicles and axis of orientation of division defects in Mt interfollicular epidermis.

NuMA (Nuclear Mitotic Apparatus antigen) stain (red) and Hoescht (blue) nuclear staining showing mitotic figures and cell divisions parallel (A) and perpendicular (B) to the basement membrane (dotted line). Note the suprabasal figures in (A, upper left, B, upper right). The ratio of perpendicular to parallel divisions is abnormal in Mt epidermis (C). p63 staining (green) is expanded to suprabasal layers (K1, red) (D) in the Mt (bottom) epidermis compared to Wt (top). CD34 expression is found in a similar domain in Wt (E) and Mt (F) follicles. No interfollicular epidermal CD34 is observed Scale bar is 40 μm.

of mitotic cells in the suprabasal expanded layer of the cilia mutants, which is not found in normal wildtype skin. Thus, the misorientation of the cell divisions along with altered proliferative regulation in the skin is similar to what occurs with cyst formation in the kidneys of cilia mutants and suggests that there may be a common mechanism connecting these phenotypes. Furthermore, these finding may have implications for ciliary function in regulating or maintaining the interfollicular stem cell or progenitor populations, which has also been reported for neural progenitors (Chizhikov et al., 2007).

Stem Cell Markers in Cilia Disrupted Epidermis

Consistent with these NuMA findings was expansion of the p63 cell population in the conditional mutant epidermis (Figure 5d). p63 contributes to the maintenance of basal layer progenitor/stem cells and contributes to the regulation of epidermal proliferation and differentiation. Increased levels of p63 can also block differentiation of basal keratinocytes (Honeycutt et al., 2004; Koster and Roop, 2004). Loss of p63 expression results in defects in proliferation and mitotic spindle orientation where all divisions occur parallel to the basement membrane preventing stratification, and is a microRNA target for repression of "stemness" (Lechler and Fuchs, 2005; Yi et al., 2008). In contrast to the interfollicular progenitor and stem cell pool, the follicular stem pool, as labeled by CD34, (Figure 5e-f) is not similarly expanded in the conditional mutants. This may suggest against a role for epidermal cilia in follicular stem cell maintenance.

Phenotypes Observed in Activated Smoothened Compared to Mice with Conditional Loss of IFT Function

Several of the phenotypes that we observed are consistent with the idea that loss of cilia may be associated with an increase in Shh and/or canonical Wnt signaling. To begin analyzing this possible connection, we compared the phenotype of the cilia mutants to that observed when smoothened is ectopically activated in the basal layer of the epidermis. We crossed *K14-cre* mice with the mouse strain *K5-flxGFP-M2SMO* (hereafter referred to as *K5-M2SMO*) which expresses an activated form of Smo in the basal cell population when exposed to Cre activity. There were remarkable similarities observed with the cilia mutants and activated Smo mice. Both activated smoothened and conditional IFT disrupted epidermis ectopically express K17 (Figure 4). Additionally both mice have an expanded K5 layer (Figure 5 and (Hoseong Yang et al., 2008)), a "scruffy" appearance, and ectopic hair bud formation both interfollicularly and in association with nearby follicles (Figure 6).

β-catenin Signaling in Epidermal Cilia Disrupted Mutants and Activated Smoothened Mice

β-catenin is normally expressed at the membrane in the interfollicular epidermis with little detectable nuclear accumulation. In contrast, nuclear β-catenin is normally present in developing hair follicle placodes and associated dermal condensates. However, both *K5-M2SMO* mice and mice with activation of the Wnt pathway develop different ectopic hair bud phenotypes which both exhibit nuclear β-catenin localization (Gat et al., 1998; Van Mater et al., 2003; Lo Celso et al., 2004; Hoseong Yang et al., 2008). Furthermore, several

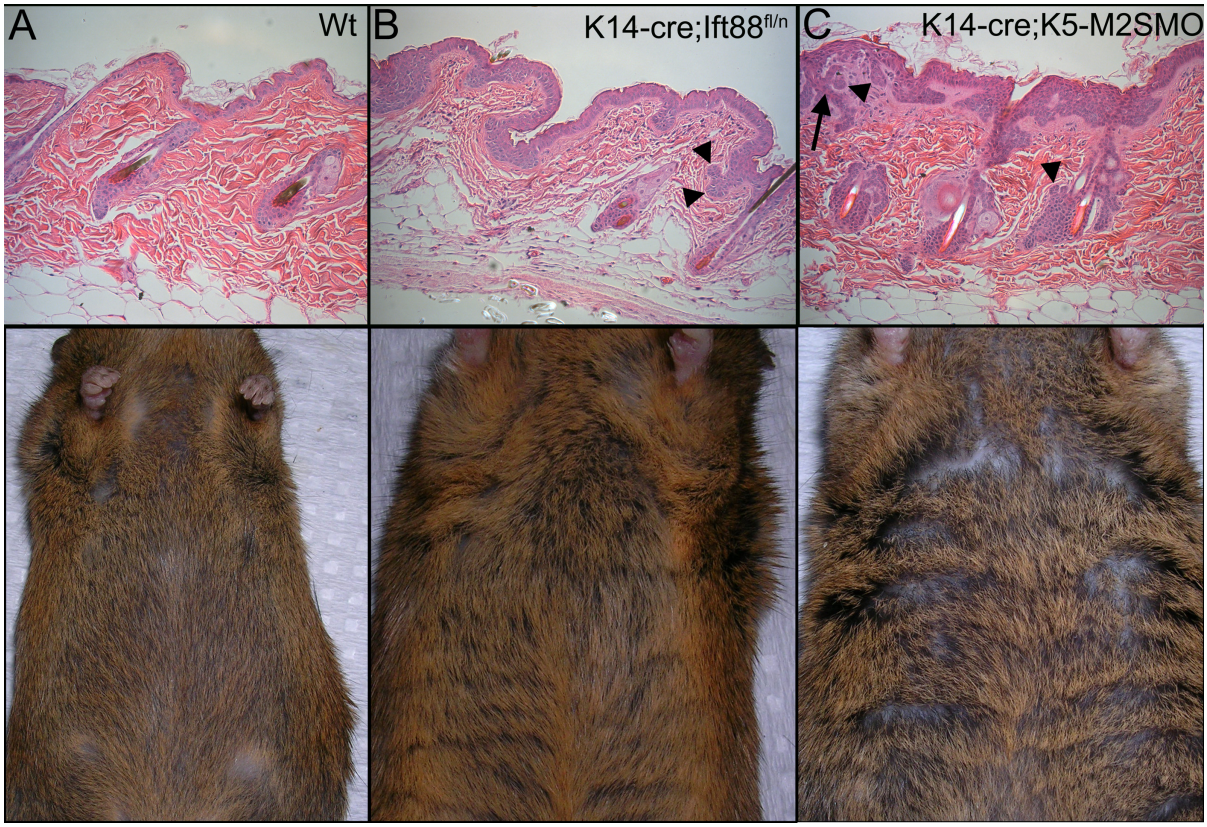

Figure 6 Comparison of an activated Smo phenotype and the disrupted *Ift88* phenotype with identical Cre drivers.
Wildtype epidermis (A, top) is thin with normal appearing follicles. The wildtype ventral pelage coat appears lush (A,bottom). Epidermal ciliary disrupted mice (B, top) have epidermal hyperplasia and hair bud like ingrowths (arrowheads). The ventral coat (B, bottom) appears ruffled and disorganized. Mice with activated Smo (*K14-cre;K5-M2SMO*) expression in the epidermis (C, top) have a thickened epidermis with ectopic hair buds (arrowheads) and follicular growths (arrow). Ventral fur is also somewhat disheveled and has mild alopecia (C, bottom).

