HHS Public Access

Author manuscript

J Invest Dermatol. Author manuscript; available in PMC 2016 March 01.

Published in final edited form as:

J Invest Dermatol. 2015 September ; 135(9): 2162–2172. doi:10.1038/jid.2015.147.

Roles of *GasderminA3* in catagen- telogen transition during hair cycling

Xiufeng Bai[1], **Mingxing Lei**[2,3,4,*], **Jiazhong Shi**[1], **Yu Yu**[1], **Weiming Qiu**[1], **Xiangdong Lai**[2,3], **Yingxin Liu**[1], **Tian Yang**[1], **Li Yang**[2,3], **Randall Bruce Widelitz**[4], **Cheng-Ming Chuong**[4], and **Xiaohua Lian**[1,*]

[1]Department of Cell Biology, the Third Military Medical University, Chongqing 400038, People's Republic of China

[2]Key Laboratory of Biorheological Science and Technology, Ministry of Education, Bioengineering College, Chongqing University, Chongqing 400044, China

[3]'111' Project Laboratory of Biomechanics and Tissue Repair, Bioengineering College, Chongqing University, Chongqing 400044, China

[4]Department of Pathology, University of Southern California, Los Angeles, CA 90033, USA

Abstract

Hair follicles undergo cyclic behavior through regression (catagen), rest (telogen) and regeneration (anagen) during postnatal life. The hair cycle transition is strictly regulated by the autonomous and extrinsic molecular environment. However, whether there is a switch controlling catagen-telogen transition remains largely unknown. Here we show that hair follicles cycle from catagen to the next anagen without transitioning through a morphologically typical telogen after *Gsdma3* mutation. This leaves an ESLS (epithelial strand-like structure) during the time period corresponding to telogen phase in WT mice. Molecularly, *Wnt10b* is upregulated in *Gsdma3* mutant mice. Restoration of *Gsdma3* expression in AE (alopecia and excoriation) mouse skin rescues hair follicle telogen entry and significantly decreases the *Wnt10b*-mediated Wnt/β-catenin signaling pathway. Overexpression of *Wnt10b* inhibits telogen entry by increasing epithelial strand cell proliferation. Subsequently, hair follicles with a *Gsdma3* mutation enter the second anagen simultaneously as WT mice. Hair follicles cannot enter the second anagen with ectopic WT *Gsdma3* overexpression. A luciferase reporter assay proves *Gsdma3* directly suppresses Wnt signaling. Our findings suggest *Gsdma3* plays an important role in catagen-telogen transition by balancing the Wnt signaling pathway, and that morphologically typical telogen is not essential for the initiation of a new hair cycle.

Correspondence: Xiaohua Lian, Department of Cell Biology, the Third Military Medical University, Chongqing 400038, China. 129hx@sina.com; Mingxing Lei, Key Laboratory of Biorheological Science and Technology, Ministry of Education, Bioengineering College, Chongqing University, Chongqing 400044, China. lmxgoodluck@163.com.

CONFLICT OF INTEREST

The authors state no conflict of interest.

INTRODUCTION

Hair cycle activity is dependent on interactions of cyclic micro- and macro-environmental signals, showing dynamic morphologies at different cyclic phases (Muller-Rover *et al.*, 2001). At catagen phase, apoptotic signals from the hair matrix region, induce apoptosis in the whole hair bulb and hair shaft, leaving an epithelial strand structure, characteristic of the mid-catagen phase. As the epithelial strand retracts via apoptosis, the dermal papilla (DP) is pulled toward the base of the bulge where hair stem cells reside. Hair follicles then enter telogen phase, with the newly formed second hair germ (SHG) bridging the DP and bulge (Greco *et al.*, 2009). After a quiescent period, the hair follicle reenters the next anagen phase. During these transitions, the hair stem cell-derived fast-cycling progeny home back to the niche while the slow-cycling progeny contribute to the next hair cycle (Hsu *et al.*, 2011).

Although catagen-telogen transition occurs very rapidly from its initiation, development through termination, it is strictly regulated by signaling involving DKK1 (Kwack *et al.*, 2012), EGF (Philip *et al.*, 1985), HGF (Lindner *et al.*, 2011), IGF-I receptor (Rudman *et al.*, 1997), TGF-β1 (Foitzik *et al.*, 2000), TNF-a (Ruckert *et al.*, 2000), etc. On the other hand, balanced interactions between Wnt/β-catenin and BMP signaling pathways help to drive hair follicles from telogen to anagen (Kobielak *et al.*, 2007; Plikus *et al.*, 2008). Previous studies show that the Wnt/β-catenin signaling pathway crucially maintains hair follicle homeostasis (Andl *et al.*, 2004; Ito *et al.*, 2007; Stenn, 2001). During embryonic skin development, Wnt/β-catenin signaling directs the skin epithelium to differentiate towards the hair follicle lineage (Fu *et al.*, 2011; Huelsken *et al.*, 2001; Lei *et al.*, 2013b; Millar, 2002). In the first postnatal anagen, Wnt/β-catenin signaling stimulates hair follicle stem cell activation and differentiation (Blanpain and Fuchs, 2009). In addition, FGF7 and FGF18 are reported to be involved in telogen-anagen transition (Greco *et al.*, 2009; Kimura-Ueki *et al.*, 2012).

A number of questions remain to be further understood. Does Wnt/β-catenin signaling play an essential role in catagen progression? How is the duration of the catagen phase regulated? How is the initiation and termination of telogen regulated? Is a typical telogen required for second anagen reentry?

The *Gsdma3* gene is located on mouse chromosome 11 and is a key member of the gasdermin gene family (Lunny *et al.*, 2005; Runkel *et al.*, 2004; Saeki *et al.*, 2000). *Gsdma3* is expressed in mouse skin keratinocytes (Runkel *et al.*, 2004; Tanaka *et al.*, 2013; Tanaka *et al.*, 2007) and takes part in regulating skin differentiation and inflammation (Lunny *et al.*, 2005; Runkel *et al.*, 2004; Saeki *et al.*, 2000). Eight *Gsdma3* mutations were reported to cause alopecia (Kumar *et al.*, 2012; Lei *et al.*, 2011; Li *et al.*, 2010; Lunny *et al.*, 2005; Runkel *et al.*, 2004; Saeki *et al.*, 2000; Tanaka *et al.*, 2013; Zhou *et al.*, 2012b). Mice with a *Gsdma3* mutation demonstrate alopecia and excoriation and are referred to as AE mice (Li et al., 2010; Zhou et al., 2012). These mutant mice also display hair cycle disorders with an especially prolonged catagen phase (Ruge *et al.*, 2011) which could be due to reduced skin keratinocyte apoptosis as shown in our previous studies (Lei *et al.*, 2012; Lei *et al.*, 2011). However, the molecular mechanisms are poorly understood. Later, we revealed that β-catenin was significantly elevated in AE mice (Lei *et al.*, 2013a). Since β-catenin is a key

factor in the Wnt pathway, it is rational to speculate that *Gsdma3* may interact with the Wnt signaling pathway.

To further explore this, in the current study we monitored morphological and molecular changes of hair follicles across two hair cycles in AE mice and dissected key steps during hair regression and regeneration. *Wnt10b* was increased at catagen in AE mice, leading to high levels of epithelial strand cell proliferation. We noted that hair follicles didn't enter a morphologically typical telogen but initiated the second anagen directly from the first catagen phase, indicating that telogen is not required for transition to anagen. We reveal that *Gsdma3* is a key modulator inhibiting the Wnt/β-catenin signaling pathway.

