HEDGEHOG-WNT INTERACTIONS DURING PATHOLOGIC EPITHELIAL BUD DEVELOPMENT AND SKIN TUMORIGENESIS

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Cellular and Molecular Biology)
in The University of Michigan
2008

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2008

Acknowledgements

I am grateful to have had a wonderful mentor and committee chair, Anj Dlugosz. I thank him for all his endless support, training, ideas, guidance, friendship, and instilling his tireless love and passion for science. Anj gave me the freedom and autonomy to explore and develop my own ways to approach a research problem, but yet his door was always open and he was always there to provide incredible amounts of guidance and advice. He truly is a wonderful 'boss' to work for, and a colleague for years to come. Research is a life-long learning experience, and I have gained a strong foundation in the scientific method and the way to approach a research problem under his tutelage.

I also have to thank my thesis committee members, Drs. Eric Fearon, Tom Glaser, Deb Gumucio, and Theo Ross. They have been incredibly supportive and I have appreciated their advice and guidance throughout my Ph.D. training. I am also incredibly indebted to all the wonderful members of the Dlugosz laboratory, both past and present: Marina Grachtchouk, Jenny Ferris, Evan Michael, Anna Wang, Jianhong Liu, Alex Ermilov, Lijyun Syu, Jim Diener, Monique Verhagen, and Mark Hutchin for all their support and wonderful memories. I also send out my special thanks to Karen Myers, Penny Morris, and Ellen Elkin for their assistance. I also would like to thank Dr. Sewon Kang for his generosity and especially for setting up a meeting with Anj during my M2 year in medical school. Special thanks to Drs. Elia Duh and Don Zack, my mentors at the

Wilmer Eye Institute at Johns Hopkins School of Medicine, for providing mentorship and guidance years after I have left Hopkins.

I also would like to thank Drs. Sarah Millar and Thomas Andl at U. Penn., who have been incredibly generous and helpful throughout my graduate career, and also for providing *TRE-Dkk1* transgenic mice. I thank the Center for Organogenesis Training Grant and Deb Gumucio; the Department of Dermatology; and MSTP for the funding support.

I am especially grateful for my parents, Seungjin and Jeongok Yang, for their support and endless love throughout my life and during my graduate career. They have sacrificed so much for my well-being, taught me the value of tirelessly working hard to achieve my goals, and led by example how to live life and always strive to be a better person. Thank you.

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Abstract

The Hedgehog (Hh) and canonical Wnt/β-catenin signaling pathways are involved in various embryonic processes, and when aberrantly activated after birth in certain cell types or settings, are associated with the development of neoplasia. Although the Hh and Wnt pathways can be activated concurrently during tumorigenesis, the functional significance of signaling crosstalk in tumor initiation and progression has not been established.

Constitutive Hh signaling underlies several human tumors, including basal cell carcinoma (BCC) and basaloid follicular hamartoma in skin. Intriguingly, superficial BCCs arise as *de novo* epithelial buds resembling embryonic hair germs, collections of epidermal cells whose development is regulated by canonical Wnt/β-catenin signaling. Similar to embryonic hair germs, human BCC buds exhibited increased levels of cytoplasmic and nuclear β-catenin, a marker of canonical Wnt/β-catenin signaling, and expressed early hair follicle lineage markers. We also detected canonical Wnt/β-catenin signaling in epithelial buds and hamartomas from mice expressing an oncogene, *M2SMO,* leading to constitutive Hh signaling in skin. Conditional overexpression of the Wnt pathway antagonist Dkk1 in M2SMO-expressing mice potently inhibited epithelial bud and hamartoma development without affecting Hh signaling, indicating pathologic Hh signaling brings about these changes indirectly, via canonical Wnt/β-catenin pathway. My findings uncover a previously unknown requirement for ligand-driven, canonical Wnt/β-catenin signaling for Hh-driven tumorigenesis, identify Wnt ligands as potential

pharmacological target for these neoplasms, and establish the molecular basis for the well-known similarity between early BCCs and embryonic hair germs.

I also describe my preliminary findings showing that epithelium-specific activation of Hh signaling is sufficient to reactivate growth of resting hair follicles, a process which is normally controlled by the Wnt/β-catenin pathway. In addition, I show that epithelial Hh signaling leads to robust activation of melanogenesis, and this can be blocked by inhibiting Wnt ligand function with Dkk1. Taken together, these studies establish that a variety of responses to deregulated Hh signaling in skin, involving both epithelial cells and melanocytes, are strictly dependent on canonical Wnt/β-catenin signaling. Moreover, they set the stage for studies further examining the biology and molecular basis of Hh-Wnt crosstalk in skin, and its potential relevance in other neoplasms.

Chapter I

Introduction

The Hedgehog (Hh) and Wnt/β-catenin signaling pathways play essential regulatory roles in a vast array of developmental and biological processes during both embryogenesis and adult life, and when deregulated, contribute to the tumorigenesis of many organs. Similar to other organs, hair follicle development is dependent upon the coordinated and complementary functions of multiple signaling pathways in both the epidermal and mesenchymal compartments, including the Hh and Wnt/β-catenin signaling pathways. Wnt signals are believed to be necessary and sufficient for the initial stages of hair follicle development, including the formation of epithelial buds, while Hh signaling subsequently promotes the rapid proliferation of follicle epithelium needed to assemble a mature follicle. Interestingly, skin cancers driven by uncontrolled Hh signaling, superficial basal cell carcinomas (BCC), arise as *de novo* epithelial buds resembling embryonic hair germs, collections of epidermal cells whose development is regulated by Wnt/β-catenin signaling. This intriguing morphological similarity was first observed by pathologists nearly 80 years ago, but the molecular basis for this phenomenon has been lacking.

For my thesis project, my objective was to gain further molecular insights into the functions of the Hh and Wnt embryonic signaling pathways in cell fate decisions and cancer, using the skin as a model system. Based on the morphological similarities

between Hh-driven ectopic epithelial buds and canonical Wnt-driven embryonic hair buds, I hypothesized that cross-talk between Hh and Wnt pathways plays important roles during Hh-driven skin tumorigenesis. In this thesis, I describe the experimental designs and scientific methods I undertook to test my hypothesis, utilizing transgenic mouse models in which the functions of Hh and Wnt pathways, in skin, had been genetically altered. Utilizing these mouse models, I demonstrate that over-expressing M2SMO, an oncogenic mutant of the Hh effector protein Smoothened (Smo) resulting in constitutive Hh signaling in the skin, leads to the development of *de novo* epithelial buds and skin tumors called follicular hamartomas. I report the evidence of β-catenin-dependent Wnt pathway activation in these Hh-induced lesions, including upregulation of genes encoding multiple Wnt ligands, nuclear and cytoplasmic localization of β-catenin, and expression of several endogenous Wnt target genes. Remarkably, I also demonstrate that transgenic conditional over-expression of the Wnt pathway antagonist Dkk1 in M2SMO skin potently inhibited M2SMO-driven epithelial bud and follicular hamartoma development without affecting Hh signaling. The work described here uncovers a novel requirement for ligand-driven, canonical Wnt/β-catenin signaling for Hh pathway-driven tumorigenesis in skin; identifies Wnt ligands as potential pharmacological targets for neoplasms with deregulated Hh signaling; and establishes the molecular basis for the well-known similarity between early BCCs and embryonic hair germs. Furthermore, these studies raise the possibility that Wnt signaling may be similarly important in other neoplasms associated with deregulated Hh signaling.

Although ectopic Hh signaling drives the formation of epithelial buds resembling hair germs these do not go on to form mature hair follicles, probably because of the

absence of a mesenchymal component required for follicle development. Utilizing a mouse model that expresses a stabilized, truncated β-catenin mutant that results in constitutive activation of Wnt/β-catenin signaling, I also demonstrate that M2SMO-driven follicular hamartomas retain the capacity to differentiate and express multiple late-stage hair follicle lineage markers. Furthermore, I demonstrate that activation of this stabilized β-catenin mutant is sufficient to induce *de novo* formation of mature hair follicles and sebaceous glands even in hairless volar skin of adult mice, demonstrating that forced canonical Wnt/β-catenin signaling can reprogram even mature epidermis to form hair follicles.

Finally, I also describe my preliminary findings which suggest that activation of Hh signaling in the epithelium of quiescent, resting hair follicles is sufficient to trigger new follicle growth. In addition, I show that constitutive activation of Hh signaling in skin results in ectopic pigment accumulation, with an increase in the number of melanocytes and their progenitors as well as upregulation of multiple melanocytic markers, possibly by an indirect mechanism via activation of canonical Wnt/β-catenin signaling.

Morphogenesis of the Hair Follicle: Wnt and Hh Pathways Play Sequential and Distinct Roles

The hair follicle is the most prominent miniorgan of the skin, and the epidermis and its appendages play many critical roles in homeostasis and sustainment of life, including providing an essential protective barrier from a constant array of assaults and damages from ultraviolet radiation from sun exposure and microbes; wound healing;

keeping body fluids in; and helping maintain a constant body temperature against ever-fluctuating environmental conditions (Fuchs, 2007). During embryogenesis, the epidermis and early hair follicles develop from pluripotent progenitor cells (Millar, 2002) (Figure 1-1). While the embryonic surface ectoderm stratifies to form the epidermis, a subset of surface ectodermal cells develops into hair follicles and other ectoderm-derived appendages (Mikkola and Millar, 2006). It is believed that the initial signal to drive hair follicle development originates from the dermis, which then induces the formation of local thickenings (placodes) of epithelial cells, called hair buds, that grow and proliferate into the underlying dermis (Hardy, 1992). It is not clear whether the initiating dermal signal is uniform throughout the developing epidermis, or produced individually by early dermal condensates to influence the development of each hair bud , but the final location of hair buds appears to be influenced by reaction-diffusion mechanisms involving competing epithelial signals (Jiang et al., 2004; Sick et al., 2006). In mice, different types of hair follicles form at different stages during embryogenesis, and the molecular interactions among the competing signals result in the formation of a hexagonal array of primary hair buds that appears at approximately embryonic day 14.5 (E14.5). These primary hair buds give rise to the large guard hairs, and subsequently secondary hair buds are initiated in several waves between E15.5 and birth, and develop into hair follicles that produce three morphologically distinct types of hairs, designated awl, auchene, and zigzag (Schmidt-Ullrich and Paus, 2005).

Like other organs, hair follicle development is orchestrated by a series of precise molecular signals traveling between overlying epithelial follicle progenitor cells and underlying mesenchymal condensate, which together ultimately give rise to a mature hair

follicle (Fuchs, 2007). The presence of these molecular inductive signals that travel between the epidermis and dermis was established by pioneering studies performed nearly 60 years ago, with mesenchymal-epithelial tissue recombination studies using chick and mouse skin at different stages of embryonic development [reviewed in (Hardy, 1992; Olivera-Martinez et al., 2004)]. More recently, the molecular identities of some of these signals has been established, and include the bone morphogenic proteins (BMPs) and their inhibitors, which negatively regulate hair bud formation and compete with Eda in establishing a regularly patterned placodal array; Tumor Necrosis Factor (TNF)-family member ectodysplasin-A (Eda), which signals via its receptor EdaR and NFκB to regulate primary hair bud formation; transforming growth factors-β; fibroblast growth factors; Wnt/β-catenin; and Sonic hedgehog (Shh) [reviewed in (Schmidt-Ullrich and Paus, 2005; Fuchs, 2007). In this thesis, the roles of Wnt and Hh pathways during hair follicle development and tumorigenesis will be the major focus.

Canonical Wnt/β-catenin signaling is both necessary and sufficient for the initial stages of hair follicle development (Gat et al., 1998; Huelsken et al., 2001; Andl et al., 2002; Lo Celso et al., 2004), including the formation of the hair buds (Figure 1-1). The hair bud, once formed, then transmits a signal of epithelial origin to the underlying dermis to form a mesenchymal condensate, which eventually develops into the dermal papilla. Subsequently, Hh signaling promotes the massive proliferation of follicle epithelium needed to assemble a mature follicle, and the proliferating epithelial cells in the follicle epithelium envelop the developing dermal papilla to form the follicle bulb (St-Jacques et al., 1998; Chiang et al., 1999). Wnt signals then again play an important role in the differentiation of committed epithelial progenitors in the hair bulb to form late-

stage hair follicle lineages to give rise to the seven distinct concentric layers of terminally differentiating cells in the mature hair follicle (Fuchs, 2007; Millar, 2002; DasGupta and Fuchs, 1999). These studies demonstrate that while both canonical Wnt/β-catenin and Hh signaling pathways play essential roles during hair follicle development, they serve largely distinct, non-overlapping and sequential roles.

Numerous studies have demonstrated the central roles of Wnt and Hh signaling during hair follicle development. The developing embryonic hair buds and mesenchymal condensate express multiple Wnt ligands, including *Wnts 10a* and *10b*, and also exhibit nuclear and cytoplasmic β-catenin localization, an indication of activated canonical Wnt/β-catenin signaling (DasGupta and Fuchs, 1999; Noramly et al., 1999; Reddy et al., 2001). Furthermore, embryonic hair buds and dermal papillae show positive β-galactosidase expression of the Wnt reporter gene TOPgal (TCF/Lef Optimal Promoter β-galactosidase), where β-galactosidase expression is under the control of enhancer composed of multimerized TCF/Lef1 binding sites (DasGupta and Fuchs, 1999). In addition, Huelsken et al. showed that skin-specific deletion of β-catenin results in a complete lack of hair bud formation in mice (Huelsken et al., 2001). Similarly, Andl et al. showed that the constitutive over-expression of the secreted canonical Wnt inhibitor Dkk1 (see below for details) in skin prevents hair follicle morphogenesis from the earliest stages (Andl et al., 2002), and reduced hair follicle morphogenesis was also observed in *Lef1* knockout mice (van Genderen et al., 1994), further suggesting that canonical Wnt/β-catenin signaling is required for hair bud development in skin. Conversely, Gat et al. showed that transgenic mice expressing a truncated, constitutively activated form of β-catenin that lack the critical phosphorylation sites on the N-terminus that are needed for

its degradation in skin develop numerous *de novo* hair follicles (Gat et al., 1998). Similarly, Lo Celso et al. showed that the conditional over-expression of N-terminally truncated, stabilized β-catenin in postnatal epidermis is sufficient to drive *de novo* hair follicle morphogenesis (Lo Celso et al., 2004).

During embryonic development, Sonic hedgehog (Shh) is produced and secreted by the developing hair bud. Initial studies that mapped the expression patterns of Hh target genes reveal that Shh signals to both the epithelial and mesenchymal components of the developing hair follicle (Mill et al., 2003; Oro et al., 1997; St Jacques et al., 1998; Chiang et al., 1999). Analysis of Shh-deleted skin revealed that while the initial bud development was not impaired in $Shh^{-/-}$ skin, these buds failed to proliferate and grow to form mature hair follicles (Chiang et al., 1999; St-Jacques et al., 1998), demonstrating the critical role of Hh signaling in promoting the proliferation of the growing follicle epithelium. Taken together, these studies show that Wnt and Hh signaling pathways play essential but sequential roles during normal hair follicle development, with the Wnt pathway being necessary and sufficient for initiation of hair bud development.

Hair follicles grow and proliferate at an angle to the skin, pointing from anterior to posterior. Interestingly, a body of evidence also suggests a role for canonical Wnt and Hh signaling pathways in regulating the polarity of hair follicles. Shh expression in hair and feather follicles exhibits an asymmetric pattern of localization (Gat et al., 1998; Morgan et al., 1998), and overexpression of Shh in embryonic chick skin causes the formation of feather buds that have lost their normal orientation (Ting-Berreth and Chuong, 1996). Similarly, over-expression of Wnt7a, or stabilized β-catenin mutant in embryonic chick skin, and over-expression of Lef1 or stabilized β-catenin in transgenic

mouse skin resulted in altered follicular polarity (Zhou et al., 1995; Gat et al., 1998; Noramly et al., 1999; Widelitz et al., 1999; Widelitz et al., 2000), including the loss of asymmetrical Shh expression, suggesting that canonical Wnt/β-catenin signaling lies upstream of Shh in controlling polarity.

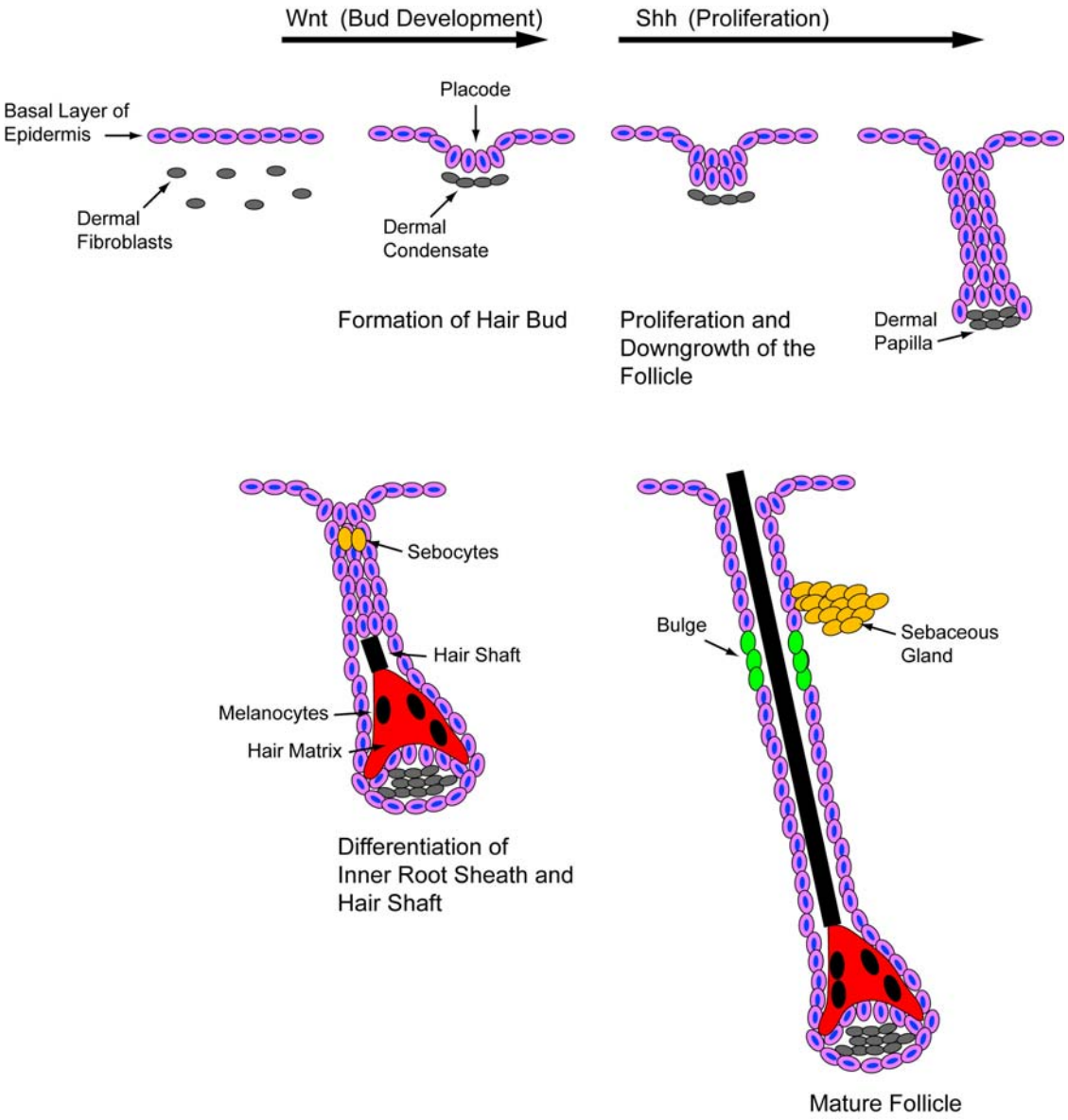

Figure 1-1. Model for hair follicle morphogenesis. Hair follicle morphogenesis occurs during embryogenesis and is initiated by the formation of hair bud, a localized collection of epidermal cells. Canonical Wnt/β-catenin signaling plays an essential role during the initial hair bud development (see text). Subsequently, Hh signaling plays a pivotal role in driving the proliferation and downgrowth needed to assemble a mature follicle. The dermal condensate is enveloped by the invading epithelial cells and forms the dermal papilla. Canonical Wnt/β-catenin signaling mediates the differentiation of distinct hair follicle cell types in the newly formed follicle, and the sebaceous gland develops concurrently. Melanocytes arising from the neural crest populate the matrix and produce melanin to generate pigmented hair shaft. The bulge region of the mature follicle serves as the stem cell niche for the follicle. Adapted from SE Millar, 2002.

The Hair Cycle

Hair follicle morphogenesis occurs during embryogenesis, and once formed, the number of hair follicles at birth remains constant throughout adult life (Hardy, 1992). The hair follicle is one of few organs that undergoes cyclic transformations for its entire life, from periods of regeneration and rapid growth (anagen), where key aspects of its embryonic development are recapitulated, to periods of massive regression via apoptotic mechanisms (catagen), followed by periods of rest and quiescence (telogen) [reviewed in (Stenn and Paus, 2001; Paus and Cotsarelis, 1999) and (Figure 1-2)].

These unique and remarkable features of the hair follicle, in addition to its relative abundance and easy accessibility, make it an extremely attractive model system. In mice, hair cycling is synchronized in the dorsal skin for the first 2-3 months of life, and the postnatal hair cycle during the synchronized period is well characterized in C57/BL6 mice (Paus et al., 1999b) (Figure 1-2). During each new hair growth cycle, molecular events that occur during embryonic hair follicle morphogenesis are recapitulated, including the interplay between inductive signals that travel between the follicular epidermal and dermal cells. *Wnts 10a* and *10b* are expressed in follicle epithelial cells adjacent to the dermal papilla at anagen onset (Reddy et al., 2001), and the Wnt-reporter TOPgal gene expression is also seen in the bulge region of hair follicles at anagen onset (DasGupta and Fuchs, 1999). In addition, activation of canonical Wnt signaling in telogen skin by expressing a 4-hydroxytamoxifen (4-OHT)-inducible stabilized, truncated β-catenin mutant is sufficient to trigger a new hair growth cycle (Lo Celso et al., 2004; Van Mater et al., 2003). Conversely, onset of the first postnatal anagen did not occur in

mice that progressively lose β-catenin from the skin and hair follicles (Huelsken et al., 2001), suggesting that canonical Wnt/β-catenin signaling plays an important role in providing the initiating signal to trigger anagen. Intriguingly, adenovirus-mediated ectopic expression of *Shh* (Sato et al., 1999; Wang et al., 2000b), as well as topical application of synthetic Hh agonist (Paladini et al., 2005) were also sufficient to induce resting telogen hair follicles to re-enter anagen, suggesting that activation of the Hh pathway in resting hair follicles is also sufficient to induce new hair growth. Similar to the growing epithelium during embryogenesis, once a new hair cycle is initiated by Wnt/β-catenin signaling, Hh signaling again plays an indispensable role in driving the proliferation of follicle epithelium (St-Jacques et al., 1998; Sato et al., 1999; Wang et al., 2000b; Altaba et al., 2002b).

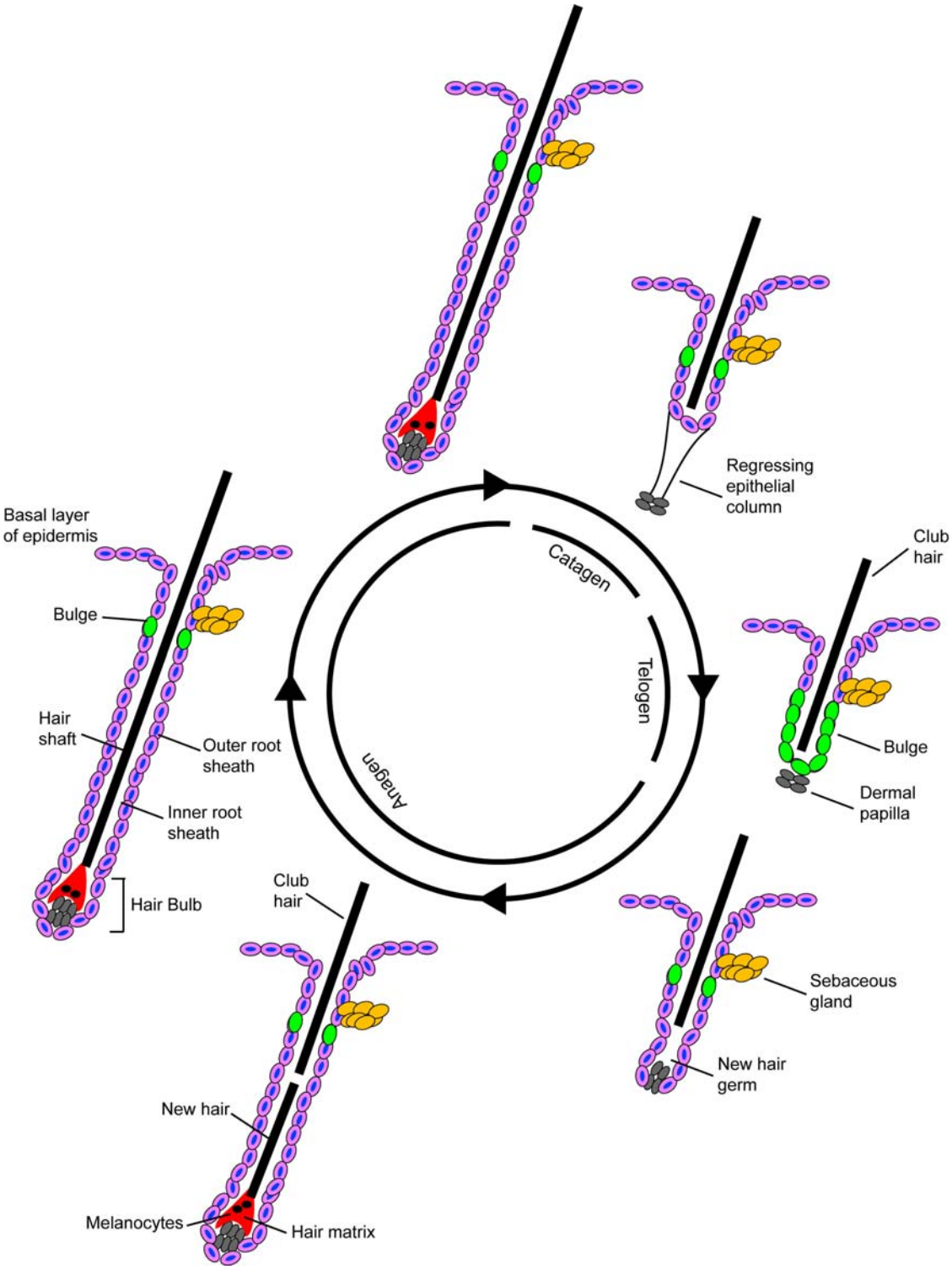
Catagen
Telogen
Anagen
Regressing
epithelial
column
Club
hair
Bulge
Dermal
papilla
Sebaceous
gland
New hair
germ
Club
hair
New hair
Melanocytes
Hair matrix
Basal layer
of epidermis
Bulge
Hair
shaft
Outer root
sheath
Inner root
sheath
Hair Bulb

Figure 1-2. The hair cycle. Once formed, postnatal hair follicles cycle through periods of rest (telogen), growth (anagen), and regression (catagen). In mice, hair follicles reside in telogen for a variable amount of time, anagen for 19-21 days, and catagen for 1-2 days (Schmidt-Ullrich and Paus, 2005). At the beginning of each new cycle, a signal from the dermal papilla activates the stem cells in the bulge (green). This leads to rapid proliferation of the follicle epithelium and invagination into the dermis. The deepest portion of the new growing anagen follicle contains highly proliferative matrix cells (red) which give rise to multiple cell lineages in the inner root sheath and hair shaft. During catagen, proliferation ceases, and there is massive apoptosis, leaving behind a relatively small number of non-proliferative epithelial cells surround the telogen hair shaft. It is within this residual epithelial cell compartment (green), that stem cells capable of giving rise to all cell lineages in the hair follicle, sebaceous gland, and epidermis, reside. Adapted from Fuchs 2007.

Hair Follicle Bulge: The Stem Cell Compartment

Hair follicles contain a population of epithelial stem cells in a region known as the bulge (Cotsarelis et al., 1990; Taylor et al., 2000; Blanpain et al., 2004; Tumbar et al., 2004; Morris et al., 2004) (Figure 1-2). Stem cells in the hair follicle are needed for skin homeostasis, wound-healing, cyclic postnatal follicle growth, and are the likely progenitor cells for a variety of skin tumors. The cells in the bulge region divide less frequently than other epithelial cells in the hair follicle, but are activated at each anagen onset to produce a population of rapidly-dividing transit-amplifying cells (Cotsarelis et al., 1990). The dermal papilla is believed to play a critical role in triggering new anagen by directly signaling to the bulge region (Stenn and Paus, 2001), due to its proximity to the bulge in the telogen hair follicle. This process of bulge-cell activation has been proposed to also recapitulate many of the signaling pathways during embryonic hair follicle development. However, despite many similarities, there also seem to be differences, and an area of active research in the field is in trying to gain a deeper insight into the relationships between the embryonic hair bud and the adult bulge. Recently, an exploding body of new knowledge from multiple laboratories has shed new light on the complex properties of the hair follicle stem cell compartment. Microarray profiling analysis studies have revealed a distinct set of genes that are preferentially expressed in the bulge (Blanpain et al., 2004; Tumbar et al., 2004; Morris et al., 2004), compared to other epithelial cells of the hair follicle. Interestingly, while many of the bulge cells express TCF3, TCF4, and several Frizzled proteins, thus suggesting that many Wnt-responsive genes are expressed in the bulge cells, they also express proteins that are

associated with inhibition, not activation, of Wnt signaling (Tumbar et al., 2004; Nguyen et al., 2006). Additional studies have demonstrated that TCF3 can function as a repressor of Wnt signaling in the absence of β-catenin (Merrill et al., 2001; Nguyen et al., 2006). Studies have demonstrated that in their quiescent resting stage, there is an active suppression of the Wnt/β-catenin signaling in the bulge cells to maintain their undifferentiated state, and subsequently upon a Wnt activation signal, stem cells are activated and a new hair growth cycle is induced (Lo Celso et al., 2004; Van Mater et al., 2003; Lowry et al., 2005). Taken together, current data suggest that the activated β-catenin/TCF/Lef1 in the early hair germ persists in the hair bulb (Figure 1-2) of the mature follicle throughout anagen, and the close proximity and interaction of this follicle epithelium with the neighboring dermal papilla is critical for the proper initiation of anagen and proliferation of epithelial cells known as the matrix cells. As the follicle epithelium rapidly proliferates, the dermal papilla moves away from the bulge, and the rapidly proliferating matrix cells in the follicle base near the dermal papilla then undergo terminal differentiation to give rise to the late stage hair follicle lineages. While there remain many unanswered questions on the biology and mechanism of bulge stem cell activation, these intriguing findings may have profound clinical applications and significance, as it was recently observed that human bulge stem cells share highly similar properties as mouse bulge stem cells (Ohyama et al., 2006). It is likely that better understanding of follicle stem cell biology from mouse models may yield new therapeutic applications against hair loss and other hair follicle-related disorders.

Canonical Wnt Signaling

The Wnt family of secreted glycoproteins mediates cell-cell interactions during cell growth and differentiation in both embryos and adults by binding to receptors on the cell surface to mediate signaling transduction events to the cytoplasm and nucleus (Willert et al., 2003). To date, genome sequencing has revealed 20 distinct Wnt proteins in vertebrates. Wnt signaling is involved in a variety of processes throughout the body such as generation of cell polarity and specification of cell fate, and when deregulated, contributes to tumorigenesis in several organs (Clevers, 2006; Cadigan and Liu, 2006; Hoppler and Kavanagh, 2007). It is now known that different Wnt ligands may participate in distinct signaling pathways, with the main ones broadly classified as "canonical" and "non-canonical" Wnt signaling pathways. The "canonical" Wnt signaling pathway is a major focus of this thesis and will be discussed in detail.

Activation of the canonical Wnt signaling pathway results when a subset of secreted Wnt proteins bind to Frizzled receptors and essential low-density lipoprotein receptor-related protein (LRP) co-receptors (Cadigan and Liu, 2006). β-catenin is the key mediator of the canonical Wnt pathway, and has dual, non-overlapping functions in cell-cell adhesion and signal transduction (Brembeck et al., 2006). There exist two main pools of β-catenin in epithelial cells: one pool forms a stable complex with the cytoplasmic domain of E-cadherin at cell-cell borders and tethers it to the actin cytoskeleton via α-catenin, and the other pool of β-catenin is found within the cytoplasm and/or nucleus. In the absence of Wnt ligands, "free" cytoplasmic β-catenin is

phosphorylated by a multi-protein complex known as β-catenin destruction complex comprised of glycogen synthase kinase 3 (GSK-3β), adenomatous polyposis coli protein (APC), Axin, and Casein Kinase Iα (CKIα). Under basal conditions, β-Transducin Repeat Containing Protein (β–TRCP) interacts with phosphorylated β-catenin, which is then ubiquitinated by E3 ubiquitin ligase complex and targeted for rapid proteasome-mediated degradation mediated by 26S proteosome machinery (Aberle et al., 1997).

Upon the binding of Wnt ligands to their Frizzled and LRP 5/6 co-receptors, a number of changes in the intracellular signaling machinery of responding cells take place. First, the cytoplasmic protein Dishevelled is phosphorylated and function of the GSK-3β/APC/Axin/CKIα destruction complex is inhibited, leading to inhibition of β-catenin phosphorylation and its subsequent stabilization. Several studies have suggested that Dishevelled inhibits the activities of β-catenin destruction complex by interacting with Frequently Rearranged in Advanced T-cell Lymphomas (FRAT)/GSK-3β Binding Protein (GBP) (Li et al., 1999; Farr, III et al., 2000; Salic et al., 2000). Once stabilized in the cytoplasm, β-catenin then translocates into the nucleus, where it complexes with members of the T-Cell Factor/Lymphoid Enhancer Factors (TCF/LEF) family of DNA binding factors to regulate the transcription of Wnt target genes (Figure 1-3). While multiple genes regulated by canonical Wnt/β-catenin signaling have been identified, *Axin2* (Jho et al., 2002) and *Sp5* (Weidinger et al., 2005) appear to be faithful target genes that are reliable markers of canonical Wnt signaling activaty. In mammals, there exist 4 members of the TCF/LEF family of transcription factors: TCF1, TCF2 (also known as LEF1), TCF3, and TCF4 (Clevers, 2006). Intriguingly, when β-catenin is not present in the nucleus, TCF/LEF transcription factors bind to transcriptional repressors such as

Groucho to inhibit the transcription of Wnt target genes (Roose et al., 1998; Cavallo et al., 1998).

While it is clear that the canonical Wnt signaling pathway is tightly regulated via an elaborate and extensive intracellular regulatory machinery outlined above, a variety of extracellular proteins have also been identified that bind to the secreted Wnt ligands and/or receptors to regulate canonical Wnt signaling. One such family of Wnt ligand binding proteins are the secreted frizzled-related proteins (sFRPs), which are extracellular factors that bind to the Wnt ligands to modulate their association with Frizzled/LRP 5/6 co-receptors (Rattner et al., 1997). Five *sFRP* genes have been discovered in verterbrates, named *sFRP1-5*; interestingly, sFRPs seem to be able to both inhibit and potentiate canonical Wnt signaling depending on the concentration and individual sFRP protein (Kawano and Kypta, 2003). For example, while Sfrp1 has been shown to primarily inhibit Wnt signaling at high concentrations, in certain contexts Sfrp1 is capable of potentiating Wnt signaling at low concentrations (Uren et al., 2000).

Another family of secreted proteins that regulate Wnt signaling extracellularly is the Dickkopf (Dkk) proteins. Dkk1, originally identified as an inducer of head formation in *Xenopus* embryos (Glinka et al., 1998), is a potent and specific endogenous secreted Wnt inhibitor (Bafico et al., 2001; Mao et al., 2001; Semenov et al., 2001; Niehrs, 2006). How does Dkk1 inhibit canonical Wnt signaling? It was found that Dkk1 inhibits canonical Wnt signaling by binding to the LRP co-receptors required for activation of canonical Wnt signaling, and the inhibition of Wnt signaling by Dkk1 also requires Kremen1 and Kremen2, transmembrane proteins that form a ternary complex with Dkk1 and LRP 5/6 co-receptors (Mao et al., 2002). The formation of the Dkk1-LRP5/6-

Kremen ternary complex rapidly induces internalization and depletion of cell-surface LRP5/6 co-receptors and blockade of canonical Wnt signaling (Figure 1-4) (Mao et al., 2002). As mentioned previously, over-expression of Dkk1 in the basal layer of mouse epidermis effectively inhibited canonical Wnt signaling and blocked hair follicle morphogenesis (Andl et al., 2002). In this mouse model, since Dkk1 is a secreted protein, it is likely that Dkk1 expressed in the basal cells also blocked canonical Wnt signaling in adjacent dermal cells in addition to the cells in the epidermis.

While the crucial role of LRP 5/6 co-receptors in canonical Wnt signaling has been established, the function of each Frizzled receptor, as well as each individual Wnt ligand in the canonical Wnt signaling pathway, has not been elucidated in full detail. Establishing the functions of each molecule in Wnt signaling is no trivial task, as a large number signaling components exist in vertebrates, including 20 Wnts, 10 Frizzled receptors, 2 LRP co-receptors, 5 Sfrp genes, 4 Dkk genes, as well as several other modifiers. Furthermore, multiple Wnt ligands seem to be capable of binding to and interacting with multiple Frizzled receptors (Bhanot et al., 1996), and these promiscuous ligand-receptor interactions also add to the complexity of the Wnt signaling pathway.

Furthermore, some of the Wnt ligands may signal through alternate "non-canonical" pathways involving Frizzled receptors but not LRP co-receptors, β-catenin, or TCF/LEF factors [reviewed in (Katoh, 2005; Kohn and Moon, 2005), and may even have antagonistic effects on canonical Wnt signaling, adding another layer to the complex nature of Wnt signaling pathway. An excellent web-based online database of Wnt signaling, its components and target genes, etc can be found online: (http://www.stanford.edu/~rnusse/wntwindow.html).

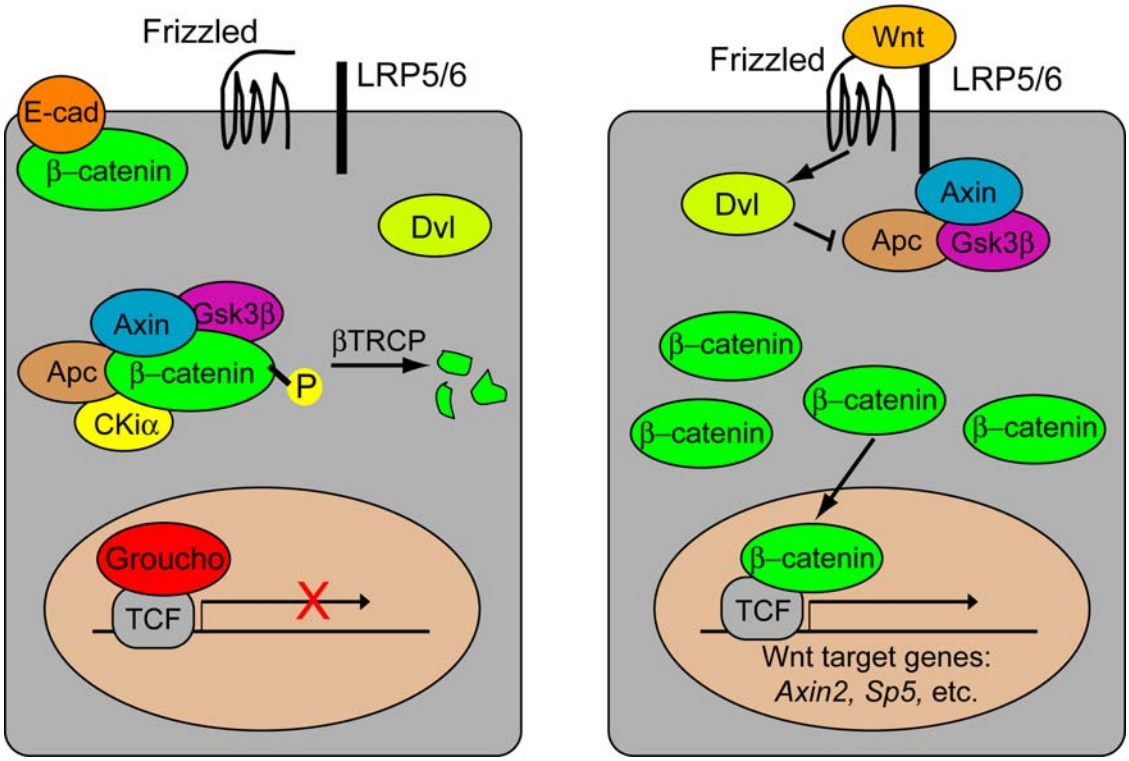

Figure 1-3. The canonical Wnt signaling pathway. In the absence of a Wnt signal (left), free cytoplasmic β-catenin is rapidly phosphorylated by a complex of proteins composed of GSK-3β, APC, Axin, and CKIα. This facilitates binding of the β-TRCP, which subsequently mediates the ubiquitinylation and proteosomal degradation of β-catenin. In the absence of β-catenin, TCF/LEF transcription factors bind to transcriptional repressor Groucho to repress target gene transcription. Interaction of a Wnt ligand (right) with Frizzled and LRP5/6 co-receptors triggers the activation of Dishevelled (Dvl) which results in the inactivation of the β-catenin destruction complex. β-catenin is no longer phosphorylated, and this allows unphosphorylated, stabilized β-catenin to accumulate and enter the nucleus, where it interacts with members of the TCF/LEF family to mediate the transcription of Wnt target genes such as *Axin2* and *Sp5*.