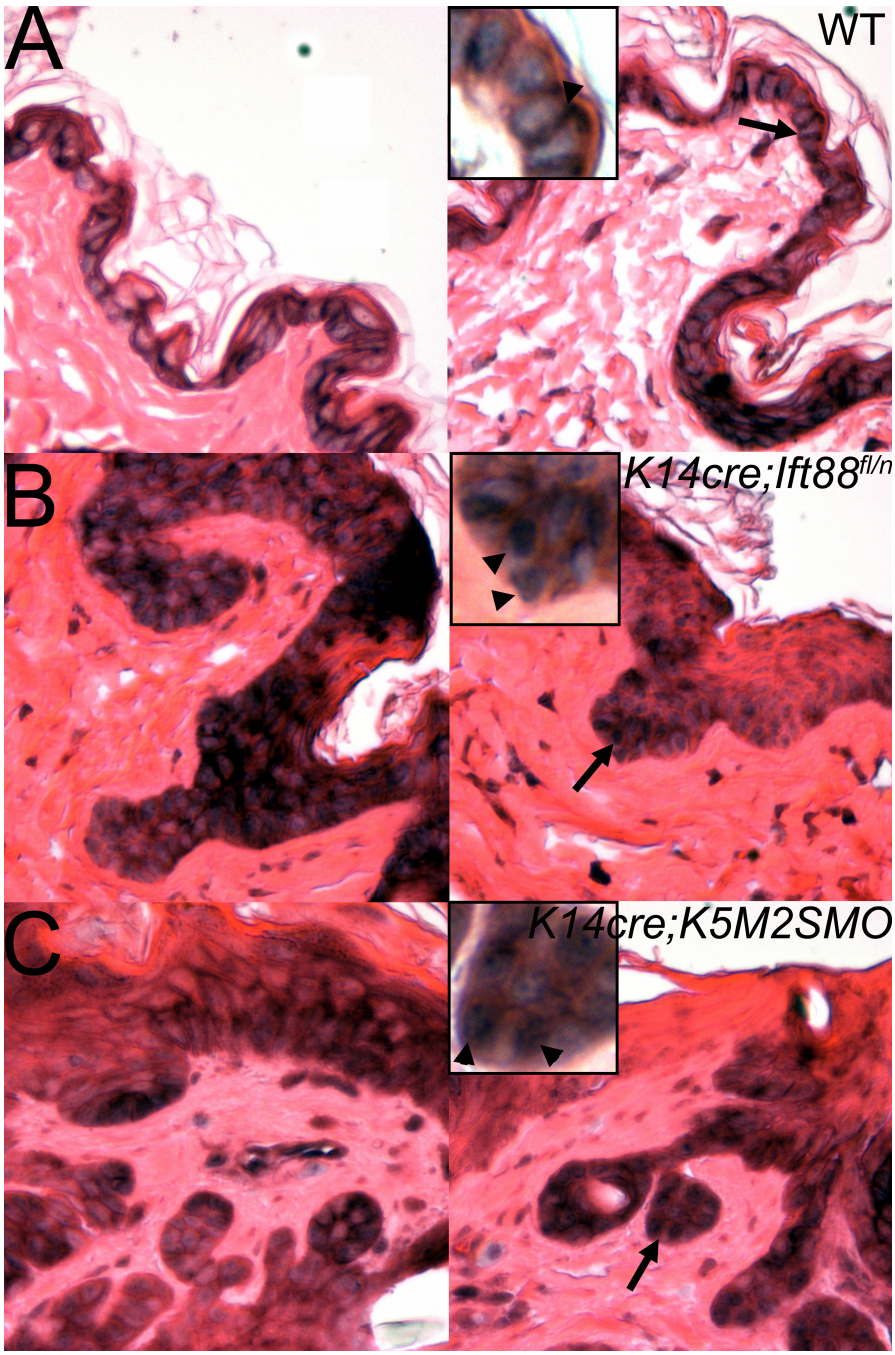

Figure 7 Immunohistochemistry staining for β-catenin shows increased nuclear staining in regions of both *K14cre;Ift88*$^{fl/n}$ and *K14cre;K5-M2SMO* epidermal ingrowths. Immunohistochemistry staining (Nickel enhanced DAB)for β-catenin in WT (A), *K14cre;Ift88*$^{fl/n}$ (B), and *K14cre; K5M2SMO* mice (C) shows only membrane staining in interfollicular WT epidermis (A,inset) with nuclear staining present in the IFT disrupted (B, inset) and activated smoothened (C,inset) ectopic hair buds. Section is eosin counterstained.

studies have demonstrated that cilia play an important role in regulation of canonical and noncanonical Wnt signaling activities (Germino, 2005; Gerdes et al., 2007; Corbit et al., 2008). These data led us to evaluate whether there are changes in β-catenin signaling associated with the epidermal cilia mutants using immunohistochemistry to stain for β-catenin (Figure 7). Our data indicate that either disruption of IFT in the basal cell population or activation of Smo leads to a marked increase in the accumulation of interfollicular nuclear activated B-catenin. These positive cells are particularly noticeable in ectopic hair buds observed in both *K14cre; Ift88*$^{fl/n}$ and in *K14cre;K5M2SMO* mice.

DISCUSSION

Potential Roles for Epidermal Cilia in Epidermal Homeostasis

Primary cilia have been identified previously in mammalian interfollicular epidermis, but their functional role and the full extent of ciliation in the epidermis has not been fully appreciated. Previous work in our laboratory has identified primary cilia as being present in epidermal cells of the hair follicle and interfollicular epidermis (Lehman et al., 2008a; Lehman et al., 2008b). This is in agreement with previous studies showing primary cilia present in epidermis and other stratified epithelium, particularly in the basal layers of stratified epithelium (Elofsson et al., 1984; Wandel et al., 1984; Warfvinge and Elofsson, 1988). This has led to speculation on their function ranging from light sensation to a contribution to control of cell division (Warfvinge and Elofsson, 1988). However, the early

embryonic lethality associated with IFT88 mutants and cilia loss in keratinocyte *in vitro* systems have prevented studies evaluating epidermal consequences of loss of cilia. Our analyses of mice with conditional disruption of IFT in the epidermis illustrate that primary cilia have roles in interfollicular homeostasis and in long term maintenance of the hair follicle unit.

The primary observed phenotypes in the conditional epidermal mutants consist of progressive ventral alopecia leading to a ridged or "scruffy" hair appearance, basaloid hyperplasia with alterations in the axis of division, and ectopic hair placode like structures arising from follicular or interfollicular epidermis. These phenotypes may reflect intrinsic roles for the primary cilia and IFT, as well as roles of IFT in signaling pathways involved in skin homeostasis such as the Shh and canonical Wnt pathways.

Characterization of the Hair Phenotypes Seen Associated With Loss of Epidermal IFT

The relatively mild follicular phenotype is surprising considering that multiple groups have linked Shh signaling and primary cilia. Data from dermal fibroblast , neuronal cell populations, and cell culture models suggest that primary cilia are essential for Shh responsiveness and Smo activation (Corbit et al., 2005; Haycraft et al., 2005; Liu et al., 2005; May et al., 2005). Intact Shh signaling is well established as being vital for normal hair follicle morphogenesis and cycling (St-Jacques et al., 1998; Gritli-Linde et al., 2007). Thus, based on the established literature, loss of epidermal IFT function should result in profound alopecia or hypotrichosis.

We are unable to completely describe the mechanistic reasons for the unexpectedly mild hair phenotype. One exciting, but controversial possibility is that primary cilia are relatively dispensable in epidermal Shh signaling. Unfortunately, there have not been well characterized systems for the analysis of *in vitro* Shh activity in epidermal cells, and most epidermal cilia are lost in culture (Strugnell et al., 1996). Thus it is not known whether cilia are identically required for Shh signaling in the epidermis versus other tissues.

Other mouse models support the concept that cilia are somewhat dispensable in the epidermis. *Msx2-cre Ift88*$^{fl/n}$ mice with expression in most follicular matrix cells and portions of the interfollicular epidermis have a similar interfollicular phenotype in areas, but do not have the same extent of macroscopic alopecia seen in *K14cre:Ift88*$^{fl/n}$ animals (Supplemental figure 1) suggesting again that matrix cilia may be somewhat dispensable. Additionally, *Shh-cre; Ift88*$^{fl/n}$ animals with high levels of Shh-Cre expression in the developing and growing hair follicle do not have macroscopic hair phenotypes and have a normal pelage coat (data not shown). The fact that the *Tg(KRT14-cre)1Amc/J;Ift88*$^{fl/n}$ mice with early Cre expression share the mild alopecia phenotype also supports this interpretation of the data.

A corresponding alternative explanation for these phenotypes is that only a very small number of epidermal cells may need to be ciliated to maintain a hair follicle unit. The hair follicle is a robustly cycling structure and two thirds of the hair follicle is completely replenished from stem cells each hair cycle. The putative hair follicle stem cell population does not seem to be adversely affected in most epidermal conditional mutant follicles. CD34 positive cell levels looked comparable between mutant and wildtype follicles (Figure 5). This suggests against a direct effect of IFT loss on stem cell maintenance in the hair follicle

niche. The stem cell population may not be highly exposed to Cre activity and would therefore replenish the matrix cell population each new hair cycle. The progressive nature of the alopecia observed in these animals is consistent with this hypothesis. In this model, the progressive alopecia results from gradual loss of functional hair follicle units due to defects in Shh stimulated proliferation of matrix cells. This would prevent a subset of follicles from growing normally and lead to gradual loss of hair follicles along with accompanying cystic structures. This model does not address the question of follicle morphogenesis, however, and is thus unable to explain the mild alopecia in the *Tg(KRT14-cre)1Amc/J;Iift88*$^{fl/n}$ mice.

Comparing Different Hyperplasia Phenotypes and Signaling Pathway Activity

The hyperplasia observed in IFT disrupted epidermis is a basaloid hyperplasia with an expansion primarily of the basal layers with more differentiated cell layers relatively normal. This differs from many common hyperplasias that have defects or expansion in multiple layers of the epidermis, such as psoriasis. Other hyperplasias may present similarly with defects or delays in differentiation leading to an expansion of a particular layer of the epidermis. One signaling pathway which has been implicated in hyperplasia is Shh signaling.

Shh signaling is involved in proliferation in many organ systems and likely contributes to hyperplasia phenotypes by stimulating proliferation, though loss of Shh expression in the interfollicular epidermis usually does not result in profound interfollicular phenotypes and Shh signaling is not required for interfollicular development (St-Jacques et

al., 1998). The role of epidermal Smo in hair follicle morphogenesis and hyperplasia is particularly intriguing. Transgenic activation of Smo using a constitutively active mutation of Smo leads to abnormal ectopic hair placode formation, hyperplasia, and activation of the β-catenin pathway (Hoseong Yang et al., 2008). Surprisingly, ablation of Smo activity in the epidermis also leads to abnormal ectopic follicle formation due to active signaling in the dermis, and hyperplasia along with a complete loss of mature hair follicle formation(Gritli-Linde et al., 2007).