RESULTS

Gsdma3 is expressed in cycling hair follicles

Immunostaining shows nuclear Gsdma3 was expressed at all stages of hair follicles (Figure 1a). At anagen, Gsdma3 is widely expressed in the hair matrix, elevated in the inner root sheath (IRS) and reduced in the outer root sheath (ORS) region. When hair follicles enter catagen, Gsdma3 is strongly expressed at the epithelial strand, and weakly at the upper bulge region. At P24 (telogen), Gsdma3 is mainly expressed in the SHG region but expands throughout the distal hair follicle as the new anagen starts at P29.

Histological Analysis of the hair cycle in AE Mice

To investigate the role of *Gsdma3* in hair follicle cycling, we monitored morphological changes in hair follicles from the first anagen to the second anagen of WT and AE mice (Figure 1b and Supplementary Figure 1a). H&E staining shows a delayed catagen phase to at least P24 in mutant mice. However, at P29 (Figure 1b–c), when WT mouse hair follicles start to initiate the second anagen, AE mouse hair follicles remain in early catagen phase, with a long ESLS between the bulge and DP. WT hair follicles need 6 days to regenerate to the second mid-anagen phase at P35. Surprisingly, AE mouse hair follicles are also morphologically analogous to the second anagen phase at P35. Versican immunostaining and Alkaline Phosphatase staining shows that the DP of AE mouse hair follicles is normal compared with those in WT hair follicles (Supplementary Figure 2a–b). Besides, DAPI staining shows that AE mouse vibrissae have normal morphology during cycling (Supplementary Figure 1c). These observations indicate that dorsum hair follicles of AE mice transit from a delayed catagen to the second anagen without a morphologically typical telogen (Figure 1d and Supplementary Figure 1b).

Upregulated Wnt signaling in AE mice

To evaluate the molecular mechanisms underlying these phenotypes, we first used PCR to screen and compare gene expression in WT and AE mouse skin. Interestingly, among multiple Wnt ligands, *Wnt4*, *Wnt7b* and *Wnt10b* are dramatically upregulated during the catagen-telogen-anagen transition (Figure 2a). *Wnt10b* is significantly increased from P17 in AE mouse skin, and *Wnt7b* is increased from P24. Some Wnt-related genes including receptors (*Frizzled4* and *Lrp6)*, key effectors (β-*catenin* and *Lef1*) and downstream targets *(Cyclin D1)* are also significantly enhanced from P9 to P35 in AE mice (Figure 2b–d).

Conversely, many Wnt inhibitors (*Catnbip1, Sfrp4, Sfrp5, Shisa3*) are significantly decreased at P17 (Supplementary Figure 3a), and *Axin2, BMP2* and *BMP4* are significantly decreased at P24 in AE mouse skin (Figure 2e). Not all Wnt-related genes were influenced by the *Gsdma3* mutation (Supplementary Figure 3a–d). A summary diagram compares gene expression profiles from the first anagen to the second anagen during hair cycling between WT and AE mice (Supplementary Figure 4).

Of the candidate Wnt ligands, *Wnt7b* is already known to affect the hair follicle cycle (Kandyba and Kobielak, 2014; Kandyba *et al.*, 2013) and *Wnt10b* is upregulated more than *Wnt4* in AE mice. We chose to test the role of Wnt10b in hair cycling. Immunostaining confirmed that at P17 representing catagen phase of WT mice, Wnt10b and Lef1 are still highly expressed in the matrix region of AE mouse hair follicles (Figure 2f and Supplementary Figure 5a). Further, fluorescent immunostaining shows that β-catenin colocalizes with nuclear *Gsdma3* in the hair matrix region of P17 AE mouse hair follicles (White arrows in Figure 2g). In addition, BrdU+ cells are significantly increased in the hair bulb and shaft region of AE mouse hair follicles (Supplementary Figure 5a). While TUNEL staining (Supplementary Figure 5b–c), P53 and Fas immunostaining (Supplementary Figure 5d) show apoptosis is significantly decreased in AE mouse hair follicles during catagen phase. In summary, these results suggest Wnt signaling is increased and apoptosis was decreased after *Gsdma3* mutation.

Gsdma3 overexpression in AE mice promotes hair follicle entry into telogen

No typical telogen phase is observed in AE mouse skin. Ectopically expressing WT *Gsdma3* (AdGsdma3) by subcutaneous injections at P18 and P20 (Figure 3a–b and Supplementary Figure 6a) diminishes the ESLS visualized by H&E staining at P24 and restores the WT phenotype. This indicates *Gsdma3* is required for catagen-telogen transition. Interestingly, Wnt10b, β-catenin and Lef1 are significantly decreased both at mRNA and protein levels and the number of BrdU+ cells is reduced after *Gsdma3* overexpression (Figure 3c–e). Ectopically expressing a mutant *Gsdma3* form by subcutaneously injecting AdmGsdma3 into AE mouse skin at P18 and P20, blocks hair follicles from reaching a complete telogen at P24 (Supplementary Figure 6c), and mRNA levels of *Wnt10b* are unchanged compared to controls (Supplementary Figure 6d). These data indicate that *Gsdma3* inhibits Wnt10b/β-catenin signaling during catagen-telogen transition.

Increased Wnt10b inhibits catagen-telogen transition

To verify whether increased *Wnt10b* can prolong catagen phase, *Wnt10b* was overexpressed in early and middle catagen of WT mice (Supplementary Figure 6b). H&E staining shows that hair follicles in the AdWnt10b-treated group still maintain catagen phase at P23, while WT hair follicles already entered telogen phase (Figure 3f). After Wnt10b overexpression, immunostaining shows Wnt10b and β-catenin are significantly enhanced and the number of BrdU+ cells is increased compared to the AdGFP-treated group (Supplementary Figure 6e). These results further demonstrate that increased *Wnt10b* expression could disturb telogen entry.

Anagen reentry is not influenced by the *Gsdma3* mutation

To better understand how hair follicles transit from the first catagen to the second anagen in AE mice, we traced hair cycle progression using pigmentation to assess the onset of anagen III within the dorsal skin (Supplementary Figure 7a). A conversion of pink telogen to black anagen occurs both in WT and AE mouse skin, suggesting anagen reentry is not affected by the *Gsdma3* mutation. Surprisingly, WT hair follicles have a SHG between the bulge and DP but AE hair follicles maintain their ESLS representing mid-catagen phase at P29 (Figure 4a). Intriguingly, both WT and AE hair follicles gradually enter anagen III by P32. Whereas the proximal WT hair follicle envelops the DP to form the hair bulb, at anagen III, the ESLS continues to exist between the bulge and hair bulb of AE hair follicles at P30 and P32 and the hair follicles enter mid-anagen by P34. These results confirm that hair follicles enter anagen after a delayed but prolonged catagen phase without transitioning through a morphologically typical telogen phase.

At the molecular level, we first examined whether the ESLS were composed of differentiated IRS cells. Immunostaining shows AE15 positive cells are absent at P29 and only begin to appear at P32, which suggests the ESLS are not derived from IRS cells (Supplementary Figure 7c). We find that K15, the hair stem cell marker, is also present in the ESLS of AE mouse hair follicles in addition to its bulge region, suggesting similarities of ESLS cells to bulge stem cells. To further track how AE mouse hair follicles regenerate, we compared their cell proliferation pattern with the established pattern found in WT mice during hair regeneration (Figure 4b). In P29 AE mice, BrdU+ cells mainly localize to the ESLS region (Figure 4b), and their number gradually decreases from P29 to P34 (Figure 4b–c). A BrdU+ cell group emerges above the DP at P30, then gradually increases in the hair follicle bulb region (Figure 4b and d). The K15+ cells in the AE mouse bulge region also have a higher proliferation rate than in those of WT mice (Figure 4b and e). Interestingly, P-cadherin as a SHG marker is expressed in many ESLS cells at P24 and becomes confined to a group of cells above the DP at P29 in AE hair follicles (Supplementary Figure 7b).