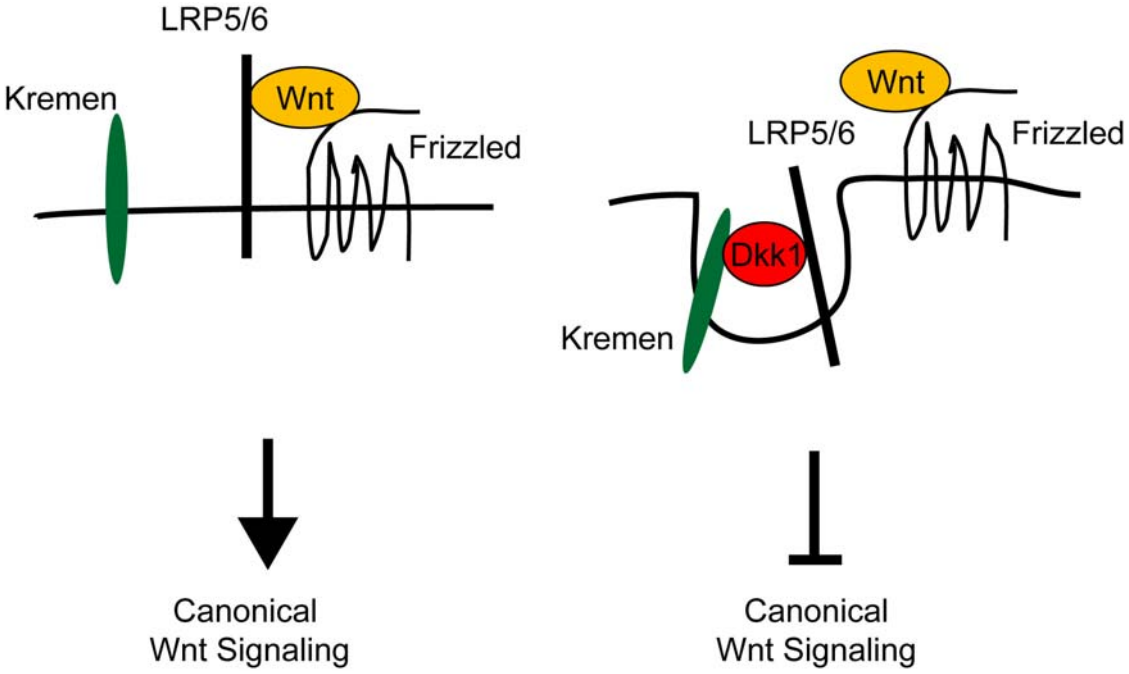

Figure 1-4. Dkk1 and Kremen inhibit canonical Wnt/β-catenin signaling by removing LRP6 from the cell surface. A model for Dkk1/Kremen action. LRP5/6 act as co-receptors for the canonical Wnt/β-catenin pathway, and the binding of a Wnt ligand by Frizzled and LRP5/6 co-receptors activates the canonical Wnt/β-catenin pathway (left). Dkk1, LRP5/6 and Kremen form a ternary complex that triggers rapid internalization and depletion of cell-surface LRP5/6 receptors, thus blocking the canonical Wnt signaling (right).

Hedgehog Signaling

First identified in genetic studies of embryonic segmentation and imaginal disk specification in *Drosophila melanogaster*, Hh proteins are expressed in many tissue types during development and influence patterning through their effects on proliferation, cell death, and lineage selection in *Drosophila* and higher organisms [reviewed in (Lee et al., 1992; McMahon et al., 2003; Ingham and Placzek, 2006)]. Proper Hh signaling is required for normal embryonic development, as fetuses with defects in Hh signaling, specifically involving Shh, fail to survive, and exhibit dramatic birth defects such as cyclopia, which is a form of holopropsencephaly (Chiang et al., 1996; Belloni et al., 1996; Roessler et al., 1996). The development of various other structures and tissues is also severely impaired in mice with Shh deficiency (Litingtung et al., 1998; St-Jacques et al., 1998; Chiang et al., 1999; Ramalho-Santos et al., 2000; Dassule et al., 2000; Pepicelli et al., 1998). Hh proteins are secreted morphogens with different amounts of signal causing the recipient cells to adopt distinct cell fates. Hh signaling is highly conserved from insects to vertebrates, and vertebrates have multiple homologues of a number of components of the pathway, including three Hh proteins in mammals: Shh, mentioned earlier, Indian hedgehog (Ihh), and desert hedgehog (Dhh) (Hooper and Scott, 2005). Of the three, Shh is the most potent in biossays (Pathi et al., 2001) and most broadly expressed in embryonic and adult tissues (Altaba et al., 2002b; Altaba et al., 2002a), and will be the focus of discussion here.

Shh protein undergoes extensive auto-processing and post-translational modifications which are essential for proper function and full activation of Hh pathway

(Porter et al., 1995; Porter et al., 1996). Shh protein, initially synthesized as a 45kD precursor protein, is autocleaved to generate a 19kD N-terminal signaling peptide and a 25 kD C-terminal fragment (Porter et al., 1995). The N-terminal signaling peptide is further processed and results in the palmitoylation of its N-terminus and covalent attachment of a cholesterol moiety at the C terminus (Shh-N) (Mann and Beachy, 2004). The fully processed, activated Shh protein is highly hydrophobic, and subsequently diffuses out to establish gradients of signaling via several mechanisms, including participating in long range signaling by multimerizing at lipid rafts with the lipid adducts sequestered at the centers (Zeng et al., 2001). An alternative mechanism to control the extracellular transport of Shh by diffusion is via interactions with heparin sulphate proteoglycans (Rubin et al., 2002). Because of these diffusion properties of Shh protein, in many tissues during development the cells that synthesize Shh are distinct from cells that respond to the signal, and while the responding cells may be adjacent to the cells producing the signal, they could also be a significant distance away.

Secreted Hh ligands frequently act as paracrine signaling effectors, altering the functions of responsive target cells by a highly conserved signaling pathway which is normally maintained in an "off" state by the Hh receptor Ptch1, a 12-span transmembrane protein (Ingham and Placzek, 2006). In the absence of Hh ligand, Ptch1 represses Smo, a 7-span transmembrane, G-protein-coupled receptor (GPCR)-like protein that is an obligatory transducer of Hh signaling in target cells (van den and Ingham, 1996; Alcedo et al., 1996). Although the exact mechanism is not yet fully clear, it is hypothesized that Ptch1 represses Smo by preventing Smo to localize to the cell surface [reviewed in (Rohatgi and Scott, 2007)]. Binding of Shh ligand to its receptor Ptch1 blocks the latter's

function, thus resulting in the de-repression of Smo which ultimately leads to an altered pattern of gene expression in the nucleus mediated by zinc-finger proteins of the Gli family via actions of several intracellular proteins. While multiple Hh/Gli target genes have been described, *Gli1* and *Ptch1* are considered 'classical' Hh target genes whose expression is consistently upregulated in response to Hh signaling, making them reliable markers for Hh pathway activation (McMahon et al., 2003).

While significant insight into the molecular mechanisms regulating Hh signaling have been achieved in recent years, many aspects of Hh signaling are still not well understood. For example, it is still not fully clear how the binding of Shh to Ptch1 de-represses Smo, although increasing evidence shows that Ptch1 functions to inhibit Smo via transport of a small molecule intermediate (Bijlsma et al., 2006; Corcoran and Scott, 2006). The indirect interaction between Ptch1 and Smo to regulate Hh signaling has gained further evidence by several studies that examined the role of cholesterol metabolites in Hh signaling. Bijlsma et al. showed that Ptch1 induces the secretion of (Pro-) Vitamin D3 from cells, and both (Pro-) Vitamin D3 and Vitamin D3 can inhibit Smo directly (Bijlsma et al., 2006). Corcoran et al. showed that oxysterols, which lie downstream of (Pro-) Vitamin D3 in the cholesterol synthesis pathway, can activate Smo (Corcoran and Scott, 2006). Several small molecules exist that modulate Smo activity, including purmorphamine, a Hh agonist (Sinha and Chen, 2006) and cyclopamine, a Hh antagonist (Chen et al., 2002). While these *in vitro* studies need to be confirmed in a more vigorous *in vivo* setting, these data suggest that in the absence of Shh, high levels of Vitamin D3 and low levels of oxysterol inhibit Smo, whereas when Shh binds Ptch1,

reduced transport of Vitamin D3, and increased synthesis/transport of oxysterols activate Smo.

In addition, the exact molecular mechanism of how Smo signals through to the Gli transcription factors to mediate the activation of Hh signaling is not fully understood. In *Drosophila*, Costal-2, a kinesin-like protein, directly interacts with Smo and recruits downstream regulators as well as the transcription factor Cubitus interruptus (Ci) to mediate signal transduction (Robbins et al., 1997; Sisson et al., 1997). However, the role of Kif7 and Kif27, homologs of Drosophila Costal-2, in vertebrate Hh signaling is more controversial. A study by Varjosalo et al. showed that in marked contrast to *Drosophila*, major Costal-2-like functions are lost in mammalian cells; instead, Suppressor of Fused (SuFu), a negative regulator of the pathway that interacts with Gli proteins, plays an essential role in signal transduction by mediating Gli2 and Gli3 localization (Varjosalo et al., 2006). Other negative regulators of Gli protein include protein kinase A (PKA) which phosphorylates Gli3, leading to proteosomal-mediated cleavage of Gli3 to a truncated form (Wang et al., 2000a; Chen et al., 1998; Hammerschmidt et al., 1996). Hh signaling is also regulated via extracellular mechanisms, as the amount of Shh available to bind Ptch1 is tightly regulated by Hh-binding proteins, such as Hh-interacting protein (Hip), which sequester Hh (Chuang and McMahon, 1999).

If the Costal-2 homolog sKif2 and Kif27 do not play essential roles in vertebrates, then what else could be mediating Hh signaling transduction from the plasma membrane to the cytoplasm? Several studies have suggested the critical role of the primary cilium in vertebrate Hh signaling (Corbit et al., 2005; Haycraft et al., 2005; Huangfu and Anderson, 2005; Huangfu et al., 2003; Liu et al., 2005). Cilia are microtubule-based cell-

surface extensions built from the basal body through an intra-flagellar transport (IFT) assembly process. Interestingly, initial IFT knockout studies in mice showed that these mice exhibited Hh-signaling-related phenotypes: for example, in the limb, where antagonism of Gli3 repressor plays a predominant role in digit specification, extra digit formation was seen in IFT mutants, but in contrast, ventral cell identities within the neural tube that require Gli1 activator forms were absent in IFT-defective mice (Haycraft et al., 2005; Huangfu and Anderson, 2005; Huangfu et al., 2003; Liu et al., 2005). In addition, almost all of Hh signal transduction seem to require IFT machinery, as epistatic analyses studies have shown that IFT proteins act downstream of Smo and upstream or at the level of Gli proteins (Huangfu and Anderson, 2005; Liu et al., 2005). Strikingly, Smo, Gli1/2/3 and SuFu proteins are localized to primary cilia, and it was shown that their accumulation in this organelle is responsive to Hh stimulation (Corbit et al., 2005; Haycraft et al., 2005). Taken together, evidence gained so far suggests the primary cilia are of critical importance for mediating vertebrate Hh signaling, and may provide a function analogous to that of Costal-2 in vertebrates, linking Smo to the modulation of Gli factors.

In vertebrate Hh signaling, Gli-interacting protein SuFu also appears to be critically important, as SuFu null mutant mouse embryos died at embryonic day E9.5 with a gain-of-function phenotype comparable to that of Ptch1 null embryos (Varjosalo et al., 2006; Cooper et al., 2005). Furthermore, it was shown that $SuFu^{+/-}$ mice develop spontaneous skin tumors, as observed in $Ptch^{+/-}$ mice (Svard et al., 2006). Interestingly, while SuFu plays an essential role in mediating vertebrate Hh signaling, Fused (Fu)

mutants are essentially normal and do not exhibit obvious Hh-related phenotypes in mice (Chen et al., 2005; Merchant et al., 2005).

To mediate physiological Hh signaling, Gli transcription factors undergo post-translational processing and ultimately translocate into the nucleus where they can bind to their promoter recognition sites to activate transcription by direct association with a consensus binding site (5'-TGGGTGGTC-3') located in the promoter region of target genes. While the signaling events downstream of Smo and the exact mechanism of Gli activation are not fully understood, the regulation of Gli activation likely requires multiple proteins, as has been shown with SuFu which acts as a negative regulator by binding to Gli to prevent the activation of Hh target genes (Kogerman et al., 1999). In *Drosophila*, PKA was shown to induce proteosome-mediated cleavage of Gli to a truncated form by phosphorylating Ci (Chen et al., 1998), and Wang et al showed that PKA-dependent processing of vertebrate Gli3 in developing limb similarly generates a potent repressor in a manner antagonized by apparent long-range signaling from posteriorly localized Shh protein (Wang et al., 2000a). In mammalian cells, there exist three zinc finger family transcription factors, Gli1, Gli2, and Gli3. Gli1 and Gli2 are thought to serve mainly as positive transcriptional activators whereas Gli3 acts as a transcriptional repressor (Ingham and McMahon, 2001). Interestingly, Gli1 deficient mice are essentially normal, suggesting that Gli1 function is dispensable in the presence of normal Gli2 expression (Park et al., 2000; Bai et al., 2002). Gli3 contains both activation and repression domains, but in most settings where Hh ligand is not present, acts as a transcriptional repressor. For example, genetic studies using *Gli3*$^{-/-}$ mice have

showed that loss of Gli3 can partially or completely rescue neural and limb development defects in *Shh*$^{-/-}$ mice (Litingtung and Chiang, 2000; Rallu et al., 2002).

The primary activating effector of mammalian Hh signaling is Gli2. *Gli2*$^{-/-}$ mice exhibit significant developmental anomalies in multiple organs, including the central nervous system, skeletal system, and the hair follicle (Mo et al., 1997; Matise et al., 1998; Mill et al., 2003), reminiscent of Hh deficiency. These findings suggest that Gli2 is the primary transcriptional effector mediating physiologic responses to Shh and other Hh ligands. How is Gli2 transcriptional activity regulated? Recent studies suggest that Gli2 exists primarily in a full length activator form, rather than a processed repressor form, and PKA/CKI-driven phosphorylation of C-terminal serine residues in Gli2 allows Gli2 to directly interact with β-TrCP, resulting in ubiquitination and proteosomal degradation of Gli2 (Bhatia et al., 2006; Pan et al., 2006). In addition, it has been shown that Sufu regulates vertebrate Gli protein subcellular distribution and transcriptional activity (Kogerman et al., 1999; Cheng and Bishop, 2002; Dunaeva et al., 2003; Ding et al., 1999), and the actin-binding protein Missing in Metastasis (MIM)/BCC_enriched gene 4 (BEG4) has also shown to be a regulator of Gli transcription by binding to Sufu and Gli to potentiate Gli-dependent transcription (Callahan et al., 2004).

Interestingly, while Wnt and Hh pathways clearly exhibit distinct roles and features, the two pathways share a number of similarities and signaling components, suggesting that certain aspects of the two pathways could have a common evolutionary origin (Nusse, 2003). For example, both Wnt and Hh ligands require palmitoylation by acyl transferases (Wnt proteins by Porcupine and Hh proteins by Skinny Hh), and both require heparin-sulphate-modified proteoglycans for transport to their respective

receptors Fz and Ptch (Takei et al., 2004). Smo and Fz are both 7 transmembrane receptors and have considerable amino-acid sequence similarity. Furthermore, each pathway is mediated by a cytoplasmic complex that can be scaffolded to bring it together with Smo or Fz. These complexes regulate phosphorylation, degradation and nuclear access of a transcriptional regulator (Ci/Gli for Hh, β-catenin for Wnt). In each case, GSK3β and CKI target the transcriptional regulator for ubiquitination by Slimb; a crucial step for blocking signaling in the absence of ligand. Furthermore, SuFu is found in complexes with β-catenin as well as Gli, and functions as a negative regulator in both pathways (Meng et al., 2001), further suggesting the similarity between canonical Wnt and Hh signaling pathways.

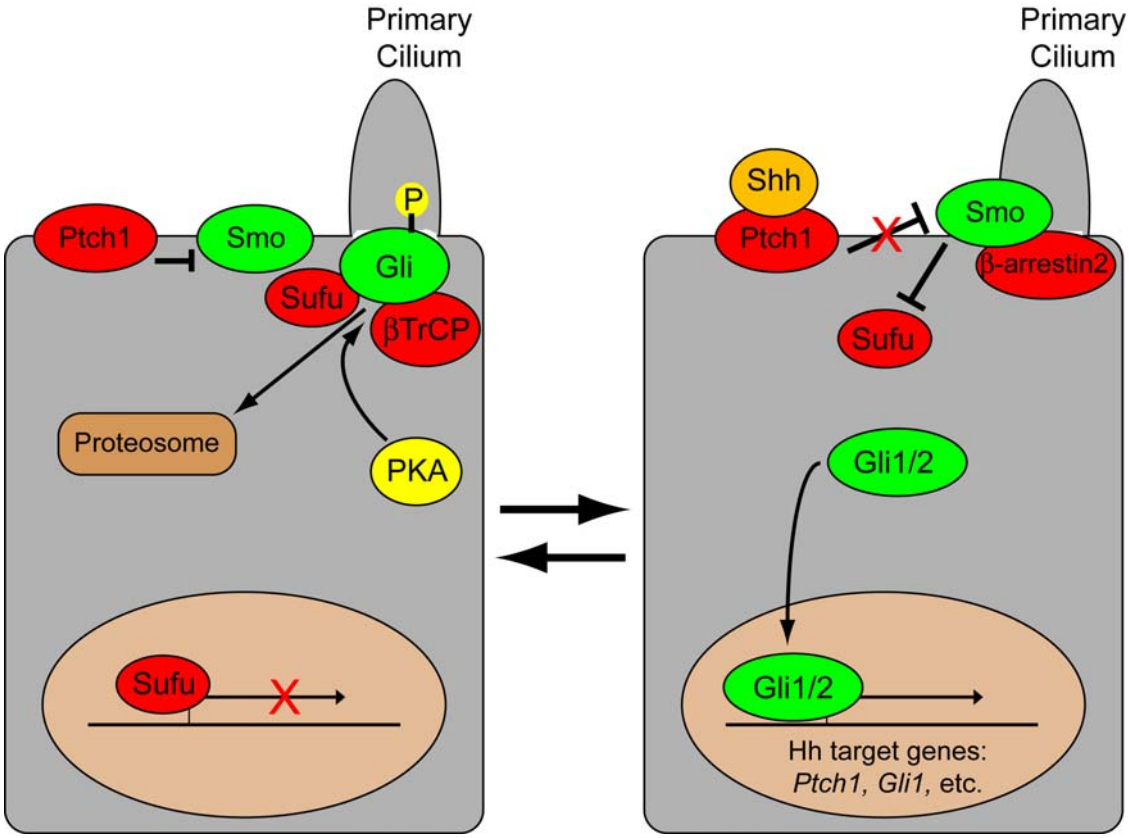

Figure 1-5. The Hedgehog signaling pathway. In the absence of Hh ligand, the Hh pathway is maintained in an "off" state by the inhibitory effects of Ptch1 on Smo. Sufu either binds to Gli proteins, sequestering them in the cytoplasm, or binds to Gli-responsive promoter elements, inhibiting transcription, or both. PKA phosphorylates Gli proteins, leading to βTrCP-mediated uniquitination and proteosomal degradation. In the presence of Hh ligand, the binding of Shh to Hh receptor Ptch1 results in the de-repression of Smo, leading to translocation of Smo to the primary cilium. Smo then associates with β-arrestin2 to be endocytosed. Smo-mediated activation of Gli proteins results in translocation of the active forms of Gli1 or Gli2 to the nucleus, resulting in transcription of Hh target genes. In physiologic Hh signaling, this process is reversible.

Wnt Signaling in Disease

Both the Hh and Wnt pathways, with such crucial functions in regulating cell proliferation and differentiation programs, have been implicated in the pathogenesis of several types of tumors when mutated or otherwise deregulated. The initial data implying Wnt signaling in tumorigenesis was the discovery of *Wnt1* (originally termed *int1*) as an oncogene that can transform mouse mammary epithelial cells (Nusse and Varmus, 1982). Subsequently, the association of aberrant Wnt signaling with human cancer, particularly colorectal cancer has been extensively established. In the early 1990s, the adenomatous polyposis coli (APC) gene was discovered in a hereditary cancer syndrome called familial adenomatous polyposis (FAP) (Kinzler et al., 1991; Nishisho et al., 1991), and subsequently it was determined that a germline APC mutation is the genetic cause of FAP. FAP patients are born with one defective APC allele, and develop a large number of colon adenomas, or polyps starting from early adulthood. The polyps in FAP patients are benign, clonal outgrowths of epithelial cells in which the second APC allele has been inactivated, and eventually progress into malignant adenocarcinomas. A majority of malignant colon cancers exhibit the loss of both APC alleles (Kinzler and Vogelstein, 1996) and subsequent inappropriate activation of β-catenin. Korinek and colleagues showed that TCF reporter constructs, which are normally transcribed only upon Wnt signaling activation, are aberrantly transcribed in APC mutant cancer cells through the action of both β-catenin and TCF4 (Korinek et al., 1997), further linking colon cancer with aberrant Wnt signaling activation. Furthermore, a number of colorectal cancers were found to contain mutations in Axin2 (Liu et al., 2000), and patients with hereditary

Axin2 mutations display a predisposition to colon cancer (Lammi et al., 2004). In addition, activating, stabilizing point mutations in β-catenin have been implicated in colorectal tumorigenesis (Morin et al., 1997), again illustrating that an 'activating' defect at any of multiple levels of the Wnt signaling transduction pathway may lead to colon cancer development.

Mounting evidence also indicates the role of aberrant Wnt signaling in the pathogenesis of several other types of tumors, including hair follicle-derived tumors. In the hair follicle, the transient-amplifying (TA) cells in the hair matrix compartment appear to be the target for neoplastic transformation by aberrant activation of canonical Wnt signaling. Expression of constitutively active, oncogenic β-catenin in mice led to the development of hair follicle-derived trichofolliculomas and pilomatricoma-like lesions (Gat et al., 1998; Lo Celso et al., 2004). In addition, Chan et al. reported that most human spontaneous pilomatricomas carry activating mutations in β-catenin (Chan et al., 1999). In another study, Lo Celso et al. showed that expression of tamoxifen-inducible, activated β-catenin mutant in adult mouse skin again induced trichofolliculomas (Lo Celso et al., 2004). In contrast to hair follicle-derived tumors, approximately one-third of a series of human sebaceous tumors carry LEF1 mutations, impairing LEF1 binding to β-catenin and subsequent transcriptional activation (Takeda et al., 2006). This observation suggests that in the absence of Wnt signaling to induce early progenitors cells into a hair follicle fate, progenitors may be erroneously redirected toward a sebocyte lineage. During embryonic hair follicle development, sebocytes are derived from cells within the superficial hair follicle, and are the final differentiated cells to appear in the developing follicle (Figure 1-1 and (Paus et al., 1999a)). Before birth,

oil-rich sebocytes form a gland located outside of the hair follicle and release their contents into the hair canal. Interestingly, a recent report by Malanchi et al. demonstrated the critical role of Wnt/β-catenin signaling in squamous cell carcinoma, another type of epithelial skin cancer which expresses lineage markers of interfollicular epidermis (Malanchi et al., 2008).

Activating canonical Wnt pathway mutations have been also detected in a variety of other cancers [reviewed in (Reya and Clevers, 2005; Clevers, 2006)] . Loss-of-function mutations in Axin have been detected in hepatocellular carcinomas (Satoh et al., 2000), and aberrant Wnt signaling activation has been reported in hematological malignancies such as chronic myelogenous leukemia (Jamieson et al., 2004), and neuronal tumors such as a subset of medulloblastoma (Guessous et al., 2008; Eberhart et al., 2000; Koch et al., 2001).

Interestingly, it has also been shown that several cancers feature elevated levels of nuclear/cytoplasmic β-catenin, a hallmark of activated canonical Wnt signaling, but in the absence of detectable APC, β-catenin, or Axin mutations. How is this possible? Increasing evidence suggests that this may occur via epigenetic silencing of genes encoding natural Wnt pathway inhibitors such as sFRP (Fukui et al., 2005; Lee et al., 2004; Liu et al., 2006) and Wnt Inhibitory Factor (WIF) (Ai et al., 2006; Batra et al., 2006; Wissmann et al., 2003), or increased expression of pathway components such as Wnt ligands (Clement et al., 2006; Katoh et al., 2001; Milovanovic et al., 2004; Rhee et al., 2002; Sen et al., 2001), FZD receptors (To et al., 2001; Kirikoshi et al., 2001), and Dvl family members (Uematsu et al., 2003a; Uematsu et al., 2003b).

It is clear that aberrant activation of canonical Wnt signaling plays a major role in the pathogenesis of a broad spectrum of human cancers and diseases, and further insight into the molecular pathogenesis will enable the discovery of novel drugs and therapies for clinical use.

Hh Signaling in Disease

In addition to the critical role Hh signaling plays during embryonic development [reviewed in (McMahon et al., 2003)], Hh signaling also directs the formation or the persistence of certain stem- and precursor-cell populations in adults (Ahn and Joyner, 2005; Baron, 2003; Machold et al., 2003; Palma and Altaba, 2004; Adolphe et al., 2004). Mutations in Hh pathway involving multiple key components have been implicated in several human anomalies, including holoprosencephaly and the nevoid basal cell carcinoma syndrome (NBCCS) [reviewed in (Mullor et al., 2002b)]. Furthermore, aberrant, constitutive activation of Hh pathway can contribute to tumorigenesis in multiple organs, including basal cell carcinoma (BCC) and basaloid follicular hamartoma in skin, medulloblastoma in brain, and embryonal rhabdosarcoma in muscle (Altaba et al., 2002b; Altaba et al., 2002a; Mullor et al., 2002a; Wetmore, 2003). Moreover, Hh pathway has also been implicated in tumors arising in the lungs, digestive and urogenital tracts, and hematopoietic system (Berman et al., 2003; Karhadkar et al., 2004; Thayer et al., 2003; Watkins et al., 2003; Dierks et al., 2007), further demonstrating that the Hh pathway exerts its effects in a wide variety of tissues and organs, and its deregulation plays a critical role in the pathogenesis of multiple tumor types. Several Hh target genes, such as *Bcl2*, *Cyclins D1* and *D2*, and *FoxM1* are directly involved in cell cycle

regulation and cell survival, and it is conceivable that the activation of these genes directly contributes to the initiation and proliferation of Hh pathway-associated tumors.

BCC is the most common type of cancer in light-skinned individuals with more than a million new cases diagnosed in the US each year, and although it rarely metastasizes, BCC can cause significant morbidity due to local invasion (Tang et al., 2007). A predisposition to skin cancer is associated with several genetic disorders including albinism, xeroderma pigmentosum and the NBCCS, also known as the Gorlin syndrome (ya-Grosjean and Couve-Privat, 2005). Gorlin syndrome, inherited in an autosomal dominant manner, is characterized by multiple developmental anomalies and predisposition to several cancers (Gorlin, 1995). Clinical features include odontogenic keratocysts, facial dysmorphism with macrocephaly, skeletal abnormalities, basal cell nevi, ovarian fibromas, medulloblastomas, neuroectodermal tumors and BCCs in skin (Manfredi et al., 2004). Analysis of Gorlin syndrome patients helped establish the molecular basis of BCC tumorigenesis. Linkage analysis revealed the locus for the tumor suppressor gene *PTCH1*, the human homologue of *Drosophila Ptc* located on chromosome 9q22-31, was implicated in Gorlin syndrome (Farndon et al., 1992; Gailani et al., 1992). Germline mutations of *PTCH1* observed in Gorlin patients (Hahn et al., 1996; Johnson et al., 1996) as well as frequent loss of heterozygosity at 9q22 found in other Gorlin syndrome-associated tumors (Vorechovsky et al., 1997b; Vorechovsky et al., 1997a) clearly linked aberrant Hh pathway with BCC tumorigenesis. Furthermore, mutations affecting several genes leading to aberrant Hh pathway activation have been identified in sporadic BCCs, including loss-of-function mutations in *PTCH1* and gain-of-function mutations in *SMO* (Hahn et al., 1996; Johnson et al., 1996; Xie et al., 1998).

Moreover, nearly all human BCCs exhibit evidence of elevated Hh signaling activity, further implicating the obligatory role of activated Hh signaling in BCC development and maintenance, regardless of the underlying genetic defect.

Genetically engineered mice have been essential and invaluable scientific tools for dissecting out the molecular mechanisms of disease. Not surprisingly, both gain-of-function as well as loss-of-function genetic studies utilizing transgenic mouse models with targeted manipulation of Hh signaling mediators at multiple levels – Shh, Ptch, Smo and Gli – have uncovered important insights into the role of Hh signaling in skin diseases, and these animal models support the notion that uncontrolled Hh pathway activation is sufficient to drive BCC- or BCC-like tumorigenesis in mice (Oro et al., 1997; Xie et al., 1998; Aszterbaum et al., 1999; Grachtchouk et al., 2000; Nilsson et al., 2000; Grachtchouk et al., 2003; Hutchin et al., 2005). Oro and his colleagues showed that over-expression of Shh in the skin of transgenic mice using a *keratin* 14 (K14) promoter induced the development of early BCC-like tumors (Oro et al., 1997). However, *K14-Shh* mice die shortly after birth, and it is unclear whether these early BCC-like tumors can progress to form full-blown BCCs in adult life, or whether activation of Shh expression in postnatal adult skin is also sufficient to drive BCC-like tumor development. Fan et al. showed that the regenerated human skin transgenic for Shh when grafted onto immune deficient mice displayed abnormal BCC-like tumors (Fan et al., 1997). In addition, Xie et al. showed that the expression of M2SMO under the 5.2 kb *keratin* 5 (K5) promoter was sufficient to induce development of BCC-like tumors in newborn skin (Xie et al., 1998), although these mice also failed to survive after birth. Work from several laboratories has also shown that over-expression of *Gli1* or *Gli2* in transgenic

mouse skin is sufficient to drive BCC or BCC-like tumor development, implicating Gli transcription factors as mediators of oncogenic Hh signaling. Mice expressing Gli2 driven by a K5 promoter develop numerous BCCs on their skin within 3 months of age (Grachtchouk et al., 2000). *K5-Gli2* BCCs share similar gross and histological appearances with human BCCs, and also exhibit a high level of Hh pathway activation as assessed by robust *Gli1* and *Ptch1* expression (Grachtchouk et al., 2000). Similarly, Nilsson and colleagues showed that expression of human *GLI1* in mice under K5 promoter induced development of a variety of skin tumors, incuding trichoepitheliomas and, rarely, BCCs (Nilsson et al., 2000).

Interestingly, analysis of adult transgenic mice expressing a constitutively activated mutant form of Smo, M2SMO, revealed that these mice did not develop BCC, but rather developed benign follicular hamartoma, a benign, slow-growing, and relatively undifferentiated skin tumor that expresses BCC protein markers and expands into the underlying dermis (Grachtchouk et al., 2003). The lack of BCC development in M2SMO-expressing mice may be explained by the lower level of Hh signaling activation in mice expressing M2SMO relative to mice expressing Gli2, as assessed by relatively lower levels of expression of Hh target genes *Gli1* and *Ptch1* (Grachtchouk et al., 2003).

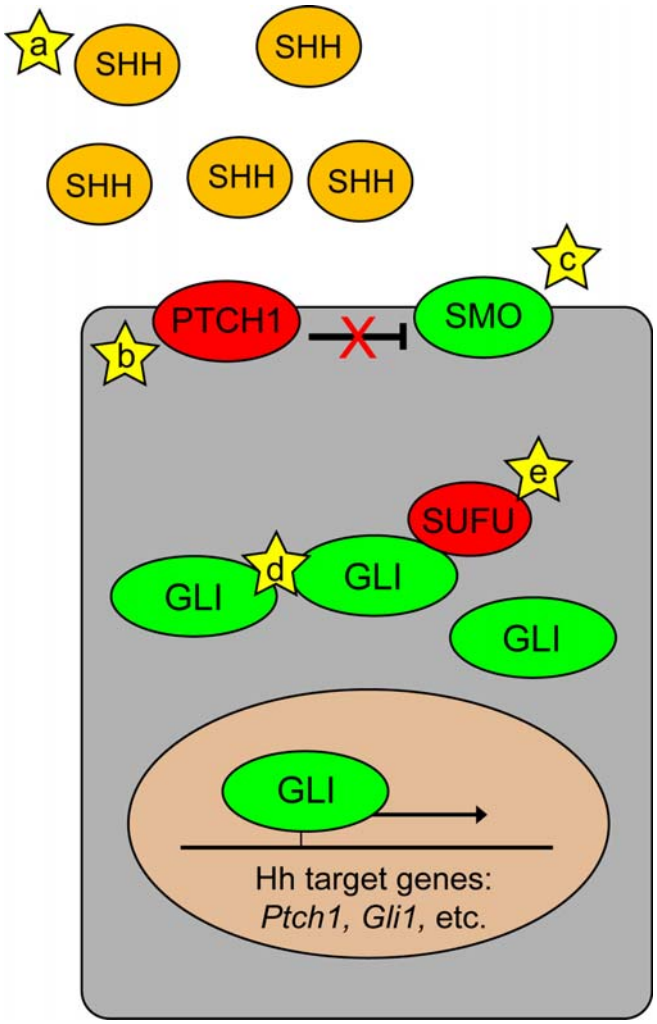

Figure 1-6. Models of Hh pathway activation in human cancers.. (**a**) HH ligand overexpression is associated with esophageal, gastric, pancreatic, breast, prostate, small-cell lung cancer, and B cell neoplasms. (**b**) Loss of function mutations in *PTCH1* are seen in basal cell carcinoma and medulloblastoma. (**c**) Pathway-activating *SMO* mutations have been found in basal cell carcinomas and medulloblastomas, as well. These mutations render SMO insensitive to PTCH1 inhibition, irrespective of HH ligand status. (**d**) GLI2 amplification has been reported in squamous cell carcinoma (Snijders et al., 2005). (**e**) Loss of function mutations of SUFU are seen in medulloblastoma and BCC. Modified from (Rubin and de Sauvage, 2006).

Unanswered Questions

BCC is classified histologically according to morphology, and includes nodular, micronodular, infiltrative and superficial forms (Sexton et al., 1990; Fuchs, 2008). In particular, superficial BCC is characterized by epithelial buds of proliferating basal cells that grow down from the epidermis into the superficial dermis, maintaining their attachment to the basal cell layer. Intriguingly, these Hh-driven superficial BCC share a striking morphological similarity with embryonic Wnt-driven hair germs (Lever, 1948; Montgomery, 1935; Sellheyer and Krahl, 2008; El-Bahrawy et al., 2003; Kumakiri and Hashimoto, 1978), but the molecular basis for this similarity is not known. Do superficial BCCs resemble embryonic hair buds because similar pathways are utilized during BCC development and early stages of hair follicle morphogenesis? Is the Hh-driven ectopic bud development a direct effect of Hh signaling, or is it indirect? With two distinct signaling pathways giving rise to similar-appearing epithelial structures, it is conceivable that ectopic Hh pathway activation drives de novo bud development indirectly, via the canonical Wnt pathway. This possibility is strengthened by a previous report in *Xenopus* showing that Gli proteins activate expression of Wnt ligands (Mullor et al., 2001). Does Hh signaling similarly influence expression of Wnt genes and canonical Wnt signaling in skin? Perhaps most importantly, is canonical Wnt signaling required for Hh-mediated development of epithelial hair buds, follicular hamartomas, or BCCs that arise from constitutive Hh signaling in skin? What are the effects of heightened Hh or canonical Wnt signaling on hairless epidermis? I was in a unique position to address

these questions utilizing the powerful genetic mouse models in which the functions of Hh and canonical Wnt signaling pathways have been altered.

In addition, while previous reports have found that canonical Wnt activation in resting telogen hair follicles is sufficient to trigger a new hair follicle growth cycle (Van Mater et al., 2003; Lo Celso et al., 2004), other studies have indicated that 'unscheduled' Hh pathway activation is also sufficient to induce a new hair growth cycle in mice (Sato et al., 1999; Paladini et al., 2005). However, in these studies, Hh activation was likely induced in both the epidermal and dermal cells, and it is unclear in which cell population Hh signaling must be activated to trigger the anagen growth phase. Several important questions remain to be answered stemming from these observations. What is the molecular explanation for this phenomenon? Could Hh activation be triggering a new growth cycle indirectly by stimulating Wnt signaling pathway in resting hair follicles? Does Shh act directly on hair follicle stem cells to reactivate growth and trigger a new growth phase? Is epithelial-specific Hh signaling sufficient to initiate new follicle formation? Utilizing novel mouse models that allow precise spatial and temporal control of Hh signaling activity in the cutaneous epithelium, I describe results that address these questions and provide new insights into hair follicle biology.

Summary

The Hh and canonical Wnt/β-catenin signaling pathways play pivotal roles in a variety of developmental and biological processes during embryogenesis and adult life, and when deregulated, contribute to the pathogenesis of many tumors. In this thesis, I address the aforementioned questions utilizing constitutive as well as inducible transgenic

mouse models in which I have modulated the Hh and Wnt signaling pathways. In Chapter II, I describe our findings as we investigated the potential involvement of Wnt signaling in superficial human BCCs and a mouse model expressing the Hh pathway effector, M2SMO. I show that similar to embryonic human hair germs, BCC buds exhibited elevated levels of nuclear and cytoplasmic β-catenin, a marker of canonical Wnt signaling, and expressed the early hair follicle lineage markers Sox9, K17, and CCAAT displacement protein (CDP). Similar changes, along with expression of multiple Wnt genes, *Tcf4*, activated β-catenin, and the Wnt target genes *Axin2* and *Sp5*, were detected in M2SMO-expressing mouse skin containing epithelial buds and follicular hamartomas. I also show that by selectively blocking canonical Wnt signaling by conditional over-expression of the potent Wnt inhibitor Dkk1, epithelial bud and hamartoma development in M2SMO mice was blocked, despite continued Hh signaling. Thus, I demonstrate an essential function for the canonical Wnt pathway downstream of pathologic Hh signaling in skin.

In Chapter 3, I show that forced activation of canonical Wnt signaling is sufficient to drive advanced hair follicle differentiation in both normal and M2SMO-skin by taking advantage of Cre-inducible *Ctnnb1*$^{(Ex3)fl/fl}$ transgenic mice (Harada et al., 1999). *Ctnnb1*$^{(Ex3)fl/fl}$ mice express a stabilized, mutant β-catenin that lacks the critical phosphorylation sites on exon 3 necessary for its degradation when crossed with a taxomifen-inducible, skin-specific Cre-driver *K5-CreERT2* (Indra et al., 1999) mice. Utilizing doxycycline-inducible *K5-rtTA* (Diamond et al., 2000);*TRE-SmoA1* (developed by fellow MSTP student Evan Michael in the Dlugosz laboratory) bitransgenic mice, I also show that epithelial-specific Hh activation in quiescent resting telogen follicles is

sufficient to trigger new anagen growth cycle (Chapter 4). Finally, I also present data showing that ectopic Hh signaling in murine skin leads to *de novo* melanogenesis and accumulation of pigment, and that this process may be mediated indirectly by activation of the canonical Wnt signaling pathway (Chapter 5).

By examining the complex interactions between Hh and Wnt pathways during pathogenesis of skin tumors driven by ectopic Hh signaling, my thesis work uncovers a novel requirement for canonical Wnt signaling downstream of pathologic Hh signaling in skin. The results described in this thesis provide novel insights into how the precisely-regulated interactions between the Hh and canonical Wnt pathways are perturbed in pathology, and provides exciting relevance for investigating the potential Hh-Wnt crosstalk in other neoplasms.

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Chapter II

Pathological responses to oncogenic Hedgehog signaling in skin are dependent on canonical Wnt/β-catenin signaling*

Summary

Constitutive Hh signaling underlies several human tumors (Rubin and de Sauvage, 2006), including basal cell carcinoma (BCC) and basaloid follicular hamartoma in skin (Grachtchouk et al., 2003; ya-Grosjean and Couve-Privat, 2005). Intriguingly, superficial BCCs arise as *de novo* epithelial buds resembling embryonic hair germs (Lever, 1948; Montgomery, 1935; Sellheyer and Krahl, 2008), collections of epidermal cells whose development is regulated by canonical Wnt/β-catenin signaling (Andl et al., 2002; Huelsken et al., 2001). Similar to embryonic hair germs, human BCC buds exhibited increased levels of cytoplasmic and nuclear β-catenin, and expressed early hair follicle lineage markers. We also detected canonical Wnt/β-catenin signaling in epithelial buds and hamartomas from mice expressing an oncogene, *M2SMO* (Xie et al., 1998), leading to constitutive Hh signaling in skin. Conditional overexpression of the Wnt pathway antagonist Dkk1 in M2SMO-expressing mice potently inhibited epithelial bud and hamartoma development without affecting Hh signaling. Our findings uncover a hitherto unknown requirement for ligand-driven, canonical Wnt/β-catenin signaling for Hh

* Most of this chapter represents a subset of the data from a published manuscript: Yang, S.H., et al. (2008). "Pathological responses to oncogenic Hedgehog signaling in skin are dependent on canonical Wnt/β-catenin signaling." Nat Genet (in press).

pathway-driven tumorigenesis, identify a new pharmacological target for these neoplasms, and establish the molecular basis for the well-known similarity between early BCCs and embryonic hair germs.

Introduction

As described in Chapter I, hair follicle development is orchestrated by a series of inductive signals involving epithelial and mesenchymal hair follicle progenitors and their progeny, and includes the BMPs and their inhibitors, transforming growth factors-β, fibroblast growth factors, and the Wnt, Eda/Edar, and Hedgehog (Hh) pathways (Fuchs, 2007). Canonical Wnt signaling is both necessary and sufficient for the initial stages of hair follicle development (Andl et al., 2002; Gat et al., 1998; Huelsken et al., 2001; Lo Celso et al., 2004), including the formation of embryonic hair buds, while Hh signaling subsequently promotes the massive proliferation of follicle epithelium needed to assemble a mature follicle (Chiang et al., 1999; St-Jacques et al., 1998). Wnt signals then play an important role in the differentiation of committed epithelial progenitors to form late-stage hair follicle lineages (DasGupta and Fuchs, 1999; Fuchs, 2007;Millar, 2002).

In addition to its functions in hair follicle development, Wnt signaling is involved in multiple other processes throughout the body, and when deregulated, contributes to tumorigenesis in several organs (Cadigan and Liu, 2006; Clevers, 2006; Hoppler and Kavanagh, 2007). Binding of a subset of secreted Wnt proteins to Frizzled receptors and low-density lipoprotein receptor-related protein (LRP) co-receptors activates a conserved "canonical" Wnt signaling pathway (Cadigan and Liu, 2006). β-catenin is a critical mediator of the canonical Wnt pathway, a protein that has dual, non-overlapping

functions: cell-cell adhesion and signal transduction (Brembeck et al., 2006). There exist two main pools of β-catenin in epithelial cells: one pool forms a stable complex with the cytoplasmic domain of E-cadherin at cell-cell borders, and the other pool is within the cytoplasm and/or nucleus. In the absence of Wnt ligands, "free" cytoplasmic β-catenin is phosphorylated by glycogen synthase kinase 3 (GSK-3β), which is complexed with adenomatous polyposis coli protein (APC), and Axin. Under basal conditions, βTrCP interacts with phosphorylated β-catenin, which is ubiquitinated and targeted for rapid proteasome-mediated degradation. When Wnt proteins bind to their Frizzled/LRP co-receptors, the cytoplasmic protein Dishevelled is phosphorylated and function of the GSK-3β/APC/Axin destruction complex is inhibited, leading to inhibition of β-catenin phosphorylation and its stabilization. In the nucleus, β-catenin complexes with members of the LEF/TCF family of DNA binding factors to regulate transcription of Wnt target genes. While multiple canonical Wnt target genes exist, *Axin2* (Jho et al., 2002) and *Sp5* (Weidinger et al., 2005) have shown to be faithful target genes, and along with nuclear/cytoplasmic β-catenin localization, may be used as reliable markers for canonical Wnt pathway activation.