The hyperplasia phenotype observed in the IFT disrupted animals has multiple similarities to mice with basal layer activated Smo expression. These similarities include an expanded basal layer and ectopic expression of the Shh responsive K17 marker. This pattern of increased K5 and K17 distribution is also seen in the hedgehog based carcinoma, basal cell carcinoma (BCC) (Yoshikawa et al., 1998). Interfollicular β-catenin levels are also elevated in both the activated Smo and disrupted IFT models, particularly in regions resembling follicular ingrowths. These findings suggest an increase in either Shh or β-catenin signaling levels may contribute to this hyperplasia.

Differences Between the Hair Buds in Epidermal Conditional Mutants and Activated Signaling Pathway Mice

Unlike the hyperplasia and ingrowth phenotypes observed in the Smo conditional knockdown mouse models (Gritli-Linde et al., 2007), the ingrowths in both the IFT disrupted and activated smoothened animals lack associated dermal condensates (as seen by H&E

(figure 2 and 4) (Hoseong Yang et al., 2008). This also differs from the ectopic hair bud phenotypes observed with LEF1 or direct β-catenin activation in the epidermis which form ectopic hair buds complete with adjacent mesenchymal condensates. One major difference between the ectopic hair buds produced by IFT mutants and activated Smo or β-catenin models is that the IFT growths do not expand into tumors. Activated Smo mice develop ectopic hair buds without associated dermal condensates which progress to form hamartomas. Hamartomas are relatively undifferentiated hair follicle tumors (Hoseong Yang et al., 2008). Mice with prolonged activated β-catenin form hair buds which associate with dermal condensates and form rudimentary hair and trichofolliculomas and pilomatricomas, well –differentiated hair follicle tumors (Hoseong Yang et al., 2008). By contrast, the conditional epidermal cilia mutants develop increasing numbers of hair follicle buds, but never develop tumors, differentiated or otherwise, even over a 2 year period. This is an important distinction from these other models and suggests that the cilia conditional mutants have a unique signaling environment. This may reflect only mild activation of β-catenin or Shh signaling which is insufficient to result in tumor progression. Another likely possibility is that primary cilia interact with both activator and repressor elements of the Shh pathway. The phenotype associated with disruption of cilia in the epidermis would then more closely mimic dysregulation of Shh signaling as opposed to a pure activation phenotype such as the Activated Smo mice.

Abnormal regulation or activation of the β-catenin pathway is associated with a variety of primary cilia phenotypes. Abnormal β-catenin signaling is associated with PKD, and activation of β-catenin in the kidney is sufficient to cause cystic kidney disease (Saadi-Kheddouci et al., 2001). Additionally, the Shh and β-catenin pathways crosstalk in early

follicular development and studies on an activated Smo model found that the ectopic hair bud phenotype was due to indirect effects of the Shh activation on the canonical Wnt pathway (Hoseong Yang et al., 2008). This data combined with the nuclear β-catenin staining observed in the conditional epidermal cilia mutants suggests that the hair bud phenotypes observed in these mutants stem from abnormal β-catenin activation, possibly due to defective inhibition of canonical Wnt signaling by the non-canonical pathway as in Corbit et al. (Corbit et al., 2008).

A role for Primary cilia in Planar Cell Polarity and Axis of Division in the Epidermis

A potential contributor to the observed basal hyperplasia is an expansion of the progenitor cell or a stem cell pool. The expansion of p63 positive cells in the MT compared to wildtype (see figure 5) along with the expanded K5 population and suprabasal mitotic figures suggests that not only might there be a delay in differentiation, but also that there may be an expansion of the proliferating interfollicular stem cell population. One theory for the maintenance of the interfollicular stem cell population relies upon control of the axis of division of basal keratinocytes (Lechler and Fuchs, 2005).

Our analysis of NuMA staining in the epidermal IFT disrupted mutants is compatible with this theory and shows a dramatic difference in axis of orientation of divisions between epidermis with disrupted IFT, and those found in wildtype mice older than e12.5 (Lechler and Fuchs, 2005). This randomization of division frequency is abnormal in the mouse epidermis and only in early mouse epidermal development (~E12.5) is there a greater than

fifty percentage frequency of parallel division in the basal epidermis. This data supports the hypothesis that primary cilia and IFT are involved in regulation of the axis of division in a potentially organ system independent fashion. This data correlates well with data in a kidney system model showing defects in axis of orientation and planar cell polarity. In the case of the kidney model system, the randomized orientation resulted in an increased number of perpendicular divisions, leading to cystic structure formation from kidney tubules (Fischer et al., 2006; Patel et al., 2008). In the IFT disrupted epidermis, the normal state is a high degree of perpendicular division leading to a small progenitor/stem cell population, but randomization of orientation of division leads to an expanded progenitor cell population and subsequent hyperplasia. Wnt signaling has also been implicated in this process and it is not possible to completely differentiate potential Wnt effects on axis of orientation from independent IFT or cilia based effects.

A Role for Cilia in Stem Cell Maintenance

There are a variety of signaling pathways with an influence on the maintenance and differentiation of the interfollicular stem cell compartment. It is interesting to note that multiple other models involve an overproliferation of stem cells leading to hyperplasia followed by “burn out”, leaving a thin weak epidermis unable to adequately maintain the stem cell population (Waikel et al., 2001). This effect is not seen in mice with disrupted IFT, nor do these mice exhibit ulcerations or other signs of a defective interfollicular epidermis.

This suggests that the stem cell compartment is genuinely expanded as opposed to prematurely differentiating to result in hyperplasia.

The corresponding explanation for the non-depletion of the stem cell population is that the loss of cilia impacts the axis of orientation of division as stated previously and thus grows the stem cell pool. These larger numbers of progenitor cells "crowd" the epidermis leading to the basaloid hyperplasia and palisading morphology changes observed. It remains to be evaluated as to whether these changes and hyperplasia may predispose these animals for an increased susceptibility to chemically or radiation induced tumors. However, no animals evaluated developed signs of skin tumor formation even after approximately 2 years time, suggesting that the disruption of IFT is not innately tumorigenic.

A Comparison Between Dermal and Epidermal Disruption of IFT

There are dramatic phenotypic differences in mice with loss of IFT in the epidermis versus the dermis. Loss of cilia in the dermis leads to profound hypotrichosis with hair follicle phenotypes that closely resemble loss of Shh activity in the skin. Changes in canonical Wnt were not observed in this population. There were no observed interfollicular ingrowths or hyperplasia, though mild fibrosis was observed (Lehman et al., 2008a).

Disruption of IFT in the epidermis leads to a mild alopecia phenotype with an associated hyperplasia and ectopic hair bud formation in nearby follicles and the interfollicular epidermis. These differences are not entirely unexpected considering that while the dermal and epidermal populations in the skin communicate extensively, there are

intrinsic differences between the cell types and differential Cre effects may also contribute to the observed differences. For example, the dermal condensate is a largely non-proliferative cell population. Loss of Shh responsiveness in most of this cell population, which does not renew, is sufficient to prevent effective downstream signaling and is responsible for the Shh associated hypotrichosis phenotype observed in the dermal IFT disrupted mice. By contrast, the epidermal cells affected by the K14-cre are highly proliferative and contain a renewing stem cell population. Loss of even 80% of the Shh responsiveness of this cell population is not sufficient to prevent normal hair follicle cycling. Differences between the effects of cilia loss on these two cellular populations may also reflect differential expression of target genes such as Wnt5a, which is expressed in the dermal condensate, but not in the neighboring epidermal placodes.

Tying Together the Roles of Epidermal Cilia in Skin/Hair Homeostasis

Primary cilia have effects on many different pathways including Shh and canonical and non-canonical Wnt, which also crosstalk extensively in the epidermis. However, these data suggest that primary cilia play important roles in the epidermis and continue to mediate portions of signaling pathways in the epidermis resulting in ectopic hair buds, basaloid hyperplasia, and ventral alopecia. .

The ectopic hair bud formation phenotype is associated with activation of β-catenin and suggests that IFT is functioning in a similar capacity in the epidermis as in the kidney and other areas to regulate the balance between canonical and non-canonical Wnt pathway

activation. The "tilting" of the balance towards canonical activation would be sufficient for ectopic hair bud formation and is consistent with the observed data. The hair follicle phenotypes were unusually mild, considering the expected vital role of primary cilia in hedgehog signaling. The IFT disrupted phenotypes observed in the epidermis are also milder than most of those seen in activated Shh pathway mutants. A likely explanation may stem from the inherent redundancy of the epidermal hair follicle system with a renewing stem cell population and a highly proliferative matrix cell population, which may compensate for non complete loss of Shh reception. The phenotypes observed are also milder than those observed in activated transgenics or knockouts involving the Shh or Wnt pathways. This is not unexpected and reflects the fact that cilia may function at multiples points in these crosstalking pathways and a disruption of IFT would lead to a dysregulation of the two pathways as opposed to absolute repression or activation.

These data establish the vital role of the primary cilia in interfollicular homeostasis and regulation of the Wnt pathway in the epidermis. They also establish a link between polarity and axis of division studies performed in the kidney with the role of cilia/IFT in coordinating the axis of division in the stratification of the epidermis. This suggests at a potential intrinsic function of primary cilia in tissue organization in multiple organ systems and raises a new perspective on the primary cilium in the epidermis.