During anagen reentry, β-catenin is first activated at the SHG in WT hair follicles (Figure 4f). However, in AE mice, many sites of nuclear β-catenin coincide with BrdU+ cells and are significantly increased in the ESLS region at P29 (Figure 4f), which then gradually become confined to the matrix region of regenerating hair follicles (Figure 4f and h). The ESLS, hair matrix and DP regions show stronger Wnt10b and Lef1 expression in AE mouse compared to WT mouse hair follicles (Supplementary Figure 7d–e). These results further demonstrate that the Wnt10b/β-catenin pathway is elevated after *Gsdma3* mutation.

Ectopic *Gsdma3* expression blocks hair regeneration by repressing Wnt/β-catenin signaling

We further investigated the relation between *Gsdma3* and Wnt signaling during hair regeneration (Supplementary Figure 8a). When we inject both AdWnt10b + AdRFP or only AdWnt10b into the refractory telogen of WT mice, hair follicles could reenter anagen phase. However, when we inject AdWnt10b + AdGsdma3 or AdRFP, the hair follicle couldn't enter anagen (Figure 5a). PCR, western blot and immunostaining show in AdWnt10b + AdRFP or only AdWnt10b-treated groups, that Wnt10b, β-catenin, and Lef1 are

significantly increased compared to the AdWnt10b + AdGsdma3 or AdRFP-treated groups (Figure 5b–d and Supplementary Figure 8b).

We employed a depilation-induced hair cycling model (Muller-Rover *et al.*, 2001) to prove that Gsdma3 could inhibit Wnt signaling (Supplementary Figure 8d). Both mRNA and protein levels of Wnt signaling are significantly decreased in the AdGsdma3-treated group compared to the AdGFP-treated group (Figure 5f and Supplementary Figure 8e–h). Sox9 and BrdU double staining show no stem cell activation (Figure 5f). BrdU+ and Ki67+ cells are dramatically decreased (Figure f–h and Supplementary Figure 8c).

To further confirm that *Gsdma3* inhibits the Wnt signaling pathway, we detected Wnt pathway activation using the TOP/FOP luciferase reporter assay in pEGFP-*Gsdma3* plasmid transfected mouse keratinocytes. Transfected cells show high GFP intensity (Figure 5i), dramatically higher *Gsdma3* expression (Figure 5j–k and Supplementary Figure 8i–j) and display significantly decreased luciferase activity compared to the control group (Figure 5l), although a promoter reporter assay shows *Wnt10b* promoter activity was not changed after *Gsdma3* overexpression (Supplementary Figure 8k). These results convincingly confirm *Gsdma3* could inhibit the Wnt/β-catenin signaling pathway.

Discussion

In the C57BL/6J mouse strain, the hair cycle is highly synchronized, showing 3 days of catagen and 9 days of telogen in the first hair cycle (Muller-Rover *et al.*, 2001). However, whether these phases are required for the hair cycle and what controls the cycle transition remains incompletely understood. In this study, we show that hair follicles transit from catagen to the second anagen without a morphologically typical telogen phase after *Gsdma3* mutation. Specifically, we demonstrate that *Gsdma3* represses *Wnt10b*-induced Wnt signaling to balance these transitions.

Morphologically, AE mouse hair follicles show an ESLS, indicative of catagen phase extending through the time of telogen phase in WT mice. This allows us to assess whether catagen regression and telogen formation are required for hair cycling. Immunostaining shows this ESLS is K15 positive. Under normal conditions, K15 is expressed in the bulge and SHG regions at telogen phase (Liu *et al.*, 2003), indicating these cells might have arisen from SHG cells. However, the normal SHG appears at late catagen or early telogen and doesn’t proliferate until the next anagen initiates (Greco *et al.*, 2009), but the cell population observed in AE mice remain highly proliferative during the catagen-anagen transition. Nevertheless, at P29, cells adjacent to the DP region become intensely proliferative, which suggests that they might arise from induced SHG cells.

How do AE mice form ESLS during catagen? We previously reported that ectopic *Wnt10b* could promote stem cell migration from the bulge area, but not induce their differentiation into ORS cells (Lei *et al.*, 2014). Here, we show that *Wnt10b* is significantly increased in hair follicles after *Gsdma3* mutation which might promote bulge stem cell migration and maintain their progenitor fate. This may also explain why bulge stem cells are significantly decreased in AE mouse hair follicles (Zhou *et al.*, 2012a). *Wnt10b* also promotes cell

proliferation (Lei *et al.*, 2014; Ouji *et al.*, 2012). The enhanced *CyclinD1* expression and increased ESLS cell proliferation seen here are likely due to increased *Wnt10b* expression, which coincides with nuclear β-catenin expression. Moreover, the decreased apoptotic signals in AE mouse hair follicles (Lei *et al.*, 2011) indicate those ESLS cells might be *Wnt10b*-enhanced proliferative cells. In addition to the ESLS cells, we observe some bulge stem cells in AE mice are still proliferating, indicating they are active during catagen-anagen transition, probably due to Wnt pathway activation. This might cause depletion of the stem cells necessary to support ESLS during aging, which is consistent with previous studies (Zhou *et al.*, 2012b). Thus we believe *Wnt10b*-induced Wnt signaling could play an important role(s) in catagen-telogen transition.

The second hair cycle initiates at similar times in wild type and mutant mice. Interestingly, *Gsdma3* mutant mice continue to display catagen morphology even at the onset of second anagen. How do hair follicles enter the second anagen phase after *Gsdma3* mutation? How do they know when anagen should start? Under normal conditions, two pathways participate in guiding hair follicles from telogen to anagen. One central pathway is Wnt signaling, which has emerged as the dominant pathway controlling the periodic initiation of hair follicles (Gat *et al.*, 1998; Huelsken *et al.*, 2001; Kratochwil *et al.*, 1996; Lowry *et al.*, 2005; Silva-Vargas *et al.*, 2005; van Genderen *et al.*, 1994; Van Mater *et al.*, 2003; Zhang *et al.*, 2008; Zhou *et al.*, 1995). Our previous studies showed overexpression of *Wnt10b* in telogen resulted in anagen onset (Lei *et al.*, 2014; Li *et al.*, 2013). Equally important is BMP signaling, which cycles out of phase with Wnt signaling. Waves of BMP expression in the dermis divide telogen into refractory (High BMP) and competent (Low BMP) phases (Kandyba *et al.*, 2013; Kobielak *et al.*, 2007; Plikus *et al.*, 2008). Our current work demonstrates that Wnt10b, β-catenin and Lef1, are all increased at the start of the second anagen in WT mice; Meanwhile, *BMP4* is decreased at P24 and *BMP2* is decreased at P29 in *Gsdma3* mutant mice. Other secreted Wnt inhibitors including *IGFBP4* and *Sfrp5* are also decreased in AE mice at P29. Therefore, it is tempting to speculate that high activators and low inhibitors in the micro- and microenvironments may force the catagen follicles to reenter the next anagen phase directly (Figure 6).

How does the ESLS progress in AE mice during catagen-anagen transition? At P34 when hair follicles enter mid-anagen phase, AE mouse ORS cells are still highly K15 positive, suggesting that not all of these cells have adopted ORS or hair matrix cell fates. To untangle the relative contributions of ESLS versus bulge cells to the prospective ORS and hair bulb cells, live imaging of cell motility is required (Deschene *et al.*, 2014; Rompolas *et al.*, 2013).