A number of extracellular proteins have been identified that bind to the secreted Wnt ligands and/or receptors to regulate canonical Wnt signaling, including Dkk1. Dkk1, originally identified as an inducer of head formation in *Xenopus* embryos, is a potent and specific endogenous secreted Wnt inhibitor, and inhibits canonical Wnt signaling by binding to the LRP co-receptors required for activation of canonical Wnt signaling (Bafico et al., 2001; Mao et al., 2001). The inhibition of Wnt signaling by Dkk1 also requires Kremen1 and Kremen2, transmembrane proteins that form a ternary complex

with Dkk1 and LRP 5/6 co-receptors (Mao et al., 2002). The formation of the Dkk1-LRP5/6-Kremen ternary complex rapidly induces internalization and depletion of cell-surface LRP5/6 co-receptors and blockade of canonical Wnt signaling (Mao et al., 2002).

Hh ligands are expressed in many embryonic tissues and influence patterning through their effects on proliferation, cell death, and lineage selection (Ingham and Placzek, 2006; Rubin and de Sauvage, 2006). After birth, proper Hh signaling is required to maintain normal cell proliferation and survival in adult tissues, and deregulated Hh signaling is associated with the development of several types of neoplasms (Roessler et al., 1996). Secreted Hh ligands frequently act as paracrine signaling effectors, altering the function of responsive target cells by a highly conserved signaling pathway which is normally maintained in an "off" state by the Hh receptor Ptch1 (Ingham and Placzek, 2006). In the absence of Hh ligand, Ptch1 represses Smoothened (Smo), an obligatory transducer of Hh signaling in target cells. Binding of Hh ligand to Ptch1 blocks its function, resulting in derepression of Smo which ultimately leads to an altered pattern of gene expression mediated by zinc-finger proteins of the Gli family (Gli1, Gli2, and Gli3) (ya-Grosjean and Couve-Privat, 2005). *Gli1* and *Ptch1* are considered 'classical' Hh target genes whose expression is consistently upregulated in response to Hh signaling, making them reliable markers for Hh pathway activation (McMahon et al., 2003).

In hair follicles, Hh pathway plays an indispensable role in driving proliferation of follicle epithelium during development and the postnatal hair cycle (Altaba et al., 2002; Sato et al., 1999; St-Jacques et al., 1998; Wang et al., 2000), which include phases of growth (anagen), regression (catagen), and rest (telogen). Normally, Hh signaling in hair follicle epithelium is tightly regulated and restricted to the initial development of the

follicle, and the growth phase of the hair cycle after birth. In contrast, sustained Hh signaling is associated with the development of several types of 'follicular' tumors, including BCC, trichoepithelioma, and basaloid follicular hamartoma.

BCC is the most common type of cancer in light-skinned individuals with more than a million new cases diagnosed in the US each year, and although it rarely metastasizes, BCC can cause significant morbidity due to local invasion (Tang et al., 2007). Mutations affecting several genes leading to aberrant Hh pathway activation have been identified in BCC, including loss-of-function mutations in *PTCH1* and gain-of-function mutations in *SMO* (Hahn et al., 1996; Johnson et al., 1996; Xie et al., 1998). Moreover, nearly all human BCCs exhibit evidence of elevated Hh signaling activity, and several animal models support the notion that uncontrolled Hh pathway activation is sufficient to drive BCC- or BCC-like tumorigenesis in mice (Aszterbaum et al., 1999; Grachtchouk et al., 2000; Grachtchouk et al., 2003; Hutchin et al., 2005; Nilsson et al., 2000; Oro et al., 1997; Xie et al., 1998).

BCC is classified histologically according to morphology, and includes nodular, micronodular, infiltrative and superficial forms (Sexton et al., 1990). In superficial BCC, epithelial buds of proliferating basal cells grow down from the epidermis into the superficial dermis, maintaining their attachment to the basal cell layer. Pathologists have long noted the morphological similarity between focal epidermal downgrowths in BCC and embryonic hair germs (El-Bahrawy et al., 2003; Kumakiri and Hashimoto, 1978; Lever, 1948; Montgomery, 1935), raising the possibility that: A) similar pathways are utilized during BCC development and early stages of hair follicle morphogenesis; and B) canonical Wnt signaling is involved indirectly in pathologic responses to deregulated Hh

signaling in skin. While some prior studies have identified coordinate changes in the Hh and canonical Wnt pathways in BCCs and other neoplasms (El-Bahrawy et al., 2003; Li et al., 2007; Mullor et al., 2001; Pasca di Magliano et al., 2007; Saldanha et al., 2004; Taipale and Beachy, 2001; Yamazaki et al., 2001), direct evidence establishing the functional significance of Wnt signaling in Hh pathway-driven pathology *in vivo* is lacking.

Using *M2SMO*-expressing transgenic mice as a model system, we tested whether pathological Hh signaling in skin leads to the formation of hair-bud like structures indirectly, via canonical Wnt signaling, the physiological stimulus for hair bud formation in embryos. We show upregulation of multiple canonical Wnt genes, nuclear and cytoplasmic redistribution of β-catenin, and upregulation of endogenous Wnt target genes *Axin2* and *Sp5* in M2SMO-expressing mouse skin. Moreover, using a doxycycline-regulated transgenic mouse expressing the canonical Wnt inhibitor Dkk1, we show that ectopic bud formation and expansion require canonical Wnt signaling activity. Our findings establish a pivotal role of Hh-Wnt interactions in the pathogenesis of a epithelial buds and hamartomas driven by aberrant Hh signaling, and provide a molecular explanation for the morphological and biochemical similarity between early BCC and embryonic hair germs.

Materials and Methods

Transgenic mice and conditional transgene activation.

To focally activate Hh signaling in skin, we crossed *K5-flxGFP-M2SMO* mice (Allen et al., 2003), which harbor a Cre-inducible *M2SMO* allele, with tamoxifen-

inducible *K5-CreERT2* (Indra et al., 1999) mice (provided by Pierre Chambon and Daniel Metzger). Low level ("leaky") recombinase activity of CreERT2, in the absence of tamoxifen, resulted in ectopic expression of M2SMO in skin (Allen et al., 2003). To generate triple transgenic mice with epithelial expression of M2SMO and doxycycline-inducible Dkk1 expression, *ΔK5-M2SMO* mice (Grachtchouk et al., 2003) were first crossed with *K5-rtTA* (Diamond et al., 2000) mice. *ΔK5-M2SMO;K5-rtTA* mice were then crossed with *TRE-Dkk1* (Chu et al., 2004) mice, to generate *ΔK5-M2SMO;K5-rtTA;TRE-Dkk1* mice, designated M2SMO+Dkk1 when treated with doxycycline to induce Dkk1 expression. To induce Dkk1 expression in M2SMO+Dkk1 mice, doxycycline (20 mg/ml) was administered in drinking water with 5% sucrose, and in doxycycline-containing chow (Bio-serve, 200 mg/kg). After three days, mice were maintained on doxycycline-containing chow but received normal drinking water. All mice were housed and maintained according to University of Michigan institutional guidelines, as stipulated by the University Committee on the Use and Care of Animals.

Tissue harvesting and wholemount preparation.

Human superficial BCCs were obtained from the Cutaneous Surgery and Oncology Unit, Department of Dermatology, University of Michigan Medical School, according to an IRB-approved protocol (IRBMED 2000-0015). Embryonic human skin was obtained from Advanced Bioscience Resources. For hematoxylin and eosin (H&E) staining and immunohistochemistry, human and mouse skin samples were fixed in neutral-buffered formalin (NBF) overnight, transferred to 70% ethanol, processed, and embedded in paraffin. Mouse skin was also embedded in OCT Compound (Tissue-Tek)

for frozen sections. To prepare whole-mounts of volar skin, mice were euthanized and ventral hind limb skin was removed. Volar skin was microdissected and whole-mount preparation was performed essentially as described previously for tail skin (Braun et al., 2003). Transilluminated whole mount images were captured with a digital camera (Diagnostic Instruments Spot RT3) mounted on a dissecting stereomicroscope (Leica MZFL3), using Spot Software Version 4.6 (Diagnostic Instruments).

Immunostaining.

The following primary antibodies were used for immunostaining: K1 (rabbit polyclonal, 1:1,000, Covance); K5 (rabbit polyclonal, 1:2,000, Covance); K17 (rabbit polyclonal, 1:4,000, gift from P. Coulombe); β-catenin (mouse monoclonal, 1:1,000, Sigma); Sox9 (rabbit polyclonal, 1:1,000, Chemicon); CDP (rabbit polyclonal, 1:100, Santa Cruz); Ki67 (rabbit polyclonal, 1:500, Vector Laboratories); AE13 (1:10, gift from Henry Sun); AE15 (1:50, gift from Henry Sun); P-cadherin (rat monoclonal, 1:500, Zymed); E-cadherin (rat monoclonal, 1:1,000, Zymed); Cyclin D1 (rabbit polyclonal, 1:1,000, Neomarkers). For immunohistochemistry, 8 μm sections were cut in a parasagittal plane. For all antibodies except for AE13, AE15, P- and E-cadherin, immunoreactivity of antigens was restored by immersing slides in boiling 0.01 M citrate buffer, pH ~6, for 10 min. Blocking was performed using 1.5% normal goat serum in phosphate-buffered saline (PBS), and tissue sections were incubated with primary antibodies diluted in PBS containing 1% bovine serum albumin, typically for 1-3 h at room temperature (21-23 ºC). Subsequent immunostaining procedures were performed using peroxidase Vectastain ABC kit (Vector Laboratories) and 3,3'-diaminobenzidine

(DAB) as a substrate, according to the manufacturer's protocol. A M.O.M. kit (Vector Laboratories) was used for immunostaining with mouse primary monoclonal antibodies according to the manufacturer's protocol. Sections were counter-stained with hematoxylin and mounted using Permount (Fisher Scientific). For immunofluorescence staining, sections were cut from OCT-embedded blocks and fixed in cold acetone for 10 minutes. FITC-conjugated secondary antibodies (Jackson ImmunoResearch) were used at 1:75 dilution, and 100 ng/ml 4-diamidino-2-phenylindole (DAPI, Merck) was used for nuclear counterstaining. Endogenous alkaline phosphatase activity was visualized using Alkaline Phosphatase Substrate Kit I (Vector Laboratories) and incubating tissue sections with substrate solution for 2-4 h at room temperature.

Immunoblotting.

Tissue was homogenized in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5 mM EDTA, 1 mM sodium orthovanadate, 200 mM sodium fluoride) using tissue microgrinder (Kontes 749520), vortexed vigorously, cleared by centrifugation for 15 min at 12,000 x g, and denatured by heating at 98 ºC for 5 min. 10 µl of protein extract was resolved on a 10% (w/v) denaturing SDS poly-acrylamide gel (BioRad) and transferred to nitrocellulose membrane. "Activated" β-catenin was detected by probing with α-activated β-catenin (αABC) antibody (mouse monoclonal, 1:1,000, Upstate). Pan-actin antibody (mouse monoclonal, 1:1,000, Labvision) was used to detect actin for loading control. Peroxidase-conjugated secondary antibodies were used and visualized by enhanced chemiluminescence using an ECL Plus kit (Amersham Biosciences).

RNA isolation and semiquantitative RT-PCR.

Volar, tail, and postnatal day 8 dorsal skin were homogenized in Trizol (Invitrogen) using a mechanical grinder (PowerGen700, Fisher Scientific), and lysates stored at -80°C until further processing. RNA isolation, first strand cDNA synthesis, and RT-PCR were performed as described previously, with minor modifications(Allen et al., 2003). Primer sequences are listed in Supplementary Table 1.

Supplementary Table 1. Primer sequences used for semiquantitative RT-PCR analysis.

Gene	Forward Primer	Reverse Primer	Size (bp)
Actin(Walterhouse et al., 1993)	TACCACAGGCATTGTGATGGA	CAACGTCACACTTCATGATGG	421
Gli1 (Grachtchouk et al., 2000)	GTCGGAAGTCCTATTCACGC	CAGTCTGCTCTCTTCCCTGC	364
Ptch1(Takabatake et al., 1997)	AACAAAAATTCAACCAAACCTC	TGTCTTCATTCCAGTTGATGTG	243
Tcf4	CAGCTCAAAGCATCAGGACTC	CTGTTGATCAAGGCCAAAGCGC	338
Axin2(Kim et al., 2005)	CAGGAGCCTCACCCTTCG	ACGCCGAGGTGCTTGCCC	402
Sp5	ACACCAGGGTACTTGCCATC	AATCGGGCCTAGCAAAAACT	305
Cyclin D1(Berman et al., 2002)	CTCTGGCTCTGTGCCTTTCT	CCGGAGACTCAGAGCAAATC	646
Wnt2(Liu et al., 2003)	CGGCCTTTGTTTACGCCATC	TGAATACAGTAGTCTGGAGAA	496
Wnt3(Liu et al., 2003)	GCCGACTTCGGGGTGCTGGT	CTTGAAGAGCGCGTACTTAG	320
Wnt4(Liu et al., 2003)	TGTACCTGGCCAAGCTGTCAT	TCCGGTCACAGCCACACTT	330
Wnt5a(Liu et al., 2003)	TCCTATGAGAGCGCACGCAT	CAGCTTGCCCCGGCTGTTGA	230
Wnt7b(Liu et al., 2003)	ACCGTCTTCGGGCAAGAACT	CCTGGCGTTCTTTTTGATCT	260

Wnt10a(Liu et al., 2003)	AAAGTCCCCTACGAGAGCCC	CAGCTTCCGACGGAAAGCTT	180
Wnt10b(Liu et al., 2003)	CGGCTGCCGCACCACAGCGC	CAGCTTGGCTCTAAGCCGGT	180
Wnt11(Liu et al., 2003)	GCCATGAAGGCCTGCCGTAG	GATGGTGTGACTGATGGTGG	160
Wnt13(Liu et al., 2003)	TGTACTCTGCGCACCTGCT	TGCACTCACACTGGGTGAC	322

Results

Expression of similar lineage markers in human superficial BCCs and embryonic hair buds

Both human superficial BCCs and embryonic hair buds, comprised a focal grouping of epidermal cells protruding into the underlying dermis (Figure 2-1), and expressed early-stage follicle lineage markers, including the outer root sheath markers K17 (Jih et al., 2003) and Sox9 (Oseroff et al., 2005), and the hair matrix/inner root sheath marker CDP (Callahan et al., 2004) (Figure 2-1c,d,g-j). However, in contrast to the non-overlapping distribution of cells expressing Sox9 and CDP in embryonic hair buds (brackets in Figure 2-1h,j), these lineage markers appeared to be co-expressed in cells of human superficial BCC (Figure 2-1g,i). Epithelial cells in both the superficial BCCs and embryonic hair buds were more proliferative, based on Ki67-immunostaining, than adjacent epidermis (Figure 2-1k,l) and did not express the epidermal differentiation marker K1 (Figure 2-1e,f). In contrast to embryonic hair buds (Figure 2-1b, arrowhead), superficial BCCs did not exhibit a morphologically recognizable mesenchymal papilla (Figure 2-1a), which is required for later stages of hair follicle morphogenesis.

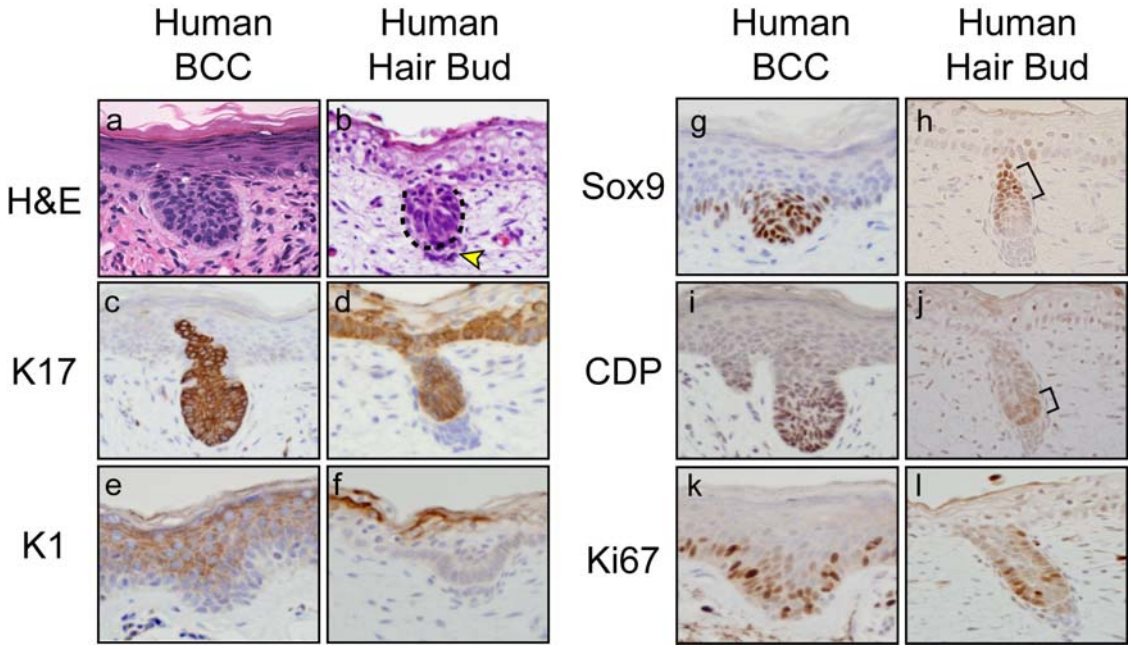


Figure 2-1. Human superficial basal cell carcinoma expresses hair bud lineage markers. (**a,b**) H&E-stained sections of early human superficial BCC and embryonic hair buds. Dashed line in (**b**) indicates epithelial component of embryonic hair follicle, with adjacent mesenchymal condensate marked with an arrowhead. Note absence of morphologically recognizable mesenchymal condensate near superficial BCC. (**c,d**) Hair placode marker K17 is expressed in superficial BCC and hair bud. At this stage of embryonic development, surrounding epidermis (**d**) also expresses this marker. (**e,f**) The suprabasal cell marker K1 is not expressed in superficial BCC or in hair bud. (**g-j**) Outer root sheath marker Sox9 and hair matrix/inner root sheath marker CDP are expressed in superficial BCC and hair bud. Note largely non-overlapping expression patterns of Sox9 and CDP in hair bud (brackets in **h,j**), compared to overlapping expression in superficial BCC. (**k,l**) Increased proliferation in superficial BCC and hair bud relative to adjacent epidermis, assessed by Ki67 staining.

Ectopic Hh signaling in mouse epidermis produces superficial BCC-like downgrowths resembling embryonic hair buds

De novo epidermal downgrowths have been noted in several mouse models with deregulated Hh signaling activity in skin (Allen et al., 2003; Grachtchouk et al., 2003; Nilsson et al., 2000; Oro et al., 1997; Vidal et al., 2005). We examined epithelial bud development in greater detail in mice expressing a gain-of-function *SMO* allele (*M2SMO)* following Cre-mediated recombination (Allen et al., 2003). We then examined epithelial bud development in mice with focally-activated Hh signaling in skin, achieved using the M2SMO oncogene (Xie et al., 1998). We restricted much of our analysis to a triangular region of volar skin completely devoid of follicles or other skin appendages, enabling us to study the effects of deregulated Hh signaling in a morphogenetically naive epidermis (Figure 2-2). Constitutive activation of Hh signaling using M2SMO resulted in *de novo* epithelial bud initiation in this normally hairless region (Figure 2-3a-d). Similar to human BCC (Figure 2-1), the M2SMO-induced buds expressed several lineage markers seen in embryonic mouse hair buds (Figure 2-5), including the outer root sheath markers K17 and Sox9, and the hair matrix/inner root sheath marker CDP (Figure 2-4 and 2-6). In addition, M2SMO-induced ectopic buds expressed Epcam (Figure 2-4c), 38kD glycoprotein that is specifically expressed in embryonic hair buds, as well as secondary hair germs in telogen hair follicles and BCC (Klein et al., 1990). Similar to human superficial BCC, epithelial cells in M2SMO-induced buds co-expressed Sox9 and CDP, while embryonic hair buds showed non-overlapping distribution of cells expressing these markers (brackets in Figure 2-6f,h). Expression of the suprabasal marker K1 was not seen in M2SMO buds or embryonic hair buds (Figure 2-6i,j), whereas proliferation

(Ki67-immunostaining) (Figure 2-6k,l) and P-cadherin expression (Figure 2-6m,n) were both increased relative to adjacent epidermis. Notably, down-regulation of E-cadherin, a characteristic alteration in embryonic hair buds that may contribute to a shift from membrane-bound to cytoplasmic and nuclear β-catenin (Mullor et al., 2002), was not apparent in M2SMO-induced buds (Figure 2-6o,p), arguing that changes in E-cadherin expression levels are not required for ectopic hair bud development in this setting. As in human BCC buds, M2SMO-induced ectopic buds were not associated with a morphologically-detectable mesenchymal component (Figure 2-6a,b) or expression of the mesenchymal condensate/papilla marker alkaline phosphatase (Figure 2-3e,f). Ectopic, cell-autonomous activation of Hh signaling in mouse epidermis thus mimics early stages of human superficial BCC development by reprogramming epidermis to form epithelial invaginations similar to embryonic hair buds, with the notable absence of an associated mesenchymal component and impaired segregation of epithelial cells into distinct Sox9- and CDP-expressing compartments.

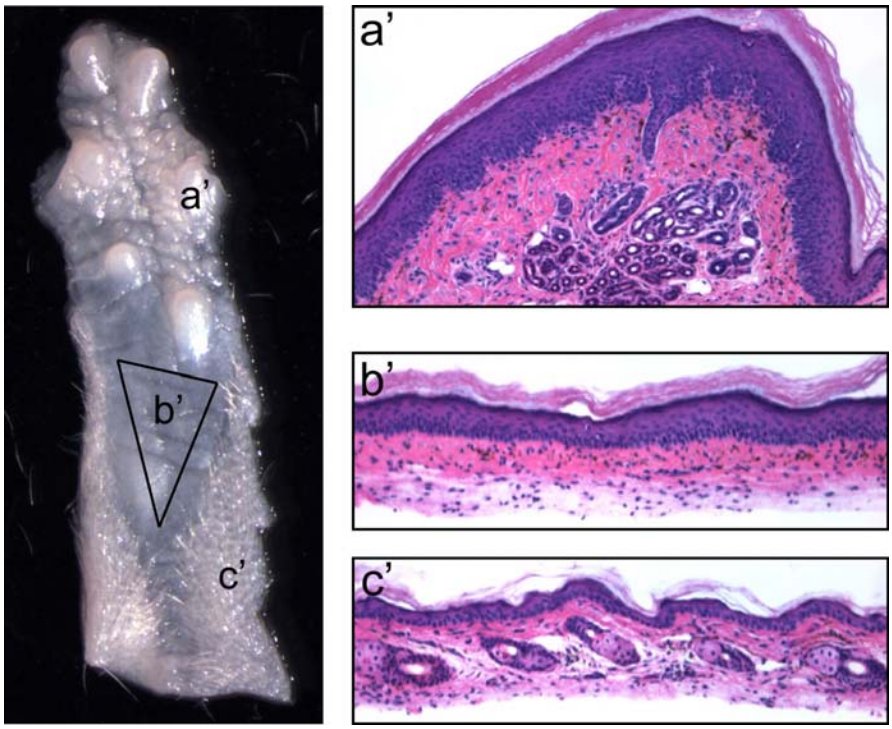


Figure 2-2. Hairless region of mouse volar skin. Whole-mount view of control skin from ventral aspect of mouse hind limb, with H&E-stained sections from indicated regions. Triangle outlines a region completely devoid of hair follicles or other skin appendages (**b'**), compared to eccrine glands in the footpad region (**a'**) and hair follicles at the periphery (**c'**).

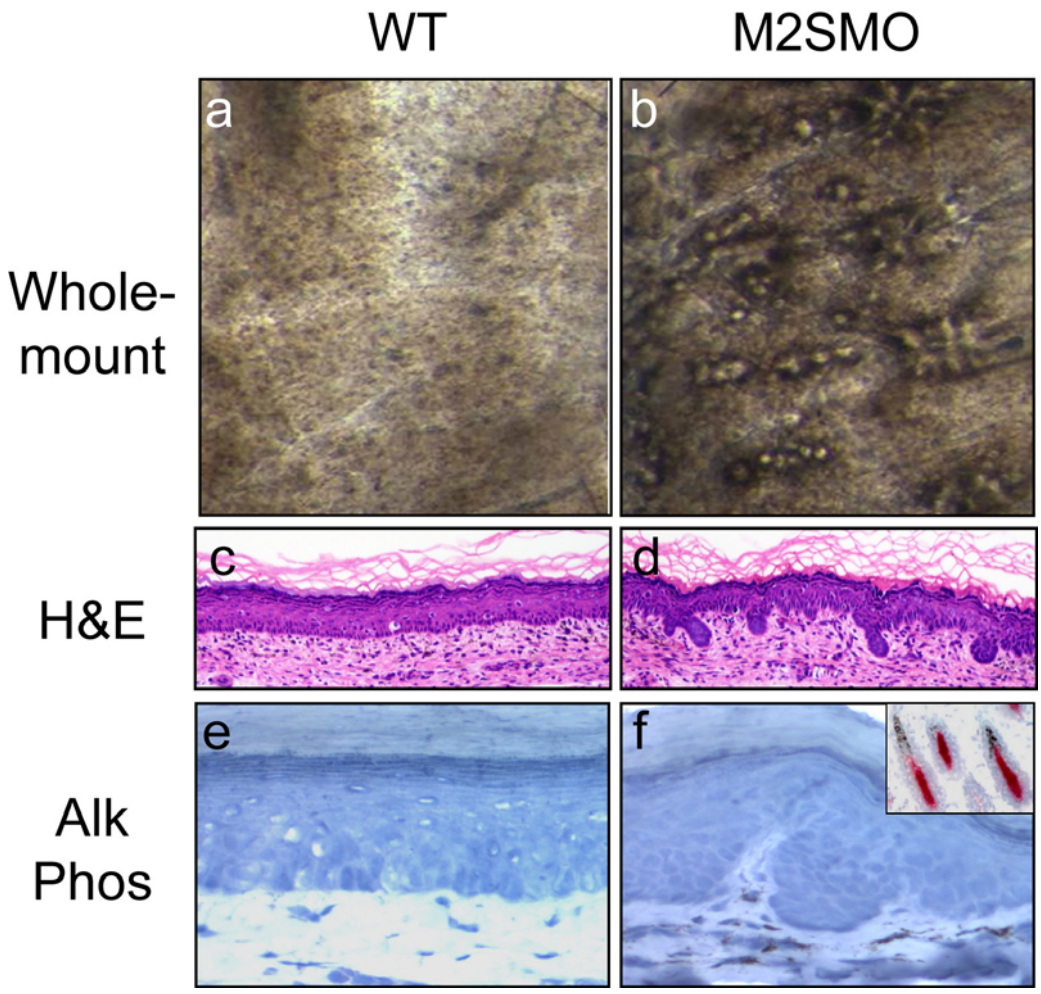


Figure 2-3. Ectopic Hh signaling in M2SMO-expressing hairless mouse skin drives superficial BCC-like downgrowths resembling hair buds. Histology, marker expression and increased proliferation rate of ectopic epithelial buds in volar skin from M2SMO-expressing mice and hair bud from E16.5 mouse embryo. (**a,b**) Wholemount view of hairless control (WT) and M2SMO transgenic volar skin. Activation of Hh signaling using M2SMO results in the appearance of scattered buds and hyperpigmentation in hairless skin (**b**). (**c,d**) H&E-stained sections showing distribution of buds in M2SMO volar skin (**d**). (**e,f**) Endogenous alkaline phosphatase, a marker for follicle-associated mesenchymal condensates, is not detected in control or M2SMO volar skin. Inset in **(f)** shows alkaline phosphatase-positive (red) dermal papillae in hair follicles.

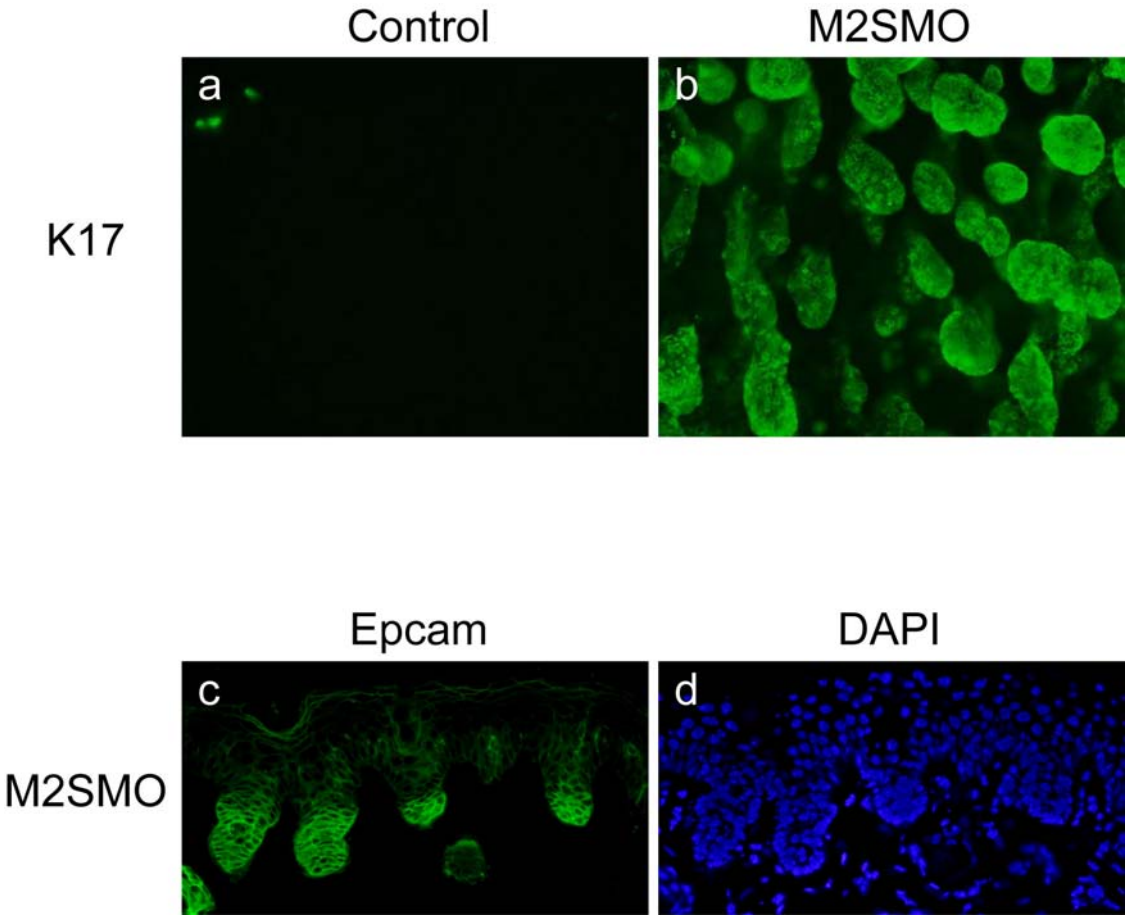


Figure 2-4. Ectopic Hh-induced buds in hairless volar skin express hair bud markers K17 and Epcam. (**a,b**) Wholemount K17 immunoflourescence staining reveals K17-positive ectopic buds in M2SMO volar skin at a slightly later developmental time period than M2SMO volar skin shown in Figure 2-3. Note only occasional K17-positive cells are seen in control volar skin. (**c**) M2SMO-induced buds express Epcam, hair bud specific marker (Klein et al., 1990). (**d**) DAPI immunoflourescence image of (**c**) for nuclei staining.

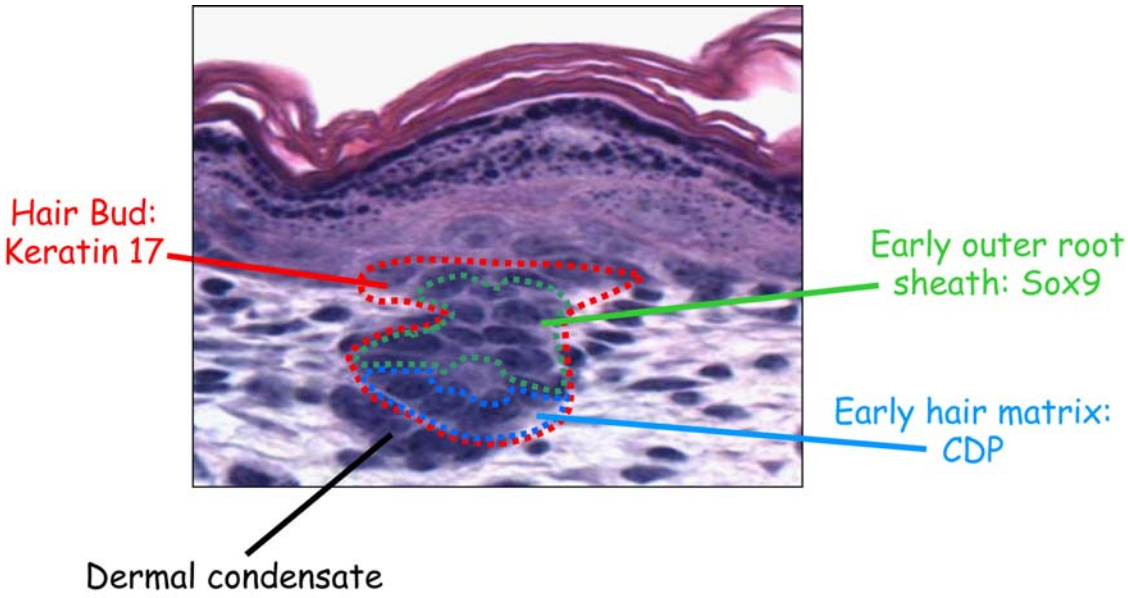


Figure 2-5. Mouse hair bud cellular compartments and epithelial lineage markers. Cartoon illustrating early embryonic mouse hair bud compartments and epithelial lineage markers. Red dashed line indicates Keratin 17 (hair bud marker) expression, green dashed line indicates Sox9 (outer root sheath marker) expression, and blue dashed line indicates CDP (matrix/inner root sheath) expression. Note that Sox9 and CDP expression are compartmentalized and do not overlap.

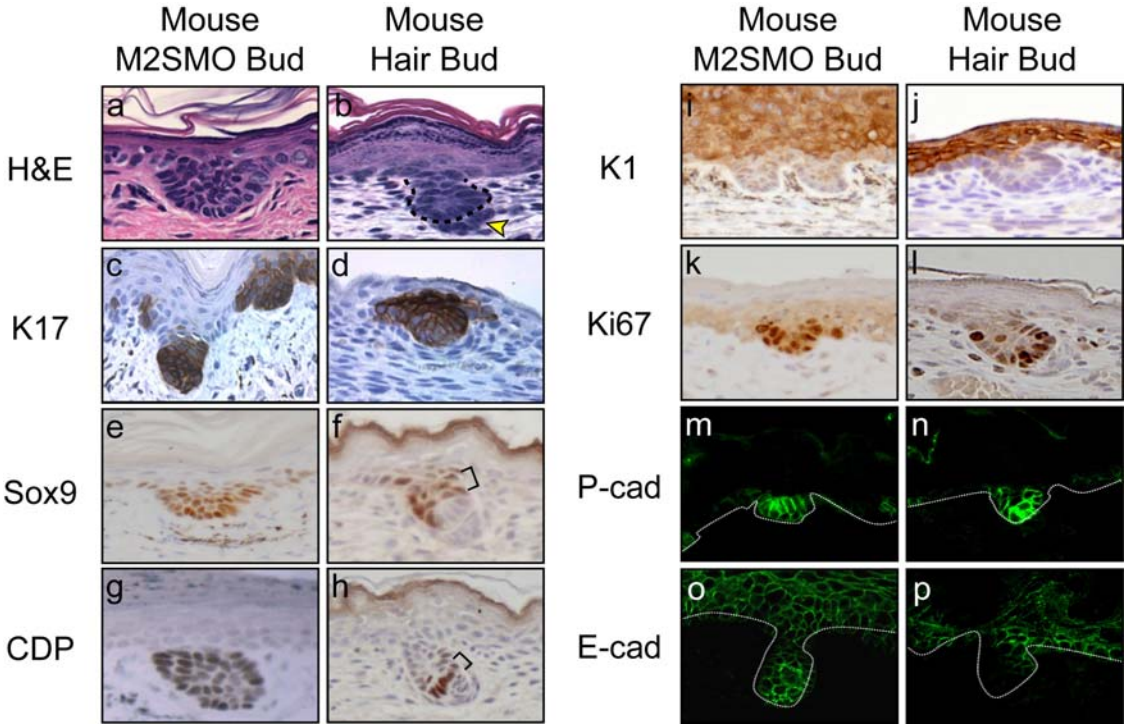


Figure 2-6. M2SMO-induced ectopic epithelial buds express hair bud lineage markers. (**a,b**) H&E-stained sections of *de novo* epithelial bud in M2SMO volar skin and embryonic mouse hair bud. The mesenchymal condensate neighboring normal hair buds [arrowhead in (**b**)] is not detected in association with the M2SMO-induced bud (**a**). Immunostaining revealed expression of hair follicle outer root sheath markers K17 and Sox9 (**c-f**), and the hair matrix/inner root sheath marker CDP (**g,h**) in ectopic buds and embryonic hair buds. Note non-overlapping expression of Sox9 and CDP in hair buds (brackets in **f,h**), compared to M2SMO-induced bud. (**i,j**) The suprabasal marker K1 is not expressed in M2SMO-induced buds or embryonic hair buds, while proliferation (**k,l**) is increased in both. (**m,n**) The hair bud marker P-cadherin is up-regulated in both M2SMO and embryonic hair buds, whereas down-regulation of E-cadherin is detected only in hair buds (**o,p**). White dotted line shows epidermal-dermal junction.

Canonical Wnt signaling in human and murine Hh pathway-driven epithelial buds

The formation of *de novo* epithelial buds in response to ectopic Hh signaling in epidermis was intriguing, given the compelling evidence pointing to the canonical Wnt pathway as the initiator of skin appendage development (Andl et al., 2002; Gat et al., 1998; Huelsken et al., 2001; Ito et al., 2007; Lo Celso et al., 2004). We therefore determined whether pathological Hh signaling in this setting was associated with activation of the canonical/β-catenin-dependent Wnt pathway. Increased levels of β-catenin in the cytoplasm and nucleus were seen in human embryonic hair buds (Figure 2-7b), consistent with the notion that β-catenin-dependent Wnt signaling is activated during early stages of human follicle development, as previously shown in mouse hair follicles (Figure 2-7d). We also detected increased levels of β-catenin in the cytoplasm and nucleus of neoplastic cells in human superficial BCCs and in M2SMO-induced mouse epithelial buds (Figure 2-7a,c), suggesting activation of the canonical Wnt pathway in response to ectopic Hh signaling. Furthermore, non-phosphorylated (active) β-catenin was detected in lysates from M2SMO transgenic mouse skin by immunoblotting, with little or no expression in control, non-transgenic volar skin (Figure 2-7e). Although nuclear β-catenin was seen in clusters of mesenchymal cells associated with human and murine embryonic hair follicles (Figure 2-7b,d), there was no detectable β-catenin in mesenchymal cells adjacent to human or murine epithelial buds driven by aberrant Hh signaling (Figure 2-7a,c).

To determine if aberrant Hh signaling may be influencing the canonical Wnt/β-catenin pathway through alterations in *Wnt* ligand expression, we performed

semiquantitative RT-PCR on RNA isolated from volar skin of M2SMO-expressing mice and controls (Figure 2-8). As expected, mRNA encoding the Hh target genes *Gli1* and *Ptch1*, indicating Hh pathway activation, was detected in samples from M2SMO volar skin, with negligible levels in controls. In addition, transcripts encoding multiple *Wnt* ligands (*Wnt3, 4, 5a, 7b, 10a* and *10b*), and the transcriptional coactivator *Tcf4*, were coordinately induced in M2SMO volar skin. Expression of endogenous Wnt target genes (Clevers, 2006) *Axin2* and *Sp5*, and the indirect target *Cyclin D1*, was also upregulated in M2SMO-expressing skin, indicating activation of a transcriptional program associated with canonical Wnt signaling.

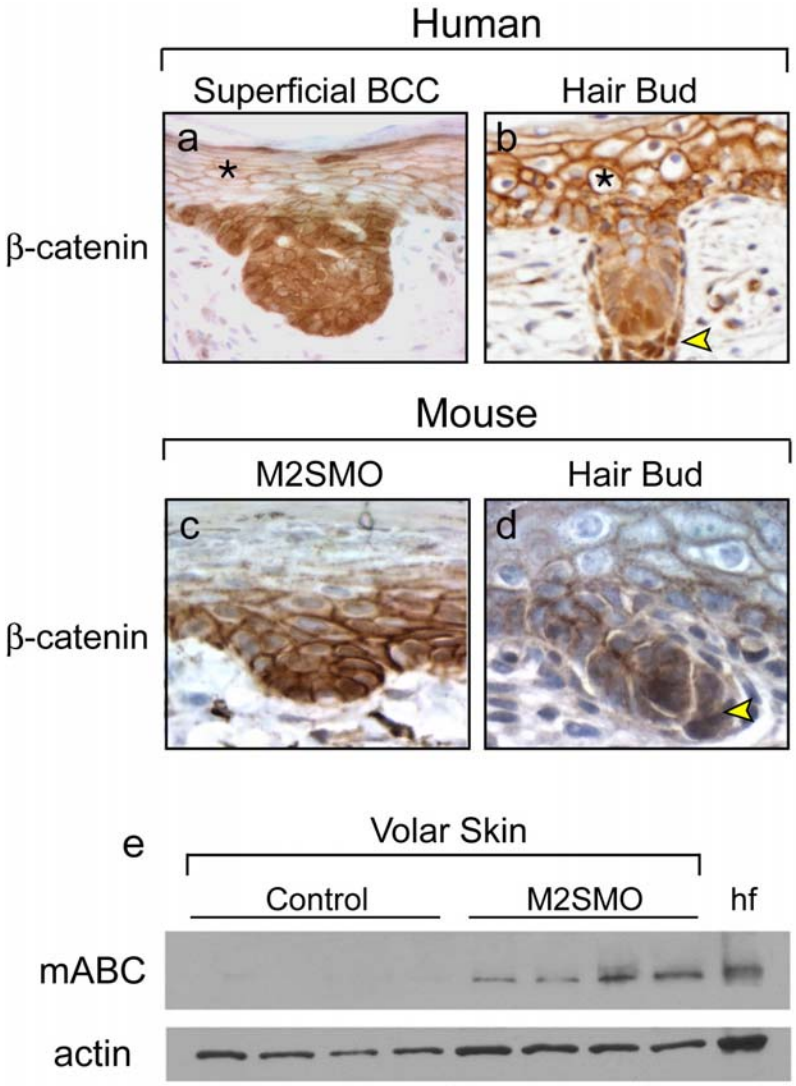


Figure 2-7 Canonical Wnt signaling is activated in human superficial BCC and epithelial buds in M2SMO-expressing hairless mouse skin. (**a,b**) β-catenin immunostaining reveals nuclear/cytoplasmic β-catenin localization in human superficial BCC and hair bud, consistent with activation of canonical Wnt signaling. Note intense nuclear staining in mesenchymal cells adjacent to hair bud [arrowhead in (**b**)] but not BCC (**a**). Adjacent epidermis (*) exhibits β-catenin localized to the cell membrane. (**c,d**) Similarly, β-catenin immunostaining reveals nuclear/cytoplasmic localization in mouse M2SMO bud cells arising from hairless volar skin and E16.5 mouse embryonic hair bud, with stained mesenchymal cells also noted adjacent to the hair bud (arrowhead). (**e**) Immunoblotting using mABC antibody showing presence of non-phosphorylated (active) β-catenin in lysates from M2SMO volar skin, but not hairless control volar skin. Skin lysate from postnatal day 8 mouse with actively-growing hair follicles (hf) was used as a positive control.