METHODS

Mice

The *Ift88* conditional allele, *Ift88*^{fl} (*Ift88*^{tm1Bky}), was generated at the University of Alabama at Birmingham and was described previously ((Haycraft et al., 2007)). The conditional *Kif3a* allele, *Kif3a*^{fl} (*Kif3a*^{tm2Gsn}), was obtained from Dr. Goldstein (UCSD) (Marszalek *et al.*, 2000). The *FVB-Tg(K14-Cre)8Brn* is referred in the text as *K14-cre* and was obtained from NCI (Jonkers et al., 2001). *Tg(KRT14-cre)1Amc/J* mice were obtained from JAX and have been extensively characterized previously ((St-Jacques et al., 1998; Gritli-Linde et al., 2007). *K5-flxGFPM2SMO* mice were obtained from Andrezj Dlugosz and generated as indicated (Allen et al., 2003). Conditional cilia mutant mice were analyzed on a mixed C57BL/6J × 129P2/OlaHsd genetic background. The experimental mice were generated using male *K14-cre* mice to avoid possible germline activity of K14-cre. Mice were genotyped by tail PCR as previously described (Yoder *et al.*, 1997; Murcia *et al.*, 2000). All animals in this study were maintained in AAALAC accredited mouse facilities at UAB and in accordance with IACUC regulations and protocols at the University of Alabama at Birmingham (UAB).

Immunofluorescence and Immunohistochemistry

Hair and skin tissue were obtained and analyzed via immunofluorescence microscopy as previously described (Lehman et al., 2008a). Antibodies used include anti-polaris/IFT88 (B1700, 1:1,000, (Haycraft *et al.*, 2007)), anti-acetylated α-tubulin (Sigma, St. Louis, Missouri, catalog no. T6793, 1:1,000), anti-β-catenin (Sigma catalog no. C2206, 1:1000), anti-K5 (Covance, Berkeley, CA, catalog no. PRB-160P, 1:1000), anti-K1 (Covance catalog

no. PRB-165P, 1:500), anti-K17 (IHC) 1:5000 (Pierre Coulombe) and anti-loricrin (Covance, catalog no. PRB-145P, 1:500). Nuclei were stained with Hoechst 33258 (Sigma). Immunohistochemistry was performed using the Vectastain ABC kit.

β-galactosidase assays

The location of Cre recombinase activity was analyzed in sections of mice doubly transgenic for the Cre construct of interest and the ROSA26 Cre-reporter, *R26R* (Zambrowicz *et al.*, 1997), using X-gal as a substrate.

NuMA assessment of mitotic figures

Mitotic figures were assessed as perpendicular or parallel divisions using the criteria described by Lechler et. al (Lechler and Fuchs, 2005).

Conflict of Interest

The authors state no conflict of interest.

Acknowledgements

We would like to thank the UAB Comparative Pathology Core for histology services. We thank Pierre Coulombe and Michael Ruppert for rabbit polyclonal anti-K17 antibody. We would also like to thank Andrezj Dluogosz for the generous gift of *K5-flxGFPM2SMO* mice.

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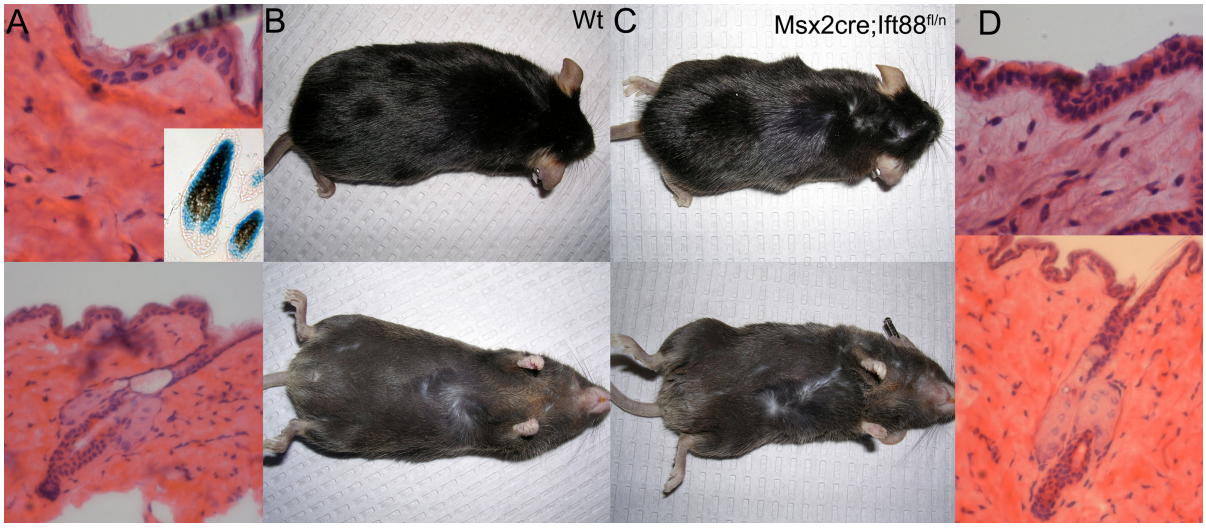

Supplemental Figure 1
Msx2-cre conditional cilia disrupted mice have Cre activity throughout the matrix cells of the hair follicle (A, inset). Wildtype mice have thin, normal epidermis (A, top) with hair follicles (A, bottom). Their dorsal and ventral pelage coat is lush and normal. *Msx2cre;Ift88*$^{fl/n}$ mice also have a normal appearing pelage coat (C), but have regions of hyperplasia (D, top) and epidermal ingrowths (not shown). Numerous normal hair follicles are also visible (D, bottom).

SUMMARY

Primary cilia are microtubule based organelles present on most cells in the human body. These organelles were initially described as an evolutionary throwback without function or implications for human health (Afzelius, 1995). This view changed dramatically with the identification of a link between defects in primary cilia function and polycystic kidney disease, one of the most common potentially lethal genetic diseases (Murcia et al., 2000; Pazour et al., 2000). The discovery of the essential nature of *Tg737/Ift88,* and the study of the embryonic lethal phenotypes when this gene is disrupted expanded the scope of the organs studied in association with IFT and cilia (reviewed in (Adams et al., 2007; Badano et al., 2006; Hildebrandt and Zhou, 2007; Lehman et al., 2008)). These studies also connected ciliary signaling to a variety of pathways including Shh, PDGFR-αα, and canonical/noncanonical Wnt. However, there were still several organ systems which had not been well characterized and for which the ciliary localization, or even whether cilia were present, was not well known. Studies in our lab and others implicated primary cilia in Shh signaling in the limb (Haycraft et al., 2007; Liu et al., 2005), so we evaluated unexplored organ systems in which Shh signaling plays a vital role. The hair follicle was an excellent example of such an organ system.

Despite some pioneering studies by Elofsson et al. , there was considerable controversy and much contradictory literature describing the extent of ciliation in the

epidermis (Elofsson et al., 1984; Roperto et al., 1999; Wandel et al., 1984). There was also very little characterization of cilia in the prototypical mammalian hair follicle. The hair follicle is also a system with extensive crosstalk and recurrent mesenchymal-epithelial interactions. This meant that understanding a possible role for the primary cilium in the hair follicle was an excellent research opportunity to expand our understanding of the contribution of primary cilia both to the hair follicle and mesenchymal-epithelial interactions in general.

Characterization of Primary Cilia Localization in the Hair and Skin

The overall goal of this work was to begin to characterize the roles of primary cilia in the hair and skin. My initial hypothesis based on the literature was that primary cilia would play important roles in signal transduction in the hair and skin. The vital first step in examining this hypothesis was to determine if IFT proteins were expressed in the skin and hair and if primary cilia were present. We initially utilized the *Ift88*^{tm1Rpw} mice which contain a lacZ knockin reporter for expression of IFT88. We found β-gal staining was present in most cell types of the hair and skin. Now that the expression pattern of IFT88, a vital component of primary cilia, was known in the skin, we were able to evaluate the presence of cilia directly using immunofluorescent antibodies against the mature ciliary tubulin, acetylated α-tubulin, and IFT88.

The hair follicle is a relatively large cellular structure and is approximately 40μm wide. The primary cilium is a small, singular organelle approximately 2-3 μm

long and may have many different orientations. This made it technically impossible to use even small section immunofluorescence microscopy to evaluate the extent of ciliation in the hair follicle. This required us to use new scientific tools to meet the technical challenge of reliably detecting a small primary cilium in a complex and large 3-D structure such as the hair follicle. Using confocal microscopy, Voxx2 3-D visualization software, and thick section immunofluorescence protocols optimized for the skin, primary cilia were found to be present throughout epidermal and dermal populations of the hair follicle. We were also able to identify differences in orientation between epidermal and dermal cilia. These data provided a road map for the further goal of determining the function of these primary cilia in cell populations of the hair follicle and skin.

The Roles of Primary Cilia in the Skin and Hair

A first step in identifying the function of an organelle or gene such as the primary cilia is to disrupt the gene or organelle function. Disrupting expression of IFT88 or the motor protein KIF3A is sufficient to disrupt the function of the entire organelle. As described above, IFT88 and cilia function are essential for normal embryogenesis. Thus, we used the conditional Cre-lox system to disrupt cilia in only specific cell populations. The two primary cell populations responsible for the morphogenesis of the hair follicle are the epidermal placode and derivatives, and the dermal condensate which becomes the dermal papilla. Disrupting either of these cell populations allows us to assess the role of

primary cilia in hair follicle morphogenesis and any signaling changes in either neighboring cell population. The (*Tg*(*Prx1-cre*)^{1Cjt}) mice which were used for the study of primary cilia and skeletal and limb development have early expression in the ventral dermis and limb bud (Haycraft et al., 2007; Logan et al., 2002). This mouse was crossed to a floxed *Ift88* or *Kif3a* containing mouse line and used to study the effects of ciliary disruption in dermal cells, including the dermal condensate, while a *K14-cre* with expression in the epidermis and hair follicles was used to study cilia and the epidermal portions of the hair and skin.