What is the relation between *Gsdma3* and Wnt signaling? In the present study, restoring *Gsdma3* expression in AE mouse catagen skin diminishes Wnt signaling activity and hair follicles regress to telogen. Also, AdWnt10b-mediated Wnt signaling activation in WT mouse catagen follicles confirm that increased Wnt signaling leads to telogen deletion. In addition, when *Gsdma3* is overexpressed in *Wnt10b*-increased skin, hair follicles can’t enter anagen. These results demonstrate *Gsdma3* inhibits Wnt signaling during hair cycle transitions. Moreover, our luciferase reporter assay further confirms the direct inhibitory effect between *Gsdma3* and Wnt signaling.

Bioinformatics analysis reveals that *Gsdma3* contains three coiled coil domains with a leucine zipper zone, which was predicted to function as a transcription factor (Lunny *et al.*, 2005; Saeki *et al.*, 2000). Accordingly, we show that *Gsdma3* is localized to the nucleus in hair follicles. The results from our functional study make it tempting to speculate that *Gsdma3* may regulate the Wnt signaling pathway. Suppressing Wnt pathway molecule mRNA levels by *Gsdma3* overexpression further supports this prediction. However, our promoter reporter assay shows overexpression of Gsdma3 didn't change Wnt10b promoter activity. Where Gsdma3 directly targets the Wnt signaling pathway needs further study.

In conclusion, our findings demonstrate that hair follicles do not have to go through telogen in order to transition to a new anagen. Normal catagen termination requires *Gsdma3*-induced apoptosis and decreased *Wnt* signaling. Therefore, *Gsdma3* inhibits the Wnt/β-catenin pathway and promotes the catagen-telogen transition (Figure 6).

Materials & Methods

Mice

AE mice (gift from Dr. Gao Xiang, Nanjing University, China)(Zhou *et al.*, 2012b) and C57BL/6 mice were used for these studies. ENU mutagenesis of C57BL/6 mice produced the AE mouse. Genome sequencing revealed that nucleotide 1112 of Gsdma3 gene was replaced (T-C) in the mutant mouse. All experiments were conducted in accordance with the guidelines for studies with laboratory animals of the Third Military Medical University Ethics Committee.

Adenoviruses and plasmids

pEGFP-N1 and pEGFP-*Gsdma3* plasmids were constructed as previously described (Lei *et al.*, 2012). Adenoviruses including AdGFP (control), AdRFP (Adenovirus-induced red fluorescent protein, control), AdWnt10b, AdGsdma3 and AdmGsdma3 (Adenovirus-induced expression of the AE mouse mutant form of *Gsdma3*) were generated and propagated according to previous studies (Luo *et al.*, 2007).

Histology analysis and immnofluorescence

Primary antibodies used for immnofluorescence include β-catenin (1:200; Beyotime, Nantong, China), anti-K15 (1:100, Sangon Biotech, Shanghai, China), BrdU (1:500; Abcam, Cambridge, UK), Wnt10b (1:100; Santa Cruz, Dallas, USA), Lef1 (1:100; Santa Cruz, Dallas, USA), Ki67 (1:100; Santa Cruz, Dallas, USA), and anti-Sox9 (1:100; Santa Cruz, Dallas, USA). Gsdma3 antibody was collaboratively (1:100, GL Biochem, Shanghai, China) made against a synthesized Gsdma3 peptide antigen (231-TFPKIRRVPCSA-242). Secondary antibodies were Cy3 (1:200, Beyotime, Nantong, China) or Alexa Fluor 488 (1:500, Invitrogen, Carlsbad, USA) conjugated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This study was supported by grant 81171515 from the National Nature Science Foundation of China. CMC and RBW are supported by US NIH grant AR 42177 and AR 60306.

Abbreviations

AE	alopecia and excoriation
ES	epithelial strand
ESLS	Epithelial strand-like structure
Gsdma3	gasdermin A3
IRS	inner root sheath
ORS	outer root sheath
SHG	secondary hair germ

REFERENCE

Andl T, Ahn K, Kairo A, et al. Epithelial Bmpr1a regulates differentiation and proliferation in postnatal hair follicles and is essential for tooth development. Development. 2004; 131:2257–2268. [PubMed: 15102710]

Blanpain C, Fuchs E. Epidermal homeostasis: a balancing act of stem cells in the skin. Nature Reviews Molecular Cell Biology. 2009; 10:207–217. [PubMed: 19209183]

Deschene ER, Myung P, Rompolas P, et al. beta-Catenin activation regulates tissue growth non-cell autonomously in the hair stem cell niche. Science. 2014; 343:1353–1356. [PubMed: 24653033]

Foitzik K, Lindner G, Mueller-Roever S, et al. Control of murine hair follicle regression (catagen) by TGF-beta1 in vivo. FASEB J. 2000; 14:752–760. [PubMed: 10744631]

Fu J, Ivy Yu H-M, Maruyama T, et al. Gpr177/mouse Wntless is essential for Wnt-mediated craniofacial and brain development. Developmental Dynamics. 2011; 240:365–371. [PubMed: 21246653]

Gat U, DasGupta R, Degenstein L, et al. De Novo hair follicle morphogenesis and hair tumors in mice expressing a truncated beta-catenin in skin. Cell. 1998; 95:605–614. [PubMed: 9845363]

Greco V, Chen T, Rendl M, et al. A Two-Step Mechanism for Stem Cell Activation during Hair Regeneration. Cell Stem Cell. 2009; 4:155–169. [PubMed: 19200804]

Hsu Y-C, Pasolli HA, Fuchs E. Dynamics between Stem Cells, Niche, and Progeny in the Hair Follicle. Cell. 2011; 144:92–105. [PubMed: 21215372]

Huelsken J, Vogel R, Erdmann B, et al. beta-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. Cell. 2001; 105:533–545. [PubMed: 11371349]

Ito M, Yang Z, Andl T, et al. Wnt-dependent de novo hair follicle regeneration in adult mouse skin after wounding. Nature. 2007; 447:316–320. [PubMed: 17507982]

Kandyba E, Kobielak K. Wnt7b is an important intrinsic regulator of hair follicle stem cell homeostasis and hair follicle cycling. Stem Cells. 2014; 32:886–901. [PubMed: 24222445]

Kandyba E, Leung Y, Chen YB, et al. Competitive balance of intrabulge BMP/Wnt signaling reveals a robust gene network ruling stem cell homeostasis and cyclic activation. Proceedings of the National Academy of Sciences of the United States of America. 2013; 110:1351–1356. [PubMed: 23292934]

Kimura-Ueki M, Oda Y, Oki J, et al. Hair Cycle Resting Phase Is Regulated by Cyclic Epithelial FGF18 Signaling. J Invest Dermatol. 2012; 132:1338–1345. [PubMed: 22297635]

Kobielak K, Stokes N, de la Cruz J, et al. Loss of a quiescent niche but not follicle stem cells in the absence of bone morphogenetic protein signaling. Proc Natl Acad Sci U S A. 2007; 104:10063–10068. [PubMed: 17553962]

Kratochwil K, Dull M, Farinas I, et al. Lef1 expression is activated by BMP-4 and regulates inductive tissue interactions in tooth and hair development. Genes & Development. 1996; 10:1382–1394. [PubMed: 8647435]

Kumar S, Rathkolb B, Budde BS, et al. Gsdma3(I359N) is a novel ENU-induced mutant mouse line for studying the function of Gasdermin A3 in the hair follicle and epidermis. J Dermatol Sci. 2012; 67:190–192. [PubMed: 22682752]