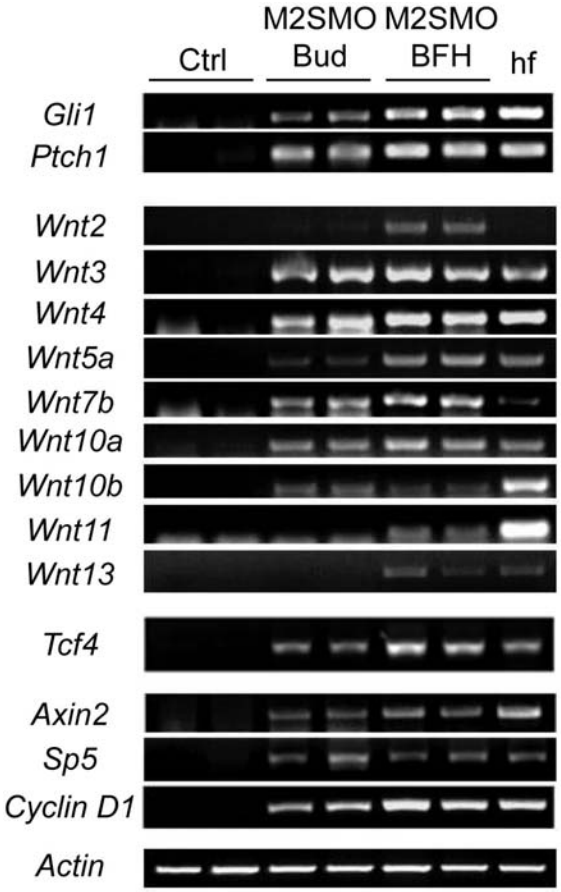


Figure 2-8. Multiple Wnt ligands, Tcf4, and Wnt target genes *Axin2, Sp5* and *Cyclin D1* are expressed in M2SMO volar skin. Semiquantitative RT-PCR was performed on RNA isolated from control volar skin (Ctrl), early bud-stage M2SMO transgenic volar skin (M2SMO Bud), and later, basaloid follicular hamartoma-containing M2SMO volar skin (M2SMO BFH), with postnatal day 8 skin as a positive control (hf). Upregulation of *Gli1* and *Ptch1* expression in M2SMO skin confirms activation of Hh signaling. M2SMO-expressing skin expresses multiple Wnt genes, *Tcf4*, and endogenous Wnt target genes *Axin2*, *Sp5*, and *Cyclin D1*. In control volar skin, there is negligible expression of *Gli1*, *Ptch1*, and Wnt pathway-associated genes.

SMO*-induced hair buds form follicular hamartomas instead of hair follicles

In contrast to embryonic hair buds, M2SMO-driven *de novo* buds did not develop into mature hair follicles, which express distinct markers in each of its seven cell lineages (Figure 2-9). Instead, they formed undifferentiated basaloid follicular hamartomas (Grachtchouk et al., 2003) which contained two of the seven cell lineages present in normal mature hair follicles (Figure 2-10). M2SMO-driven hamartomas expressed the outer root sheath markers K17 (McGowan and Coulombe, 1998) and Sox9 (Vidal et al., 2005) (Figure 2-11a,c) and the hair matrix/inner root sheath marker CDP (Ellis et al., 2001) (Figure 2-11e). In this setting, since an inner root sheath is not present, CDP expression likely reflects a hair matrix-like differentiation program. While the majority of epithelial cells comprising M2SMO-induced buds initially co-expressed Sox9 and CDP (Figure 2-6e,g) and exhibited nuclear and cytoplasmic localization of β-catenin (Figure 2-7c), expression of lineage markers and β-catenin was compartmentalized in hamartomas (Figure 2-11a,c,e,g,i,k). The majority of cells in hamartomas expressed K17, with cells at the lesion periphery frequently expressing reduced levels of Sox9 (Figure 2-11c). In contrast, cells expressing CDP were in most cases restricted to the periphery of these lesions (Figure 2-11e). Unlike normal hair follicles, M2SMO-driven hamartomas did not express late stage follicle markers specific for inner root sheath (trichohyalin, detected with AE15 antibody (O'Guin et al., 1992) or hair shaft (type I hair keratins, detected using AE13 antibody (Lynch et al., 1986)) (Figure 2-11m,o). Thus, the bulk of cells in follicular hamartomas expressed outer root sheath hair follicle markers while cells at the periphery of these lesions exhibited some features analogous to hair matrix cells. These undifferentiated tumors are thus distinct from the well-differentiated hair follicle

tumors, trichofolliculomas and pilomatricomas, that arise in mice expressing stabilized forms of β-catenin (Gat et al., 1998; Lo Celso et al., 2004), and human pilomatricomas associated with *CTNNB1* mutations (Chan et al., 1999). Potential explanations for the lack of late-stage follicle lineage markers in M2SMO-induced hamartomas may include an insufficiently high level of canonical Wnt/β-catenin signaling for trichogenic differentiation, absence of an inductive mesenchyme/dermal condensates/dermal papillae, which may provide an essential maturation signal for follicular epithelium, or expansion of restricted progenitor cells with limited developmental potential, in response to M2SMO.

Immunostaining for Ki67 revealed that in many of the hamartomas, proliferating cells were concentrated at the periphery where cells expressing the matrix marker CDP were located (Figure 2-11e,g). Similarly, immunostaining for β-catenin showed cytoplasmic and nuclear accumulation preferentially in these outer-most cells, compared with cells in more central regions of the hamartomas (Figure 2-11i), and Cyclin D1 was also localized to the hamartoma periphery (Figure 2-11k). Since Cyclin D1 is a Wnt target (Tetsu and McCormick, 1999), it may provide the link between compartmentalized expression of cytoplasmic/nuclear β-catenin, and increased proliferation, seen at the periphery of follicular hamartomas.

Semiquantitative RT-PCR was used to examine alterations in gene expression associated with progression from M2SMO-induced hair buds to follicular hamartomas. Expression of Hh target genes *Gli1* and *Ptch1* was increased in volar skin with hamartomas (M2SMO BFH) relative to hair buds (M2SMO Bud, Figure 2-8), likely reflecting an expansion of the transgene-expressing population of cells with activated Hh

signaling. The levels of *Wnt5a* and *Tcf4*, previously shown to be Hh-responsive (Pasca di Magliano et al., 2007; Reddy et al., 2001), were increased further in hamartomas, whereas expression of *Wnts 3, 4, 7b*, and *10a* was essentially unaltered, and *Wnt 10b* levels appeared slightly reduced (Figure 2-8). Interestingly, relatively low-level expression of transcripts for *Wnts 2, 11, and 13* was detected in follicular hamartomas but not epithelial buds (Figure 2-8): the presence of *Wnt11* in hamartomas mimics the relatively late appearance of this Wnt in maturing hair follicles (Reddy et al., 2001). The expression level of Wnt target genes *Axin2* (Jho et al., 2002) and *Sp5* (Weidinger et al., 2005) was not appreciably different in volar skin with hair buds versus hamartomas (Figure 2-8). Thus, the evolution from hair buds to follicular hamartomas is associated with continued or newly-induced expression of transcripts encoding several Wnt ligands and Wnt target genes, and expansion of a population of undifferentiated, proliferative, matrix-like epithelial cells at the tumor periphery, expressing cytoplasmic/nuclear-localized β-catenin and Cyclin D1.

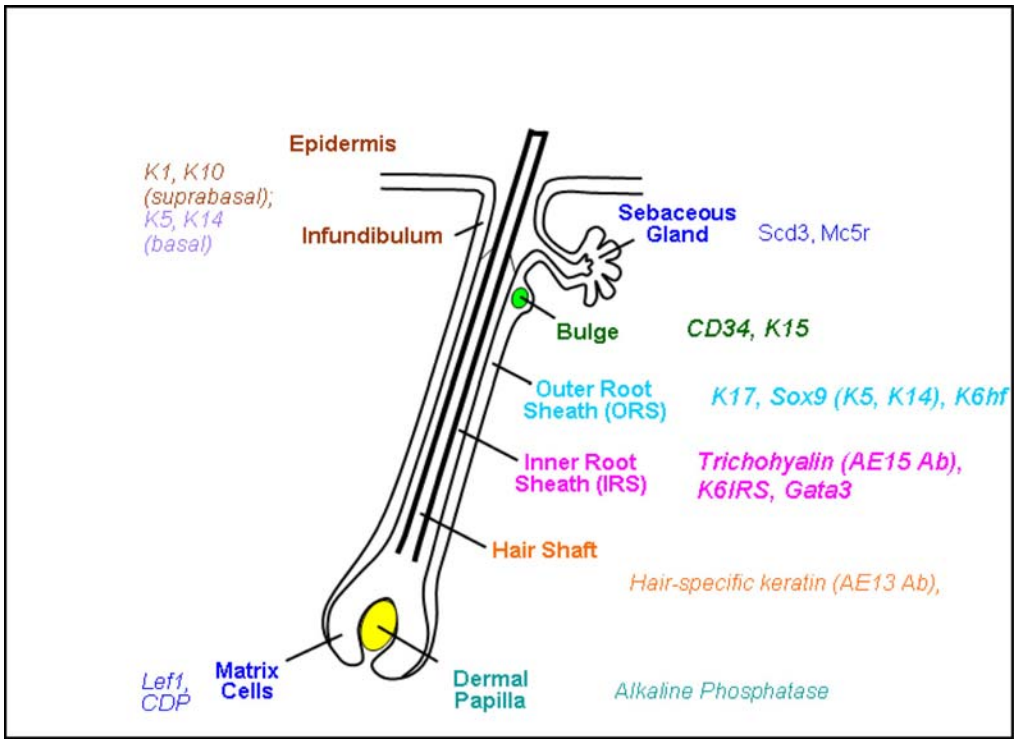


Figure 2-9. Hair follicle cell lineages. Cartoon showing follicle cellular compartments and lineage markers in the growing phase (anagen) of the hair cycle.

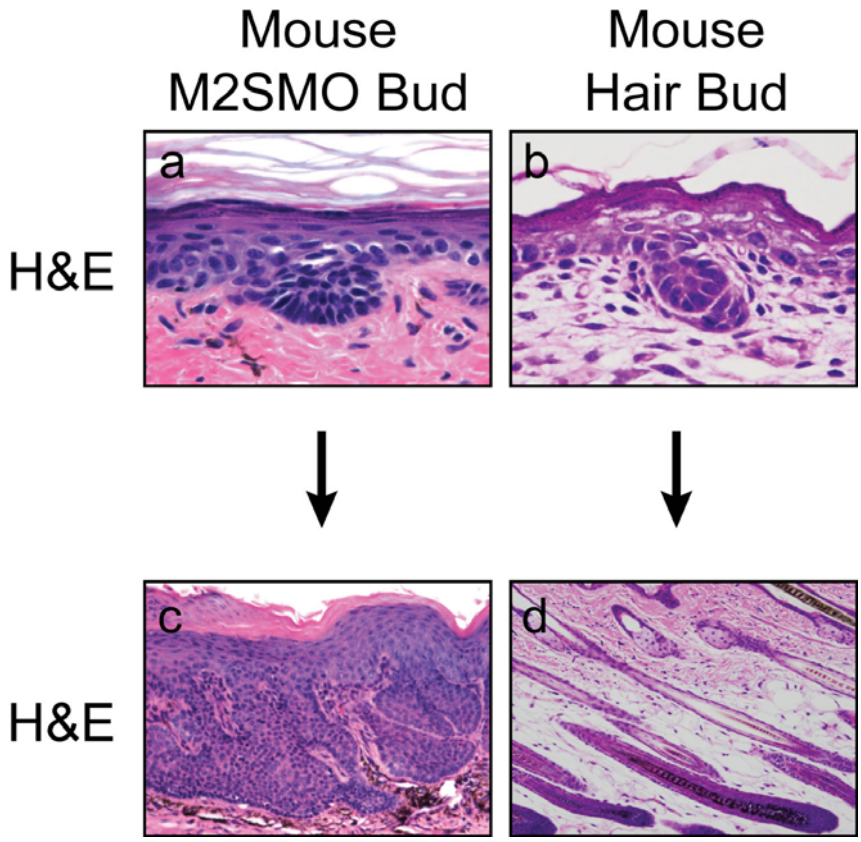


Figure 2-10. Hh-induced epithelial buds expand to form basaloid follicular hamartomas with sustained Wnt signaling. (**a,b**) H&E-stained sections of early-stage M2SMO bud arising from hairless volar skin, and embryonic hair bud. (**c,d**) M2SMO skin from mice 3 weeks of age and older contains basaloid follicular hamartomas, whereas embryonic hair buds form mature hair follicles.

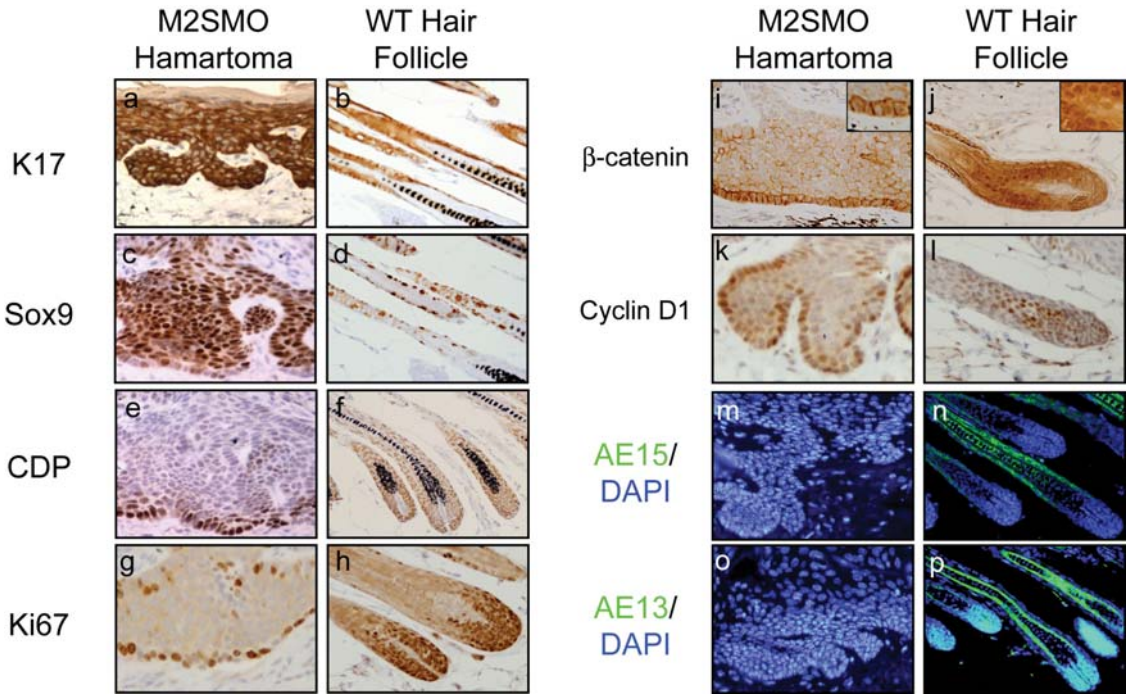


Figure 2-11 Marker expression of basaloid follicular hamartomas in M2SMO-expressing mice. (**a-d**) Follicular hamartomas and anagen hair follicles express hair follicle outer root sheath markers K17 and Sox9, with cells at the periphery of hamartomas frequently expressing reduced levels of Sox9. (**e,f**) The majority of cells expressing the hair matrix/inner root sheath marker CDP in hamartomas are restricted to the periphery of these lesions. The peripheral cells in hamartomas are also more proliferative (**g,h**), exhibit nuclear/cytoplasmic localization of β-catenin (**i,j**), and express higher levels of the Wnt target Cyclin D1 (**k,l**) than cells in the central regions of hamartomas. (**m-p**) In contrast to hair follicles, hamartomas do not express late-stage follicle differentiation markers trichohyalin, detected using AE15 antibody, or hair-specific keratin, detected with AE13 antibody.

Development of M2SMO-induced epithelial buds and hamartomas is blocked by Dkk1

Using a transgenic mouse model where doxycycline-regulated activation of the secreted Wnt inhibitor Dkk1 could be achieved in skin (Chu et al., 2004), we next tested whether canonical Wnt signaling was required for development of epithelial buds and their expansion to form hamartomas. For these experiments, we combined M2SMO-expressing transgenic mice [(*ΔK5-M2SMO*) (Grachtchouk et al., 2003)], keratin 5-targeted reverse-tetracycline transactivator mice [(*K5-rtTA*) (Diamond et al., 2000)], and mice carrying a doxycycline-regulated transgene [*TRE-Dkk1,* (Chu et al., 2004)], enabling expression of a secreted inhibitor of canonical Wnt signaling, Dkk1. We designated these triple-transgenic mice (*ΔK5-M2SMO;K5-rtTA;TRE-Dkk1*) M2SMO+Dkk1, for the sake of brevity. In M2SMO+Dkk1 mice, Dkk1 expression could be induced by administration of the tetracycline analog doxycycline, leading to inhibition of canonical Wnt signaling at different time points during the development of ectopic buds and follicular hamartomas.

We first induced Dkk1 in M2SMO-expressing mice (M2SMO+Dkk1) during embryogenesis, by administering doxycycline to pregnant dams at embryonic day 16.5. (Figure 2-12a). Blockade of canonical Wnt signaling at this stage led to nearly complete inhibition of M2SMO-driven ectopic bud development in volar skin, as assessed by whole-mount analysis and histology (Figure 2-12b) of skin on postnatal day 7 (P7). Dkk1 induction at P1 (Figure 2-13a) also led to a dramatic inhibition of M2SMO-mediated hair bud development, and a profound suppression of follicular hamartoma

formation, both in volar (Figure 2-13b and 2-16d) and tail (Figure 2-13c and 2-16b) skin examined at P35. Interestingly, the strikingly increased pigmentation in M2SMO volar and tail skin was also blocked in M2SMO+Dkk1 mice (For further discussion see Chapter V). Although expression of *Wnt* genes was similarly elevated in M2SMO and M2SMO+Dkk1 skin (Figure 2-14b), translocation of β-catenin to the cytoplasm and nucleus was suppressed in M2SMO+Dkk1 mice (Figure 2-14a), and expression of the Wnt target genes *Axin2* and *Sp5* was negligible, indicating effective blockade of canonical Wnt signaling by Dkk1 (Figure 2-14b). In contrast, expression of the Hh target genes *Gli1* and *Ptch1* was similar in M2SMO and M2SMO+Dkk1 skin, indicating that M2SMO-driven constitutive Hh signaling was unaffected by Wnt pathway inhibition (Figure 2-14b). Similarly, ectopic expression of the Hh-responsive keratin, K17 (Callahan et al., 2004), was seen in both M2SMO and M2SMO+Dkk1 volar skin (Figure 2-15b,c), whereas expression of hair bud and follicular hamartoma markers Sox9 and CDP, and increased cell proliferation, were no longer detected in M2SMO+Dkk1 mice (Figure 2-15f,i,l). The profound blockade of M2SMO-induced hair bud and follicular hamartoma development, by Dkk1, establishes that biological responses to ectopic Hh signaling in skin are largely mediated indirectly, via the canonical Wnt/β-catenin signaling pathway.

Notably, buds and hamartomas also developed in dorsal skin of M2SMO mice, but in this location they were not suppressed in M2SMO+Dkk1 mice, and β-catenin remained localized to the nucleus, suggesting that Dkk1 was not effectively inhibiting canonical Wnt signaling at this site. This may be due to insufficiently high expression of Dkk1 in dorsal skin or a relative deficiency in the level of Kremens 1/2 (Mao et al., 2002),

which facilitate Dkk1's ability to block Wnt signaling. Bud and hamartoma development was also not inhibited in small regions of volar skin near footpads (arrowhead in Figure 2-13b and 2-16d**)**, and here again the presence of nuclear β-catenin indicated inefficient blockade of canonical Wnt signaling (Figure 2-16d and insets). These results further underscore the tight correlation between inhibition of canonical Wnt/β-catenin signaling and suppression of Hh pathway-induced bud and hamartoma development.

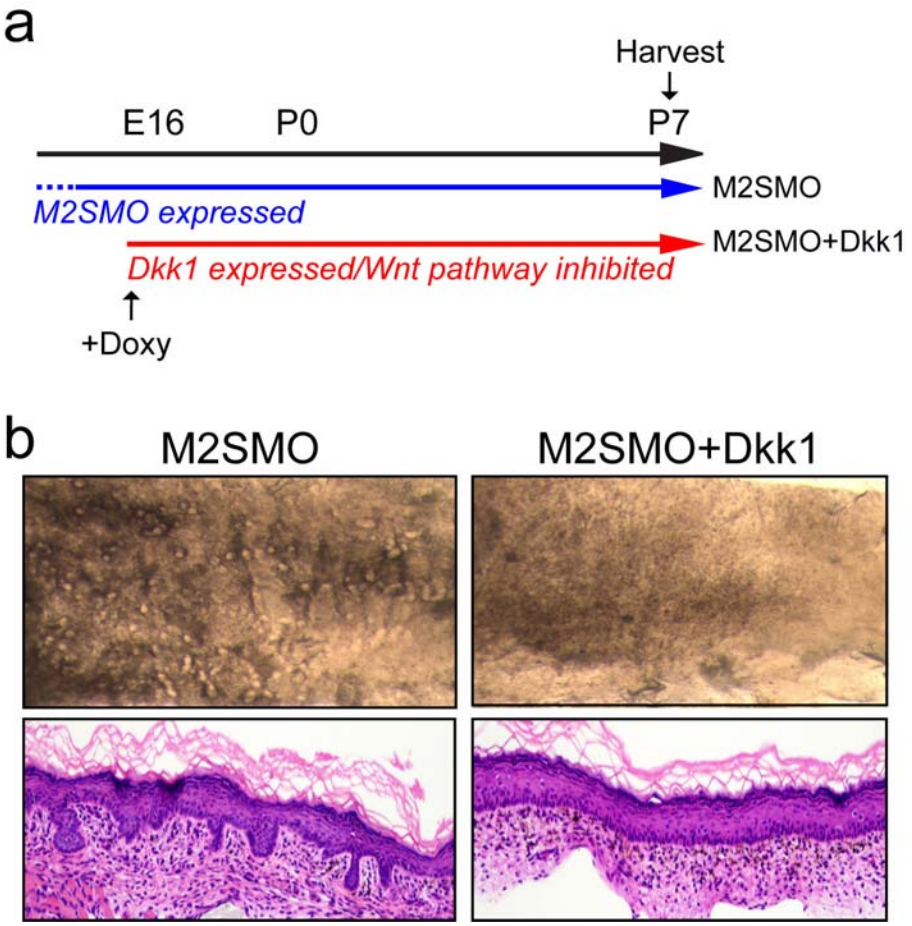
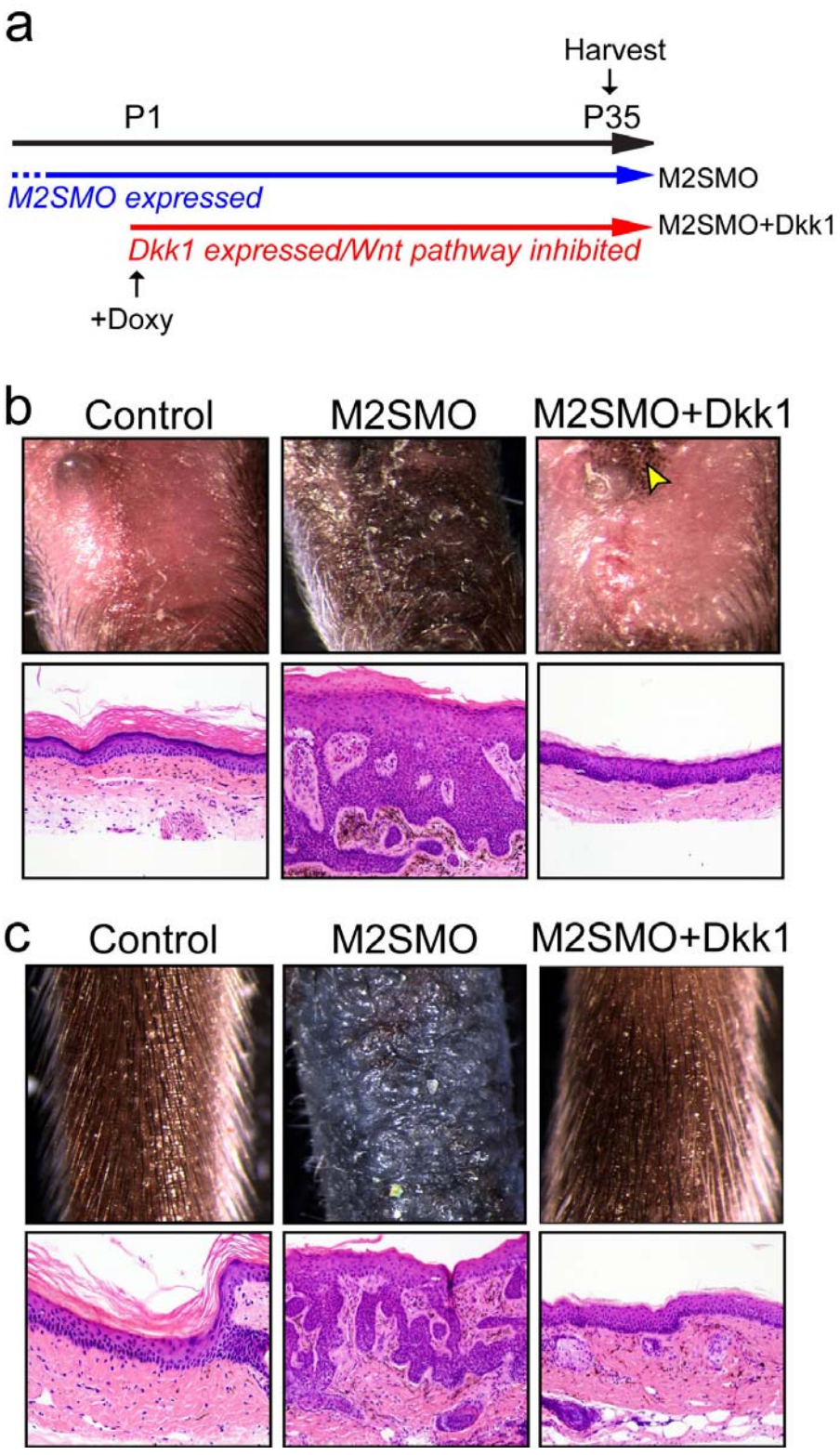


Figure 2-12. Inhibition of canonical Wnt signaling with Dkk1 blocks M2SMO-induced development of epithelial buds. (**a**) Experimental time-line for induction of Dkk1 expression in M2SMO+Dkk1 mice during embryogenesis (E16.5) with analysis at postnatal day 7. (**b**) Expression of Dkk1 blocks development of *de novo* buds seen in whole-mount or H&E-stained sections.

Figure 2-13. Inhibition of canonical Wnt signaling with Dkk1 blocks M2SMO-induced development of epithelial buds and follicular hamartomas. (**a**) Experimental time-line for Dkk1 induction at postnatal day 1 with analysis at postnatal day 35 to assess effects on hamartoma development. (**b**) Gross view and H&E-stained sections of volar skin from control, M2SMO, and M2SMO+Dkk1 mice. Note increased pigmentation and hamartoma development in M2SMO skin, compared to control. The M2SMO-induced phenotype is dramatically inhibited by coexpression of Dkk1. Foci of hyperpigmentation were sometimes detected in M2SMO+Dkk1 volar skin near footpads (arrowhead in **b**), and histology in these regions revealed buds and/or hamartomas with nuclear β-catenin (not shown), indicating incomplete blockade of canonical Wnt signaling in these regions. (**c**) Gross view and H&E-stained sections of tail skin from control, M2SMO, and M2SMO+Dkk1 mice. Note disrupted hair formation, increased pigmentation, and hamartomas in M2SMO skin, compared to control. The M2SMO-induced phenotype is again dramatically inhibited by coexpression of Dkk1.


a
Harvest
P1
P35
M2SMO
M2SMO expressed
M2SMO+Dkk1
Dkk1 expressed/Wnt pathway inhibited
+Doxy
b
Control
M2SMO
M2SMO+Dkk1
c
Control
M2SMO
M2SMO+Dkk1

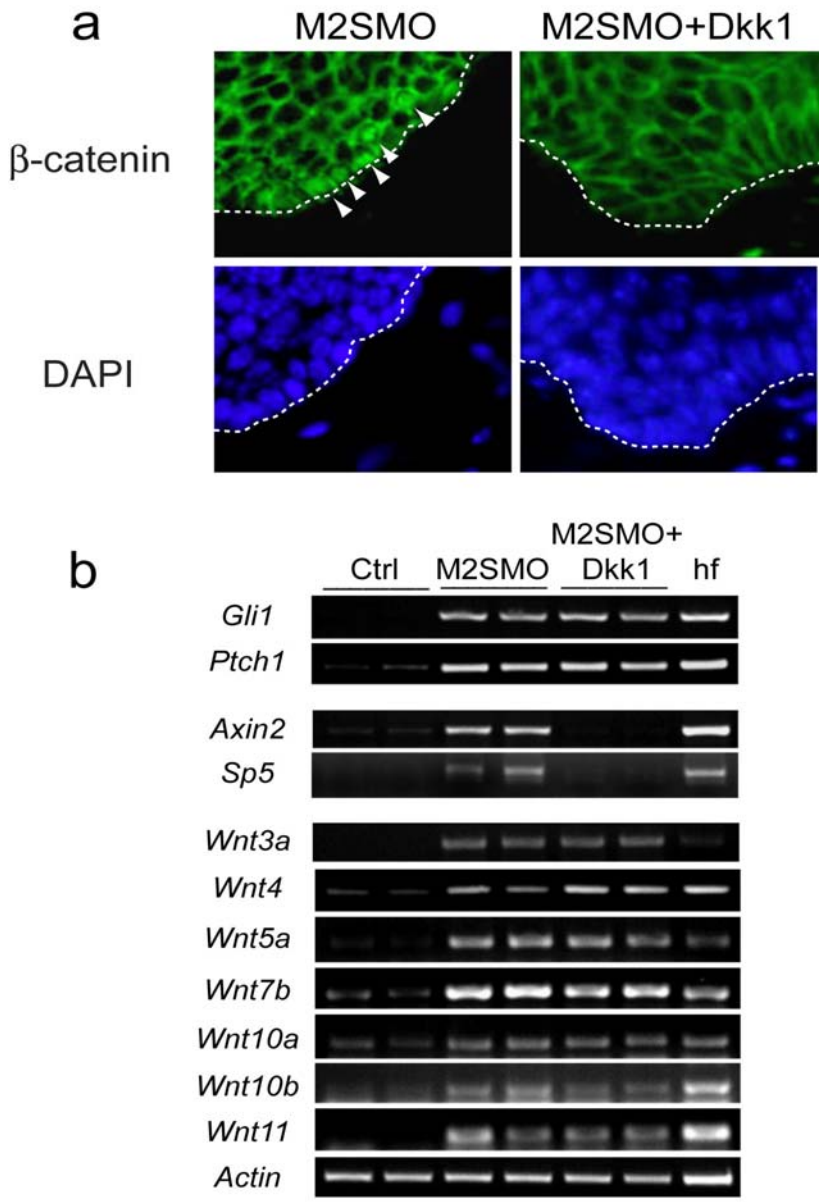


Figure 2-14. Conditional Dkk1 overexpression inhibits canonical Wnt/b-catenin signaling without affecting Hh signaling. (**a**) Immunoflourescence for β-catenin in volar skin. Cells with nuclear β-catenin in M2SMO skin, indicating activation of canonical Wnt signaling, are marked with arrowheads. Inhibition of nuclear β-catenin localization in M2SMO+Dkk1 mice, indicating effective blockade of canonical Wnt signaling. White dotted lines represent epidermal-dermal junction. (**b**) Semiquantitative RT-PCR using RNA from control, M2SMO and M2SMO+Dkk1 tail skin reveals down-regulation of *Axin2* and *Sp5* in M2SMO+Dkk1 volar skin, confirming blockade of canonical Wnt signaling. In contrast, expression of Hh target genes *Gli1* and *Ptch1*, as well as Wnt genes, is essentially unaltered in M2SMO+Dkk1 skin compared to M2SMO skin.

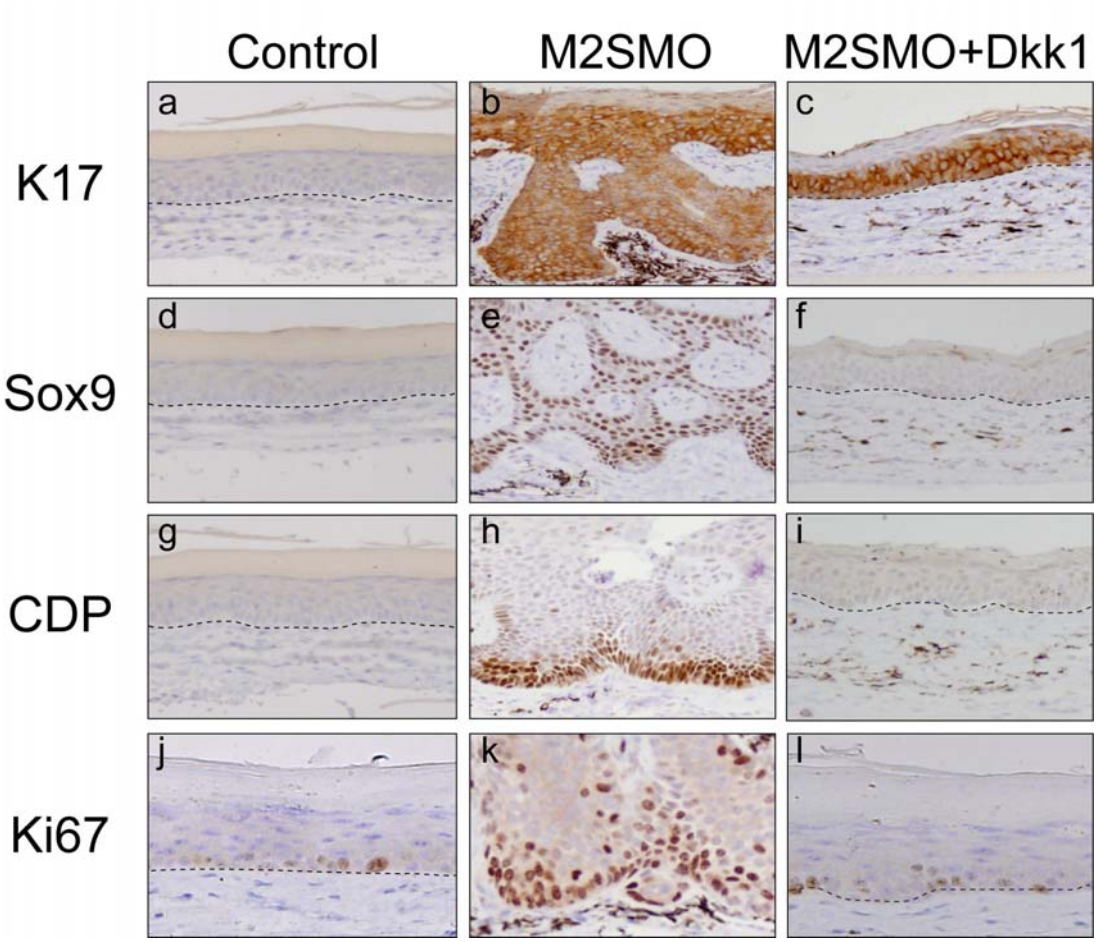


Figure 2-15. Hh-responsive keratin K17 is expressed in both M2SMO and M2SMO+Dkk1 skin, but bud lineage marker Sox9 and CDP expression, as well as proliferation are lost in M2SMO+Dkk1 skin. (**a-c**) The Hh-responsive keratin, K17, is not detected in control volar skin, but ectopically expressed in both M2SMO and M2SMO+Dkk1 volar skin, in keeping with sustained activation of Hh signaling in these mice. (**d-l**) M2SMO-associated expression of Sox9, CDP, and Ki67 are blocked or inhibited in mice also expressing Dkk1.

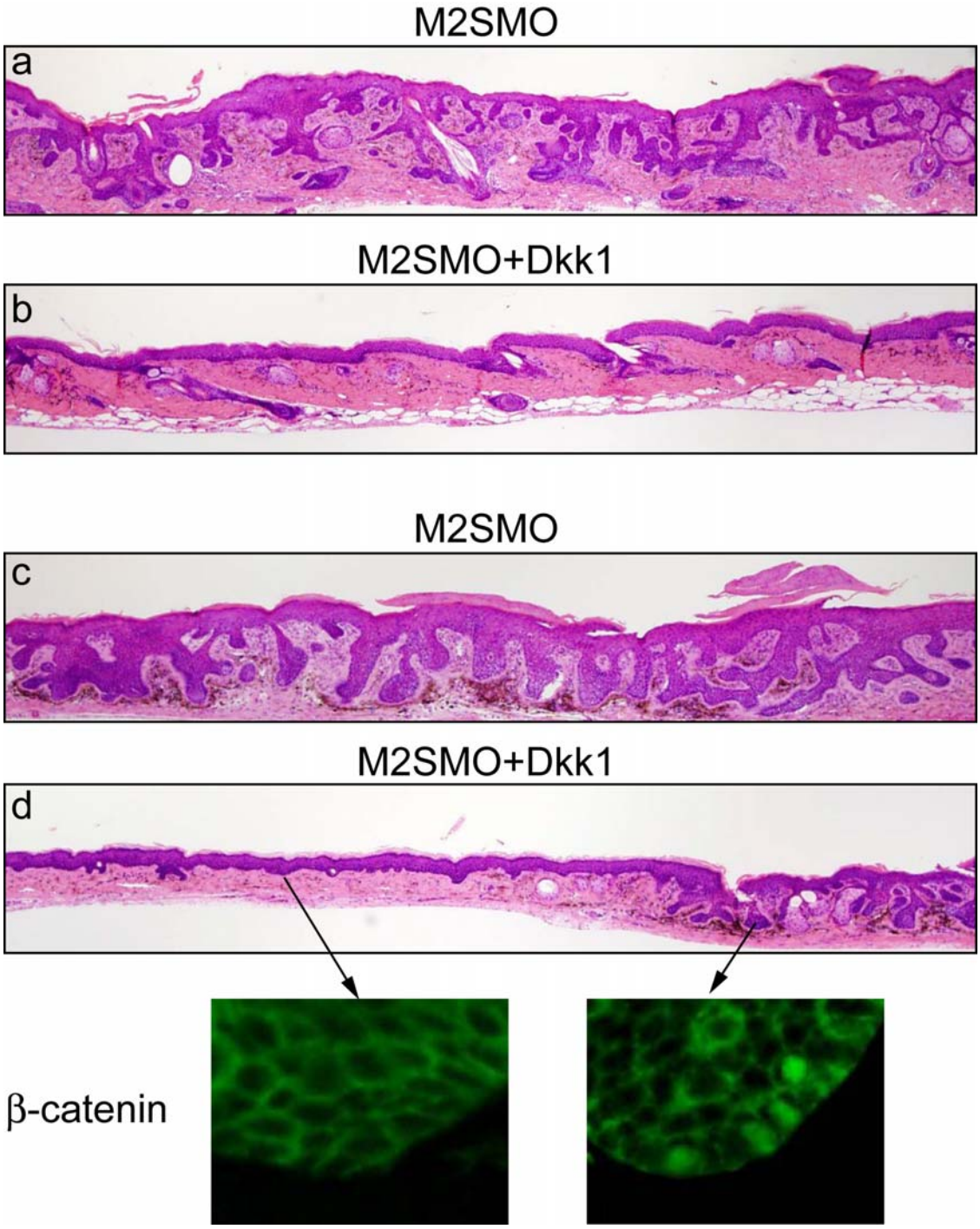


Figure 2-16. Inhibition of M2SMO-induced epithelial bud and hamartoma development by Dkk1 is uniform in tail skin, but not in volar skin. (**a**) H&E-stained section of low magnification view of tail skin in M2SMO mice at postnatal day 35. Note

extensive hamartoma development in M2SMO tail skin. (**b**) The M2SMO-induced phenotype is dramatically inhibited in M2SMO+Dkk1 tail skin, and the inhibition of hamartoma development is uniform throughout the tail skin. Nuclear and/or cytoplasmic β-catenin localization was not detected (not shown). (**c**) H&E-stained sections of low magnification view of volar skin in M2SMO mice at postnatal day 35. Note extensive pigmentation and hamartoma development in M2SMO volar skin. (**d**) Blockade of M2SMO-phenotype and canonical Wnt signaling by Dkk1 is incomplete and non-uniform in the volar skin of M2SMO+Dkk1 mice, as a small foci of hyperpigmentation and hamartoma development is visible adjacent to regions with dramatic M2SMO-phenotype inhibition. β-catenin immunostaining revealed nuclear β-catenin localization in these small regions (lower right box), indicating incomplete blockade of canonical Wnt signaling.