An Essential Dermal Role for Primary Cilia in Hair Follicle Morphogenesis

These mice with dermally disrupted IFT88 or KIF3A developed a profound hypotrichosis, or reduction in hair formation phenotype. Most follicles in the Cre expression region are arrested at stage 2 of development (hair germ) and fail to develop hair. They also had severe defects in dermal condensate maintenance, suggesting that cilia were important for the signaling which maintains this cell population. This data showed that dermal primary cilia are essential for normal hair follicle development.

This stage 2 follicular arrest was a striking phenotype considering that it mirrored phenotypes seen in mice with disrupted Shh signaling (Mill et al., 2003; St-Jacques et al., 1998). However, previous literature had suggested that Shh downstream signaling was only important in the epidermis. This was based on studies using a disrupted *Gli2* mouse which normally results in an arrested follicle phenotype. However, this phenotype is

rescued when transgenic Gli2 is expressed solely in the epidermis (Mill et al., 2003). Our data suggested that dermal primary cilia were also important in Shh reception in the dermis and reduced Gli1 levels were observed by QRT-PCR in microdissected dermal condensates from dermal cilia disrupted mutants. This data suggests that there may be a Shh mediated signal to the dermis which is important for dermal condensate maintenance and hair follicle development, but which does not require Gli2. One possible target based on Shh studies and expression patterns is Wnt5a (Reddy et al., 2001). The complete crosstalk and signaling interactions between primary cilia and the hair follicle remain incompletely understood, but this data established the dermal cilia as a vital player in the signaling and morphogenesis of the hair follicle.

The Role of Epidermal Cilia in the Hair and Skin

Epidermal cilia were disrupted by crossing the *FVB-Tg(K14-Cre)8Brn* K14-cre mouse with mice with floxed or deleted *Ift88* or *Kif3a* alleles. The mice were born with a normal appearance, but progressively developed a constellation of phenotypes including ventral progressive alopecia, epidermal ingrowths resembling ectopic hair buds, and interfollicular basaloid hyperplasia (expansion of the basal layer of the epidermis).

The relatively mild alopecia phenotype was particularly interesting considering that it is well established that epidermal Shh signaling is vital for hair follicle morphogenesis (Corbit et al., 2005; Haycraft et al., 2005; Liu et al., 2005; May et al.,

2005). Initially, we hypothesized that the relatively mild hair follicle phenotype was due to inefficient Cre activity. However, after crossing these mice with a alternative K14-cre made by Andy McMahon with early and well characterized Cre expression (Gritli-Linde et al., 2007; St-Jacques et al., 1998) the observed phenotype was extremely similar with only mild, primarily ventral, alopecia.

One intriguing interpretation of this data is that primary cilia may be somewhat dispensable for Shh signaling in the epidermis. This hypothesis is consistent with the observed mild phenotypes, but contrasts dramatically with the phenotype seen in an identical Cre used by McMahon's group to show that Smo was essential for hair follicle morphogenesis (Gritli-Linde et al., 2007). However, there have not been well characterized systems for the analysis of *in vitro* Shh activity in epidermal cells, and most epidermal cilia are lost in culture (Strugnell et al., 1996). Additionally the primary experiments showing a requirement for cilia in hedgehog signaling were performed in neuronal cells or fibroblasts (Chizhikov et al., 2007; Haycraft et al., 2005; Houde et al., 2006; Liu et al., 2005; Rohatgi et al., 2007). Thus it is not known whether cilia have identically required for Shh signaling in the epidermis.

The interfollicular hyperplasia phenotype was also unexpected and similarities in K5 and K17 labeling between epidermal IFT disrupted mice and mice with an activation of the Shh pathway suggest that Shh signaling may even be elevated in the epidermal mutants. This would be indicative of a dysregulation of Shh signaling as opposed to a classical repression or activation of the pathway. Wnt signaling also plays a role in the observed ectopic hair bud phenotype and nuclear β-catenin signal was observed at the hair buds of both Activated Smo and cilia disrupted mice. This shows cilia in the

epidermis are playing a role in the suppression of canonical Wnt signaling. This is similar to the schema described by Corbit et al. (Corbit et al., 2008) with cilia repressing canonical Wnt signaling by allowing its repression by non canonical Wnts.

The ectopic hair bud phenotype warrants special mention. Both activated β-catenin models and activated Shh models develop ectopic hair buds, but both models progress towards tumorigenesis. The β-catenin models progress towards a differentiated trichofolliculoma, while the Smo progress towards a less well differentiated follicular hamartoma (Hoseong Yang et al., 2008). Epidermal cilia mutants do not progress towards tumorigenesis, even after two years. This suggests that the loss of primary cilia may somehow dysregulate these pathways in such a fashion as to result in mild activation, but prevent or repress full activation. This phenotype should be explored further and has implications for our understanding of the primary cilia and carcinomas or even carcinoma treatment.

Another contributor to the hyperplasia is an aberrant axis of division phenotype which occurs in these mutants. Their basal keratinocytes divide in a parallel fashion more often than is normal, which over-replenishes the stem cell population and leads to basaloid hyperplasia and expansion of the potential progenitor/stem cell population as seen by an expansion of p63 immunostaining. This randomization of division is similar to that observed in the kidney with other IFT disrupted models and potentially places the primary cilia in a vital position in stem cell regulation in the epidermis.

Conclusions and Future Directions

This work set forth to illuminate an unknown facet of cilia biology in the hair and skin. Herein we have established that most cells in the hair follicle are ciliated and utilized 3-D confocal microscopy to visualize cilia and their orientation in the hair in three dimensions for the first time. We have also shown that dermal primary cilia are essential for hair follicle development and that epidermal cilia have different but important roles in interfollicular homeostasis. However, there are still many experiments to be done.

It is known that primary cilia are essential for hair follicle morphogenesis and that they function via a Shh mechanism in the dermis, but the precise interplay of signaling molecules responsible for the dermal Shh phenotype is still not understood. Further characterization by assessing the expression of Wnt5a and PDGFRα could be useful, though both are Shh targets. Epistasis experiments with activated Gli2 in the epidermis and disrupted cilia in the dermis could also help characterize the interaction between the epidermis and the dermis, at least as regards the Shh pathway.

Another important experiment would be to replicate the prx1-cre experiments with a more specific cre promoter such as Alx4, which has skin expression primarily in the dermal papilla (Hudson et al., 1998). This would completely determine if the phenotype is due to defective recruitment to the dermal papilla as well as loss of Shh responsiveness in this population. Use of a tamoxifen responsive system would determine if cilia are necessary during cycling of the hair follicle, as opposed to solely during hair follicle morphogenesis.

There are multiple interesting questions to be explored with epidermal cilia disrupted mice as well. These mice have an ectopic hair bud phenotype which does not precisely match either the phenotypes seen in activated Shh pathway or β-catenin models. The loss of primary cilia seems to results in an intermediate phenotype which never progresses to tumorigenesis. Assessing the Shh signal status of these mice via QRT-PCR or semi-quantitative PCR would be an important step in determining the reasons behind the mild phenotype. This result leads to the conclusion that primary cilia may be coordinating these two pathways in the epidermis or other cell populations. One method of testing this hypothesis would be to mate these epidermal cilia disrupted mutants with a DKK1 (a Wnt antagonist) expressing line. This would abrogate Wnt signaling and determine if the ectopic hair placodes or hyperplasia phenotypes change with Wnt signaling.

A faster and more convenient method to assess this Wnt/Shh question would be to move to cell culture, at least in dermal cells. Transfected Wnt or Shh reporters on dermal or epidermal cells could be used to assess the response of ciliated and non-ciliated cells to Wnt or Shh ligand in culture. With the combination of the *Prx1-cre* and *K14-cre*, raft culture would become possible and signaling changes in one cell population could be evaluated when the other cell population loses its cilia These studies could help to further our understanding of the relationship of cilia to Wnt and Shh signaling.

The biggest potential expansion on the epidermal work may come from evaluating the impact of loss of primary cilia on tumorigenesis and carcinogenesis models. The epidermal cilia disrupted mutant mice have an expanded potential progenitor cell population and hyperplasia. An expanded stem cell population may

predispose these animals to develop carcinoma either in response to an initiating mutation or chemical mutagenesis, or in response to a tumor promoting agent. Alternatively, mice with disrupted cilia in the epidermis may have a resistance towards the development of carcinoma in response to chemical or UV treatment. This work would be particularly relevant since many carcinomas are ciliated, including basal cell carcinoma (Wilson and Mc, 1963). It is important and valuable to know what signaling contribution these cilia make in these carcinomas. Depending on the results of this study, further characterization could proceed by crossing cilia deleted mice to cancer models in the epidermis such as activated Gli2 transgene expressing mice. A highly efficient Cre driver would be necessary for this experiment and it would be complicated by the fact that the cells in portions of the mouse may only express one transgene and "escape" the potentially anti or pro tumorigenic effects of loss of primary cilia.