Kwack MH, Kim MK, Kim JC, et al. Dickkopf 1 Promotes Regression of Hair Follicles. J Invest Dermatol. 2012; 132:1554–1560. [PubMed: 22358062]

Lei M, Bai X, Yang T, et al. Gsdma3 is a new factor needed for TNF-alpha-mediated apoptosis signal pathway in mouse skin keratinocytes. Histochem Cell Biol. 2012; 138:385–396. [PubMed: 22585037]

Lei M, Gao X, Yang L, et al. Gsdma3 gene is needed for the induction of apoptosis-driven catagen during mouse hair follicle cycle. Histochem Cell Biol. 2011; 136:335–343. [PubMed: 21805153]

Lei M, Guo H, Qiu W, et al. Modulating hair follicle size with Wnt10b/DKK1 during hair regeneration. Exp Dermatol. 2014; 23:407–413. [PubMed: 24750467]

Lei M, Yang T, Lai X, et al. Upregulation of interfollicular epidermal and hair infundibulum beta-catenin expression in Gsdma3 mutant mice. Acta Histochem. 2013a; 115:63–69. [PubMed: 22694914]

Lei MX, Chuong CM, Widelitz RB. Tuning Wnt Signals for More or Fewer Hairs. J Invest Dermatol. 2013b; 133:7–9. [PubMed: 23299447]

Li J, Zhou Y, Yang T, et al. Gsdma3 is required for hair follicle differentiation in mice. Biochem Biophys Res Commun. 2010; 403:18–23. [PubMed: 20977888]

Li YH, Zhang K, Yang K, et al. Adenovirus-Mediated Wnt10b Overexpression Induces Hair Follicle Regeneration. J Invest Dermatol. 2013; 133:42–48. [PubMed: 22832493]

Lindner G, Horland R, Wagner I, et al. De novo formation and ultra-structural characterization of a fiber-producing human hair follicle equivalent in vitro. Journal of Biotechnology. 2011; 152:108–112. [PubMed: 21277344]

Liu Y, Lyle S, Yang Z, et al. Keratin 15 Promoter Targets Putative Epithelial Stem Cells in the Hair Follicle Bulge. J Invest Dermatol. 2003; 121:963–968. [PubMed: 14708593]

Lowry WE, Blanpain C, Nowak JA, et al. Defining the impact of beta-catenin/Tcf transactivation on epithelial stem cells. Genes & Development. 2005; 19:1596–1611. [PubMed: 15961525]

Lunny DP, Weed E, Nolan PM, et al. Mutations in gasdermin 3 cause aberrant differentiation of the hair follicle and sebaceous gland. J Invest Dermatol. 2005; 124:615–621. [PubMed: 15737203]

Luo J, Deng Z-L, Luo X, et al. A protocol for rapid generation of recombinant adenoviruses using the AdEasy system. Nat Protoc. 2007; 2:1236–1247. [PubMed: 17546019]

Millar SE. Molecular Mechanisms Regulating Hair Follicle Development. J Invest Dermatol. 2002; 118:216–225. [PubMed: 11841536]

Muller-Rover S, Handjiski B, van der Veen C, et al. A Comprehensive Guide for the Accurate Classification of Murine Hair Follicles in Distinct Hair Cycle Stages. J Invest Dermatol. 2001; 117:3–15. [PubMed: 11442744]

Ouji Y, Ishizaka S, Yoshikawa M. Dermal Papilla Cells Serially Cultured With Wnt-10b Sustain Their Hair Follicle Induction Activity After Transplantation Into Nude Mice. Cell Transplantation. 2012; 21:2313–2324. [PubMed: 22490342]

Philip G, Moore M, Panaretto BA, et al. Epidermal Hyperplasia and Wool Follicle Regression in Sheep Infused with Epidermal Growth Factor. Journal of Investigative Dermatology. 1985; 84:172–175. [PubMed: 3871824]

Plikus MV, Mayer JA, de la Cruz D, et al. Cyclic dermal BMP signalling regulates stem cell activation during hair regeneration. Nature. 2008; 451:340–344. [PubMed: 18202659]

Ruckert R, Lindner G, Bulfone-Paus S, et al. High-dose proinflammatory cytokines induce apoptosis of hair bulb keratinocytes in vivo. British Journal of Dermatology. 2000; 143:1036–1039. [PubMed: 11069516]

Rompolas P, Mesa KR, Greco V. Spatial organization within a niche as a determinant of stem-cell fate. Nature. 2013; 502:513–518. [PubMed: 24097351]

Rudman SM, Philpott MP, Thomas GA, et al. The Role of IGF-I in Human Skin and its Appendages: Morphogen as Well as Mitogen? Journal of Investigative Dermatology. 1997; 109:770–777. [PubMed: 9406819]

Ruge F, Glavini A, Gallimore AM, et al. Delineating immune-mediated mechanisms underlying hair follicle destruction in the mouse mutant defolliculated. J Invest Dermatol. 2011; 131:572–579. [PubMed: 21160494]

Runkel F, Marquardt A, Stoeger C, et al. The dominant alopecia phenotypes Bareskin, Rex-denuded, and Reduced Coat 2 are caused by mutations in gasdermin 3. Genomics. 2004; 84:824–835. [PubMed: 15475261]

Saeki N, Kuwahara Y, Sasaki H, et al. Gasdermin (*Gsdm*) localizing to mouse Chromosome 11 is predominantly expressed in upper gastrointestinal tract but significantly suppressed in human gastric cancer cells. Mammalian Genome. 2000; 11:718–724. [PubMed: 10967128]

Silva-Vargas V, Lo Celso C, Giangreco A, et al. β-Catenin and Hedgehog Signal Strength Can Specify Number and Location of Hair Follicles in Adult Epidermis without Recruitment of Bulge Stem Cells. Developmental Cell. 2005; 9:121–131. [PubMed: 15992546]

Stenn KS. Insights from the asebia mouse: a molecular sebaceous gland defect leading to cicatricial alopecia. Journal of Cutaneous Pathology. 2001; 28:445–447. [PubMed: 11553309]

Tanaka S, Mizushina Y, Kato Y, et al. Functional Conservation of Gsdma Cluster Genes Specifically Duplicated in the Mouse Genome. G3 (Bethesda). 2013; 3:1843–1850. [PubMed: 23979942]

Tanaka S, Tamura M, Aoki A, et al. A new Gsdma3 mutation affecting anagen phase of first hair cycle. Biochem Biophys Res Commun. 2007; 359:902–907. [PubMed: 17572385]

van Genderen C, Okamura RM, Fariñas I, et al. Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in LEF-1-deficient mice. Genes & Development. 1994; 8:2691–2703. [PubMed: 7958926]

Van Mater D, Kolligs FT, Dlugosz AA, et al. Transient activation of β-catenin signaling in cutaneous keratinocytes is sufficient to trigger the active growth phase of the hair cycle in mice. Genes & Development. 2003; 17:1219–1224. [PubMed: 12756226]

Zhang Y, Andl T, Yang SH, et al. Activation of β-catenin signaling programs embryonic epidermis to hair follicle fate. Development. 2008; 135:2161–2172. [PubMed: 18480165]

Zhou P, Byrne C, Jacobs J, et al. Lymphoid enhancer factor 1 directs hair follicle patterning and epithelial cell fate. Genes & Development. 1995; 9:700–713. [PubMed: 7537238]

Zhou Y, Jiang X, Gu P, et al. Gsdma3 Mutation Causes Bulge Stem Cell Depletion and Alopecia Mediated by Skin Inflammation. Am J Pathol. 2012a; 180:763–774. [PubMed: 22155111]