Discussion

The histological resemblance of superficial BCCs to embryonic hair germs had been reported as early as 1913 (El-Bahrawy et al., 2003; Kumakiri and Hashimoto, 1978; Lever, 1948; Montgomery, 1935). Here, we show that human BCC buds, similar to embryonic hair germs, exhibit nuclear and cytoplasmic β-catenin and express early hair follicle lineage markers. We describe analogous changes in mouse epidermis with ectopically activated Hh signaling activity, along with elevated expression of multiple Wnt genes, *Tcf4*, and Wnt target genes *Axin2* and *Sp5*. Furthermore, we show that selective blockade of canonical Wnt signaling with Dkk1 dramatically inhibits epithelial bud and hamartoma development in M2SMO mice, indicating that pathologic Hh signaling brings about these changes indirectly, via the canonical Wnt pathway. Our findings identify the canonical Wnt pathway as a mediator of pathological responses in skin previously attributed to the Hh pathway, help elucidate how 'follicular' tumors may arise from interfollicular epidermis, and provide a molecular explanation for the morphological and biochemical similarity between BCC buds and hair germs.

Under normal conditions, Wnt/β-catenin signaling initiates hair bud development while Hh signaling is required for subsequent proliferation of follicle epithelium leading to formation of hair matrix and multiple cell lineages comprising the mature follicle (DasGupta and Fuchs, 1999; Fuchs, 2007; Millar, 2002). Activation of canonical Wnt signaling in skin ultimately leads to induction of Hh signaling, whereas Wnt pathway blockade impairs physiological Hh signaling (Andl et al., 2002; Gat et al., 1998; Huelsken et al., 2001; Lo Celso et al., 2004). These data place Hh downstream of the

initiating Wnt signal in follicle development, but since a direct effect on expression of Hh ligand(s) or other Hh pathway components has not been demonstrated, this may merely reflect the fact that Hh signaling is part of the biochemical program of hair matrix cells. Our data indicate that this relationship is reversed in the setting of Hh-driven pathology in skin, where the initial, aberrant activation of Hh signaling in epidermis leads to canonical Wnt signaling and formation of *de novo* epithelial buds and hamartomas. In light of our findings in the setting of pathologically-activated Hh signaling in skin, it will be interesting to determine whether the Hh pathway contributes to Wnt signaling under physiological conditions, as has been proposed in other organs [e.g., (Hu et al., 2005; Madison et al., 2005)]. Since Hh signaling is activated in hair matrix cells in the maturing follicle, it may play a role in the robust activation of Wnt signaling in neighboring hair shaft progenitors.

Previous reports have documented positive interactions between the Hh and Wnt signaling pathways in several cell types and tissues and in different species, but a requirement for Wnt signaling in a Hh-driven pathological process has not previously been demonstrated *in vivo*. For example, in *Drosophila* imaginal discs, the Wnt orthologue wingless is directly regulated by the Gli orthologue Ci (Von and Hooper, 1997). Expression of *Wnt5A*, *7B, 7C, 8, 8B,* and *11* has been reported in *Xenopus* animal cap explants injected with Gli2 and Gli3 mRNA, and blockade of Wnt signaling inhibits the morphogenetic response to Gli2 in this system (Mullor et al., 2001). Also, in E1A-immortalized RK3E rat kidney cells, expression of *Wnt2b*, *Wnt4*, and *Wnt7* was induced by GLI1, and dominant-negative TCF4 inhibited GLI1-mediated focus formation in cell culture (Li et al., 2007). While these data demonstrate that Hh-Wnt crosstalk is necessary

for an embryonic process and *in vitro* transformation, respectively, our findings are the first to establish a stringent requirement for canonical Wnt/β-catenin signaling in Hh pathway-driven neoplasia.

In contrast to our findings, there are reports suggesting that Hh signaling can antagonize the canonical Wnt pathway in some settings. This has been reported in colon, where Indian hedgehog appears to block Wnt signaling in mature colonocytes (van den Brink et al., 2004). An antagonistic interaction between these pathways has also been reported in tongue, where blocking Hh signaling leads to enhanced Wnt signaling and taste papilla formation (Farndon et al., 1992). Although the mechanisms through which these two key pathways interact remain largely unexplored, it is clear that the effects of Hh signaling on the Wnt pathway are highly dependent on cell and tissue context, and will therefore need to be determined empirically in each setting.

Previous studies in normal skin have shown that in developing hair buds, canonical Wnt signaling precedes, and is required for, subsequent activation of Hh signaling (Andl et al., 2002; Gat et al., 1998; Huelsken et al., 2001; Lo Celso et al., 2004). Our data indicate that this temporal relationship is reversed in the setting of Hh-driven pathology in epidermis, where ectopic activation of Hh signaling leads to canonical Wnt signaling with resultant formation of *de novo* epithelial buds and follicular hamartomas. Several earlier reports have described links between the Hh and Wnt pathways in BCC, including upregulation of one or more Wnt genes (Mullor et al., 2001) and localization of β-catenin to the cytoplasm and/or nucleus (El-Bahrawy et al., 2003; Saldanha et al., 2004; Yamazaki et al., 2001). Furthermore, nuclear β-catenin localization has been reported in BCC-like tumors in mice with conditional homozygous inactivation of Ptch1

(Adolphe et al., 2006). Interactions between the Hh and Wnt pathways have also been proposed to operate in pancreatic cancer (Pasca di Magliano, 2007) and cigarette smoke-induced lung cancer (Manfredi et al., 2004). Our results are in keeping with these observations, and provide the first direct evidence that canonical Wnt signaling is essential for a tumorigenic response to deregulated Hh signaling in skin. Interestingly, a recent report described a role for β-catenin in cutaneous squamous cell carcinoma (Malanchi et al., 2008), which is biologically and pathogenetically distinct from BCC and not linked to aberrations in the Hh pathway, but it is not known whether signaling in squamous tumors is driven by Wnt ligands. Since Wnt ligand expression is elevated in BCCs and β-catenin distribution is cytoplasmic and nuclear, our findings raise the possibility that canonical Wnt signaling may also be required for the development and/or expansion of full-blown BCC. We have performed parallel experiments to those described here using Dkk1 in mice which develop nodular, BCC-like skin tumors, but Dkk1 was not effective in blocking canonical Wnt signaling in this setting (data not shown). Alternative approaches will therefore be needed to assess the role of β-catenin function in the initiation and maintenance of frank BCC.

Although M2SMO-induced epithelial buds expanded to form hamartomas, they failed to differentiate beyond a rudimentary stage of development, containing only two of the seven cell lineages seen in mature follicles. This may be due at least in part to the fact that they failed to produce mesenchymal condensates and papillae required for proper follicle development. The lack of condensates near ectopic buds was observed in dorsal (hairy) skin as well as hairless volar skin, arguing against the possibility that volar mesenchyme is selectively deficient in responsiveness to form this cell population.

It is also interesting that during M2SMO-induced epithelial bud neogenesis, bud-specific marker P-cadherin was up-regulated in the *de novo* bud, but E-cadherin down-regulation was not observed. E-cadherin downregulation has been reported to be critical for epithelial bud formation (Jamora et al., 2003). This suggests that at least in the context of Hh-driven epithelial bud development, E-cadherin down-regulation is not a requirement.

Furthermore, inconsistent results were seen in *TOPGal;ΔK5-M2SMO* bitransgenic skin, as well as in embryonic dorsal hair buds in *TOPGal* transgenic mice, suggesting that at least in certain contexts, *TOPGal* transgenic mice may not be a faithful reporter of TCF/Lef promoter activity/canonical Wnt pathway activation.

Taken together, our findings suggest that blockade of canonical Wnt/β-catenin signaling may be a useful strategy for treatment of neoplasms currently considered to be caused by uncontrolled Hh signaling. Because deregulated Hh signaling impacts on β-catenin signaling primarily at the level of Wnt ligands, the range of potential therapeutic strategies is considerably greater than it is for colorectal and other cancers with mutational defects in APC or β-catenin, and would likely include antibodies or other recombinant proteins that antagonize the interaction of Wnt ligands with Frizzled and LRP receptors. Future work will better clarify the utility of targeting proximal Wnt pathway components for the prevention or treatment of Hh-dependent neoplasms and other disorders.

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Chapter III

Forced activation of canonical Wnt/β-catenin signaling is sufficient to drive advanced hair follicle differentiation in normal hairless volar and M2SMO skin*

Introduction

β-catenin is one of the key downstream effectors in the canonical Wnt signaling pathway, and the critical role of Wnt pathway in both early embryonic development and tumorigenesis has been well-established (Clevers, 2006). As previously described in Chapter I, Wnt/β-catenin signaling plays a key role in early-stage hair bud initiation as well as late-stage advanced hair follicle lineage differentiation, and also in the development of hair follicle derived tumors (Fuchs, 2007; Reya and Clevers, 2005). Multiple studies have established the role of β-catenin in controlling epidermal lineage commitment in adult tissue. Reddy et al. showed that a range of Wnts, their receptors, and antagonists are expressed in the epidermis, and *Wnts 10a, 10b,* and *5a* are specifically upregulated in the developing hair placodes (Reddy et al., 2001). Epidermal expression of N-terminally truncated, constitutively activated mutant form of β-catenin using *keratin* 14 (K14) promoter led to *de novo* hair follicle development from interfollicular epidermis and pre-existing hair follicles (Gat et al., 1998; Lo Celso et al., 2004). The K14 promoter, similar to K5 promoter, is active in all basal cells of

* Portion of this chapter represent data from a previously published manuscript: Zhang. Y., Andl. T., Yang S.H., et al. (2008). "Activation of β-catenin signaling programs embryonic epidermis to hair follicle fate." Development 135, 2161-2172 (2008) doi:10.1242/dev.017459

interfollicular epidermis and along the length of the hair follicle outer root sheath (Vassar et al., 1989; Byrne et al., 1994; Wang et al., 1997). The *de novo* hair follicles formed sebaceous glands and dermal papilla, normally only established during embryonic development, and also expressed Shh, Ptch, and markers of advanced hair follicle differentiation. Interestingly, these investigators found that *de novo* hair follicle development was only seen in haired skin, such as skin from dorsal, tail, and dorsal foot region, but not in normally hairless regions such as the hairless volar skin (see Chapter I for orientation and pictures). Also, two independent studies found that a brief, transient activation of canonical Wnt signaling by expressing truncated β-catenin mutant in telogen skin was sufficient to trigger new hair growth (anagen) (Lo Celso et al., 2004; Van Mater et al., 2003). Silva-Vargas et al. further demonstrated that K14 promoter driven truncated β-catenin expression induces *de novo* hair follicle formation (Silva-Vargas et al., 2005) and these hair follicles contain clonogenic keratinocytes that express bulge-specific stem cell markers such as CD34 and keratin 15 (K15) (Morris et al., 2004; Trempus et al., 2003; Tumbar et al., 2004). In addition, β-catenin-induced *de novo* follicles contained associated dermal papilla, provide a niche for melanocytes, and undergo cycles of growth and regression, but *de novo* follicle development did not occur in hairless volar skin of these mice as well (Silva-Vargas et al., 2005).

Taken together, these studies clearly demonstrate that β-catenin can control epidermal lineage commitment in the adult tissue. However, it is not clear why *de novo* hair follicle formation was not seen in hairless volar skin in these studies. Could it be explained by the possible lack of multipotent hair follicle progenitor cells in the hairless volar skin that prevents β-catenin induced *de novo* hair follicle development?

Alternatively, are there additional inductive factors that are necessary to trigger the mutant β-catenin responses that are not present in the hairless volar skin? Hairless volar skin does not contain dermal papillae, and since the dermal papillae play a key role in hair follicle morphogenesis and cycling, could the lack of dermal papillae and its inductive signals prevent *de novo* hair follicles to form in response to mutant β-catenin expression?

The lack of mutant β-catenin-induced responses in hairless volar skin in above studies was surprising, given the robust M2SMO-induced ectopic epithelial bud and hamartoma development in hairless volar skin in M2SMO transgenic mice. Furthermore, we were also intrigued by the fact that M2SMO-induced ectopic buds in M2SMO transgenic mice did not feature associated mesenchymal condensate/dermal papillae, which are required for normal hair follicle development. Also, M2SMO-induced follicular hamartomas contained only two of seven hair follicle lineages, and did not express late stage follicle lineage markers, such as trichohyalin (specific for inner root sheath) or type I hair keratin (specific for hair shaft) . Our results indicate that canonical Wnt signaling is activated in M2SMO-induced buds and hamartomas (Chapter II). If canonical Wnt signaling is sufficient to drive late stage hair follicle differentiation, then how can one explain the lack of late-stage follicle differentiation in M2SMO-induced hamartomas? Is M2SMO-driven activation of canonical Wnt signaling enough to activate the development of ectopic epithelial buds, but is it too low to drive differentiation of advanced-stage follicle lineages?

To address these intriguing questions, I set out to test the effects of directly modulating Wnt/β-catenin activity in skin of M2SMO-expressing transgenic mice. To

achieve this, a series of crosses using several transgenic mice were required: 1) Cre-inducible M2SMO transgenic mice (*K5-flxGFP-M2SMO*) (Allen et al., 2003), which upon crossed with mice expressing a taxomifen-inducible form of Cre (CreERT2) in epidermis (K5-CreERT2) (Indra et al., 1999), develop *de novo* ectopic epithelial buds and hamartomas after birth because of low level recombinase activity in the absence of 4-hydroxytamoxifen (4-OHT) (Allen et al., 2003); 2) *Ctnnb1*$^{(Ex3)fl/fl}$ mice, in which exon 3 of β-catenin was flanked by *loxP* sequences (Harada et al., 1999), that results in the accumulation of unphosphorylated, stabilized β-catenin mutant in K5-expressing cells and their progeny when combined with *K5-CreERT2* mice upon 4-OHT treatment; and 3) *K5-CreERT2* (Indra et al., 1999) mice, which express a tamoxifen-inducible form of Cre (CreERT2) in epidermis. When *Ctnnb1*$^{(Ex3)fl/fl}$ mice were combined with Cre-inducible M2SMO and *K5-CreERT2* transgenic mice, the resulting *K5-CreERT2;K5-flxGFP-M2SMO;Ctnnb1*$^{(Ex3)fl/fl}$ triple transgenic mice had developed ectopic buds and hamartomas in their skin prior to 4-OHT treatment, and upon 4-OHT treatment, stabilized β-catenin mutant accumulated in all K5-expressing cells, including the already established buds and hamartomas. In addition, I also tested the effects of modulating Wnt/β-catenin activity on normal adult hairless volar skin by combining *Ctnnb1*$^{(Ex3)fl/fl}$ mice with *K5-CreERT2* mice.

Materials and Methods

Generation of transgenic mice and transgene activation

Generation of transgenic mice with constitutive expression of M2SMO in skin (*ΔK5-M2SMO)* has been described (Grachtchouk et al., 2003). To generate epithelial-specific Cre-inducible M2SMO mice, *K5-flxGFP-M2SMO* mice (Allen et al., 2003) were

crossed with *K5-CreERT2* (Indra et al., 1999) mice. Low level recombinase activity without 4-OHT in untreated double-transgenic M2SMO mice was described previously (Allen et al., 2003). To generate 4-OHT inducible stabilized β-catenin mutant (designated βcat*), *Ctnnb1*$^{(Ex3)fl/fl}$ (Harada et al., 1999) mice were crossed with *K5-CreERT2* mice to produce double transgenic progeny (*K5-CreERT2;Ctnnb1*$^{(Ex3)fl/fl}$). To generate triple transgenic *K5-CreERT2;K5-flxGFP-M2SMO;Ctnnb1*$^{(Ex3)fl/fl}$ mice (designated M2SMO+βcat*), *K5-flxGFP-M2SMO* mice were crossed with *Ctnnb1*$^{(Ex3)fl/fl}$ mice, and resulting double transgenic progeny were further crossed with *K5-CreERT2* mice. To induce Cre recombinase expression in 7-8 week old adult βcat* and M2SMO+βcat* mice, dorsal back hair was clipped using Oster Golden A5 hair clipper (Oster, McMinnville, TN), and 50 uL of 4-OHT dissolved in 95% ethanol (10 mg/mL, Sigma-Aldrich, St. Louis, MO) was applied on dorsal back skin. 95% ethanol vehicle alone was used as control. All mice were housed and maintained according to University of Michigan institutional guidelines, as stipulated by the University Committee on the Use and Care of Animals.

Tissue harvesting, wholemount preparation and wholemount Oil Red O staining

For hematoxylin and eosin (H&E) staining, mouse skin was fixed in neutral-buffered formalin (NBF) overnight, transferred to 70% EtOH, processed, and embedded in paraffin. Mouse skin was also embedded in O.C.T. Compound (Tissue-Tek, Torrance, CA) for frozen sections. To prepare wholemounts for mouse tail skin, mice were euthanized and tail skin was removed and cut in pieces in ~1cm length. Tail skin wholemount preparation was performed as described previously (Braun et al., 2003). To

prepare wholemounts for mouse volar skin, mice were euthanized and ventral hindlimb skin was removed. Volar skin was microdissected and wholemount preparation was performed essentially as described previously for tail skin (Braun et al., 2003). After the wholemounts were prepared, Oil Red O staining was performed as described previously (Braun et al., 2003). Transilluminated wholemount photomicrographs were captured with digital camera (Spot RT3.0, Diagnostic Instruments, Inc, Sterling Heights, MI) mounted on dissecting microscope.

Semiquantitative RT-PCR

Volar, tail, and postnatal day 8 dorsal skin were microdissected under dissecting microscope (Nikon, Japan), homogenized in Trizol (Invitrogen), and stored at -80°C until further processing. RNA isolation, first strand cDNA synthesis, and RT-PCR were performed as described previously (Allen et al., 2003).

Immunostaining and wholemount immunoflourescence staining

The following primary antibodies were used for immunostaining: K5 (Covance), 1:2000; K17 (gift from P. Coulombe); 1:4000, β-catenin (Sigma), 1:1000; CDP (Santa Cruz), 1:100; AE13 (gift from H. Sun), 1:10; AE15 (gift from H. Sun), 1:50; K6hf (gift from L. Langbein), 1:1000. For immunohistochemistry, tissues were fixed overnight in NBF, processed and paraffin-embedded, and 8 μm sagitally sections were prepared for staining. Immunoreactivity of antigens was restored by immersing slides in boiling 0.01 M citrate buffer, pH ~6, for 10 min. Blocking was performed using 1.5% NGF in PBS, and tissue sections were incubated with primary antibodies diluted in PBS containing 1%

bovine serum albumin, typically for 1-3 h at room temperature (21-23 ºC). Subsequent immunostaining procedures were performed using peroxidase Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) and 3,3'-diaminobenzidine (DAB) as a substrate, according to the manufacturer's protocol. M.O.M Kit (Vector Laboratories, Inc., Burlingame, CA) was used for β-catenin immunostaining according to manufacturer's protocol. Sections were counter-stained with hematoxylin and mounted using Permount (Fisher Scientific). For immunoflourscence, fluorescent secondary antibodies (Jackson Labs) were used at 1:75 dilution. Wholemount immunoflourescence staining was performed as described previously (Braun et al., 2003). OCT compound-embedded frozen sections were used for endogenous alkaline phosphatase staining. Endogenous alkaline phosphatase activity was visualized using Alkaline Phosphatase Substrate Kit I (Vector Laboratories, Inc., Burlingame, CA) according to manufacturer's protocol.

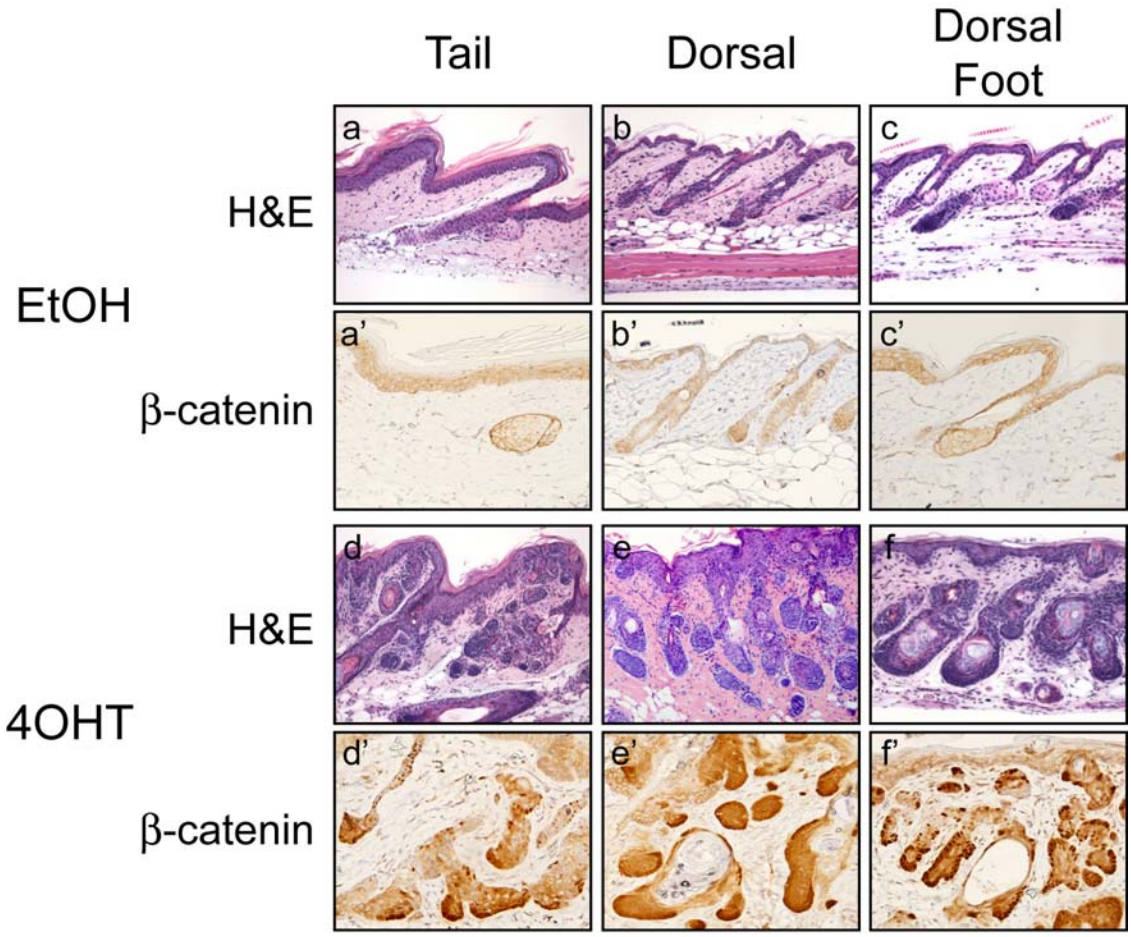
Results

Stabilized β-catenin induced in adult epidermis causes formation of functional ectopic hair follicles in multiple regions of skin

To test the effects of inducing mutation of β-catenin in adult epidermis, we used mice carrying a mutant β-catenin allele (*Ctnnb1(Ex3)*$^{fl/+}$) (Harada et al., 1999), which yields a stabilized form of β-catenin (βcat*) following Cre-mediated recombination. In the first set of experiments, *Ctnnb1(Ex3)*$^{fl/+}$ mice were crossed with mice carrying a 4-OHT-inducible *K5-CreERT2* transgene (Indra et al., 1999) to generate bitransgenic mice (Figure 3-1). Tail, dorsal, and dorsal foot skin from solvent-treated *K5-*

CreERT2;Ctnnb1(Ex3)$^{fl/+}$ mice was similar to control wild type skin, and did not contain any evidence of ectopic hair follicles or other skin appendages (Figure 3-1a-c). In contrast, tail, dorsal, and dorsal foot skin from adult mice treated with 4-OHT to activate βcat* expression rapidly developed multiple, tightly packed de novo hair follicles within seven days (Figure 3-1d-f). The *de novo* follicles developed from existing hair follicle outer root sheaths as well as interfollicular epidermis, and expressed hair follicle matrix marker CDP (Figure 3-1i, white arrowheads) and outer root sheath marker K17 (Figure 3-1j). In contrast to cell membranous β-catenin localization in control skin (Figure 3-1a'-c'), βcat* skin revealed intense nuclear and cytoplasmic β-catenin localization, indicating robust activation of canonical Wnt signaling pathway.

De novo follicle formation in 4OHT-induced *K5-CreERT2;Ctnnb1(Ex3)*$^{fl/+}$ mice could also be seen via wholemount analysis of tail skin after epidermal-dermal separation (Figure 3-1g,h), where *de novo* follicles arising from existing tail hair follicles are visible in 4-OHT treated tail skin (Figure 3-1h, yellow arrowheads).


Tail
Dorsal
Dorsal Foot
EtOH
H&E
β-catenin
4OHT
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a
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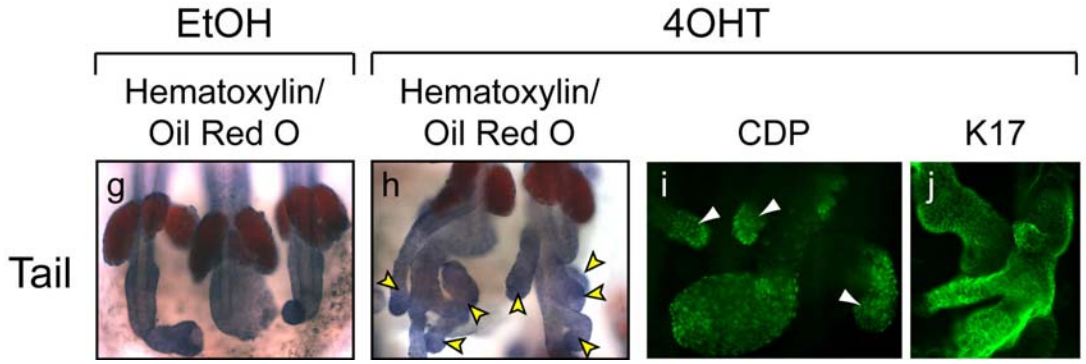
EtOH
4OHT
Hematoxylin/ Oil Red O
Hematoxylin/ Oil Red O
CDP
K17
Tail
g
h
i
j

Figure 3-1. β-catenin mediated development of *de novo* hair follicles in adult *K5-CreERT2;Ctnnb1(Ex3)$^{fl/+}$* mice. (**a-f**) H&E sections of EtOH-treated (**a-c**) and 4OHT-treated (**d-f**) tail, dorsal, and dorsal foot skin from *K5-CreERT2;Ctnnb1(Ex3)$^{fl/+}$* mice, 7 days after treatment. 4OHT-treated tail, dorsal and dorsal foot skin contain *de novo* hair follicles arising from interfollicular epidermis as well as existing hair follicles. (**a'-f'**) Immunohistochemistry for β-catenin reveals 'basket-weave'-like cell membranous localization in control (EtOH-treated) skin (**a'-c'**), while intense nuclear/cytoplasmic β-catenin localization is seen in 4OHT-treated skin (**d'-f'**). (**g,h**) Wholemount view of hematoxylin/Oil Red O-stained epidermis of tail skin after epidermal-dermal separation. *De novo* hair follicles arising from existing hair follicles are visible in 4OHT-treated tail skin (yellow arrowheads). (**i**) Wholemount immunoflourescence for matrix marker CDP. Both existing hair follicle and *de novo* follicles (white arrowheads) show CDP expression. (**j**) Wholemount immunoflourecence staining for hair follicle marker K17 reveals K17-positive *de novo* follicles arising from existing hair follicle.

Normal hairless volar skin is competent to form mature hair follicle lineages following expression of stabilized β-catenin (βcat*)

Although Hh signaling activates several early markers of follicle development in hairless volar skin, advanced stages of follicle maturation are not achieved even after prolonged intervals (Allen et al., 2003; Grachtchouk et al., 2003). To test whether volar skin is competent to respond to a potent signal for follicle development, we generated *K5-CreERT2;Ctnnb1(Ex3)*$^{fl/+}$ double transgenic offspring, then applied either solvent or 4-OHT on the dorsal skin starting at postnatal day 49 (Figure 3-2a). Volar skin was then harvested and analyzed five days after 4-OHT treatment. Volar skin from solvent-treated *K5-CreERT2;Ctnnb1(Ex3)*$^{fl/+}$ mice was similar to control volar skin, and did not contain any evidence of hair follicles or other skin appendages (Figure 3-2b,d,f,h). In contrast, volar skin from adult mice treated with 4-OHT to activate βcat* expression rapidly (within five days) developed multiple skin appendages (Figure 3-2c,d,g,i). Some of these consisted only of dense aggregates of matrix-like cells, while others comprised matrix-like cells associated either with hair shafts, sebaceous glands, or both (Figure 3-2e,g,i). Immunostaining and semiquantitative RT-PCR revealed expression of multiple hair follicle and sebaceous gland lineage markers, including the inner root sheath marker trichohyalin (detected using AE15 antibody) (O'Guin et al., 1992); hair follicle-specific keratin 6 (K6hf), a marker of the innermost layer of the outer root sheath (the companion layer) and medulla (Gailani et al., 1992); *Lef-1*, a hair matrix/pre-cortical marker; mouse hair keratin A1 (*mHK-A1*) (Kaytes et al., 1991) and *Hacl-1*, hair shaft-specific markers (Huh et al., 1994); *K6irs*, an inner root sheath specific marker (Thayer et al., 2003); and

the sebocyte marker *Mc5r* (Chen et al., 1997) (Figure 3-3e-i). In addition, the dermal condensate/papilla marker, alkaline phosphatase, was induced in mesenchymal cells adjacent to the newly-formed follicles (Figure 3-3d). Many of the epithelial cells in these follicles, as well as neighboring mesenchymal cells in the dermis, had intense nuclear β-catenin expression detected by immunostaining (Figure 3-3b). Thus, hairless volar skin remains competent to form mature hair follicles and sebaceous glands in adult mice following epidermal activation of a stabilized β-catenin mutant under the control of its endogenous promoter.

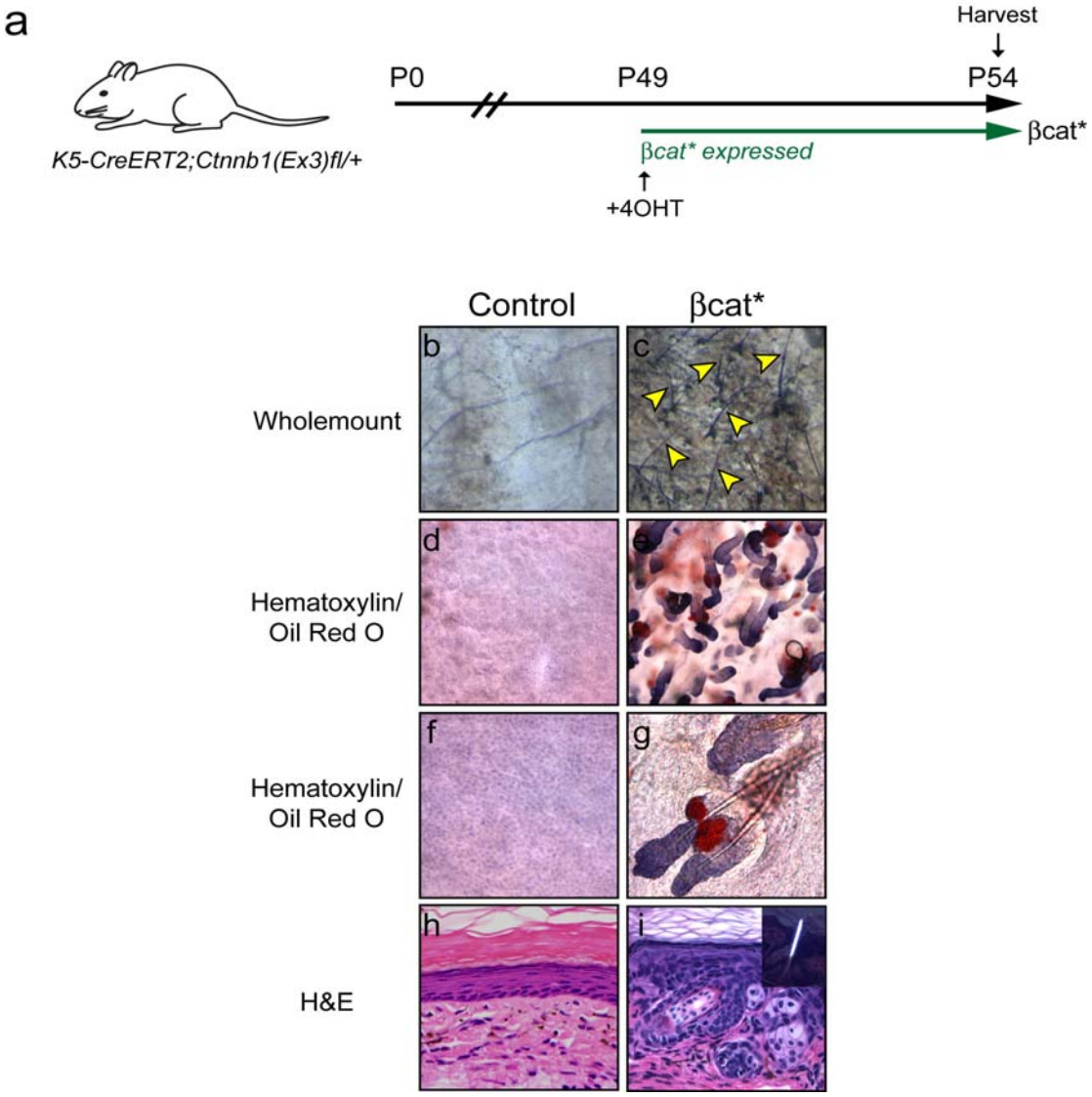

Figure 3-2. β-catenin mediated development of *de novo* hair follicles and sebaceous glands in hairless volar skin. (a) Bitransgenic *K5-CreERT2;Ctnnb1(Ex3)*$^{fl/+}$ mice and experimental design. 7 week old *K5-CreERT2;Ctnnb1(Ex3)*$^{fl/+}$ mice were given either solvent or 4OHT to induce βcat* expression. **(b,c)** Transilluminated wholemount view of hairless volar skin removed from ventral aspect of hind limb from EtOH-treated (b) and 4OHT-treated (c) *K5-CreERT2;Ctnnb1(Ex3)*$^{fl/+}$ mice, 5 days after treatment. In addition to skin folds also seen in controls (b), 4OHT-treated volar skin (c) contains multiple hair follicles which produce hair shafts (yellow arrowheads) **(d-g)** Wholemount view of hematoxylin/Oil Red O-stained epidermis after epidermal-dermal separation. *De novo* hair shafts and Oil Red O-positive sebaceous glands are visible in 4OHT-treated volar skin. **(h,i)** H&E-stained sections showing *de novo* hair follicles and sebaceous glands in 4OHT-treated volar skin. Inset: Hair shaft in de novo hair follicle is visualized using polarized lenses.

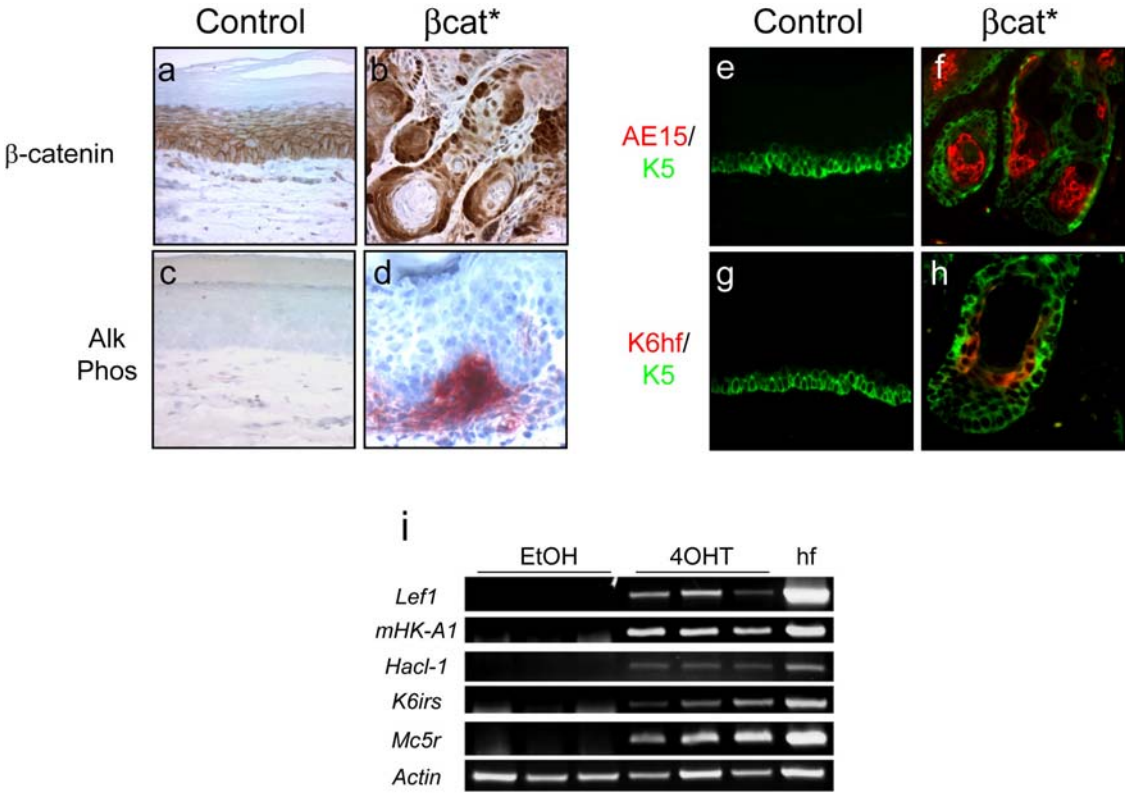

Figure 3-3. βcat*-induced *de novo* follicles express intensely nuclear and cytoplasmic β-catenin, alkaline phosphatase, and multiple late-stage follicle lineages. (**a,b**) Immunohistochemistry for β-catenin reveals 'basket-weave'-like cell membranous localization in control (EtOH-treated) skin, while intense nuclear/cytoplasmic β-catenin localization is seen in 4OHT-treated skin. (**c,d**) Endogenous alkaline phostaphase, a marker for dermal papilla cells, is detected in 4OHT-treated volar skin. (**e-h**) Immunoflourescence for hair follicle lineage markers trichohyalin (detected with AE15 antibody) and K6hf (Texas Red), and Keratin 5 (FITC), marker for the basal layer of epidermis and outer root sheath. Trichohyalin and K6hf are only detected in hair follicles in 4OHT treated volar skin. (**i**) Semiquantitative RT-PCR demonstrating induction of hair follicle (*mHA-K1, Hacl-1*, and *K6irs*) and sebocyte lineage markers (Mc5r) in volar skin of 4OHT-treated *K5-CreERT2;Ctnnb1(Ex3)*$^{fl/+}$ mice 5 days post-induction. Postnatal day 8 control dorsal skin was used as positive control (hf). Note upregulation of the Wnt target gene and hair matrix marker *Lef1* in 4OHT-treated volar skin.

βcat* expression results in preputial gland keratinization and differentiation

Preputial gland is a large exocrine gland that is composed of two leaf-like structures located subcutaneously at the base of the penis. Preputial glands are almost entirely composed of sebocyte-like cells and their progenitors, and normally do not express hair follicle or epidermal lineage markers. During our analysis of 4-OHT treated *K5-CreERT2;Ctnnb1(Ex3)*$^{fl/+}$ bitransgenic mice, we also noted significant hyperplasia of preputial glands in male *K5-CreERT2;Ctnnb1(Ex3)*$^{fl/+}$ mice expressing βcat*. Grossly, βcat*-expressed preputial glands were several-fold larger than controls, and appeared more opaque in color. Histologically, normal preputial glands are almost entirely composed of sebocyte-like cells and their progenitors (Figure 3-4a), but βcat* preputial glands exhibited a marked loss of sebocyte population and instead featured large foci of accumulated keratinized material and morphological features of advanced stage hair follicle differentiation (Figure 3-4b). β-catenin immunostaining revealed intense nuclear and cytoplasmic β-catenin localization in βcat*-expressing preputial gland (Figure 3-4d), indicating robust activation of canonical Wnt signaling pathway, but hardly any nuclear or cytoplasmic β-catenin localization was observed in control preputial gland (Figure 3-4c). Control preputial glands do not express hair follicle or epidermal lineage markers (Figure 3-4e,g), but βcat*-expressing preputial glands expressed multiple late stage hair follicle lineage markers, including the inner root sheath marker trichohyalin (detected using AE15 antibody); hair specific keratin (detected using AE13 antibody); and epidermal lineage markers K1 and K10 (Figure 3-4f,h).

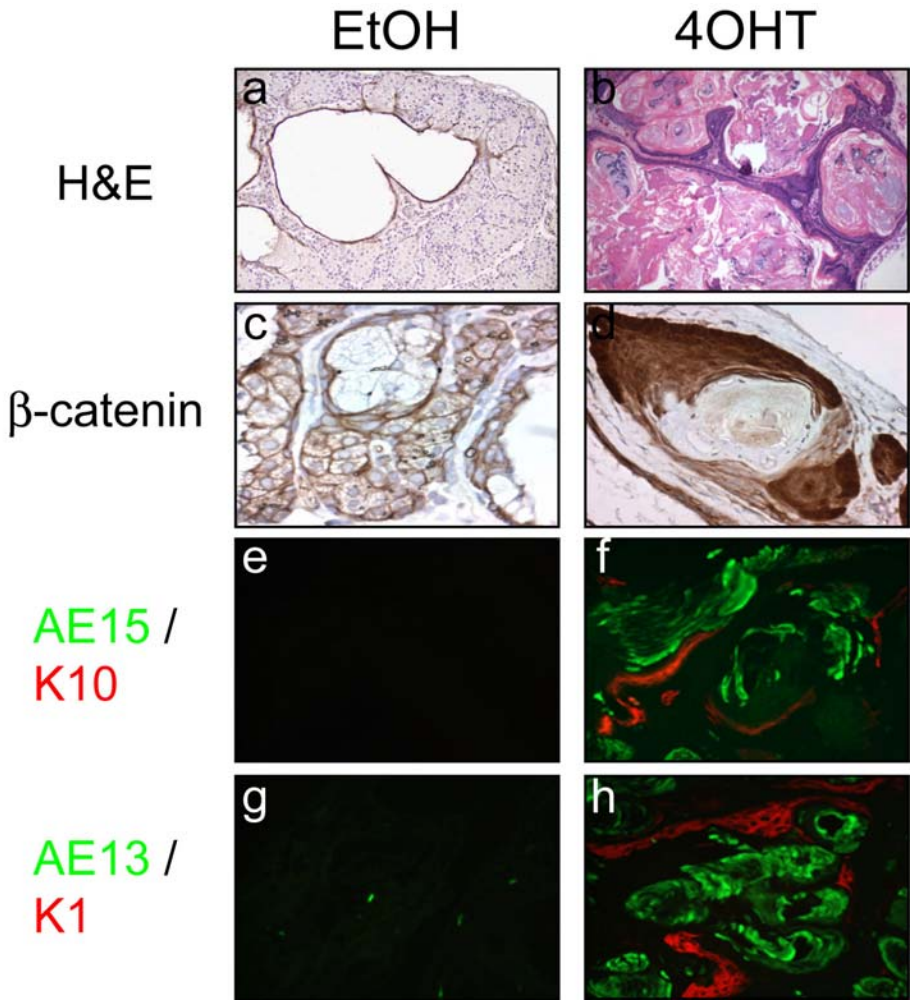

Figure 3-4. βcat* induces preputial gland keratinization and differentiation. (**a**) H&E-stained section of normal preputial gland. Note normal preputial gland is composed almost entirely of sebocyte-like cells. (**b**) H&E-stained section of βcat*-expressing preputial gland. Note extensive keratinization with accumulation of keratinized material within the gland. (**c,d**) β-catenin immunostaining reveals intense nuclear and cytoplasmic β-catenin localization in βcat*-expressing preputial gland, whereas cell membranous β-catenin localization is seen in control preputial gland. (**e,f**) βcat*-expressing preputial gland expresses late stage follicle lineage marker trichohyaline (detected by AE15 antibody, FITC), and epidermal lineage marker K10 (Texas Red). Control preputial gland does not express trichohyalin or K10. (**g,h**) βcat*-expressing preputial gland expresses late stage follicle marker hair keratin (detected by AE13 antibody, FITC), and epidermal lineage marker K1 (Texas Red). Control preputial gland does not express hair keratin or K1.