At the start of this work, the term cilia in the field of dermatology was more likely to refer to an eyelash than an organelle, but hopefully the work in this dissertation will begin the long process of bringing the primary cilium into the world of dermatology.

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APPENDIX A

The ORPK mouse has abnormalities in multiple other tissues that have yet to be analyzed. One of these phenotypes is the scruffy appearance of the fur that was noted in the initial manuscript describing the ORPK mouse (Moyer et al., 1994). Skin and hair abnormalities in ORPK mice are present at birth before onset of severe disease phenotypes in other tissues, thus, it is unlikely to be caused by other factors associated with the general health of the mutants, although health status may contribute to the expressivity. It is highly penetrant on the FVB/N background, but has variable expressivity, as seen in several other phenotypes in the ORPK mutants (Moyer et al., 1994; Sommardahl et al., 2001), with a few animals being mildly affected and others having flaky skin and sparse hair (Fig. 1).

We have determined that primary cilia are present on most cells in the developing and cycling hair and skin with the exception of more differentiated cells in the stratum corneum and granular cell layers of the epidermis and the differentiating hair shaft (Fig. 1D-G;(Lehman et al., 2008)). Similar to that seen in other tissues of the ORPK mouse, the cilia on cells in the hair follicle and skin are stunted. Histologically, the skin of ORPK mice is characterized by mild orthokeratotic hyperkeratosis and follicular dysplasia, with delayed follicular development, accumulation of keratinaceous debris in mildly cystic follicles, and sparse granulomatous or pyogranulomatous folliculitis (Fig. 2). This phenotype is evident by P17, and keratin-filled cystic follicles typically are still visible in

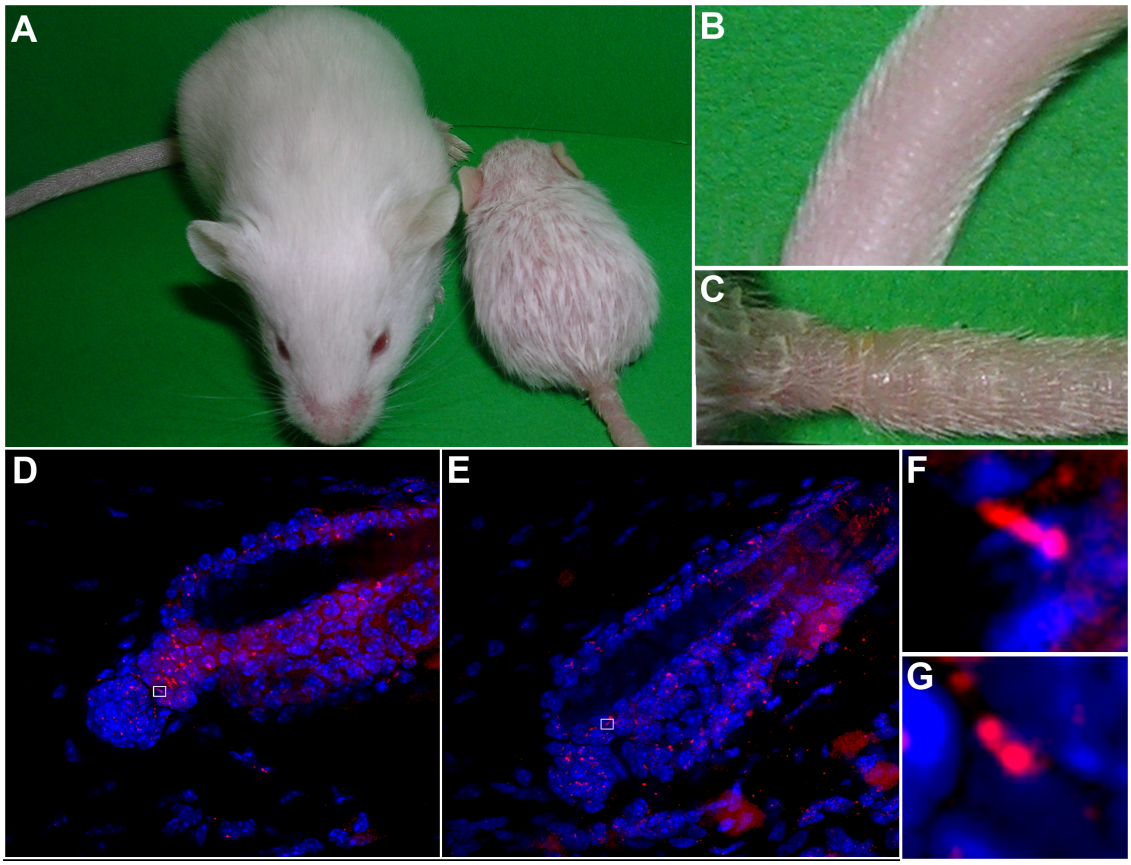

Figure 1 Gross epidermal and cilia phenotypes in the Oak Ridge Polycystic Kidney (ORPK) mouse.
A-C: Dry and flaky skin with sparse fur is noticeable in the ORPK mutant (A right, C) by postnatal day (P) 16 compared with its wild-type littermate control (A left, B). **D,E:** Primary cilia are found in the follicles of both adult wild-type (D) and ORPK mutant (E) hair follicles by three-dimensional immunofluorescence confocal imaging for IFT88 (red) and Hoescht nuclear stain (blue). **F,G:** Higher magnification images of the cilia found in the hair follicle from the boxed regions in wild-type (F) and ORPK mutants (G). As seen in other tissues, the primary cilia are stunted in ORPK mice compared with wild-type controls.

older (P25) mice (Fig. 2C, arrow). By P25, a delayed hair growth cycle also is evident in ORPK mice, in which the follicles remain in the first telogen (Fig. 2C), whereas follicles of wild-type mice have progressed to anagen (Fig. 2F). One possible explanation for the hair phenotype is that the early hair follicle delay may precurse later abnormal development that leads to hair shaft structural defects or twisted keratin fibers. These twisted fibers lead to intrafollicular keratin accumulation when the keratinocytes that form the hair shaft do not differentiate or proliferate properly, leading to follicular cysts, some of which rupture. Consistent with this hypothesis is that PDGF-$A^{-/-}$ transplanted skin develops abnormal hair shafts and keratinized cystic structures with defects in proliferation (Karlsson et al., 1999). Also, transgenic overexpression of Wnt3a in mice using the Keratin 14 promoter, which results in overactivation of β-catenin in the epidermis, also have accumulation of keratinaceous debris in the follicles and formation of follicular cysts (Millar et al., 1999). During catagen, when the hair follicle degenerates, these defects may lead to leakage of keratin, provoking a granulomatous reaction. The timing of this phenotype also suggests the possibility that the ability of the follicles to undergo the first anagen is impaired.

It is important to note that the normal transitions from anagen to catagen and telogen to anagen involve the PDGF receptor-alpha (PDGFR-α) pathway and that proper proliferation of keratinocytes during anagen requires both the PDGFR-α and Shh pathways (St-Jacques et al., 1998; Karlsson et al., 1999; Mill et al., 2003; Mill et al., 2005; Tomita et al., 2006). As discussed above, cilia are required for the normal activity of these pathways (Haycraft et al., 2005; Huangfu and Anderson, 2005; Liu et al., 2005; Schneider et al., 2005; Rohatgi et al., 2007). Indeed, real time quantitative polymerase