Zhou Y, Jiang X, Gu P, et al. Gsdma3 mutation causes bulge stem cell depletion and alopecia mediated by skin inflammation. Am J Pathol. 2012b; 180:763–774. [PubMed: 22155111]

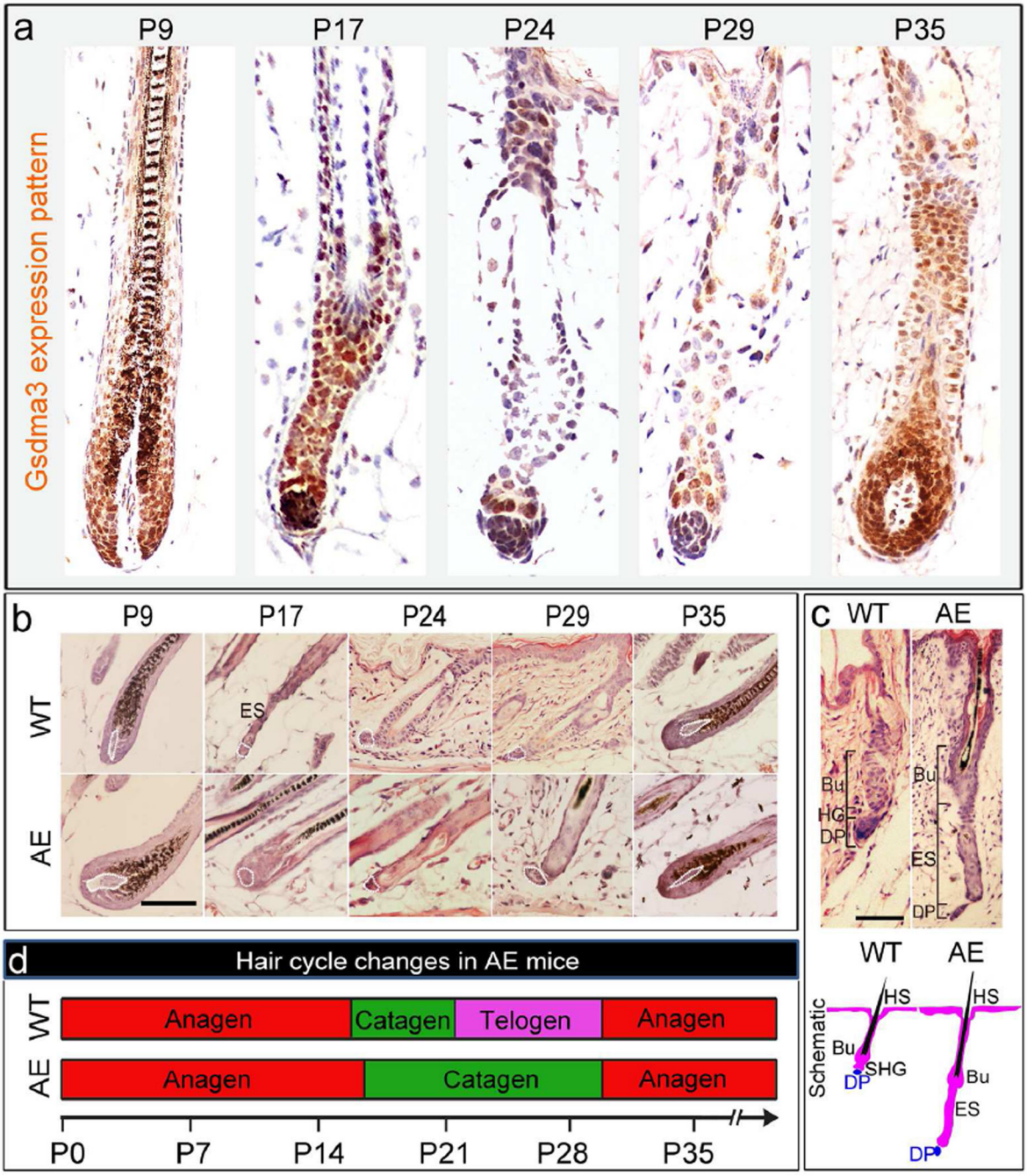

Figure 1. Gsdma3 expression pattern and morphological changes of AE mouse hair follicles during the hair cycle

(a) Gsdma3 expression pattern during the hair cycle. (b) H&E staining of back skins in WT and AE mice. White dashed lines show the DP structures. (c) H&E staining and schematic show morphology of hair follicles at P29. (d) Schematic illustration of the abnormal Hair cycle in AE mice compared with their WT littermates. n>20. AE: *Gsdma3* mutant mice; WT: Wild type. Bu: bulge; DP: dermal papilla; ES: epidermal strand; HG: hair germ; HS: hair shaft; SHG: secondary hair germ. Bar=50μm.