M2SMO-induced ectopic buds and hamartomas in volar skin retain the capacity to produce late-stage follicle lineages with expression of stabilized β-catenin

We then tested the effects of inducing βcat* expression in pre-existing follicular hamartomas in triple-transgenic (*K5-CreERT2;Cre-inducible M2SMO*; *Ctnnb1(Ex3)*$^{fl/+}$) mice (Figure 3-5a). This was feasible because the Cre-inducible M2SMO transgene undergoes limited recombination to produce follicular hamartomas in the absence of 4-OHT treatment, in *K5-CreERT2;Cre-inducible M2SMO* bitransgenic mice (Allen et al., 2003). Therefore, when 4-OHT was applied to *K5-CreERT2;Cre-inducible M2SMO*; *Ctnnb1(Ex3)*$^{fl/+}$ mice at postnatal day 49, these triple transgenic mice already had developed follicular hamartomas in their skin. Expression of βcat* in M2SMO-expressing skin starting at postnatal day 49 induced the appearance of multiple advanced-stage hair follicle lineages rapidly within 7 days (Figure 3-5b-m). Morphological changes resembling an expanded inner root sheath compartment and occasional hair shafts were evident in M2SMO+βcat* skin (Figure 3-5c), but not in skin expressing M2SMO alone (Figure 3-5b). Epithelial cells in M2SMO+βcat* skin showed dark nuclear and cytoplasmic β-catenin localization (Figure 3-5e), with a staining intensity that appeared significantly higher than that seen in skin expressing M2SMO alone (Figure 3-5d). M2SMO+βcat* skin also expressed the mesenchymal papilla marker alkaline phosphatase (Figure 3-5g), and multiple late stage epithelial follicle differentiation markers, including trichohyalin, hair shaft-specific keratin, and K6hf (Figure 3-5g,i,k,m), but skin from M2SMO mice did not (Figure 3-5f,h,j,l). These data demonstrate that undifferentiated M2SMO induced hamartomas remain competent to

produce late stage hair follicle lineages following robust activation of canonical Wnt signaling using a stabilized β-catenin mutant.

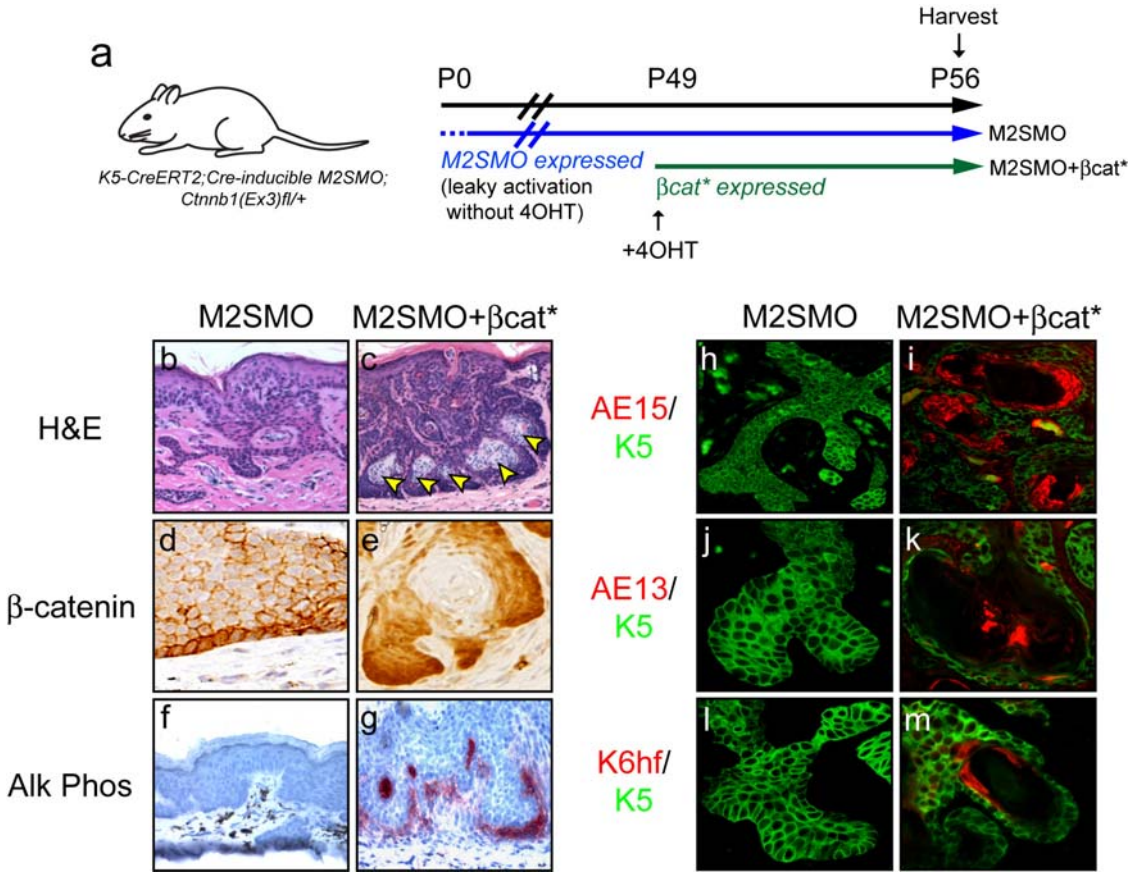

Figure 3-5. SMO*-induced hamartomas retain the capacity to produce late stage follicle lineages with expression of stabilized β-catenin.
(**a**) Triple transgenic *K5-CreERT2;Cre-inducible SMO*;Ctnnb1(Ex3)*$^{fl/+}$ mice and experimental design. Due to leaky expression of Cre-inducible SMO* transgene without 4OHT treatment, triple transgenic *K5-CreERT2;Cre-inducible SMO*;Ctnnb1(Ex3)*$^{fl/+}$ mice produce follicular hamartomas prior to 4OHT treatment. Induction of βcat* expression in 7 week old *K5-CreERT2;Cre-inducible SMO*;Ctnnb1(Ex3)*$^{fl/+}$ mice was achieved by treating the mice with 4OHT (see text for more details). (**b,c**) H&E sections of volar skin from SMO* and SMO*+βcat* mice. Note morphological changes resembling an expanded inner root sheath compartment in SMO*+βcat* skin (arrowheads), compared to hamartomas in SMO* skin. (**d,e**) Immunostaining for β-catenin reveals relatively much more intense nuclear/cytoplasmic β-catenin localization in SMO*+βcat* skin compared to SMO* alone. (**f,g**) Positive alkaline phosphatase activity in SMO*+βcat* skin, while SMO* alone does not stain for alkaline phosphatase. (**h-m**) Immunofluorescence staining for late stage follicle differentiation markers. SMO*+βcat* skin expresses AE15 (trichohyaline), AE13, and K6hf, while SMO* alone does not.

Discussion

Forced expression of a stabilized β-catenin in epidermis led to robust and rapid formation of *de novo* hair follicles which expressed multiple follicle specific markers in tail, dorsal, and dorsal foot skin (Figure 3-1). Furthermore, βcat* expression in epidermis led to the formation of alkaline phosphatase-expressing mesenchymal cells both in control and M2SMO-expressing volar skin (Figure 3-2 and 3-4). In volar skin from M2SMO mice, βcat* induced the rapid appearance of late-stage follicle lineages and rudimentary hair shafts within hamartomas, showing that these undifferentiated tumors retain the potential to undergo advanced stages of terminal differentiation when supplied with an appropriate stimulus. Since β-catenin immunostaining yields an intense signal in skin of M2SMO+βcat* mice, compared to M2SMO mice, it is possible that high-level β-catenin activity in βcat*-expressing mice is required for activation of hair lineage markers. Thus, the level of Wnt signaling in M2SMO mice may be sufficient to initiate epithelial bud development, but inadequate to stimulate terminal differentiation of hair lineages. This would be in keeping with the intense expression of X-gal staining in *lacZ*-based TCF/Lef reporter mice in hair precursor cells in mature follicles, compared to relatively low staining levels detected in hair buds (DasGupta and Fuchs, 1999), and the ability of high-level canonical Wnt signaling to drive development of follicle lineages even in glandular epithelia (Bierie et al., 2003; Miyoshi et al., 2002). Despite consistent detection of nuclear and cytoplasmic β-catenin in epithelial buds and hamartomas in M2SMO-expressing skin, we have been unable to detect TCF reporter activity using the

TOPgal mouse reporter line (DasGupta and Fuchs, 1999), arguing that in certain settings, immunolocalization of β-catenin provides a more reliable read-out of signaling activity.

As a control for experiments examining the consequences of βcat* induction in follicular hamartomas, we also examined the effects of postnatal βcat* activation in otherwise normal volar epidermis in adult mice. Within 5 days, βcat*-expressing mice developed multiple *de novo* skin appendages, some of which appeared to be relatively normal-appearing follicles with sebaceous glands and hair shafts. Particularly interesting was the concurrent appearance of other 'types' of skin appendages with seemingly different combinations of cell types: some of these follicular structures produced only hair shafts, others only a sebaceous gland, and still others exhibited little evidence of terminal differentiation, based on tissue morphology (Figure 3-2e,g). What could explain this phenomenon? Possible explanations for these findings include βcat* activation in epidermal progenitors with different developmental potential; activation of an inductive mesenchyme adjacent to only a subset of these lesions; or differential expression of required cofactors, e.g., Tcf4, in volar epidermis. It still remains to be determined if mesenchymal condensates/dermal papillae are only seen in a subset of *de novo* 'appendages,' which may help explain why hair shafts are only seen in some. Nonetheless, our findings underscore the plasticity of adult skin even from a region that is normally hairless, and although other investigators had not reported follicle development in hairless skin following Wnt pathway activation (Gat et al., 1998; Lo Celso et al., 2004), our study is the first to examine the consequences of expressing an activated β-catenin mutant from its endogenous promoter in adult mice. Although earlier studies have demonstrated expression of truncated β-catenin mutant is sufficient to

induce *de novo* hair follicle development in skin (Gat et al., 1998; Lo Celso et al., 2004; Silva-Vargas et al., 2005), these skin-targeting studies studies have generally used basal cell keratin *K14* promoter, which is either not expressed, or weakly expressed, in hair matrix and matrix progenitor cells. In addition, it is also plausible that in previous studies, K14 promoter activity could have been downregulated by mutant β-catenin, in contrast to positive feedback regulation of epithelial β-catenin mRNA levels by βcat* (Liu et al., 2007). It is also possible that there may be subtle activity differences in the mutant β-catenin protein with its first 87 N-terminal residues truncated and exon 3-deleted β-catenin mutant protein used here.

Aforementioned β-catenin mutant-induced *do novo* hair follicles in haired skin provided a niche for melanocytes, and the hair shafts were pigmented (Lo Celso et al., 2004). The βcat*-induced *de novo* follicles and associated hair shafts that formed in the hairless volar skin in our model did not contain pigment morphologically, although more rigorous biochemical assays to determine the presence of melanocytes have not been done. If βcat*-induced follicles do not contain melanocytes or pigmented hair shafts, it will be interesting to determine why *de novo* follicles that form on hairless volar skin are incompetent to accumulate pigmented hair shafts.

βcat* expression induced the formation of nearly normal-appearing hair follicles in hairless volar skin within 5 days. Because the hairless volar skin is a morphogenetically naive epidermis that normally does not contain hair follicles or follicle-associated structures, it can be considered a 'clean slate' with no expression of hair follicle lineage markers. One can therefore take advantage of this fact and perform mircroarray experiments on βcat*-induced skin at different timepoints (day 0, 1, 3, 5, etc)

to globally monitor gene expression profile changes during *de novo* hair follicle morphogenesis. It will be an important finding to compare any differences in gene expression patterns during postnatal *de novo* hair follicle morphogenesis versus normal embryonic hair follicle development. By employing the already developed epidermal-dermal separation technique that works extremely well with hairless volar skin, the βcat*-induced volar skin can even be separated into epidermal-dermal components and the microarray analysis may be done separately to dissect out the molecular signatures.

We also saw βcat*-induced keratinization and differentiation of preputial glands in 4-OHT treated male *K5-CreERT2;Ctnnb1(Ex3)*$^{fl/+}$ mice (Figure 3-4). Preputial glands are almost entirely composed of sebocyte-like cells and their progenitors, and normally do not express hair follicle or epidermal lineage markers. However, βcat* expression drove development of expression of multiple late-stage follicle lineage markers in preputial glands of *K5-CreERT2;Ctnnb1(Ex3)*$^{fl/+}$ mice. These results are in keeping with previous studies which found that activated of canonical Wnt signaling leads to transdifferentiation and development of hair follicle lineages in glandular epithelia such as prostate and mammary epithelium (Bierie et al., 2003; Miyoshi et al., 2002).

It will be interesting to examine the consequences of expressing βcat* in the suprabasal cells of the epidermis and test whether forced activation of canonical Wnt signaling will have an effect on the further differentiated suprabasal cells. To achieve this, one can utilize *K10-CreERT2* mice to generate *K10-CreERT2;Ctnnb1(Ex3)*$^{fl/+}$ bitransgenic mice, which will then allow for Cre recombination and accumulation of βcat* in the suprabasal cells of the epidermis upon 4-OHT treatment. Could βcat*

expression in the differentiated suprabasal epithelial cell population induce de-differentiation of these cells and force them to differentiate into follicle lineages?

Previous studies showed that expression of constitutively active β-catenin mutant in mouse skin induces the development of hair follicle derived tumors such as pilomatricoma and trichofolliculoma, although these tumors did not develop until the transgenic mice reached several months of age (Gat et al., 1998; Lo Celso et al., 2004). I also wanted to test whether βcat* expression would lead to tumor development in haired as well as hairless volar skin of 4-OHT treated *K5-CreERT2;Ctnnb1(Ex3)*$^{fl/+}$ mice, but no tumor development was seen in *K5-CreERT2;Ctnnb1(Ex3)*$^{fl/+}$ mice 45 days after 4-OHT treatment. It is possible that pilomatricoma or triochofolliculoma may develop in these mice if allowed to survive longer, but 4-OHT treated *K5-CreERT2;Ctnnb1(Ex3)*$^{fl/+}$ mice became sick and moribund a few weeks after 4-OHT treatment and had to be euthanized. To circumvent this problem, it may be necessary to use a much diluted concentration of 4-OHT and apply a tiny volume onto a small area on the skin (1-2 uL) to induce a localized Cre-recombination and βcat* accumulation and avoid a systemic βcat* effect. These mice should then survive for a normal life-span after 4-OHT treatment and can then be monitored for any tumor development.

βcat*-induced *de novo* hair follicles that formed in hairless volar skin looked relatively normal with sebaceous glands and hair shafts, and they also expressed the stem cell marker K15 (data not shown) (Morris et al., 2004), suggesting the establishment of stem cell niche in βcat*-induced *de novo* follicles. It would be interesting to determine whether these *de novo* follicles also undergo normal hair cycles of growth, regression and quiescence, but with the current *K5-CreERT2;Ctnnb1(Ex3)*$^{fl/+}$ system this may not be

possible as the continuous accumulation of βcat* disrupts follicle morphology and function. An alternative, inducible model of βcat* expression in skin where βcat* can be transiently expressed in the volar skin, and subsequently switched off, will be necessary to address this issue.

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Chapter IV

Epithelium-specific activation of Hh signaling is sufficient to reactivate growth of resting hair follicles

Introduction

Like other organs, developing hair follicles arise via a precisely orchestrated series of secreted signals traveling between epithelial hair follicle progenitors and an adjacent mesenchymal condensate, which together ultimately give rise to a mature hair-producing follicle. Multiple inductive signals are expressed and play a role during hair follicle morphogenesis, and in particular, Shh is produced and secreted by a group of epithelial cells in the developing hair bud. Based on initial studies mapping the expression patterns of developing hair follicle (Oro et al., 1997; St Jacques et al., 1998; Chiang et al., 1999; Mill et al., 2003), the Hh pathway is activated in epithelial cells of the hair bulb and outer root sheath, as well as in the dermal papillae (Sato et al., 1999; Oro and Higgins, 2003). Once formed, the postnatal hair follicle undergoes repeated cycles of growth, regression, and rest (Paus and Cotsarelis, 1999; Stenn and Paus, 2001), thus making it an unique and ideal model for studying fundamental questions involving cutaneous stem cell biology, organogenesis, and regeneration. The expression of Shh and Hh target genes in follicle epithelium is restricted to the active growth phase, anagen (Sato et al., 1999; Oro and Higgins, 2003). However, the precise location of Hh-activated cells in growing and resting hair follicles has not yet been established. The

deepest portion of the growing anagen follicle contains highly proliferative matrix cells which give rise to multiple cell lineages in the inner root sheath and hair shaft. During catagen, proliferation ceases, and there is massive apoptosis, leaving a relatively small number of non-proliferative epithelial cells surrounding the telogen hair shaft (Figure 1-2). It is within this residual epithelial cell compartment, often referred to as the 'bulge', that stem cells capable of giving rise to all cell lineages in the hair follicle, sebaceous gland, and epidermis, reside (Cotsarelis et al., 1990; Kobayashi et al., 1993; Oshima et al., 2001).

As mentioned in the previous chapters, it has been well-established that the canonical Wnt pathway can act as a switch to trigger new hair growth, and Wnt pathway activation in telogen mouse skin is sufficient to induce new hair growth cycle (Van Mater et al., 2003; Lo Celso et al., 2004). Subsequently, Shh acts as a powerful stimulus to drive proliferation of the growing follicle epithelium during anagen. However, adenoviral delivery of Shh (Sato et al., 1999) or topical application of a synthetic Hh agonist (Paladini et al., 2005) can also stimulate telogen follicles to enter anagen, suggesting that Hh signaling can also act as a switch for triggering follicle growth (perhaps indirectly via activation of canonical Wnt signaling, a hypothesis that will need to be explored in future studies). It should be noted that each of these approaches delivered Hh pathway activators indiscriminantly to multiple cutaneous cell types, including the follicle epithelium and mesenchyme. These studies therefore raise several fundamental questions: does Shh act directly on hair follicle stem cells to reactivate growth and trigger a new anagen phase? Is epithelial Hh signaling sufficient to initiate new follicle formation? In this chapter, I addressed these questions using novel

conditional mouse models that allow precise spatial and temporal control of Hh signaling activity in skin. One such mouse model is the tetracycline-inducible *TRE-SmoA1* mouse (developed by fellow MSTP student Evan Michael in the Dlugosz laboratory). When crossed with *K5-rtTA* (Diamond et al., 2000) mouse, resulting *K5-rtTA;TRE-SmoA1* bitransgenic mice express SmoA1, cell-autonomous activator of Hh signaling, specifically in the epithelium upon doxycycline administration. Utilizing this inducible mouse model, I was able to address the question of whether epithelium-specific Hh activation is sufficient to trigger new follicle growth.

Furthermore, it has been established that Hh signaling promotes growth of many tissues, and it has been proposed that Hh triggers proliferation by acting directly on stem cell populations to maintain normal homeostasis, or when aberrantly activated, in tumorigenesis [reviewed in (Ruiz i Altaba et al., 2002)]. However, there is limited functional data that support this theory in vertebrates and no study to date has conclusively demonstrated this. By utilizing the *keratin* 15 (K15) promoter which selectively targets transgene expression specifically to stem cells in the bulge compartment of hair follicles in postnatal mice (Liu et al., 2003; Morris et al., 2004), I was able to test the effects of modulating Hh function selectively in this stem cell population *in vivo*, to test whether stem cell compartment-specific Hh activation is sufficient to induce new follicle growth in telogen hair follicles.

The data described in this chapter and additional future work based on the data presented will enhance our understanding of how Hh signaling influences skin appendage biology and may ultimately have clinical impact affecting a large proportion of the population, given that disorders of the hair follicle and sebaceous glands are extremely

common. Understanding the molecular basis for growth and maintenance of the follicle may yield new approaches to treating these conditions.

Materials and Methods

Transgenic mice and conditional transgene activation.

To focally activate Hh signaling specifically in the epithelium, we crossed *K5-rtTA* (Diamond et al., 2000) mice with *TRE-SmoA1* (generated by Evan Michael) mice. Resulting *K5-rtTA;TRE-SmoA1* double transgenic mice were screened and used for experiments. To induce SmoA1 expression, doxycycline (20 mg/ml) was administered in drinking water with 5% sucrose, and in doxycycline-containing chow (Bio-serve, 200mg/kg). After three days, mice were maintained on doxycycline-containing chow but received normal drinking water. Duration of doxycycline administration varied with each experiment, as described in results section below. To generate triple transgenic mice with inducible expression of SmoA1 specifically in the stem cell compartment, *R26X-rtTA+GFP* (Yu et al., 2005; Belteki et al., 2005) mice were crossed with *TRE-SmoA1* transgenic mice. *R26X-rtTA+GFP;TRE-SmoA1* bitransgenic mice were then crossed with *K15-CrePR1* (Morris et al., 2004) to generate Cre- and doxy- regulated *K15-CrePR1;R26X-rtTA+GFP;TRE-SmoA1* triple transgenic mice. To induce stem cell compartment specific expression of SmoA1 transgene, triple transgenic animals were clipped, and 200 uL of RU486 (200 ug/mL) was topically applied on dorsal skin at postnatal day 51 once daily for five days, and doxycycline was administered orally as described above. All mice were housed and maintained according to University of

Michigan institutional guidelines, as stipulated by the University Committee on the Use and Care of Animals.

Tissue harvesting.

For hematoxylin and eosin (H&E) staining and immunohistochemistry, mouse skin samples were fixed in neutral-buffered formalin (NBF) overnight, transferred to 70% ethanol, processed, and embedded in paraffin. Images were captured as described previously (Chapter II).

Immunostaining.

The following primary antibodies were used for immunostaining: Sox9 (rabbit polyclonal, 1:1,000, Chemicon); K17 (rabbit polyclonal, 1:4,000, gift from P. Coulombe); K6 (rabbit polyclonal, 1:500, Covance); HA (rat polyclonal, 1:100, Roche); K15 (mouse monoclonal, 1:250, Neomarkers); CD34 (1:50, rat polyclonal, BD Biosciences); β-catenin (mouse monoclonal, 1:1,000, Sigma); Ki67 (rabbit polyclonal, 1:500, Vector Laboratories). Immunohistochemistry was performed as described previously (Chapter II).

Results

Epithelial-specific Hh activation by SmoA1 in telogen skin is sufficient to reactivate growth of resting hair follicles

First, we tested whether epithelial Hh activation in quiescent telogen skin is sufficient to trigger new follicle growth. To test this hypothesis, I utilized doxycycline-

inducible *K5-rtTA;TRE-SmoA1* bitransgenic mice, where epithelium-specific expression of *SmoA1* transgene, a constitutively activated allele of the proximal Hh effector Smo that drives cell-autonomous activation of Hh signaling, could be induced by administration of doxycycline. Postnatal hair cycling during the synchronized period is well characterized in C57/BL6 mice (Figure 4-1 and (Paus et al., 1999)), and the dorsal hair follicles enter prolonged second telogen phase at approximately postnatal day 40 and remain in telogen until about postnatal day 70. Taking advantage of this fact, *K5-rtTA;TRE-SmoA1* mice and littermate controls were allowed to mature to postnatal day 50, when the dorsal hair follicles had just entered a prolonged telogen phase. At postnatal day 50, *K5-rtTA;TRE-SmoA1* and control mice were given: A) doxycycline only; B) depilation only to induce anagen; or C) doxycycline and depilation to assess the effects of heightened Hh signaling on activated follicular cells that are undergoing active phases of growth and proliferation. Doxycycline administration was continued for 12 days, and the animals were sacrificed and skin harvested at postnatal day 62.

Depilation using a depilatory agent is a well-established method to stimulate anagen induction. As expected, depilation alone induced reactivation of follicle growth in both *K5-rtTA;TRE-SmoA1* and control mice (Figure 4-2b,e). In addition, we also saw morphological evidence of reactivated follicle growth in doxycycline-treated *K5-rtTA;TRE-SmoA1* mice, suggesting that the epithelium-specific activation of Hh signaling by SmoA1 expression was also sufficient to trigger anagen (Figure 4-2a). We also treated control mice with doxycycline to assess whether doxycycline alone has an effect on hair follicle cycling; we observed no abnormal phenotype in doxycycline-treated control skin, and the hair follicles remained in telogen (Figure 4-2d). Reactivated follicle

growth was also induced in *K5-rtTA;TRE-SmoA1* and control mice that were treated with doxycycline and depilated (Figure 4-2c,f).

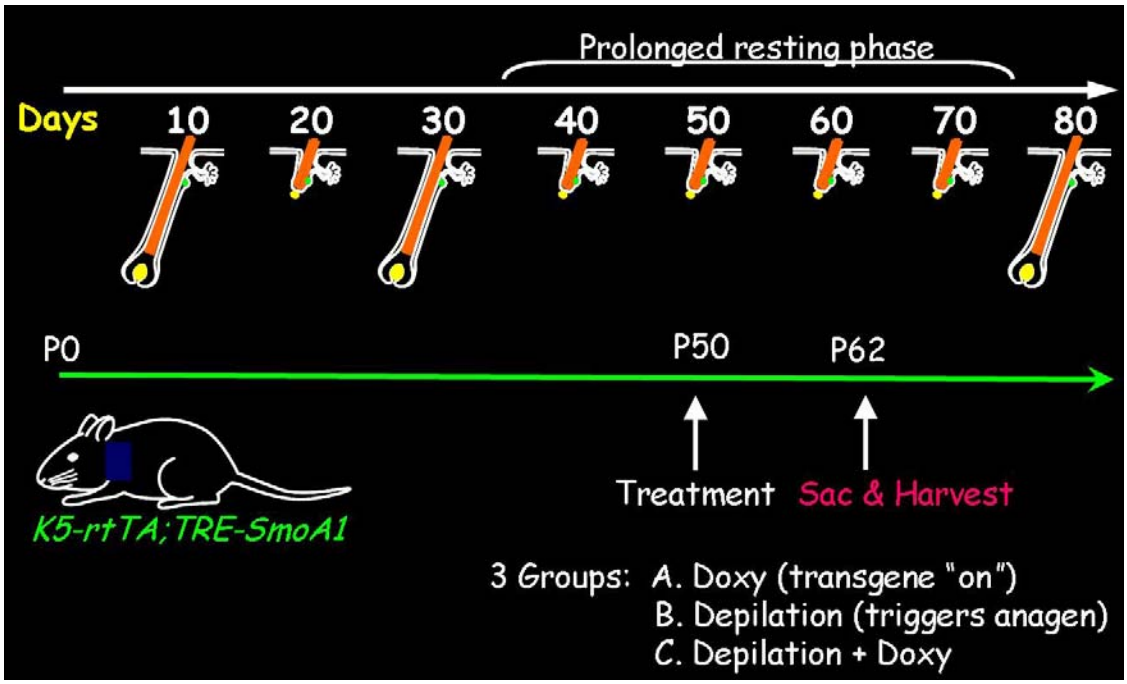


Figure 4-1. Cartoon illustrating experimental design and time-line for induction of SmoA1 expression in *K5-rtTA;TRE-SmoA1* mice during prolonged resting phase. Hair follicle cycling is synchronized in the first few months of life in mice. Taking advantage of this fact, *K5-rtTA;TRE-SmoA1* mice and littermate controls were allowed to reach postnatal day 50 (when the dorsal hair follicles are in the prolonged telogen phase), and the mice were either A) given doxy only; B) depilated only; and C) given doxy and depilated. Doxycycline was administered for 12 days, and the animals were sacrificed and skin harvested on post-natal day 62.

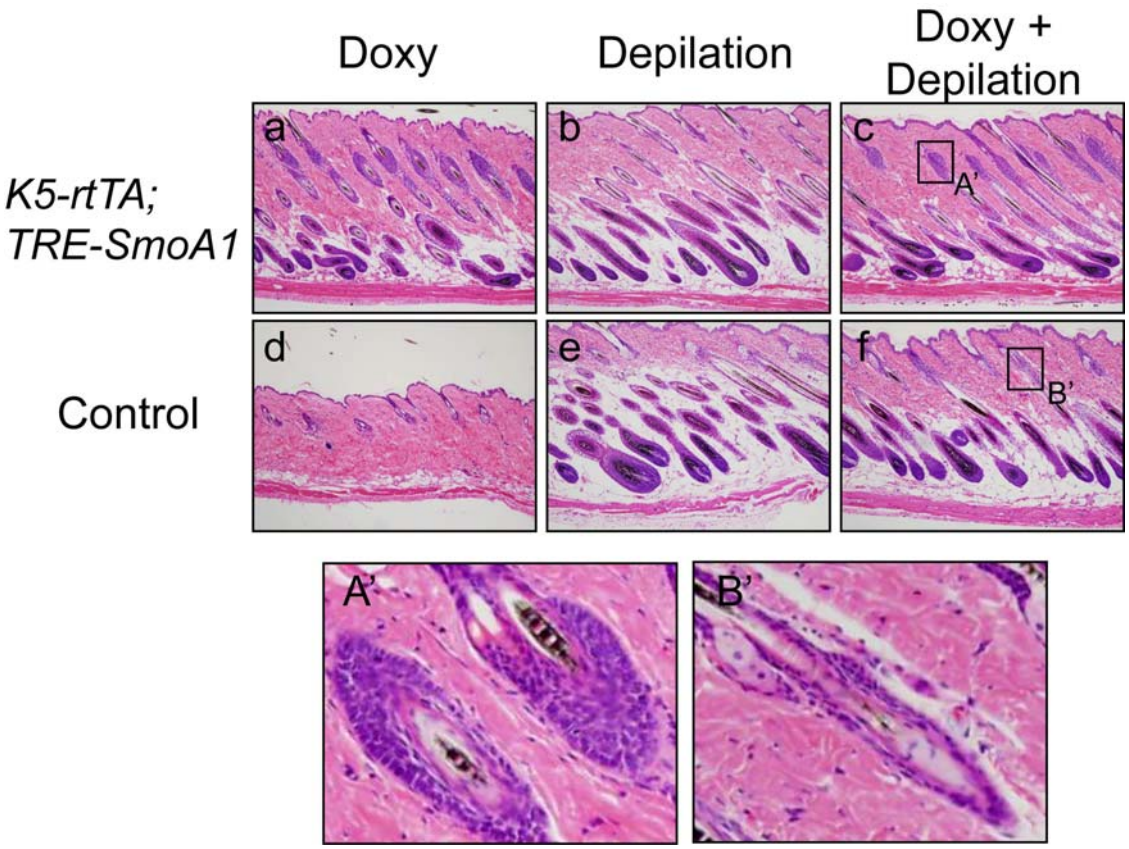


Figure 4-2. Epithelial Hh activation by SmoA1 is sufficient to trigger new hair growth, but with preferential expansion of outer rooth sheath (ORS) cells near the bulge, the stem cell compartment. (**a**) H&E-stained sections of *K5-rtTA;TRE-SmoA1* mice that were given doxy only ("Doxy"). Hair follicles are in anagen, in contrast to control (**d**), but with an expansion of ORS compartment. (**b,e**) H&E-stained sections of *K5-rtTA;TRE-SmoA1* and control mice that were depilated. Note presence of anagen follicles in both. (**c,f**) H&E-stained sections of *K5-rtTA;TRE-SmoA1* and control mice that were treated with doxycycline and depilated. Anagen follicles are seen in both, but note expansion of ORS cells near the bulge, stem cell compartment. (**A',B'**) High-powered magnification view of follicles in (**c,f**). Note expansion of ORS cells in A' compared to B'.

Preferential expansion of outer root sheath cells near follicle bulge, the stem cell compartment, in SmoA1-induced growing hair follicles

Interestingly, histological analysis revealed a preferential expansion of outer root sheath (ORS) cells in SmoA1-induced follicles just below the sebaceous gland, which corresponded to the area of the stem cell niche, the bulge. The ORS expansion was observed in both doxycycline-treated, and doxycycline-treated and depilated *K5-rtTA;TRE-SmoA1* skin (Figure 4-2a,c). The close proximity of the expanding ORS cells in SmoA1-induced follicles to the bulge might suggest that SmoA1 expression may induce an expansion of the follicular stem cells and/or their transit-amplifying progeny. Expansion of the ORS cells in SmoA1-induced follicles compared to control was easily appreciated (Figure 4-2A',B'. Immunophenotyping confirmed the expansion of ORS cells (Figure 4-3) in doxycycline-treated *K5-rtTA;TRE-SmoA1* mice. ORS-specific marker Sox9 immunostaining revealed a massive expansion of Sox9-positive ORS cells in SmoA1-induced follicles (Figure 4-3b) compared to control (Figure 4-3a).

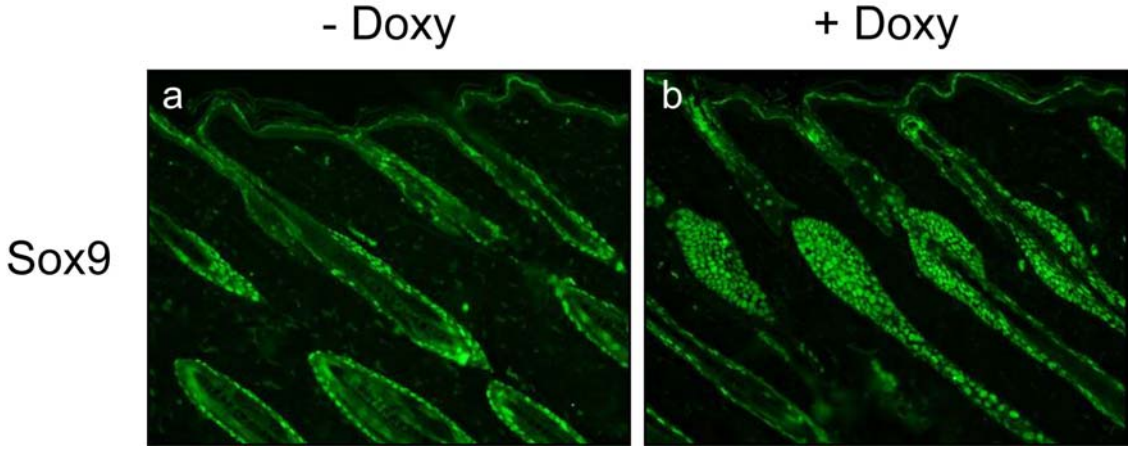
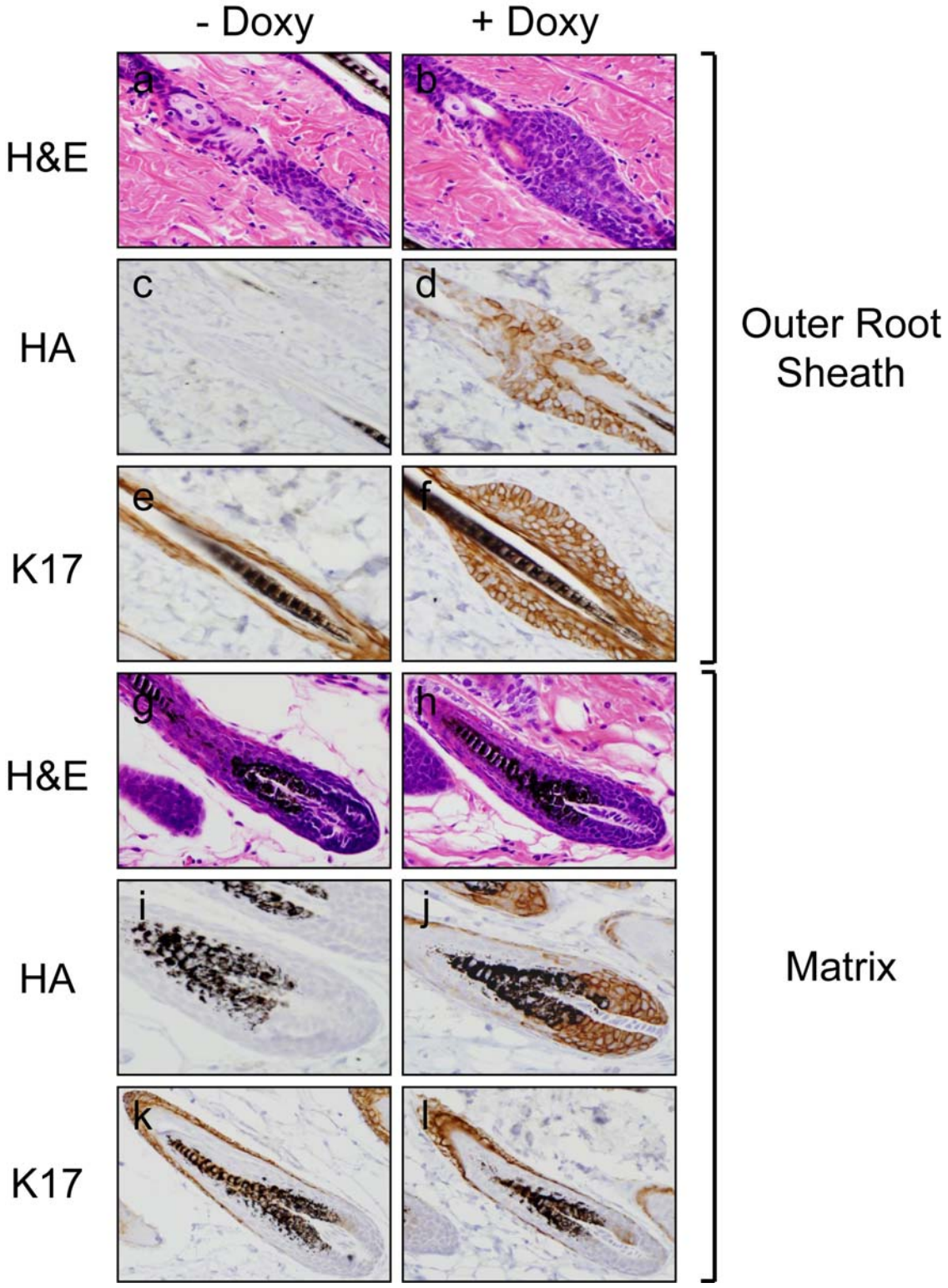


Figure 4-3. Preferential expansion of ORS compartment in SmoA1-induced follicles. (**a**) Immunofluorescence staining of control anagen follicles with ORS-specific marker Sox9. Sox9 staining reveals Sox9-positive ORS cells that are 1-2 cell layers thick. (**b**) Immunofluorescence staining of doxycycline-treated *K5-rtTA;TRE-SmoA1* follicles with Sox9. Note massive expansion of Sox9-positive cells

SmoA1 transgene is expressed in both ORS and matrix compartments, but distinct cell populations show differing responsiveness to SmoA1

Intrigued by the expansion of the ORS compartment in SmoA1-induced follicles, we then next sought to determine SmoA1 transgene expression pattern in the doxycycline treated *K5-rtTA;TRE-SmoA1* skin. Because of the presence of HA-epitope tag in the SmoA1 transgene construct, SmoA1 transgene expression could be easily detected by simple HA immunostaining. No HA expression could be detected in mice that were not treated with doxycycline, confirming the lack of SmoA1 expression and tight regulation of transgene expression in uninduced *K5-rtTA;TRE-SmoA1* bitransgenic mice (Figure 4-4c). In contrast, HA-positive cells were detected in the ORS compartment, interfollicular epidermis, and matrix compartment of doxycycline treated follicles of *K5-rtTA;TRE-SmoA1* mice. Interestingly, HA-staining was not homogeneous and uniform in SmoA1-induced follicles, but rather patchy with numerous non-HA positive cells mixed in together with HA-positive cells in the ORS compartment (Figure 4-4d). In addition, intensely HA-positive cells were detected in the matrix compartment of doxycycline treated follicles (Figure 4-4j), suggesting that robust levels of SmoA1 transgene was expressed in the matrix compartment. However, in contrast to the ORS compartment of SmoA1-induced follicles where prominent ORS expansion was apparent (Figure 4-4a,b), the matrix compartment morphologically looked similar to control (Figure 4-4g,h). In addition, Hh target gene K17 expression and localization were indistinguishable in the matrix compartment of SmoA1-induced follicles compared to control (Figure 4-4k,l), suggesting that despite robust SmoA1 transgene expression, SmoA1 had no gross effect

on the matrix compartment, which was in striking contrast to the ORS compartment (Figure 4-4e,f).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k
l
Outer Root Sheath
Matrix

Fig. 4-4. Differing responsiveness of distinct cell populations to SmoA1 transgene expression. (**a,b**) H&E-stained sections of control (**a**) and doxycycline treated (**b**) follicles, showing expansion of ORS compartment in (**b**). (**c,d**) HA immunostaining reveals patchy HA expression in ORS compartment of doxycycline treated follicles (**d**), while no HA expression is seen in control (**c**). (**e,f**) ORS-specific marker K17 immunostaining reveals expansion of the ORS compartment in SmoA1-induced follicles compared to control. (**g,h**) H&E-stained sections of the matrix compartments of control (**g**) and SmoA1-induced (**h**) follicles. Despite robust SmoA1 expression as assessed by HA immunostainig (**j**), matrix compartment of doxycycline treated follicle (**h**) morphologically looks normal and virtually indistinguishable to control (**g**). (**k,l**) Hh target gene K17 staining reveals virtually identical K17 expression in control (**k**) and doxycycline treated (**l**) follicles.

Expression of stem cell markers K15 and CD34 is lost over time in follicles reactivated to grow by SmoA1

Because the location of ORS expansion in SmoA1-induced follicles was in close proximity to the bulge, we were intrigued by the possibility that epithelium-specific Hh activation may lead to the expansion of the follicular stem cells. To begin addressing this issue, I used the follicle stem cell markers K15 (Liu et al., 2003; Morris et al., 2004) and CD34 (Trempus et al., 2003) to see whether the expanded ORS contained an increased number of cells expressing K15 and/or CD34 expression. K15 and CD34 expression and localization in the bulge region of control telogen follicles confirmed that these markers were specific for the follicle bulge (Figure 4-5a,c). Interestingly, the expanded ORS cells of SmoA1-induced follicles did not express K15 or CD34 (Figure 4-5b,d). Additional functional studies will be required to assess whether Hh signaling leads to an expansion of stem or progenitor cells, despite the apparent absence of stem cell markers in this interesting cell population.