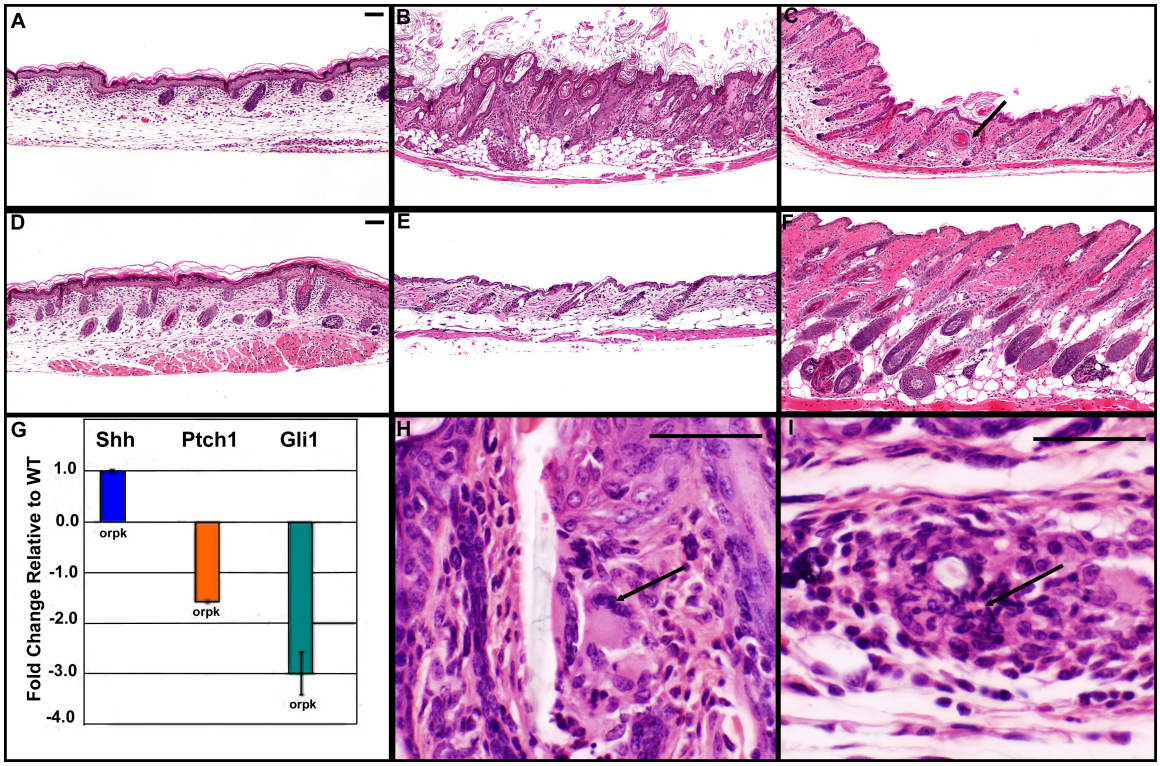

Figure 2. Skin and follicular abnormalities in the Oak Ridge Polycystic Kidney (ORPK) mouse.
A,D: At postnatal day (P) 1, ORPK mice have mild growth delay with reduced adnexa in early follicular development (A) compared with wild-type littermates (D). **B,E:** P18 wild-type littermates have normal follicles in telogen (E), whereas the skin of ORPK mice (B) is characterized by orthokeratotic hyperkeratosis, follicular accumulation of keratinaceous debris, and follicles in catagen. **H,I:** Some follicles rupture, leading to a giant cell inflammatory response (H, arrow) and foreign body granulomas (trichogranulomas; I, arrow). **C,F:** At P25, wild-type follicles (F) are in anagen, whereas the follicles of ORPK mice (C) remain in first telogen. **G:** Relative quantification of Shh pathway expression as determined by quantitative real time polymerase chain reaction standardized to 18S RNA. Relative fold change was calculated by the $2^{-\Delta\Delta CT}$ method by comparing the normalized gene expression level for each gene between the mutant and the control (±SD). *Shh* expression is identical between P14 ORPK skin and littermate controls, but downstream components of the pathway *Ptch1* and *Gli1* are down-regulated. Scale bars = 50 microns.

chain reaction (QRT-PCR) analysis of skin from P14 ORPK mice further supports this hypothesis. Although Shh expression levels are normal in the skin of ORPK mice as standardized to 18S RNA, activation of the pathway is impaired as determined by expression of downstream targets *Ptch1* and *Gli1* (Fig. 2G). These data indicate that the cilia defects in ORPK mice likely contribute to improper cycling or structural defects in the follicles. The precise molecular causes for the follicular dysplasia remain uncharacterized, but inasmuch as the ORPK mouse mutation is an *Ift88* hypomorph, it is likely that the Wnt, Shh, and PDGFRα pathways contribute to the phenotype.

These new findings strengthen the argument for clinical awareness of potential ectodermal dysplasia phenotypes in human ciliopathies. Potential examples of human ciliopathies with ectodermal dysplasia phenotypes are Ellis-van-Creveld syndrome (EVC) (Ruiz-Perez et al., 2007) and Sensenbrenner syndrome or cranioectodermal dysplasia (CED). A gene responsible for one form of EVC has recently been identified and found to encode a cilia protein (Ruiz-Perez et al., 2007), while the gene for CED has not been identified. However, the spectrum of phenotypes seen in CED patients suggests it will be an additional member of the ciliopathies. They includes tubulointerstitial renal disease, congenital hepatic fibrosis, skeletal defects, hypoplasia of the corpus callosum, and retinitis pigmentosa (Tsimaratos et al., 1998; Costet et al., 2000; Zaffanello et al., 2006). CED also has extensive ectodermal dysplasia features, including a sparse hair phenotype.

In summary, the ORPK mouse has served as a model for analyzing defects in a variety of human organ systems caused by ciliary dysfunction. In this communication, we extend this analysis to the "scruffy fur" phenotype and illustrate the continuing

applicability of this model for human disease in the rapidly expanding world of the ciliopathies.

This Appendix was excerpted from the following with license from the publisher.

Lehman JM, Michaud EJ, Schoeb TR, Aydin-Son Y, Miller M, Yoder BK. 2008. The Oak Ridge Polycystic Kidney mouse: Modeling ciliopathies of mice and men. Dev Dyn.

Format and errata corrected for dissertation

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APPENDIX B

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: March 18, 2008

TO: Bradley K. Yoder, Ph.D.
MCLM-652 0005
FAX: 934-0950

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Role of Cilia/IFT in Skin and Hair
Sponsor: NIH
Animal Project Number: 080308421

On March 26, 2008, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	A	2575

Animal use is scheduled for review one year from March 2008. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 080308421 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
B10 Volker Hall
1670 University Boulevard
205.934.7692
FAX 205.934.1188

Mailing Address:
VH B10
1530 3RD AVE S
BIRMINGHAM AL 35294-0019

FOR OFFICE USE ONLY		
Date Received:	Date Approved:	Animal Project Number (APN):

PROVIDE A COPY OF THE CORRESPONDING GRANT, CONTRACT APPLICATION, OR STUDY PLAN (PDF format is preferred), which should be consistent with the AUR but do not refer to it instead of responding to the questions below.

SECTION I: GENERAL INFORMATION (Questions 1-4)

1. Principal Investigator			
Name	Bradley K. Yoder	**Blazer ID**	Byoder
Department	Cell Biology	**Division**	
Office Address	MCLM 688	**Office Phone**	934-0994
Email	byoder@uab.edu	**FAX Number**	934-0950
Contact who should receive copies of IACUC correspondence (Optional)			
Name	**Mandy Croyle**	**Email**	**pydnam@uab.edu**
Phone	**934-0995**	**FAX Number**	**934-0950**

2. Application	
Project Title	Role of Cilia/IFT in Skin and Hair
Sponsor	NIH ACTS
Project Period (start date & end date)	04/01/2008
Previous APN	None
If part of a Program Project, SCORE, Center, etc:	
Name of PI	Yoder, Bradley K
Title	**Professor**

3. Will personnel other than the PI (e.g., faculty, staff, students, or fellows) be involved in the animal work being proposed? ☐ NO ☒ YES

If the response is YES, provide the name(s) below.

Name	Blazer ID	For Nonhuman Primate Users: Date of Confirmation of Negative TB Status
Mandy Croyle	Pydnam	n/a
Venus Roper	Vtchild	n/a
Jonathan Lehman	Lehman	n/a
Neeraj Sharma	Neeraj	n/a
Ajay Kale	Ajaykale	n/a
Erica Hamby	Ericab	n/a
Amber Oconnor	Aoconnor	n/a

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: December 3, 2007

TO: Bradley K. Yoder, Ph.D.
MCLM-652 0005
FAX: 934-0950

FROM: Judith A. Kapp
Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use COmmittee (IACUC) on December 3, 2007.

Title: Cilia and Cystic Kidney Disease

Sponsor: NIH

This institution has an Animal Welfare Assurance on file with the Office for Protection from Research Risks (Assurance Number A3255-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).

Institutional Animal Care and Use Committee
B10 Volker Hall
1670 University Boulevard
205.934.7692
FAX 205.934.1188

Mailing Address:
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1530 3RD AVE S
BIRMINGHAM AL 35294-0019

Faxed OHS 12-11-07

Page 1 of 2

Appendix G
Project Registration

OH&S USE ONLY
Project #

Instructions: Submit completed and signed form for each project to:
UAB Occupational Health and Safety, CH19 Suite 445. (FAX: 934-7487).
A notice that OH&S has received this form will be sent to the address you provide below.

A

Name (Principal Investigator/Title) Bradley K. Yoder/Professor

e-mail address byoder@uab.edu

Title of Project Cilia and Cystic Kidney Disease

Campus/Business Phone # 4-0994 **Address** MCLM 688

Location of Project MCLM 689 & 609, and LH 856 (Buildings and Rooms)

UAB Affiliation Cell Biology (Department, Center or Institute)

B

Check all that apply:
Status of this submission:

- ☐ New ☐ Continuation of OH&S # ____
- ☑ Renewal ☐ Resubmission of OH&S # ____
- ☐ Core ☐ Program Project

Check all that apply:
Funding source:

- ☐ Private Business in UAB facility; specify: ____
- ☑ External Grant, specify: NIH NIDDK
- ☐ UAB Internally funded, specify: ____

Original title if different from title in section A : ____

C

Instructions: Respond to **all** questions in Section C by checking "No" or "Yes" as it applies to **this** project.
For all "Yes" responses, complete the corresponding question on Page 2.

Does this project involve:	Check Response	
1. Radioisotopes	No ☑	Yes ☐
2. Carcinogenic/mutagenic/teratogenic chemicals	No ☐	Yes ☑
3. Highly toxic chemicals/drugs	No ☑	Yes ☐
4. Toxins or toxic products	No ☐	Yes ☑
5. Microbial agents or products (bacterial, viral, fungal, parasitic)	No ☐	Yes ☑
6. Human blood, body fluids, tissues, or human subjects	No ☑	Yes ☐
7. Recombinant DNA./RNA molecules	No ☑	Yes ☐
8. Animals (what species: Mus musculus)	No ☐	Yes ☑
9. Cell/Tissue Culture	No ☐	Yes ☑

PLEASE NOTE

Each Appendix G ***must*** *be accompanied by a brief project description that includes:*

- Overall project objective
- Specific aims
- If your Laboratory Safety Plan is not on file with OH&S, explicitly state personnel safety precautions and the reason for their selection
- A copy of the grant may be requested at a later date.