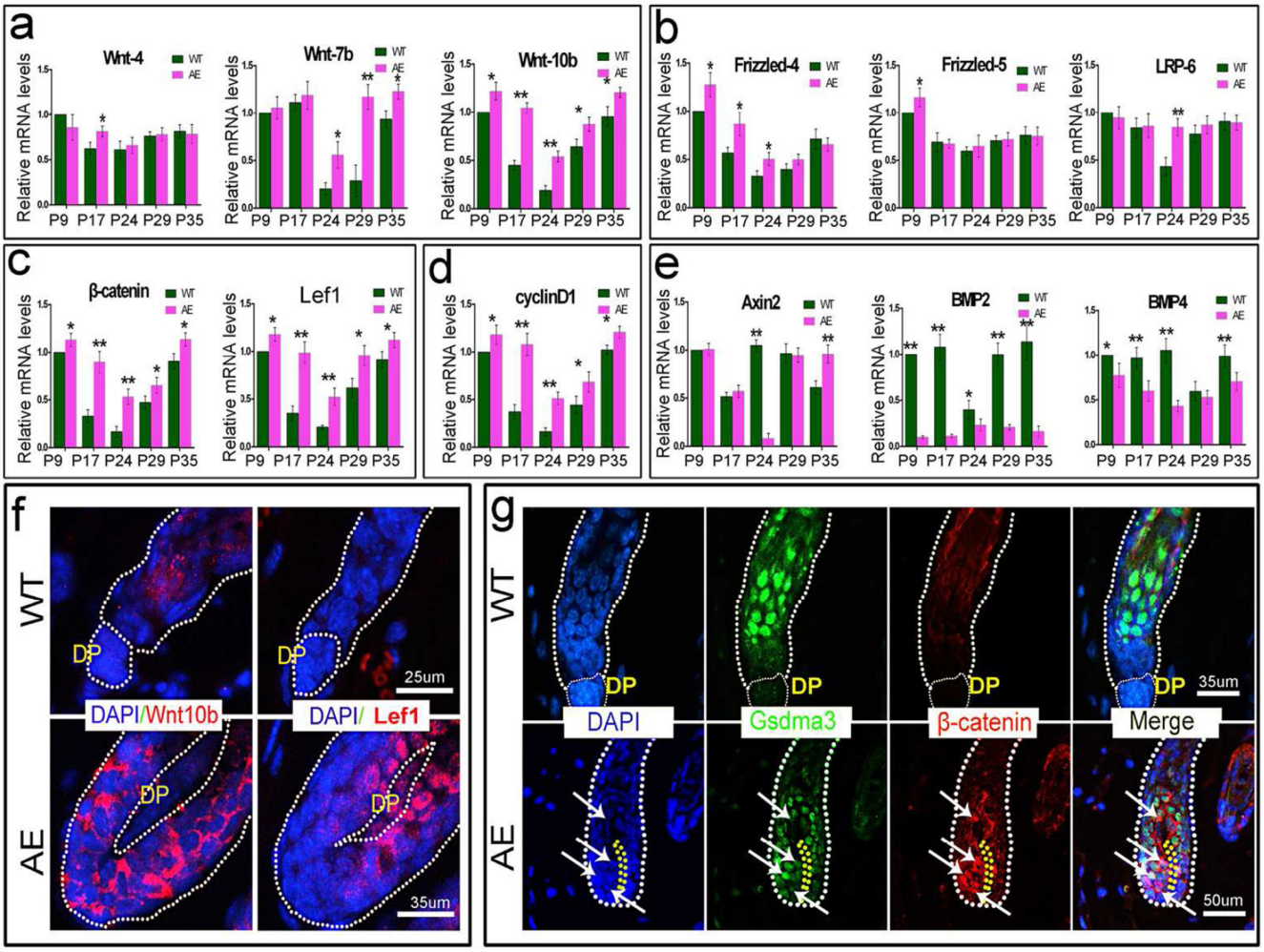

Figure 2. Wnt pathway is upregulated in AE mice

Bar charts display fold change of (a) Wnt ligand mRNA expression, (b) Wnt receptors, (c) β-catenin and Lef1, (d) Wnt targets and (e) Wnt inhibitors in WT and AE back skin (n>3) at different time points. (f) Immunofluorescence analysis of Wnt10b and Lef1 expression in WT and AE hair follicles. (g) Immunofluorescence shows the colocalization of nuclear β-catenin with Gsdma3 in the WT and AE hair matrix (arrowheads). n>10. Data are reported as average ± SD. *P<0.05, **P<0.01. AE: *Gsdma3* mutant mice; WT: Wild type. DP: dermal papilla. Bars=50μm.

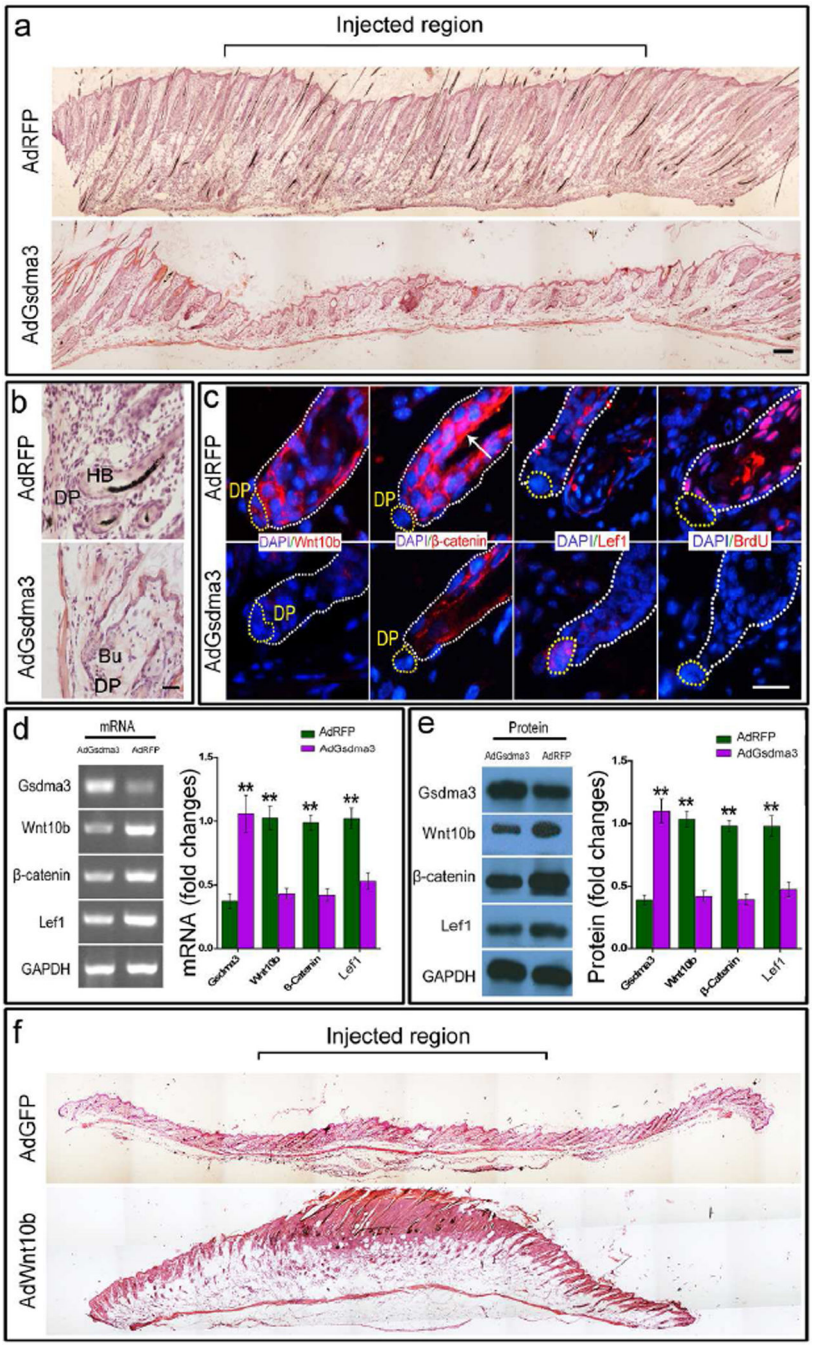

Figure 3. Gsdma3 overexpression restores the telogen phase in AE mice

(a) H&E staining shows the structural changes of AE mouse skin after AdGsdma3 compared to AdRFP injection. (b) Magnification of the hair bulb after viral treatment. (c) Immunofluorescence analysis of Wnt10b, β-catenin, Lef1 expression and BrdU positive cells in AdGsdma3 and AdRFP-injected hair bulbs (white arrow shows nuclear staining). RT-PCR and Western blotting shows the mRNA (d) and protein (e) levels of Wnt10b, β-catenin, Lef1 after AdGsdma3 and AdRFP treatment (n=3). (f) H&E staining shows hair follicles maintain catagen phase after Wnt10b overexpression. Data are reported as average

± SD. *P<0.05, **P<0.01. AE: *Gsdma3* mutant mice; WT: Wild type. DP: dermal papilla. Bars=50μm.