Next, instead of a 12 day doxycycline administration in *K5-rtTA;TRE-SmoA1* mice, they were given doxycycline for 3 days and skin harvested at day 3. Previously, it was found that application of topical Hh agonist on dorsal skin did not induce an appreciable upregulation of Hh transcriptional activity for several days (Paladini et al., 2005), suggesting that Hh pathway activation in telogen skin may be triggering anagen indirectly via a secondary signaling pathway. Given my earlier data on Hh-Wnt interactions during Hh-driven ectopic epithelial bud development, I hypothesized that forced Hh activation in follicle epithelium may trigger new follicle growth indirectly via activation of canonical Wnt pathway. *K5-rtTA;TRE-SmoA1* mice in prolonged telogen

phase (postnatal day 50) were given doxycycline for 3 days, and then were sacrificed and skin harvested at day 3. Morphologically, 3 day SmoA1-induced follicles featured expansion of the secondary hair germ at the base of follicles (Figure 4-6a,b). HA immunostaining confirmed robust expression of SmoA1 transgene in SmoA1-induced follicles, especially at the base of follicles (Figure 4-6c,d). Double immunostaining of SmoA1-induced follicles with Ki67 and HA revealed that the majority of HA-positive cells were also Ki67-positive, consistent with the possibility that SmoA1 may be driving the proliferation of follicle epithelial cells (Figure 4-6i,j).

I then examined β-catenin localization in early SmoA1-induced follicles. β-catenin immunostaining revealed robust nuclear and cytoplasmic β-catenin localization at the base of SmoA1-induced follicles (Figure 4-6f), indicating activation of canonical Wnt signaling pathway, while no nuclear/cytoplasmic β-catenin localization was seen in control (Figure 4-6e). This data suggests that epithelial Hh activation leads to canonical Wnt activation, and that Hh-mediated hair follicle growth may be mediated indirectly via activation of canonical Wnt activation. However, functional study to definitively test this hypothesis is still required (see Discussion for additional comments). Interestingly, intensely nuclear β-catenin localization was also seen in dermal papillae of SmoA1-induced follicles (Figure 4-6f), although SmoA1 transgene was not expressed in dermal papilla cells as assessed by HA staining (Figure 4-6j), suggesting a mesenchymal response to secreted Wnt ligands.

Furthermore, immunostaining with the stem cell marker K15 in 3 day SmoA1-induced follicles revealed an expansion of K15-positive cells (Figure 4-6h) compared to control (Figure 4-6g), which was in contrast to the lack of K15 positive cells in 12 day

SmoA1-induced follicles (Figure 4-5b,d). Based on these initial stem cell marker immunostaining results, it is possible that while the initial SmoA1 expression induces expansion of the follicular stem cell population, continued SmoA1 expression and sustained high level Hh activation results in the depletion of follicular stem cell reservoir.

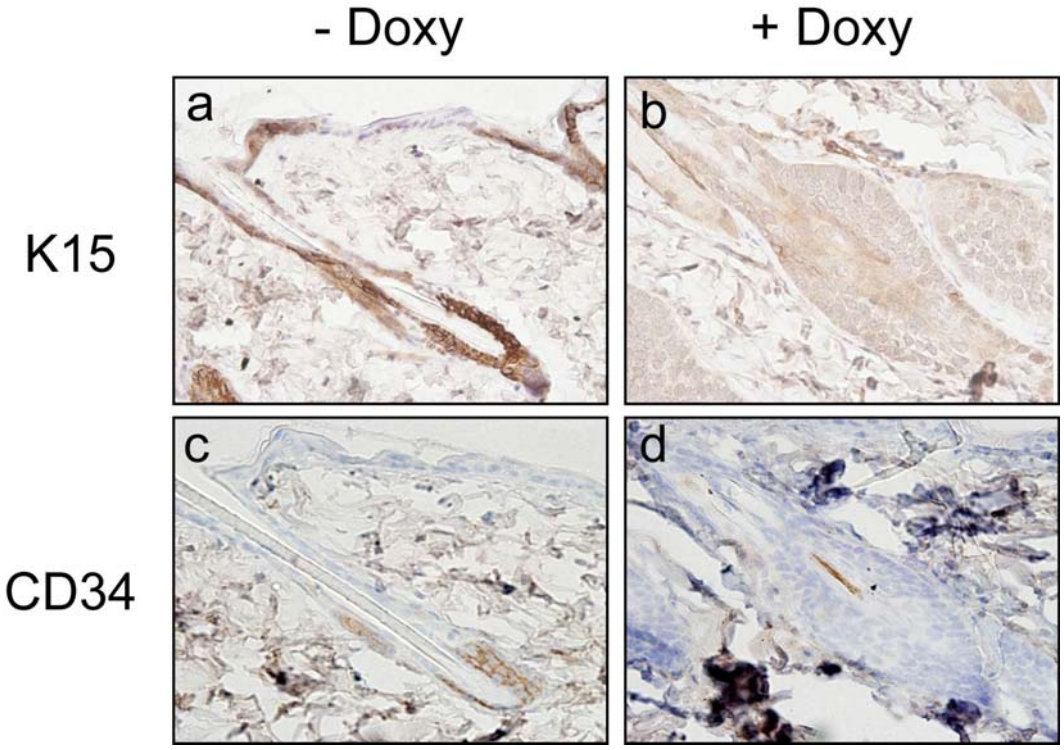

Figure 4-5. Stem cell markers K15 and CD34 are not expressed in expanded ORS cells of SmoA1-induced follicles. (**a**) K15 is expressed in the bulge region of control telogen follicle. Note patchy K15 expression also in the interfollicular epidermis. (**b**) K15 is not expressed in expanded ORS cells of SmoA1-induced follicles. (**c**) CD34 is expressed in the bulge region of control telogen follicle. (**d**) CD34 is not expressed in expanded ORS cells of SmoA1-induced follicles.

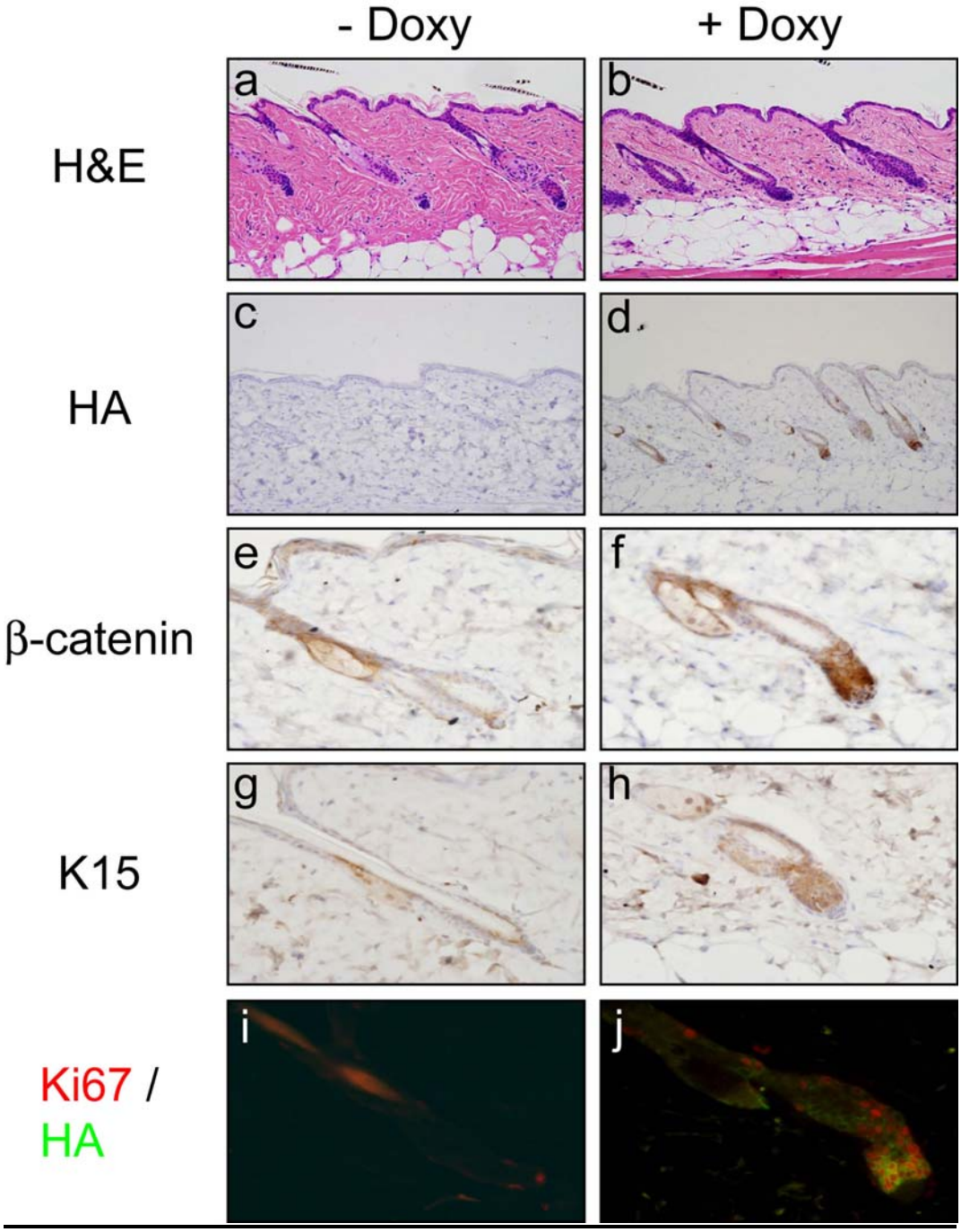
- Doxy
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Figure 4-6. 3-day SmoA1 expression in telogen skin induces canonical Wnt pathway activation and expansion of K15 positive cells. (**a,b**) H&E stained sections of dorsal skin from control (**a**) and *K5-rtTA;TRE-SmoA1* mice treated with doxycycline for 3 days (**b**). (**c,d**) As assessed by HA immunostaining, SmoA1 transgene is expressed in the hair follicles of doxycycline treated *K5-rtTA;TRE-SmoA1* mice, but not in control (**c**). (**e,f**) β-catenin immunostaining reveals robust nuclear and cytoplasmic β-catenin localization in SmoA1-induced follicle at the base and in the dermal papilla (**f**), indicating activation of canonical Wnt pathway. No nuclear/cytoplasmic β-catenin localization is seen in control follicle (**e**). (**g,h**) Expansion of K15 positive cells are seen in SmoA1-induced follicle (**h**), compared to control (**g**). (**i,j**) Double immunoflourescence staining using Ki67 and HA antibodies reveal numerous follicular cells co-expressing Ki67 and HA at the base of follicle, while no HA-positivity and occasional Ki67 positive cells in the secondary hair germ are seen in control.

Activation of Hh Signaling in the stem cell compartment does not induce anagen

Given the results above and the widely held notion that Hh signaling acts directly on stem cells during development and tumorigenesis [reviewed in (Altaba et al., 2002)], I hypothesized that Hh pathway activation specifically in quiescent hair follicle stem cells would be sufficient to reactivate hair follicle growth. To test this hypothesis, I utilized *K15-CrePR1* mice (Morris et al., 2004), which allows for specific targeting of transgene expression to stem cells within the bulge compartment of hair follicles in postnatal mice. In *K15-CrePR1;Rosa26Reporter* bitransgenic mice, topical treatment with the progesterone antagonist RU486 activates Cre function and results in recombination strikingly limited to the stem cell compartment of telogen hair follicles. In addition, fate-mapping studies using similarly-treated mice shows that progeny of these β-gal expressing cells can contribute to all epithelial cell types in the epidermis, hair follicle, and sebaceous gland, verifying that recombination/*lacZ* activation took place in multipotent progenitor cells (Morris et al., 2004). To activate Hh pathway specifically in the stem cell compartment, I generated triple transgenic *K15-CrePR1;R26X-rtTA+GFP;TRE-SmoA1* mice, which combined the Cre- and doxycycline-regulated systems to achieve tight control of transgene expression in hair follicle stem cells (Yu et al., 2005; Belteki et al., 2005). Triple transgenic mice and littermate controls in second prolonged telogen were treated with topical RU486 once daily for 5 days starting at post-natal day 51, and were given doxycycline, clipped and monitored for hair growth (Figure 4-7). Similar to the experiment outlined in Figure 4-1, triple transgenic mice and controls were also depilated only, and treated with RU486 and doxycycline, and depilated to

assess the effect of heightened Hh signaling on activated follicle stem cells and their transient-amplifying cell progeny.

As expected, new follicle growth was observed in control and triple transgenic mice that were depilated only (Figure 4-8b,e). However, follicles in doxycycline-treated triple transgenic mice remained in telogen and new follicle growth was not observed (Figure 4-8a), suggesting that Hh activation by SmoA1 specifically in the stem cell compartment is not sufficient to trigger new hair growth. In contrast, triple transgenic mice that received doxycycline and were depilated featured prominent expansion of ORS cells compared to control (Figure 4-8c,f). HA immunostaining to assess SmoA1 transgene expression revealed occasional HA-positive cells in doxycycline treated follicles (Figure 4-8g), while robust HA expression was seen in doxycycline-treated and depilated follicles (Figure 4-8i). Immunostaining with ORS-specific marker Sox9 confirmed expansion of Sox9-positive ORS cells in doxycycline-treated and depilated follicles (Figure 4-8l) compared to control (Figure 4-8k).

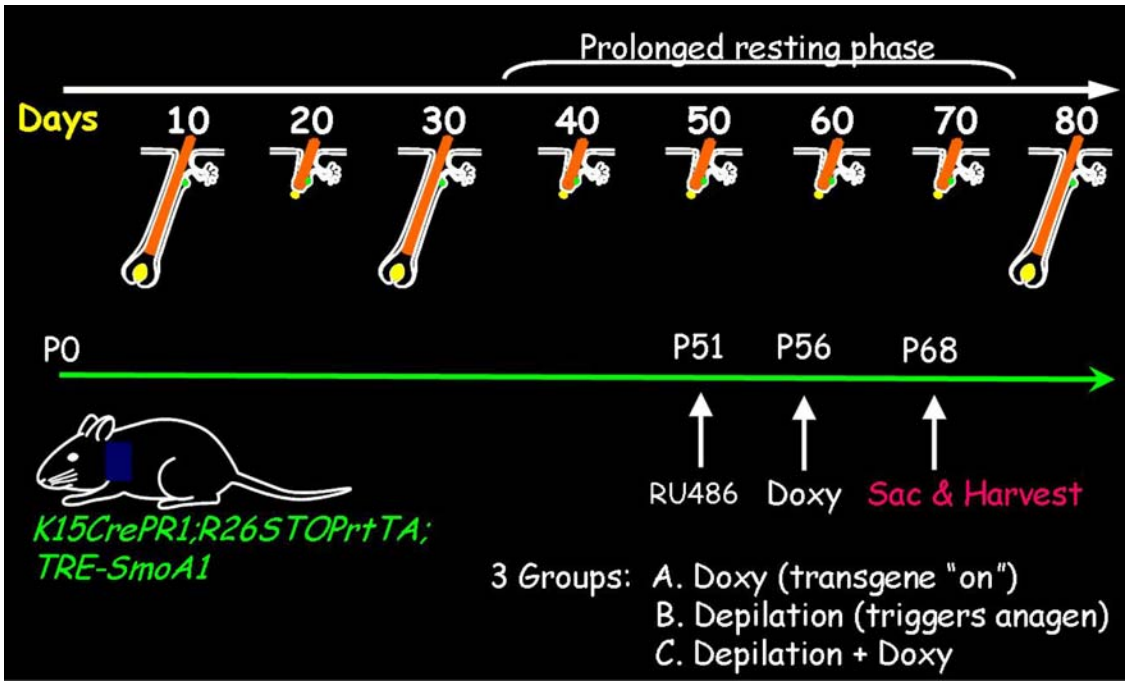

Figure 4-7. Cartoon illustrating experimental design and time-line for induction of SmoA1 expression in *K15-CrePR1;R26X-rtTA+GFP;TRE-SmoA1* mice during prolonged resting phase. *K15-CrePR1;R26X-rtTA+GFP;TRE-SmoA1* mice were allowed to age to postnatal day 51, when dorsal hair follicles were in second prolonged telogen. At postnatal day 51, triple transgenics and littermate controls were treated with topical RU486 once daily for 5 days. After 5 days (postnatal day 56), triple transgenic mice and controls were : A) given doxycycline only; B) depilated only; and C) given doxycycline and depilated. Doxycycline was administered for 12 days, and the animals were sacrificed and skin harvested on post-natal day 68.

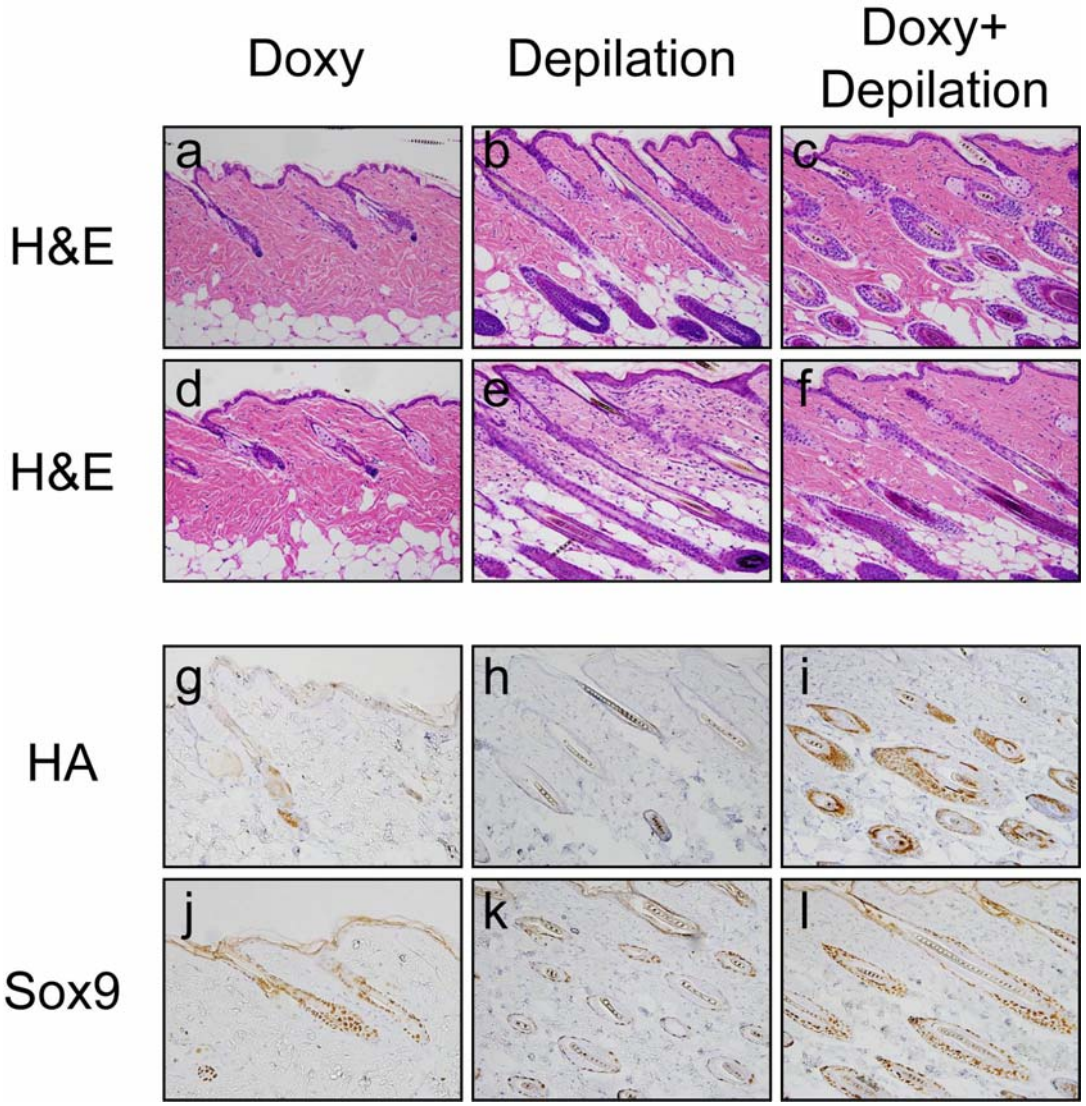

Figure 4-8. Activation of Hh Signaling in stem cell compartment does not induce anagen. (**a,d**) H&E stained sections of dorsal skin from *K15-CrePR1;R26X-rtTA+GFP;TRE-SmoA1* mice (**a**) and controls (**d**) treated with doxycycline. Note follicles are in telogen phase in *K15-CrePR1;R26X-rtTA+GFP;TRE-SmoA1* skin, suggesting that SmoA1 expression specifically in the stem cell compartment is not sufficient to trigger new follicle growth. (**b,e**) As expected, new follicle growth is seen in triple transgenic and control mice that are depilated. (**c,f**) H&E stained sections of *K15-CrePR1;R26X-rtTA+GFP;TRE-SmoA1* mice and controls that were given doxycycline and were depilated. Note ORS expansion and clear cell hyperplasia in (**c**) compared to (**f**). (**g**) HA immunostaining reveals occasional HA positive follicles in doxycycline treated skin. In contrast, expanded ORS cells in triple transgenic mice show robust but patchy HA expression (**i**). (**j,k,l**) ORS specific marker Sox9 immunostaining reveals ORS expansion in *K15-CrePR1;R26X-rtTA+GFP;TRE-SmoA1* mice (**l**) compared to control follicles (**k**).

Discussion

Adenoviral delivery of Shh (Sato et al., 1999) as well as topical treatment with Hh-agonist (Paladini et al., 2005) in mouse skin is sufficient to trigger growth of quiescent telogen follicles in mice. However, in both settings, multiple cell types including keratinocytes, fibroblasts, and other mesenchymal cells, including dermal papilla cells, may have been responding to Hh pathway activation, and it is not clear whether activation of follicle growth was due to direct stimulation of quiescent bulge stem cells, or a consequence of stimulating additional cell types which could then indirectly influence the stem cell compartment through a secondary signal(s). Utilizing novel mouse models that allow precise spatial and temporal control of Hh signaling in skin, I tested whether epithelium-specific activation of Hh signaling is sufficient to trigger follicle growth in resting telogen skin. My results with *K5-rtTA;TRE-SmoA1* bitransgenic mice show that epithelium-specific Hh activation is sufficient to trigger new follicle growth (Figure 4-2).

Interestingly, 12-day SmoA1-induced anagen follicles also featured expansion of the ORS compartment near the bulge, the stem cell niche of the hair follicle (Figure 4-4b). Furthermore, 3-day SmoA1-induced follicles exhibited expansion of stem cell marker K15-positive cells in the reactivated hair follicle, which also expressed robust levels of SmoA1 transgene and increased Ki67 expression (Figure 4-6h). In addition, a very brief doxycycline induction (12 – 24 hours) in *K5-rtTA;TRE-SmoA1* mice resulted in new follicle growth without obvious expansion of the ORS compartment (data not shown). We therefore suspected SmoA1 may drive the expansion of follicular stem cells.

However, immunostaining with stem cell markers revealed lack of K15 and CD34 expression in the expanded ORS cells of 12-day SmoA1 induced follicles. Based on these observations, it is possible that while SmoA1 expression initially induces the expansion of follicular stem cells, sustained and continued Hh activation for 12 days may deplete the stem cell reservoir. It should also be noted that the lack of positive K15 or CD34 staining does not absolutely preclude the fact that Hh signaling may influence the function and status of follicular stem cells. To address this issue, more rigorous functional assays such as fluorescence activated cell sorting analysis and *in vitro* clonogenic assays to characterize the nature of the expanded cell population in SmoA1-induced follicles will be needed, to determine whether activation of Hh signaling influences the function or number of follicle stem cells. Furthermore, in a separate experiment, *K5-rtTA;TRE-SmoA1* mice could be given doxycycline for 12 days to induce anagen, and subsequently discontinue doxycycline treatment to allow the follicles to complete a hair cycle and return to the quiescent telogen phase. If the follicular stem cells are depleted in the *K5-rtTA;TRE-SmoA1* mice, upon another depilatory challenge, these follicles will not be able to re-enter another growth phase as robustly as control follicles.

In SmoA1-induced follicles, HA immunostaining revealed robust SmoA1 transgene expression in the matrix compartment, but without obvious morphological abnormalities or Hh target gene K17 expression compared to control (Figure 4-4h,j,l). What can explain the differing responsiveness of distinct cell populations to SmoA1 transgene expression? The lack of responses in the matrix compartment may be due the fact that during periods of follicle growth, the matrix compartment already has a high

level of Hh activation. Therefore, an additional stimulus by SmoA1 may not be inducing a phenotype because the matrix cells are already saturated and "maxed out".

Interestingly, HA immunostaining in SmoA1-induced follicles also exhibited patchy HA expression. Co-immunolocalization of HA (for transgene expression) and Hh target genes (such as Gli1, Ptch1, and K17) and/or proliferation markers (such as Ki67 and PCNA) will reveal whether the HA-negative epithelial cells that surround HA-positive cells also have Hh pathway activation and a proliferative advantage or other functional alteration.

It was shown that a topical application of a synthetic Hh agonist on mouse skin is sufficient to trigger growth of telogen follicles (Paladini et al., 2005), but Hh pathway transcriptional activation did not occur for 3-4 days after topical Hh agonist treatment, a considerable delay which could implicate a secondary signal with subsequent activation of endogenous Hh signaling during the expected time-frame in early anagen. Furthermore, numerous studies are pointing to the canonical Wnt signaling as the key stimulus needed to trigger postnatal anagen onset (Van Mater et al., 2003; Lo Celso et al., 2004). These results raise the possibility that Hh agonist treatment itself is not directly triggering new follicle growth but rather the Hh agonist may be inducing anagen by stimulating a particular cell type in the skin that in turn activates another signaling pathway, such as canonical Wnt signaling pathway. This hypothesis is plausible, given 1) my previous data showing that Hh-driven ectopic epithelial bud development occurs indirectly by activation of canonical Wnt signaling pathway; 2) detection of intense nuclear and cytoplasmic β-catenin (indication of canonical Wnt activation) in the follicles 3 days after SmoA1 induction; and 3) previous studies that support the notion that

canonical Wnt plays a key role in triggering new follicle growth (Van Mater et al., 2003; Lo Celso et al., 2004). I have tried to test this hypothesis by acquiring the synthetic Hh agonist from Curis, Inc and first confirming that it can trigger new follicle growth in telogen mouse skin. A single topical application of Hh agonist was sufficient to trigger anagen in all mice tested (data not shown). However, Hh agonist treatment on *K5-rtTA;TRE-Dkk1* bitransgenic mice, which upon doxycycline administration express Dkk1 in K5-expressing cells to block the canonical Wnt pathway, still resulted in the induction of new follicle growth on dorsal skin (data not shown). Given that Dkk1 expression was not effective in blocking M2SMO-induced bud and hamartoma phenotype in dorsal skin (Chapter II), it is plausible that *K5-rtTA;TRE-Dkk1* is not an ideal system to test whether blocking the canonical Wnt pathway would inhibit Hh agonist induced anagen. Another mouse model to block canonical Wnt pathway, such as one that expresses soluble Wnt decoy receptor comprising the Frizzled8 cysteine-rich domain (CRD) fused to the human Fc domain (F8CRDhFc) (DeAlmeida et al., 2007), may be a better system to address this hypothesis and could be generated.

Immediately following the Hh agonist application on skin and several days thereafter, what cell types in the skin are responding to the Hh agonist? This is an important question that remains to be answered. In our laboratory, we have multiple Hh pathway reporter mice, such as *Gli1-lacZ* (Bai et al., 2002) and *Ptch1-lacZ* (Goodrich et al., 1997) mice, in which upon X-gal staining *lacZ* gene product β-galactosidase can be visualized as a marker of Hh pathway *in vivo*. Topical application of Hh agonist directly on *Gli1-lacZ* and/or *Ptch1-lacZ* telogen dorsal skin and identifying the specific cell types

that are responding to Hh agonist by wholemount X-gal staining or in sections will be required to identify target cells responding to this drug..

We also tested if Hh activation selectively in the follicle stem cells is sufficient to trigger new follicle growth. To address this question, I took advantage of *K15-CrePR1* mice, which allows for targeting of transgene expression to stem cells within the bulge compartment of hair follicles in post-natal mice (Liu et al., 2003; Morris et al., 2004), providing a powerful tool for modulating gene function in this stem cell population *in vivo*. By generating Cre- and doxycycline- inducible *K15-CrePR1;R26X-rtTA+GFP;TRE-SmoA1* triple transgenic mice, I was able to specifically activate Hh signaling in the stem cell niche. Activation of Hh signaling specifically in the stem cell compartment did not result in induction of anagen, suggesting that direct Hh activation on hair follicle stem cells with SmoA1 is not sufficient to initiate new follicle formation. In contrast, outer root sheath hyperplasia was observed in follicles that were treated with doxycycline and depilated, suggesting that SmoA1 expression had an effect on the progeny of activated stem cells in the bulge, including transient amplifying cells that give rise to all lineages of the hair follicle.

It should be noted, however, that the lack of anagen induction by Hh activation in stem cells may be due to a technical issue, where insufficient Cre recombination and subsequent low level SmoA1 transgene expression in the bulge may be the reason for the lack of new follicle growth. Modifying the experimental protocol may improve and yield higher Cre recombinase activity. In addition, only a subset of follicles in doxycyline treated triple transgenic mice showed HA expression (Figure 4-8g), suggesting inefficient Cre recombinase activity and low level SmoA1 expression in these follicles. However,

since a single paraffin section only exposes a small surface area of the follicle, it is possible that not all HA-positive epithelial cells are detected using conventional immunostaining methods. A wholemount HA immunostaining technique will circumvent this problem.

Taken together, data presented in this chapter suggest that epithelium-specific activation of Hh signaling by cell-autonomous Hh activator SmoA1 is sufficient to trigger new follicle growth in telogen skin. Combined with the *K15-CrePR1;R26X-rtTA+GFP;TRE-SmoA1* triple transgenic data, it may be postulated that Hh signaling in skin does not act directly on the stem cells in the hair follicle, but instead acts on their transient amplifying progeny. Additional future work will be needed to assess the biological potential of the expanded cell population in SmoA1-expressing cells, and determine whether anagen induction in Hh-activated follicle epithelium reflects a direct Hh pathway-mediated effect or activation of indirect signaling via the canonical Wnt/β-catenin pathway.

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Chapter V

Heightened Hh signaling in epidermis stimulates ectopic pigment accumulation and melanogenesis

Introduction

M2SMO-expressing mice consistently exhibited areas of increased pigmentation in multiple regions of the skin, including the tail, paws, ventral skin and ears. Increased pigmentation in other mouse models of Hh-activated skin tumors has been described (Grachtchouk et al., 2000; Sheng et al., 2002; Grachtchouk et al., 2003; Hutchin et al., 2005; Svard et al., 2006), and this is in keeping with the fact that a subset of human BCCs (but not other common epithelial tumors) may also be pigmented (Bleehen, 1975). In this chapter, I examined the Hh-induced ectopic pigment accumulation at the molecular level to gain a further understanding into the potential link between aberrant Hh signaling and 'unscheduled' melanogenesis. Understanding Hh-associated melanogenesis will help explain why a subset of human BCCs are pigmented, and may yield novel insight into the regulation of melanogenesis in general.

Melanocytes are highly specialized melanin-producing cells of the hair and skin that originate from the neural crest and migrate to the epidermis and hair follicles during embryonic development (Erickson and Reedy, 1998; Dorsky et al., 2000a). Once formed, melanocytes play a crucial role in skin by absorbing ultraviolet radiation and offering photoprotection and thermoregulation. Neural crest development is dependent on several

signal transduction pathways, including members of the canonical Wnt pathway (Dorsky et al., 2000b; Wilson et al., 2001; Garcia-Castro et al., 2002), bone morphogenetic protein families (Wilson et al., 2001), and fibroblast growth factor (Trainor et al., 2002). Neural crest cells are pluripotent cells that migrate via two distinct pathways during embryogenesis: a ventral path between the neural tube and somites for cells that will differentiate into neurons and glial cells of the peripheral nervous system, and a dorsolateral path between the ectoderm and dermomyotome of the somites for cells that will differentiate into melanocytes (Erickson and Reedy, 1998; Dorsky et al., 2000a). Melanoblasts, the precursors of melanocytes, migrate, proliferate, and differentiate en route to their destinations in the basal layer of epidermis and hair follicles. In mice, melanoblasts differentiate from pluripotent neural crest cells at about embryonic day 8.5, and by embryonic day 14.5 melanocytes exit from the overlying dermis and populate the epidermis and developing hair follicles (Mackenzie et al., 1997).

Multiple signaling pathways and transcription factors are known to play critical roles during melanocyte development and migration, including the canonical Wnt/β-catenin signaling pathway (Christiansen et al., 2000). It has been reported that endogenous Dkk1 secreted by fibroblasts in the dermis elicits the hypopigmented phenotype of palmoplantar skin in humans due to suppression of melanocyte function and growth via the regulation of two important signaling factors, microphthalmia-associated transcription factor (MITF) and β-catenin (Yamaguchi et al., 2008; Yamaguchi et al., 2007b; Yamaguchi et al., 2004). In addition, overexpression of β-catenin in zebrafish promotes melanoblast formation and reduction of neuron and glia formation (Dorsky et al., 1998). In addition, MITF (Steingrimsson et al., 2004) is also known to play a critical

role in melanocyte development. MITF expression is activated early on while neural crest cells are differentiating into melanoblasts, and is absolutely required for the survival of migrating melanoblasts (Steingrimsson et al., 2004). Furthermore, sustained expression of MITF throughout the life of an organism seems to be critical for the survival of melanocytes: mice which carry the hypomorphic mutant allele *Mitf*^{vit} have normal melanocyte development at early stages, but accelerated age-dependent graying of coat color occurs due to premature postnatal melanocyte loss (Lerner et al., 1986). c-Kit, a tyrosine kinase receptor, and its ligand Stem Cell Factor (SCF) also play important roles in permitting melanoblast survival and proliferation (Steel et al., 1992; Mackenzie et al., 1997; Jordan and Jackson, 2000). Transgenic *Scf* expression in skin using the *keratin* 14 (K14) promoter was sufficient to support melanocyte homing to the epidermis in mice, which otherwise has few epidermal melanocytes in fur-bearing regions (Kunisada et al., 1998). In addition, it was found that the use of imatinib mesylate (Gleevec), a Bcr-Abl tyrosine kinase inhibitor that also inhibits c-Kit tyrosine kinase, can result in the loss of melanocytes in human skin (Legros et al., 2005). Other molecules that are also believed to play a role in melanocyte migration and function include Endothelin 1 (ET1) and Endothelin 3 (ET3), hepatocyte growth factor (HGF) and basic fibroblast growth factor (b-FGF) (Imokawa, 2004).

Once the melanoblast migration and expansion are complete, within the postnatal hair follicle exists two discrete melanocytic populations: melanocyte stem cells that reside in the bulge, and their differentiated progeny which take residence in the bulb of the follicle to give rise to pigmented hair shafts. Under physiological conditions, melanogenesis is stimulated in association with Hh-driven proliferation of follicle

epithelium. Shortly after activation of a new hair follicle growth phase, unpigmented melanoblasts residing within the follicle bulge are stimulated to divide and migrate into the newly-formed hair bulb, where they differentiate and transfer pigment to the developing hair shaft (Nishimura et al., 2002). However, many questions still remain to be answered: What are the signals that modulate melanocyte stem cells' awakening from and return to quiescence? To what degree are the melanocyte stem cells dependent on the bulge, and what are the signals that modulate melanocyte cell death during hair follicle regression?

While multiple signaling pathways and transcription factors have been implicated in melanocyte development and melanocyte-derived pigment defect pathology, the link between aberrant Hh signaling and melanogenesis has not been clearly established. Interestingly, transgenic mouse models with epithelium-specific activation of Hh signaling, such *ΔK5-M2SMO* (Grachtchouk et al., 2003) and doxycycline-inducible *K5-tTA;TRE-SmoA1* mice, consistently exhibit increased pigmentation in various regions of skin. Furthermore, full blown BCCs that develop in *K5-Gli2* (Grachtchouk et al., 2000) and *K5-rtTA;TRE-Gli2* (Hutchin et al., 2005) mice that are bred on agouti and black founders also exhibit increased pigment. In addition, clinicians have long noted that a subset of human BCCs, tumors that are driven by aberrant Hh signaling, also contains pigment (Bleehen, 1975), thus further suggesting that oncogenic Hh signaling may be influencing on melanogenesis. How is oncogenic Hh signaling in keratinocytes giving rise to increased pigmentation? One possible explanation, given the critical role of canonical Wnt signaling in melanogenesis, is that heightened Hh signaling may be

indirectly stimulating melanogenesis and pigment accumulation by stimulating Wnt ligand expression and activating canonical Wnt signaling.

A recent paper revealed a novel surprising genoprotective role of tumor suppressor p53 in the regulation of the suntan response (Cui et al., 2007). In response to ultraviolet-induced genotoxic stress, p53 becomes activated in skin keratinocytes and stimulates transcription from the pro-opiomelanocortin (POMC) gene promoter. The POMC precursor polypeptide is then processed into several bioactive products including alpha-melanocyte stimulating hormone (α-MSH), which through a paracrine effect on epidermal melanocytes (mediated by the α-MSH receptor melanocortin-1 receptor (MC1R) and MITF), leads to melanin production and redistribution among skin cells (Cui et al., 2007). Intriguingly, these investigators also found that only those human BCCs that retain wild-type p53 are pigmented, while BCCs harboring p53 mutations are not pigmented. If this is true, then would the same finding apply to those human BCCs that feature foci of hyper-pigmentation within tumor nests without pigment accumulation? Furthermore, would ectopic pigment accumulation in M2SMO expressing skin or Gli2-driven BCCs also require normal p53 function? This could be tested with crosses of M2SMO- or Gli2- over-expressing mice onto a $p53^{-/-}$ background.

In this chapter, I describe the preliminary data that show ectopic Hh activation in mouse skin using the *keratin* 5 (K5) promoter results in a striking accumulation of grossly visible pigment, and furthermore, this increase in pigment is accompanied by a striking increase in melanoblast and/or melanocyte number as assessed using *Dct-lacZ* reporter mice, and expression of multiple transcripts encoding melanocyte markers. In addition, gene expression for several secreted factors previously implicated in

melanogenesis are also upregulated. Inhibition of canonical Wnt signaling by co-overexpression of the potent Wnt inhibitor Dkk1 in M2SMO skin blocked pigment accumulation (Chapter 2 and Fig. 2-11b), suggesting that non cell-autonomous effects of Hh-activated keratinocytes on the melanocyte lineage are dependent on canonical Wnt signals. The work described here provides the foundation for future studies exploring the molecular mechanism of aberrant melanogenesis in the setting of deregulated Hh signaling in skin and other pathological conditions.

Materials and Methods

Generation of transgenic mice

Generation of transgenic mice with constitutive expression of M2SMO in skin (*ΔK5-M2SMO*) mice has been described (Grachtchouk et al., 2003). Generation of transgenic melanoblast reporter mice by inserting a *lacZ* transgene under the control of *Trp2* promoter (*Dct-lacZ*) has been described (Mackenzie et al., 1997). *Dct-lacZ* mice were crossed with *ΔK5-M2SMO* mice to generate *Dct-lacZ;ΔK5-M2SMO* bitransgenic mice. All mice were housed and maintained according to University of Michigan institutional guidelines, as stipulated by the University Committee on the Use and Care of Animals.

Tissue harvesting, wholemount preparation and wholemount X-gal staining

For hematoxylin and eosin (H&E) staining, mouse skin was fixed in neutral-buffered formalin (NBF) overnight, transferred to 70% EtOH, processed, and embedded

in paraffin. To prepare wholemounts for mouse volar skin, mice were euthanized and ventral hindlimb skin was removed. Volar skin was microdissected and wholemount preparation was performed essentially as described previously for tail skin (Braun et al., 2003). For wholemount X-gal staining, separated epidermal and dermal sheets were fixed in fresh 4% paraformaldehyde/PBS (pH 7.0-7.5) for 1 hour at 4 °C. Following 3 washes, for 15 minutes each in wash buffer (100 mM sodium phosphate (pH 7.3), 2 mM MgCl2, 0.01% sodium deoxycholate, and 0.02% NP-40), tissues were stained in freshly prepared staining solution (wash buffer plus 5mM potassium ferricyanide, 5mM potassium ferocyanide, and 1 mg/mL X-gal solution from 25 mg/mL stock in dimethylformamide stored at -20 °C) for overnight at 37 °C. Tissues were post-fixed overnight in NBF and stored in 70% EtOH. Transilluminated wholemount photomicrographs were captured with digital camera (Spot RT3.0, Diagnostic Instruments) mounted on dissecting microscope (Leica MZFL3), using Spot Software Version 4.6 (Diagnostic Instruments).

Semiquantitative RT-PCR

Volar and postnatal day 8 dorsal skin were microdissected under dissecting microscope (Nikon, Japan), homogenized in Trizol (Invitrogen), and stored at -80 °C until further processing. RNA isolation, first strand cDNA synthesis, and RT-PCR were performed essentially as described previously (Allen et al., 2003).

Results

Heightened Hh signaling in epidermis stimulates melanogenesis

'Inappropriate' pigment accumulation has been reported in human BCCs, tumors that are driven by aberrant Hh signaling (Bleehen, 1975). Similarly, mouse BCCs and other skin tumors driven by Gli2 or Gli2ΔN2 overexpression also contain abundant pigment (Grachtchouk et al., 2000; Sheng et al., 2002; Hutchin et al., 2005). Skin from M2SMO-expressing transgenic mice also consistently exhibited increased pigmentation compared to control skin [(Figure 5-1), and (Grachtchouk et al., 2003)]. Dramatic ectopic pigmentation accumulation was easily appreciated in transilluminated wholemount M2SMO volar skin compared to control (Figure 5-1a,b), and also in H&E-stained sections (Figure 5-1c,d). H&E sections revealed the vast majority of ectopic pigment accumulation had occurred in the dermis of M2SMO volar skin (Figure 5-1d).