See OH&S web site for details
www.healthsafe.uab.edu

UAB Safety procedures applicable to the materials listed above are described in the *UAB Chemical Safety and Waste Management Manual, UAB Biosafety Manual, NIH Guidelines for Research Involving Recombinant DNA Molecules, UAB Radiation Safety Procedures Manual,* and the *UAB General Health and Safety Management Program Manual.*

The signature of the investigator below indicates that he/she is familiar with UAB safety policies relevant to any hazardous procedures or materials used in his/her laboratory and has incorporated safety training, practices, and procedures into the routine of his/her laboratory.

Date 12-11-07 **Signature** Bradley Yoder

OH&S Use Only Pending Review
RAD CHEM BIO

Revised 3-01

Appendix G – Project Registration

Personnel involved in the project Venus Childress Roper, Mandy Croyle, Neeraj Sharma, Ajay Kale, Jon lehman, Bradley K Yoder

☑ **Yes** ☐ **No** Personnel have received documented safety training.

If the response to any question in Section C of this form is "Yes", please **complete all parts** of the corresponding question in Section D. See Instructions for Completing Appendix G at our web site **www.healthsafe.uab.edu**

D

1. Radioisotopes used N/A

   Chemical Form _____ Used in: ☐ tissue culture ☐ animal tissue ☐ administered to animals

   ☐ **Yes** ☐ **No** Is this protocol on file in the UAB Radiation Safety? If yes, date approved _____

   Licensee Name _____ License Number _____ Contact _____ Phone # _____

2. Carcinogenic/mutagenic/teratogenic chemicals used Tamoxifen, BrdU (bromodeoxyuridne)

3. Highly toxic chemicals/drugs used NA

4. Toxins or toxic products used Tamoxifen, BrdU (bromodeoxyuridne)

5. Microbial agents/products used E. coli DH5alpha strain

6. ☐ Human blood ☐ body fluids ☐ unfixed tissues ☐ fixed tissue will be used.

   ☐ **Yes** ☑ **No** Is this a Human Gene Therapy protocol?

7. The host/vector system used in this project is NA.

   I have reviewed the NIH Guidelines and this project is ☐ non-exempt ☐ exempt according to Section _____ of the Guidelines. For non-exempt projects attach page 3 of Appendix H Registration Document of Recombinant DNA Research.

8. ☑ **Yes** ☐ **No** Will any of the agents listed in section C of this form be administered to animals?

   If yes describe the following:

   Agents administered Tamoxifen, BrdU

   Route of administration IP

   Place of administration (Building and room #) LH 888E, MCLM 689

   Animal Housing location LH 888E

   Precautions to be used to protect personnel and animals Lab Coats, Goggles, Face Masks, Gloves

   Animal disposal method/location BrdU - BrdU toxic waste in MCLM 609

   Bedding disposal method/location BrdU - BrdU toxic waste in MCLM 609

9. Check ✓ all that are used in this project:

☑ **Animal cell culture**	☑ **Continuous**	☑ **Primary**
☐ **Human cell culture**	☐ **Continuous**	☐ **Primary**

Complete these statements if this project involves microbial agents, recombinant DNA, human blood, body fluids, or tissues, human or animal cell culture or introduction of any of the above material(s) into animals or humans.

The laboratory biosafety containment level used for this project will be (check one):
☐ **BSL1** ☑ **BSL2** ☐ **BSL3**

The animal biosafety containment level used for this project will be (check one):
☑ **ABSL1** ☐ **ABSL2** ☐ **ABSL3**

THE UNIVERSITY OF
ALABAMA AT BIRMINGHAM

MEMORANDUM

DATE: November 22, 2006

TO: Bradley K. Yoder, Ph.D.
MCLM-652 0005
FAX: 934-0950

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on November 22, 2006.

Title of Application: Cilia and Cystic Kidney Disease
Fund Source: NIH

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW) (Assurance Number A3255-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).

Institutional Animal Care and Use Committee
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BIRMINGHAM AL 35294-0019

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Office of the Provost

MEMORANDUM

DATE: July 14, 2005

TO: Bradley K. Yoder, Ph.D.
MCLM-652 0005
FAX: 934-0950

FROM: Suzanne M. Michalek, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use COmmittee (IACUC) on July 13, 2005.

Title: The Role of Primary Cilia/Intraflagellar Transport in Skin and Hair Pathology

Sponsor: Internal

This institution has an Animal Welfare Assurance on file with the Office for Protection from Research Risks (Assurance Number A3255-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).

Institutional Animal Care and Use Committee
B10 Volker Hall
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205.934.7692 • Fax 205.934.1188
iacuc@uab.edu
www.uab.edu/iacuc

The University of
Alabama at Birmingham
Mailing Address:
VH B10
1530 3RD AVE S
BIRMINGHAM AL 35294-0019

Submitted for approval 5/17/2005

OFFICE USE ONLY (revised 05/16/01)
IACUC Approval Number:
Funded Date:
Not Funded/Delete Date:
Category

ONLINE, TYPE ONLY IN THE VISIBLE AREA

UAB Animal Use Review Form For New Proposals
General Information
Section A

Principal Investigator: Bradley K. Yoder Email: byoder@uab.edu

Department/Division: Cell Biology Extension: 934-0994

Affiliation:

Graduate School		Medicine	✔
Med. Center Adm.		Nursing	
Med. Center Joint Dept.		Optometry	
S.H.R.P.		Public Health	
Dentistry		Academic Affairs	

Campus Address (Room, Bldg.): MCLM 652

Project Title: The Role of Primary Cilia/Intraflagellar Transport in Skin and Hair Pathology

Program Project or SCORE Title (If Applicable):

Project Period: August 1, 2005 To: July 31, 2006

External Supporting Agency: Application Deadline:

Internal Supporting Agency: SDRC Pilot Application Deadline: April 1, 2005

Does this proposal recover indirect costs? Yes: No: ✔

Are all individuals having contact with the animals in this project participating in the:
(1) ARP Personnel Health Program? Yes: ✔ No:
(2) ARP Animal Care and Use Training Program? Yes: ✔ No:

Species	Number Used Per Year	Approx. No. Days Housed Per Year	Approx. Daily Census Avg. / High	Animal Source Vendor	Housing Site*
1. Mice	1500	365	250 / 500	In house Colony	LH863 and 864
2.			/		
3.			/		

* If animals will be housed or undergo procedures outside of ARP animal facilities (in laboratories or other study areas) for more than 12 hours, please indicate the building and room number here:

I certify that the information furnished herein is complete and correct to the best of my knowledge.

Investigator Signature Date

Investigators responsible for experimental animal procedures other than the principal investigator:

Name(s) Courtney J Haycraft, Ph.D. and Jon Lehman

Technical staff involved in the experimental procedures (please indicate training and experience):

Name	Hire Date	Length of Experience	Nature of Experience
Kelley Rosborough	2005	1 Year	Transgenic mice and colony maintenan

1. Briefly explain the scientific merit of your proposal to justify the use of animals to any reasonable and well-informed lay person. This explanation should include your project's relevance to human or animal health and/or the advancement of knowledge. Include the rationale for the choice of species. If necessary, attach a separate sheet.

Mice lacking the cilia genes Tg737 or KIF3A in the skin develop significant skin and hair pathology including hair loss and lack of hair development. In this grant, we are exploring the role of cilia in normal skin and hair development. Examining the role of cilia in different populations of growing hair follicles will also help to expand our knowledge of the intricate interplay between different populations of cells within hair follicles. Elucidating this connection between cilia and signaling pathways that dictate formation and cycling will have significant impact on our understanding of how the mammalian hair follicle develops and cycles throughout life and the complex cellular communications that keep this tissue growing appropriately. Mice were chosen for this study because there are several independent mutations in the Tg737 gene in this organism and there are considerable tools and protocols for the analysis and cycling of mouse hair.

2. Briefly justify the number of animals requested for the control and experimental group size and the statistical analysis planned. If necessary, attach a separate sheet.

This project involves the characterization of mice carrying conditional alleles generated in genes required for cilia assembly. We will be crossing them with several lines of transgenics that express cre recombinase in tissue/cell type specific manner to disrupt cilia assembly in different cells in a developing hair follicle. These mice will also be mated to various reporter systems to analyze signalling effects. The mutant mice and their controls starting at E14.5 through the first hair cycle (~day 28) will be analyzed for any effects on cilia formation, alterations in gene expression, and skin and hair pathologies such as hair loss or lack of development. Due to the fact that the cre mediated recombination is less than 100% efficient , that we have to establish time matings to generate the correct stage embryos, the mice will be on mixed genetic background, and the number of strains we will have to utilize a relatively large number of animals for this study. It is estimated that ~1500 mice will be needed per year to accomplish the goals of this proposal. This number is based on previous experience in a similar project conducted while I was at the Oak Ridge National Lab and ongoing projects in the lab on limb development. This number includes both the mutants and controls and mice needed to maintain the colony of cre and floxed alleles.
Mice will be used for this study due to the fact that they are an excellent model available for studying hair cycling and development and they are the only practical system for generating conditional mutants. In addition, we have been generating regents for this type of analysis in mice (antibodies against mouse proteins,etc) for over a decade and switching to another system would greatly impede the study.