To determine whether this phenotype was associated with an increased number of melanoblasts and/or melanocytes rather than just grossly increased accumulation of melanin, we crossed M2SMO mice with *Dct-lacZ* reporter mice (Mackenzie et al., 1997). *Dct-lacZ* mice are useful reporters since dopachrome tautomerase (Dct), also called tyrosinase-related protein 2 (Trp2), is a reliable marker of the melanocytic lineage, expressed in melanocyte progenitors, melanoblasts residing in adult skin, and terminally differentiated melanocytes (Tsukamoto et al., 1992; Bouchard et al., 1994; Mackenzie et al., 1997; Virador et al., 2001). Following dermal-epidermal separation and wholemount examination, epidermis from X-gal stained *Dct-lacZ;M2SMO* volar skin exhibited numerous β-galactosidase expressing cells compared to *Dct-lacZ* volar skin, where almost no positive cells were visible (Figure 5-2a,b). Surprisingly, although the majority of X-gal positive cells in *Dct-lacZ;M2SMO* skin were in the epidermis, most of the

increased pigment was again detected in the dermis (Figure 5-2b,d,f). Sections of wholemount stained *Dct-lacZ;M2SMO* volar skin showed numerous X-gal positive cells scattered among the suprabasal epithelial cells in hamartomas (Figure 5-2f), but not among the basal cell layer where they are normally distributed in regions of epidermis containing melanocytes. This overall distribution pattern of melanocytic cells is reminiscent of what has been reported during late-stage development in embryonic hair-bearing skin (Nishimura et al., 1999), in keeping with the notion that follicular hamartomas represent an expansion of primitive, follicle-like epithelium. Semiquantitative RT-PCR of volar skin from M2SMO-expressing mice revealed induction of multiple melanocyte lineage markers, including *Mitf*, a major lineage determinant which activates expression of several genes involved in melanogenesis (Levy et al., 2006). These genes include *Tyr*, *Tyrp1*, *Dct/Tyrp2*, *Pmel17*, and *Ped*, none of which are detected in control volar skin (Figure 5-2g). In addition, multiple transcripts of secreted factors involved in melanogenesis, including *Endothelin 1 (ET1), Endothelin 3 (ET3), kit ligand/stem cell factor (SCF), and basic fibroblast growth factor (b-FGF)* were also upregulated in early bud-stage as well as late hamartoma-stage M2SMO volar skin (Figure 5-2g). mRNA levels for *SCF* appeared to be expressed relatively higher in hamartoma-stage compared to bud-stage M2SMO volar skin, although quantitative PCR studies are needed to confirm this impression. If true, it would suggest that SCF is unique among the genes implicated in melanogenesis that we examined, since it is preferentially upregulated during M2SMO-driven hamartomas development and growth.

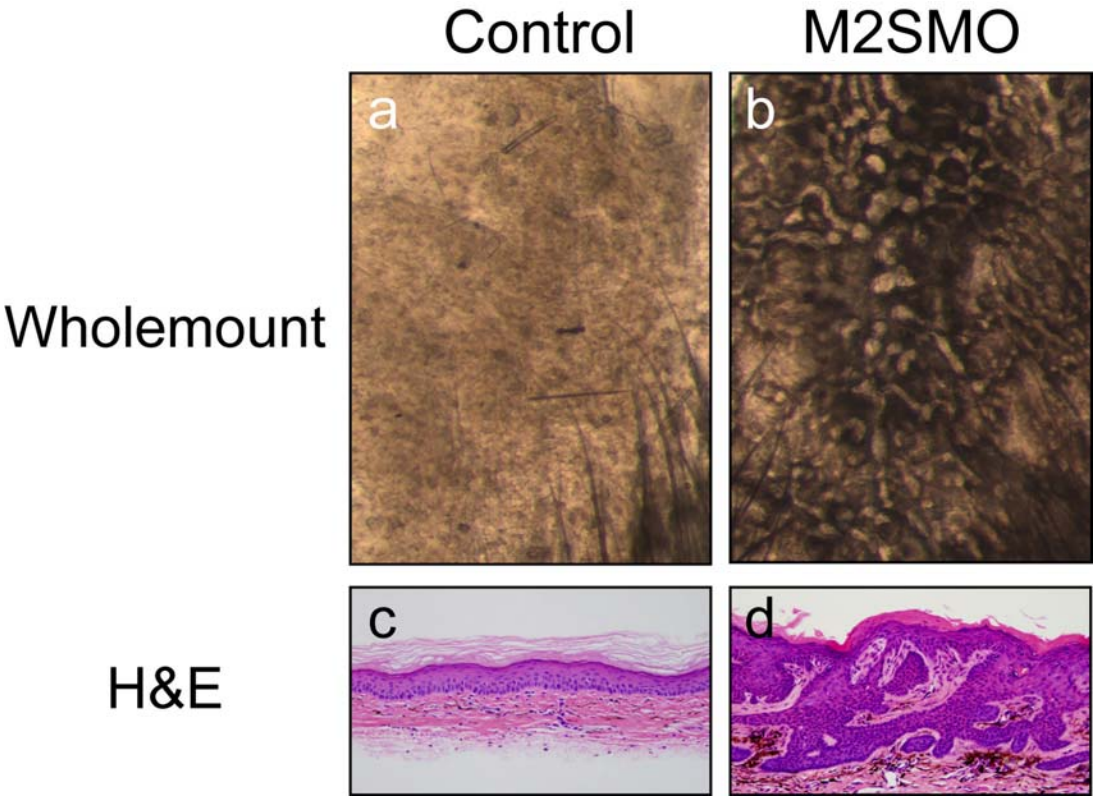

Figure 5-1. Increased pigment accumulation in M2SMO hairless volar skin. (**a**) Transilluminated wholemount image of control hairless volar skin at postnatal day 30. Hair shafts on lower right corner are from the normal adjacent hair bearing region on the ventral hindlimb. (**b**) Transilluminated wholemount image of M2SMO hairless volar skin at postnatal day 30. Note extensive hamartoma development and associated pigment accumulation, featured by disorganized cords and bands of epithelial thickening and dark pigmentation surrounding them. (**c**) H&E-stained section of control volar skin. Note the absence of hair follicles or follicle-associated structures, and minimal pigment accumulation in the dermis. (**d**) H&E-stained section of M2SMO volar skin. Note extensive hamartoma development and accompanying pigment accumulation in the dermis.

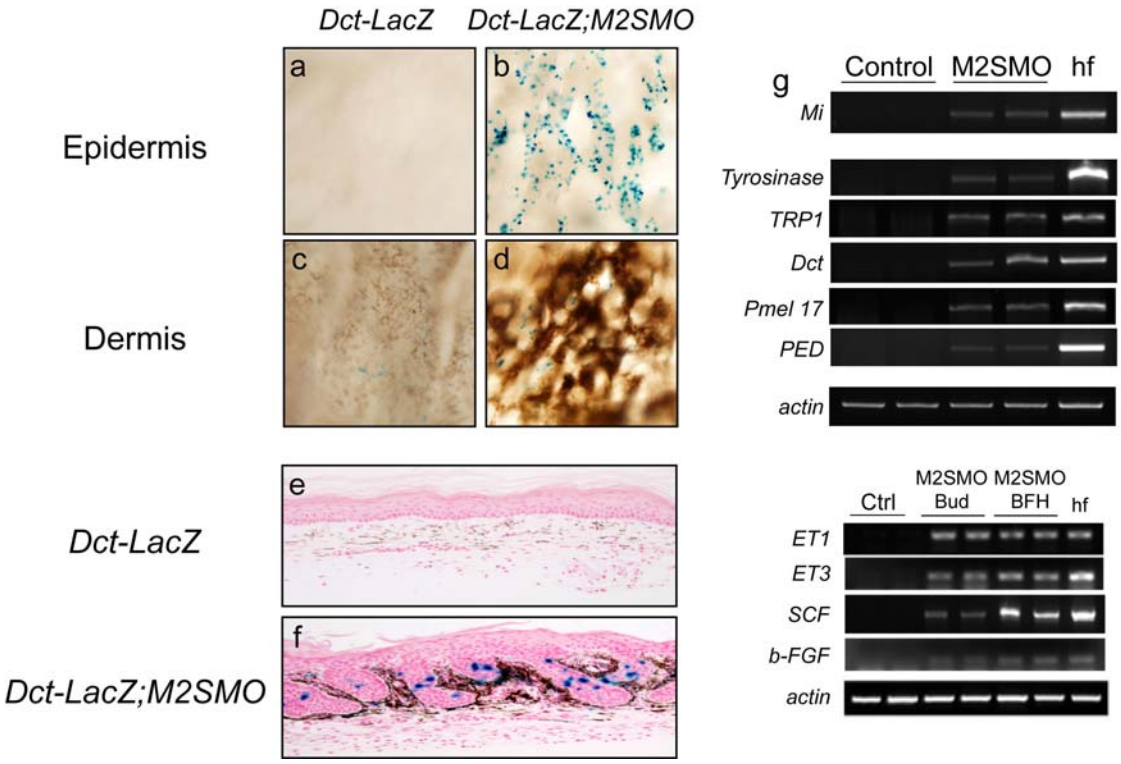

Figure 5-2. Increased pigmentation in M2SMO skin: Hedgehog signaling induces increased melanogenesis. (**a,b**) Wholemount X-gal stained epidermis of *Dct-LacZ* and *Dct-LacZ;M2SMO* volar skin. Numerous X-gal positive cells are visible in *Dct-LacZ;M2SMO* volar skin. (**c,d**) Wholemount X-gal stained dermis of *Dct-LacZ* and *Dct-LacZ;M2SMO* volar skin. Dark pigmentation in dermis is visible in *Dct-LacZ;M2SMO* skin. (**e,f**) X-gal stained sections with Nuclear Fast Red counterstain of *Dct-LacZ* and *Dct-LacZ;M2SMO* volar skin. Note X-gal positive cells in M2SMO-induced hamartomas that are scattered throughout the hyperplastic epithelium but not dermis, while no X-gal positive cells are visible in *Dct-LacZ* volar skin. (**g**) Semiquantitative RT-PCR was performed on RNA isolated from control volar skin, M2SMO volar skin and positive control (postnatal day 8 back skin, hf). Transcripts for multiple melanocytic markers are upregulated in M2SMO volar skin, including *Mi*, *Tyrosinase*, *TRP1*, *Dct*, *Pmel 17*, and *PED*. Transcripts for genes involved in melanogenesis, including *Endothelin 1 (ET1), Endothelin 3 (ET3), kit ligand/stem cell factor (SCF), and basic fibroblast growth factor (b-FGF)* are also upregulated in both early bud-stage (M2SMO Bud) and late hamartoma-stage (M2SMO BFH) skin.

Summary

M2SMO-expressing skin consistently exhibited increased pigmentation compared to control skin, as previously reported in other mouse models with elevated Hh signaling in skin and in a subset of human BCCs (Bleehen, 1975). The majority of accumulated pigment was localized in the dermis of M2SMO-expressing skin. M2SMO mice were then crossed with *Dct-lacZ* reporter mice (Mackenzie et al., 1997) to determine whether increased pigment accumulation was associated with an increased number of melanoblasts and/or melanocytes. Wholemount X-gal staining revealed an increased number of β-galactosidase expressing cells in the epidermis of *Dct-lacZ;M2SMO* volar skin compared to *Dct-lacZ* volar skin alone, supporting the notion that pigment accumulation in M2SMO mice is associated with an increase in the number of melanoblasts and/or melanocytes. While melanocytes are normally found in the basal layer of the epidermis (Yamaguchi et al., 2007a), X-gal positive cells in the epidermis of *Dct-lacZ;M2SMO* volar skin were scattered among the suprabasal epithelial cells in hamartomas.

It will be important to determine in the future the molecular mechanism for increased number of melanocytes in M2SMO-expressing skin. How does heightened Hh signaling stimulate unscheduled melanogenesis in postnatal skin? Is it possible that epithelial Hh activation leads to the activation of *Dct-lacZ* negative melanocyte stem cells in the epidermis or dermis of volar skin? Alternatively, circulating melanocyte progenitors may also be recruited and migrate in. Immunostaining with antibodies specific for MITF and Trp2 in the volar skin of doxycycline-inducible *K5-rtTA;TRE-*

SmoA1 bitransgenic animals before and after SmoA1 expression will be necessary to address this issue.

Melanogenesis is regulated through the concerted action of multiple secreted signaling molecules, including Wnts [reviewed in (Lin and Fisher, 2007)]. Specifically, *Wnt3a* leads to increased numbers of neural crest-derived melanocyte progenitors as well as terminally-differentiated melanocytes (Takeda et al., 2000; Dunn et al., 2005), raising the possibility that the elevated expression of Wnt3a and/or other Wnts is contributing to the increased number of Dct-positive cells in M2SMO-expressing skin. Other secreted factors involved in melanogenesis in various settings include ET1, ET3, HGF, b-FGF, SCF, and α-MSH, derived from the *Proopiomelanocortin* (*Pomc*) transcript (Diamond et al., 2000; Chu et al., 2004; Weidinger et al., 2005; Cui et al., 2007). Remarkably, semiquantitative RT-PCR revealed induction of *SCF*, *ET1*, and *ET3*, but not *HGF* or *Pomc* in volar skin of M2SMO-expressing mice (Figure 5-2g, and data not shown). Of these, only *SCF* appeared to be expressed at higher levels in skin containing hamartomas than in skin from younger mice containing epithelial buds (Figure 5-2g). Thus multiple secreted factors that are coordinately induced may work in concert to bring about the dramatic increase in pigmentation seen in M2SMO-expressing skin.

In addition to inhibiting epithelial changes associated with deregulated Hh signaling in skin, blockade of Wnt signaling in M2SMO+Dkk1 mice also grossly suppressed skin hyperpigmentation (Figure 2-13). These *in vivo* results suggest that non cell-autonomous effects of M2SMO-expressing epithelium on the melanocyte lineage are also likely to be mediated by canonical Wnt signals, but additional studies will be needed

to determine whether this reflects a direct effect of Wnt ligand(s) on melanocytes, or a secreted secondary factor(s).

Although my data implicate epithelial Hh signaling in ectopic melanogenesis in M2SMO-expressing mice and BCC, a recent paper by Cui et al. reported a central role for epithelial p53 function, via production of Pomc mRNA and MSH expression (Cui et al., 2007), in the suntan response of melanocytes to ultraviolet light. Moreover, data were presented in this report also implicating normal p53 function in pathological pigmentation in human BCCs, since human BCCs that are pigmented retain wild-type p53 while those BCCs harboring p53 mutations are not pigmented (Cui et al., 2007). Further studies are needed to directly test whether p53 function is implicated for melanogenesis associated with Hh pathway-driven epithelial tumorigenesis in M2SMO-expressing mice.

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Chapter VI

Summary and Future Directions

Hh→Wnt crosstalk in Hh-driven skin pathology

The Hh and canonical Wnt signaling pathways play essential regulatory roles in both development and adult life, and when deregulated, contribute to the tumorigenesis of many tissues and organs. In skin, Wnt signals are necessary and sufficient for the initial formation of hair buds (Gat et al., 1998; Huelsken et al., 2001; Andl et al., 2002; Lo Celso et al., 2004), and in the differentiation of committed epithelial progenitors to form late-stage hair follicle lineages to give rise to the seven distinct concentric layers of terminally differentiating cells in the mature hair follicle (DasGupta and Fuchs, 1999). In addition, Wnt signals provide the initial trigger to reactivate follicle growth during the regenerative phase of postnatal hair cycles (Van Mater et al., 2003; Huelsken et al., 2001; Lo Celso et al., 2004). Uncontrolled activation of canonical Wnt signaling, however, promotes the development of several hair follicle-derived tumors such as pilomatricoma and trichofolliculoma (Gat et al., 1998; Chan et al., 1999; Lo Celso et al., 2004). In skin, Hh signaling is essential for the proliferation of follicle epithelium needed to assemble a mature hair follicle during embryogenesis and postnatal hair cycling (St Jacques et al., 1998; Chiang et al., 1999; Wang et al., 2000). Physiologic Hh signaling in skin is tightly regulated, and deregulated, aberrant activation of Hh signaling results in the development

of skin tumors such as BCC and basaloid follicular hamartoma (Grachtchouk et al., 2003; ya-Grosjean and Couve-Privat, 2005).

BCC is the most common type of cancer in light-skinned individuals with more than a million new cases in the US each year, and although it rarely metastasizes, BCC can cause significant morbidity due to local invasion (Tang et al., 2007). BCC is classified histologically according to morphology, and includes nodular, micronodular, infiltrative and superficial forms (Sexton et al., 1990). In superficial BCC, epithelial buds of proliferating basal cells grow down from the epidermis into the superficial dermis, maintaining their attachment to the basal cell layer. Intriguingly, pathologists have long noted the morphological similarity between focal epidermal downgrowths in BCC and embryonic hair germs (Montgomery, 1935; Lever, 1948; Kumakiri and Hashimoto, 1978). However, the molecular explanation for this morphological similarity between two structures driven by two distinct signaling pathways has been lacking. This observation raised the possibility that pathologic Hh signaling can A) directly stimulate epidermis to form *de novo* hair bud-like structures and skin neoplasms; or B) stimulate Wnt signaling to indirectly bring about these pathologic responses to deregulated Hh signaling in skin. While some prior studies have identified coordinate changes in the Hh and canonical Wnt pathways in BCCs and other neoplasms (Pasca di et al., 2007; Taipale and Beachy, 2001; Mullor et al., 2001; Yamazaki et al., 2001; El-Bahrawy et al., 2003; Saldanha et al., 2004; Li et al., 2007), direct evidence establishing the functional significance of Wnt signaling in Hh pathway-driven pathology *in vivo* was lacking.

The primary goal of my thesis project was to gain further molecular insights into the functions of the Hh and Wnt embryonic signaling pathways in cell fate decisions and

cancer, using the skin as a model system. The hair follicle is one of few organs that undergoes cyclic transformations for its entire life, from periods of regeneration and rapid growth (anagen), where key aspects of its embryonic development are recapitulated, to periods of massive regression via apoptotic mechanisms (catagen), followed by periods of rest and quiescence (telogen) [reviewed in (Stenn and Paus, 2001; Paus and Cotsarelis, 1999) and (Figure 1-2)]. These unique and remarkable features of the hair follicle, in addition to its relative abundance and easy accessibility, make it an attractive model system to study many fundamental biological processes.

Based on the morphological similarity between Hh-driven ectopic epithelial buds and embryonic hair buds, I hypothesized that the cross-talk between Hh and Wnt pathways plays important roles during Hh-driven skin tumorigenesis. Using transgenic mice that express an oncogene *M2SMO* (Xie et al., 1998) as a model system, I tested whether pathological Hh signaling in skin leads to the formation of hair-bud like structures indirectly, via canonical Wnt signaling, the physiological stimulus for hair bud formation in embryos. I showed upregulation of multiple canonical Wnt genes, nuclear and cytoplasmic redistribution of β-catenin, and upregulation of endogenous Wnt target genes *Axin2* and *Sp5* in M2SMO-expressing mouse skin. Moreover, using a doxycycline-regulated transgenic mouse expressing the canonical Wnt inhibitor Dkk1, I showed that ectopic bud formation and expansion to form follicular hamartomas require canonical Wnt signaling activity. My findings 1) uncover a previously unknown requirement for ligand-driven, canonical Wnt signaling for Hh-driven tumorigenesis; 2) provide a molecular explanation for the morphological and biochemical similarity between early BCC and embryonic hair germs; 3) explain how 'follicular' tumors can

arise from interfollicular epidermis; and 4) identify Wnt ligands as a novel pharmacological target for prevention and/or treatment of a subset of Hh-driven neoplasms in skin.

My findings also challenge the pre-existing dogma that canonical Wnt signaling always precedes, and is required for, subsequent Hh activation in skin (Gat et al., 1998; Huelsken et al., 2001; Andl et al., 2002; Lo Celso et al., 2004). Our data indicate that this temporal relationship is reversed in the setting of Hh-driven pathology in epidermis, where ectopic activation of Hh signaling leads to canonical Wnt signaling with resultant formation of *de novo* epithelial buds and follicular hamartomas. In light of our findings in the setting of pathologically-activated Hh signaling in skin, it will be interesting to determine whether the Hh pathway contributes to Wnt signaling under physiological conditions, as has been proposed in other organs [e.g., (Hu et al., 2005; Madison et al., 2005)]. Interactions between the Hh and canonical Wnt pathways have also been proposed in pancreatic cancer (Pasca di et al., 2007) and cigarette smoke-induced lung cancer (Manfredi et al., 2004). Given the results presented herein, it will be important to test the functional role of canonical Wnt signaling in other tumors and cancers that are driven by aberrant Hh signaling.

Semiquantitative RT-PCR of M2SMO-expressing skin revealed upregulation of multiple Wnt ligand transcripts, including *Wnt3, 4,5a, 7b, 10a,* and *10b*. Mullor et al. also reported expression of multiple Wnts in *Xenopus* animal cap explants injected with Gli2 and Gli3 mRNA, as well as upregulation of multiple Wnt ligands in human BCC (Mullor et al., 2001). These observations raise several interesting questions: What is the biological significance of upregulation of multiple Wnt ligands? Is every induced Wnt

ligand playing a different role, or is a coordinated multi-Wnt ligand induction required to mediate Hh-induced responses? What are the temporal and spatial (epithelial versus mesenchymal) expression patterns of induced Wnt ligands in M2SMO-expressing skin? Does Hh activation trigger coordinate expression of multiple Wnt ligands, or are some Wnt ligands turned on secondarily, and if so, which Wnt ligands are turned on first? To address these questions, one could employ doxycycline-inducible *K5-rtTA;TRE-SmoA1* mice, which readily develop ectopic epithelial buds following postnatal activation of SmoA1 expression (data not shown).. Volar skin from doxycycline-induced *K5-rtTA;TRE-SmoA1* mice can be collected at multiple time points, from uninduced, and 12, 24, 48, 72 hours after doxycyline induction. A comprehensive panel of RT-PCR and *in situ* hybridization studies would reveal the temporal and spatial expression of Wnt ligand genes induced by SmoA1. A strong advantage of working with the volar skin system in this setting should be emphasized. Since the volar skin can be considered a morphogenetically naive "clean slate" that normally does not contain any hair follicles or other skin appendages, it provides a sensitive and convenient tool to detect changes in morphology or gene expression. Interestingly, it has been shown that relatively higher expression of endogenous Dkk1 secreted by dermal fibroblasts in human palmoplantar skin gives it the relatively hypopigmented phenotype as well as increased thickness compared to other regions of the body (Yamaguchi et al., 2004, Yamaguchi et al., 2008), suggesting that site-specific differential expression of secreted factors could explain the phenotypes observed in different regions of the skin. Furthermore, follicle density during embryogenesis is governed by competition between placode-stimulating (epidermis-restricting) and placode-restricting (epidermis-stimulating) factors [reviewed in (Fuchs

2007)]. Given these results, it is therefore also conceivable that the volar skin could be in an 'inhibited state' where placode-restricting signals, such as BMP signaling (Mou et al., 2006, Kobielak et al., 2003, Andl et al., 2004), or epidermal growth factor receptor (EGFR) signaling (Atit et al., 2003) are preferentially expressed to actively suppress hair follicle morphogenesis in this region. Conversely, it is also possible that the volar skin lacks the expression of BMP-inhibitor noggin (Botchkarev et al., 1999) or the FGF10/FGF7 receptor FGFRIIIb (Petiot et al., 2003) that are needed for hair follicle development and growth. These results may suggest that the volar skin could be an 'active' site for multiple signaling pathway interactions, rather than a strictly morphogenetically naive "clean slate" epidermis, and the morphological and gene expression changes brought about by the Hh activation in this region of skin may be also influenced by interactions with these additional signaling pathways that are known to play a role in hair follicle morphogenesis.

While the RT-PCR and *in situ* experiments outlined above will reveal the temporal and spatial expression patterns of Wnt ligands induced by Hh activation, these studies will not necessarily determine which Wnt ligand(s) among those induced by SmoA1 is the functionally critical player in activating the downstream canonical Wnt pathway machinery to initiate Hh-induced ectopic epithelial bud development. Answering this question would likely require a functional assay to block each Wnt ligand induced by SmoA1, assuming that the biological effects mediated by the induced Wnt ligands are driven solely by a single ligand, not via concerted efforts among multiple Wnt ligands working together. However, given my results that showed that a global Wnt inhibition using the Wnt inhibitor Dkk1 resulted in a dramatic blockade of M2SMO-

induced phenotype, the lack of this knowledge may not preclude the pre-clinical use of a global Wnt inhibitor (such as a decoy Wnt ligand receptor that will bind to multiple Wnt ligands) to test for inhibition of Hh-induced tumor development, at least initially. Future studies that will delineate the exact molecular mechanism of which Wnt ligands are activated upon Hh activation may allow for a much more selective and advanced drug designs that will target the specific key Wnt ligands to block Hh-induced tumorigenesis.

It will be also important to determine the exact molecular mechanisms of how activated Hh signaling induces the expression of Wnt ligands. Could the activated Gli transcription factors be binding directly onto the promoter regions of genes encoding various Wnt ligands, or could there be a yet-to-be identified secondary signal that mediates Hh-induced Wnt ligand expression? Several strategies could be employed to answer this question, one of which is via Chromatin Immunoprecipitation (ChIP) assay to determine the potential direct Gli-Wnt ligand promoter binding interactions. In an alternative approach, one could also start by screening for canonical Gli binding sites on different Wnt ligand promoters, and performing reporter assays with wild-type versus mutant promoter constructs co-transfected with SmoA1 or Gli expression plasmids.

It will also be interesting to determine whether M2SMO-driven ectopic epithelial bud development is accompanied by the formation of 'new' follicle stem cells. It has been proposed that Hh signaling promotes proliferation directly on stem cell populations during tumorigenesis (Altaba et al., 2002). Furthermore, expression of stabilized mutant β-catenin in postnatal skin was sufficient to induce *de novo* hair follicles that contained clonogenic keratinocytes that express bulge-specific stem cell markers such as CD34 and K15 (Silva-Vargas et al., 2005), and I have found that βcat*-induced *de novo* follicles

that arose from hairless volar skin also expressed the stem cell marker K15 (data not shown). These results, along with the finding that M2SMO induces canonical Wnt and β-catenin activation, might suggest that M2SMO-driven ectopic bud development may lead to establishment of a follicle stem cell niche. However, functional assays will be required to more rigorously assess the stem cell potential of cells from M2SMO-induced epithelial buds and hamartomas.

In M2SMO+Dkk1 skin, M2SMO is expressed in the epithelium only and results in cell-autonomous activation of Hh signaling. In contrast, Dkk1 expression is likely to influence both the epithelium and mesenchyme, since Dkk1 is a secreted molecule (Bafico et al., 2001; Mao et al., 2001). Since a precise temporal control of Dkk1 expression in *ΔK5-M2SMO;K5-rtTA;TRE-Dkk1* triple transgenic mice can be achieved using doxycycline, a number of additional experiments could be performed to address several interesting questions. Hutchin et al. found that while shutting off the *Gli2* transgene in established BCCs in *K5-tTA;TRE-Gli2* mice induced regression of these tumors, the regressed tumors never completely disappeared, and grew back often at an accelerated rate once *Gli2* transgene expression was restored (Hutchin et al., 2005). In the M2SMO+Dkk1 system, if Dkk1 expression is induced early on to inhibit M2SMO-induced bud and hamartoma development, and then terminated after a period of time, will the M2SMO-induced tumors appear? If hamartomas arise after removal of Dkk1 expression, this finding would have a therapeutic implication and suggest that a therapeutic blockade of canonical Wnt signaling may be required indefinitely to inhibit the growth of Hh-induced hamartomas.

On the other hand, what would happen to established Hh-driven hamartomas if the canonical Wnt signaling is shut off once they are already formed? Would inhibition of canonical Wnt signaling induce regression of the hamartomas, and if so, what will be the mechanism? Or will the hamartomas merely stop growing and expanding, but yet still persist and not regress? This question may be addressed by inducing Dkk1 expression in *ΔK5-M2SMO;K5-rtTA;TRE-Dkk1* mice at postnatal day 30 or later, when the M2SMO-induced hamartomas have been well-established in skin. Based on my results that show that only the outer-most peripheral cells of established hamartomas show nuclear and cytoplasmic β-catenin localization, as well as Ki67 and Cyclin D1 expression, I expect that the induction of Dkk1 in established hamartomas would not make the hamartomas regress, but rather stop them from expanding further by shutting off the canonical Wnt signaling in the outer-most cells of hamartomas. This issue also has therapeutic implications, since a potential canonical Wnt-inhibiting drug to block Hh-driven hamartoma development in humans would have to be initiated extremely early. Since hamartomas and skin cancers in humans are typically not detected or diagnosed until they are grossly visible, this may pose a challenge for practical applicability of Wnt-inhibiting drug to treat Hh-induced skin lesions.

Constitutive Hh signaling also underlies BCC development (ya-Grosjean and Couve-Privat, 2005), and several earlier reports have described links between the Hh and Wnt pathways in BCC, including upregulation of multiple Wnt genes (Mullor et al., 2001) and localization of β-catenin to the cytoplasm and/or nucleus in human BCCs (Yamazaki et al., 2001; El-Bahrawy et al., 2003; Saldanha et al., 2004). In addition, we have observed nuclear and cytoplasmic β-catenin localization, expression of stabilized/

unphosphorylated β-catenin, and upregulation of endogenous Wnt target gene *Axin2* in Gli2-driven mouse BCCs (data not shown), raising the possibility that canonical Wnt signaling may also be required for the development and/or expansion of full-blown BCC. To test this hypothesis we have performed experiments in which Dkk1 expression is induced in mice that develop nodular, BCC-like skin tumors mostly in dorsal skin, but Dkk1 was not effective in blocking canonical Wnt signaling in this setting (data not shown).

An alternative approach to test the functional role of canonical Wnt signaling in Hh-driven BCC development and maintenance may be to generate and utilize a transgenic mouse model which expresses a soluble Wnt decoy receptor comprising the Frizzled8 cysteine-rich domain (CRD) fused to the human Fc domain (F8CRDhFc) (DeAlmeida et al., 2007). The potent anti-tumor efficacy of F8CRDhFc has been shown using the mouse mammary tumor virus Wnt1 tumor model. F8CRDhFc seem to be capable of binding to and inhibiting functions of multiple Wnt ligands (personal communication). *In vitro* experiments using F8CRDhFc showed a robust inhibition of autocrine Wnt signaling in teratoma cell lines PA-1, NTera-2, Tera-2, and NCCIT, and systemic administration of F8CRDhFc significantly inhibited the growth of tumor xenografts derived from PA-1 and NTera-2 cell lines (DeAlmeida et al., 2007). Once transgenic mice that conditionally express F8CRDhFc in skin are generated, they can then be crossed with BCC-producing mice to test the role of canonical Wnt/β-catenin signaling in BCC initiation, expansion, and maintenance. The use of F8CRDhFc, which blocks the canonical Wnt signaling by directly binding to the Wnt ligands, may be a better approach to examine the functional role of canonical Wnt signaling in BCC

development and maintenance than with *K5-rtTA;TRE-Dkk1* model, since there may be a relative deficiency of Kremens 1/2 (Mao et al., 2002), which facilitate Dkk1's ability to block canonical Wnt signaling, or Dkk1 transgene may be insufficiently expressed in dorsal skin. In addition, transgenic F8CRDhFc-expressing mice will also be useful to cross with M2SMO-expressing mice to corroborate our M2SMO+Dkk1 data, since Dkk1 blocks canonical Wnt signaling at the level downstream of Wnt ligand expression (Niehrs, 2006), and the direct binding of Wnt ligands by F8CRDhFc would also be expected to be effective in blocking M2SMO-induced phenotype in *ΔK5-M2SMO* mice.

To independently test and confirm the functional role of canonical Wnt/β-catenin signaling in M2SMO-induced ectopic bud and hamartoma development, a substantial amount of effort was also dedicated to breed M2SMO-expressing mice onto β-catenin knockout background by crossing *ΔK5-M2SMO* mice with Cre-inducible conditional β-catenin knockout mice (Brault et al., 2001) and *Msx2-Cre* mice (Sun et al., 2000), which drives a patchy Cre-recombination in skin during embryogenesis. Unfortunately, no conclusive data could be achieved from *ΔK5-M2SMO;Msx2-Cre;β-catenin*$^{-/-}$ mice experiments, as the newborn *ΔK5-M2SMO;Msx2-Cre;β-catenin*$^{-/-}$ mice analyzed showed no phenotypic differences compared to *ΔK5-M2SMO* mice . *ΔK5-M2SMO;Msx2-Cre;β-catenin*$^{-/-}$ mice were born with severely deformed limbs (data not shown) and often did not survive for more than 24 hours, as the *Msx2* promoter is also active in the apical ectodermal ridge (Sun et al., 2000). This forced us to examine newborn mice or E19.5 embryos, but this complicated our analysis further since the skin phenotype in *ΔK5-M2SMO* mice is not readily apparent until a few days after birth (personal observation and (Grachtchouk et al., 2003)). In addition, generating M2SMO-expressing mice with

skin-specific deletion of β-catenin required quadruple-alleled transgenic mice with the probability of obtaining the correct genotype in only 1 out of every 16 offspring.

β-catenin drives advanced stages of hair follicle differentiation

Although M2SMO-induced epithelial buds expanded to form hamartomas, they failed to differentiate beyond a rudimentary stage of development, containing only two of the seven cell lineages seen in mature follicles. This may be due at least in part to the fact that they failed to produce mesenchymal condensates and papillae required for proper follicle development. The lack of condensates near ectopic buds was observed in dorsal (hairy) skin as well as hairless volar skin, arguing against the possibility that volar mesenchyme is selectively deficient in responsiveness to form this cell population.

In volar skin from M2SMO mice, βcat* induced the rapid appearance of late-stage follicle lineages and rudimentary hair shafts within hamartomas, showing that these undifferentiated tumors retain the potential to undergo advanced stages of terminal differentiation when supplied with an appropriate stimulus. Since β-catenin immunostaining yields an intense signal in skin of M2SMO+βcat* mice, relatively compared to M2SMO mice, it is possible that high-level β-catenin activity in βcat*-expressing mice is required for activation of hair lineage markers. Thus, the level of Wnt signaling in M2SMO mice may be sufficient to initiate epithelial bud development, but inadequate to stimulate terminal differentiation of hair lineages.

We also examined the effects of postnatal βcat* activation in otherwise normal volar epidermis in adult mice. Within 5 days, βcat*-expressing mice developed multiple

de novo skin appendages, some of which appeared to be relatively normal-appearing follicles with sebaceous glands and hair shafts. Our findings underscore the plasticity of adult skin even from a region that is normally hairless, and although other investigators had not reported follicle development in hairless skin following Wnt pathway activation (Gat et al., 1998; Lo Celso et al., 2004), the consequences of expressing an activated β-catenin mutant from its endogenous promoter in adult mice has not yet been described.

Analysis of transgenic mice that express Shh in skin (*K14-Shh*) revealed that early BCC-like lesions showed morphological evidence of dermal papilla and positive alkaline phosphatase activity, dermal papilla-specific marker (Oro et al., 1997). This finding suggests that Hh activation at the level of Shh ligand is sufficient to induce the formation of BCC-like lesions with associated dermal condensates. Recently, Cre-inducible Shh-overexpressing mice were developed in the Dlugosz laboratory, and I generated *K5-CreERT2;Cre-inducible Shh* bitransgenic mice to test whether overexpression of Shh in postnatal skin would result in the induction of epithelial buds with associated dermal condensates. Epithelium-specific Shh overexpression in hairless volar skin led to the development of *de novo* epithelial buds, and Shh-induced *de novo* buds expressed early hair bud-lineage markers Sox9, K17, and CDP (data not shown). However, these buds did not show morphological evidence of associated dermal papilla, suggesting that induction of Shh expression in postnatal skin is unable to induce the formation of dermal papillae (data not shown).

Epithelial Hh signaling reactivates growth of resting hair follicles

Adenoviral delivery of Shh (Sato et al., 1999), as well as topical treatment with Hh-agonist (Paladini et al., 2005) in mouse skin is sufficient to trigger growth of telogen follicles in mice. However, in both settings, multiple cell types including keratinocytes, fibroblasts, and mesenchymal cells including dermal papilla cells were subject to Hh pathway activation, and it is not clear whether activation of follicle growth was due to direct stimulation of quiescent bulge stem cells, or a consequence of stimulating additional cell types which could then indirectly influence the stem cell compartment through a secondary signal. Utilizing novel mouse models that allow precise spatial and temporal control of Hh signaling in skin, I tested whether epithelium-specific activation of Hh signaling is sufficient to trigger new follicle growth in telogen skin. The results with *K5-rtTA;TRE-SmoA1* bitransgenic mice showed that epithelium-specific Hh activation is sufficient to induce new follicle growth in telogen skin. Next, we also tested if direct Hh activation in the follicle stem cells is sufficient to trigger new follicle growth. To address this question, I took advantage of *K15-CrePR1* mice, which allows for selective targeting of transgene expression to stem cells within the bulge compartment of hair follicles in post-natal mice (Liu et al., 2003; Morris et al., 2004). By generating Cre- and doxycycline- inducible *K15-CrePR1;R26X-rtTA+GFP;TRE-SmoA1* triple transgenic mice, I was able to specifically activate Hh signaling in the stem cell niche and test whether bulge-specific Hh activation is sufficient to induce new follicle growth. My results showed that the activation of Hh signaling specifically in the stem cell compartment did not result in induction of anagen, suggesting that direct Hh activation on hair follicle stem cells with SmoA1 is not sufficient to initiate new follicle formation. Similar to the follicles in doxycycline-treated *K5-rtTA;TRE-SmoA1* bitransgenic mice,

expansion of outer root sheath cells was also observed in *K15-CrePR1;R26X-rtTA+GFP;TRE-SmoA1* follicles that were depilated and treated with doxycycline, suggesting that SmoA1 expression had an effect on the progeny of activated stem cells in the bulge, including transient amplifying cells that give rise to all lineages of the hair follicle.

Given the availability of Hh reporter mice such as *Gli1-lacZ* mice (Bai et al., 2002) that allow a precise *in vivo* visualization of Hh activation in skin, we can also examine which cell types in the skin are responding to Hh-agonist treatment to bring about the new follicle growth. Treatment of the Hh-agonist on *Gli1-lacZ* mice and examining the X-gal stained skin at several early time points (0, 6, 12, 24, 48, 72 hours post Hh-agonist treatment) will delineate the cells types that are responding to the Hh-agonist to induce new follicle growth.

A topical application of a synthetic Hh agonist on mouse skin is sufficient to trigger new growth in telogen follicles (Paladini et al., 2005), but Hh pathway transcriptional activation did not occur for 3-4 days after topical Hh agonist treatment. This result raises the possibility that Hh agonist treatment itself is not directly triggering new follicle growth but rather the Hh agonist may be inducing anagen by stimulating a particular cell type in the skin that in turn activates another signaling pathway, such as canonical Wnt signaling pathway. This hypothesis sounds plausible, given 1) my previous data that suggest that Hh-driven ectopic epithelial bud development occurs indirectly by activation of canonical Wnt signaling pathway; 2) detection of intense nuclear and cytoplasmic β-catenin (indication of canonical Wnt activation) in the follicles 3 days after SmoA1 induction in *K5-rtTA;TRE-SmoA1* mice; and 3) previous studies that

support the notion that canonical Wnt plays a key role in triggering new follicle growth (Van Mater et al., 2003; Lo Celso et al., 2004). However, Hh agonist-treated *K5-rtTA;TRE-Dkk1* bitransgenic mice still resulted in the induction of new follicle growth despite Dkk1 expression (data not shown). Interestingly, depilation-induced anagen was also not blocked in doxycycline-induced *K5-rtTA;TRE-Dkk1* bitransgenic mice, indicating that an alternative model to block canonical Wnt signaling in dorsal skin may be required. Furthermore, given that Dkk1 expression was not effective in blocking M2SMO-induced bud and hamartoma phenotype in dorsal skin (Chapter II), it is plausible that *K5-rtTA;TRE-Dkk1* is not an ideal system to test the functional role of canonical Wnt signaling in Hh-induced hair follicle growth. Another conditional mouse model to block the canonical Wnt pathway, such as one that expresses soluble Wnt decoy receptor F8CRDhFc (DeAlmeida et al., 2007), may be a better system to address this hypothesis.

Pathologic Hh signaling and melanogenesis

M2SMO-expressing skin consistently exhibited increased pigmentation compared to control skin, as previously reported in other mouse models with elevated Hh signaling in skin and in a subset of human BCCs (Bleehen, 1975). Wholemount X-gal staining revealed an increased number of β-galactosidase expressing cells in the epidermis of *Dct-lacZ;M2SMO* volar skin compared to *Dct-lacZ* volar skin alone, supporting the notion that pigment accumulation in M2SMO mice is associated with concurrent increase in the number of melanocytes and/or melanocyte progenitors. In addition, semiquantitative RT-PCR revealed induction of multiple melanocyte markers and multiple genes implicated in

melanogenesis, including *SCF*, *ET1*, and *ET3*, but not *HGF* or *Pomc,* in volar skin of M2SMO-expressing mice. Of these, only *SCF* appeared to be expressed at higher levels in skin containing hamartomas than in skin from younger mice containing epithelial buds (Figure 5-2g). Thus multiple secreted factors that are coordinately induced may work in concert to bring about the dramatic increase in pigmentation seen in M2SMO-expressing skin.

In addition to inhibiting epithelial changes associated with deregulated Hh signaling in skin, blockade of Wnt signaling in M2SMO+Dkk1 mice also grossly suppressed skin hyperpigmentation (Figure 2-13). These *in vivo* results suggest that non cell-autonomous effects of M2SMO-expressing epithelium on the melanocyte lineage are also likely to be mediated by canonical Wnt signals, working either directly on melanocytes, or indirectly through secondary signals such as Scf.

Interestingly, a recent paper reported a surprising role of tumor suppressor p53 in mediating the suntan responses to ultraviolet light, and reported that human BCCs that are pigmented retain wild-type p53, while those BCCs harboring p53 mutations are not pigmented (Cui et al., 2007). To functionally test whether Hh-induced melanogenesis may occur in the absence of p53 using genetic mouse models, M2SMO-expressing mice may be bred onto p53 knockout background and the skin examined for the status of ectopic pigment accumulation.

Taken together, the key findings presented herein raise the possibility that blockade of canonical Wnt/β-catenin signaling may be a useful strategy for treatment of at least some neoplasms currently considered to be caused by uncontrolled Hh signaling. Because deregulated Hh signaling impacts on β-catenin signaling primarily at the level of

Wnt ligands, the range of potential therapeutic strategies is considerably greater than it is for colorectal and other cancers with mutational defects in APC or β-catenin, and would likely include antibodies or other recombinant proteins that antagonize the interaction of Wnt ligands with Frizzled and LRP receptors. Future work will better clarify the utility of targeting proximal Wnt pathway components for the prevention or treatment of Hh-dependent neoplasms and other disorders. By examining the complex interactions between Hh and Wnt pathways during pathogenesis of skin tumors driven by ectopic Hh signaling, my thesis work uncovers a novel requirement for canonical Wnt signaling downstream of pathologic Hh signaling in skin, and opens exciting new avenues for similar investigations in other organs and tissues during normal development and pathology.

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