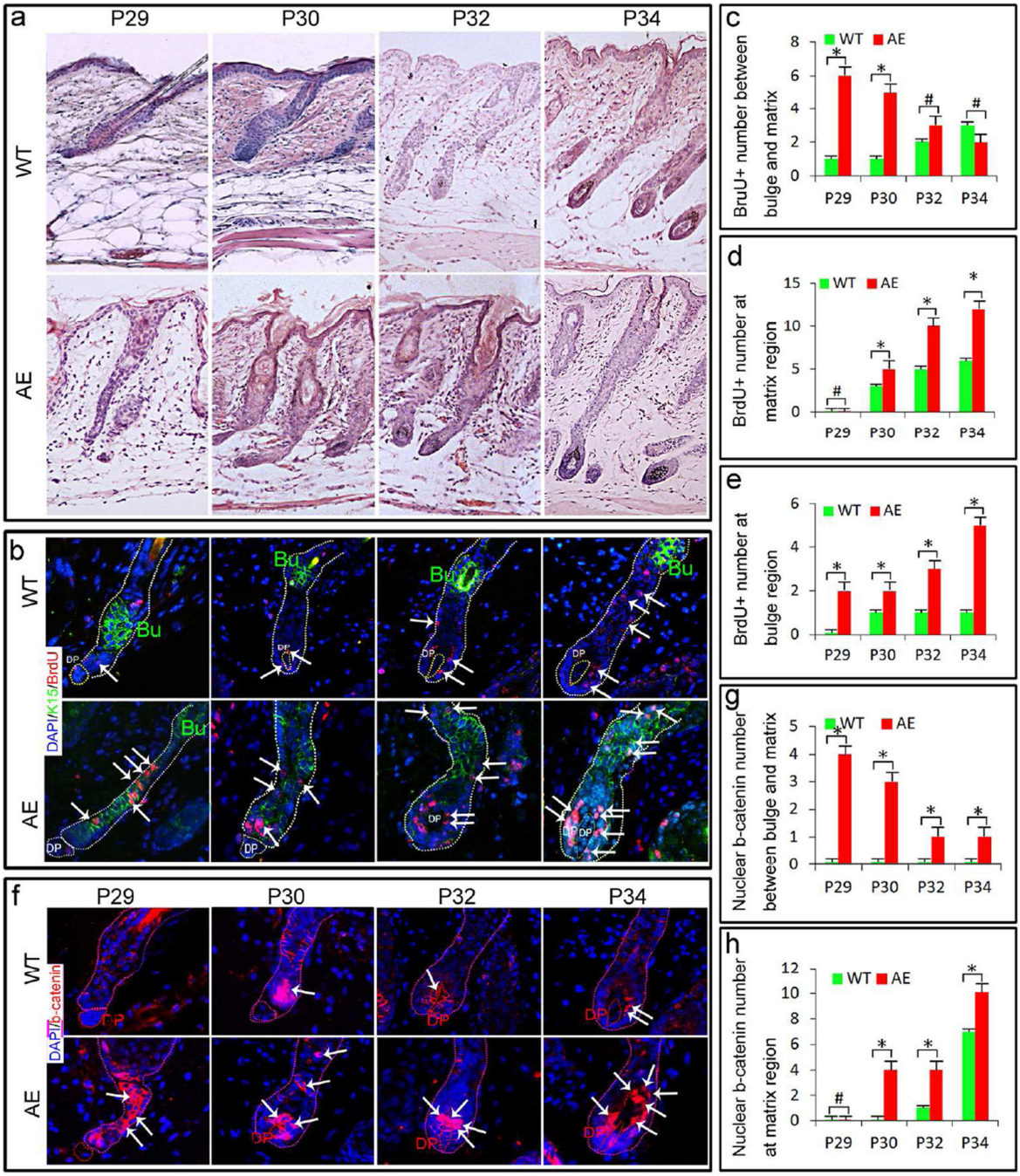

Figure 4. WT and AE mouse hair follicles start the second anagen at a similar time

(a) H&E staining shows the progression of hair follicle regeneration from the second anagen onset. (b) Immunofluorescence shows K15 and BrdU expression during hair follicle re-growth (arrows indicate BrdU positive cells). (c–e) Statistical charts show the BrdU positive cells in different hair follicle compartments during hair regeneration (n=20). (f–h) Immunofluorescence staining and statistical charts show the expression of nuclear β-catenin (n=20; white arrows). Data are reported as average ± SD. # no statistical difference, *P<0.05. AE: *Gsdma3* mutant mice; WT: Wild type. DP: dermal papilla. Bars=50μm.

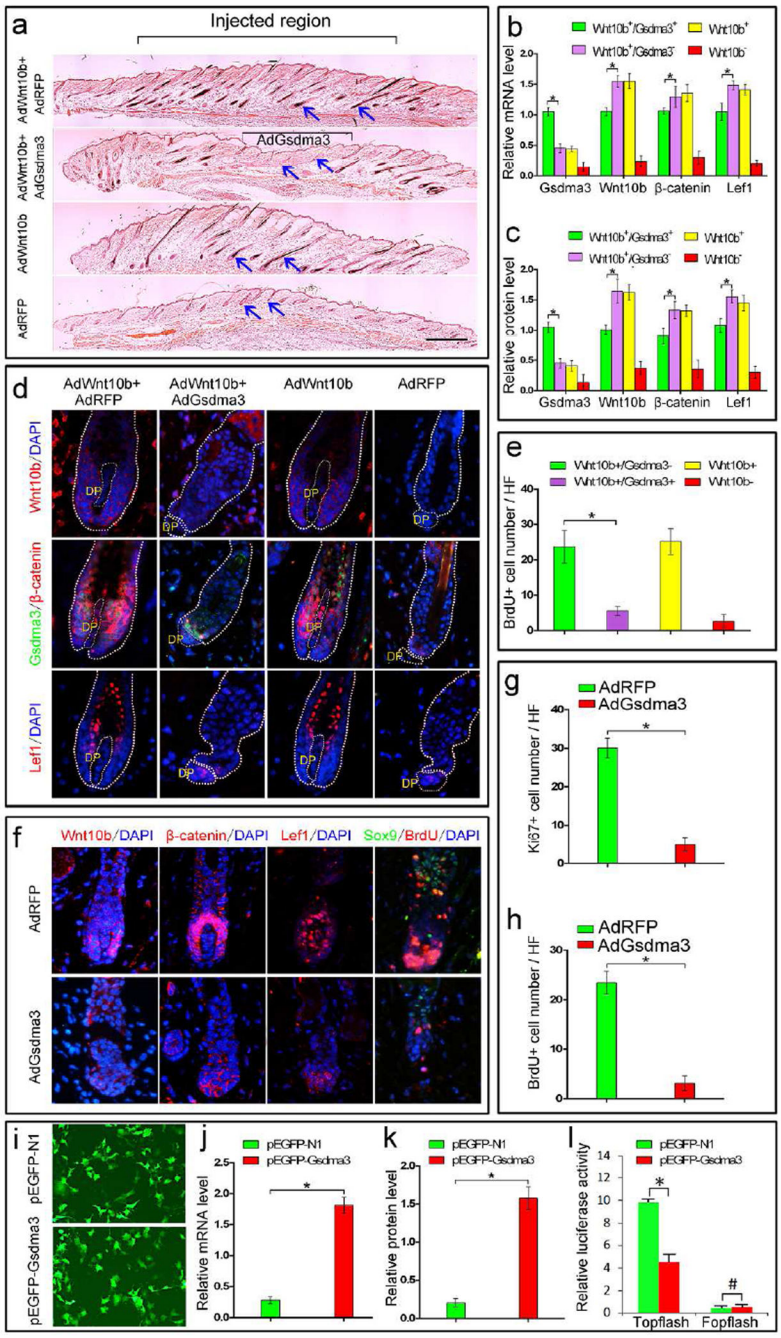

Figure 5. Gsdma3 overexpression at anagen onset blocks hair follicle regeneration by repressing Wnt signaling

(a) H&E staining shows the hair follicles (blue arrows) don't enter anagen after AdWnt10b +AdGsdma3 treatment. (b–d) Statistical charts and immunofluorescence analysis show mRNA and protein level changes after virus transduction. (e) Statistical chart of the different BrdU+ cell numbers between WT and AE mice after AdGsdma3 treatment. (f) Immunofluorescence shows Wnt signaling after AdGsdma3 injection into skin after depilation. (g–h) Statistical charts show Ki67 and BrdU+ cells in depilated skin treated with AdGsdma3. (i–l) Fluorescence and statistical charts show the GFP, mRNA, and protein

levels and TOP/FOP ratios in JB6 cells transfected with pEGFP-Gsdma3 or pEGFP-N1 (n=3). *P<0.05. Bar=500μm (a). DP: dermal papilla.

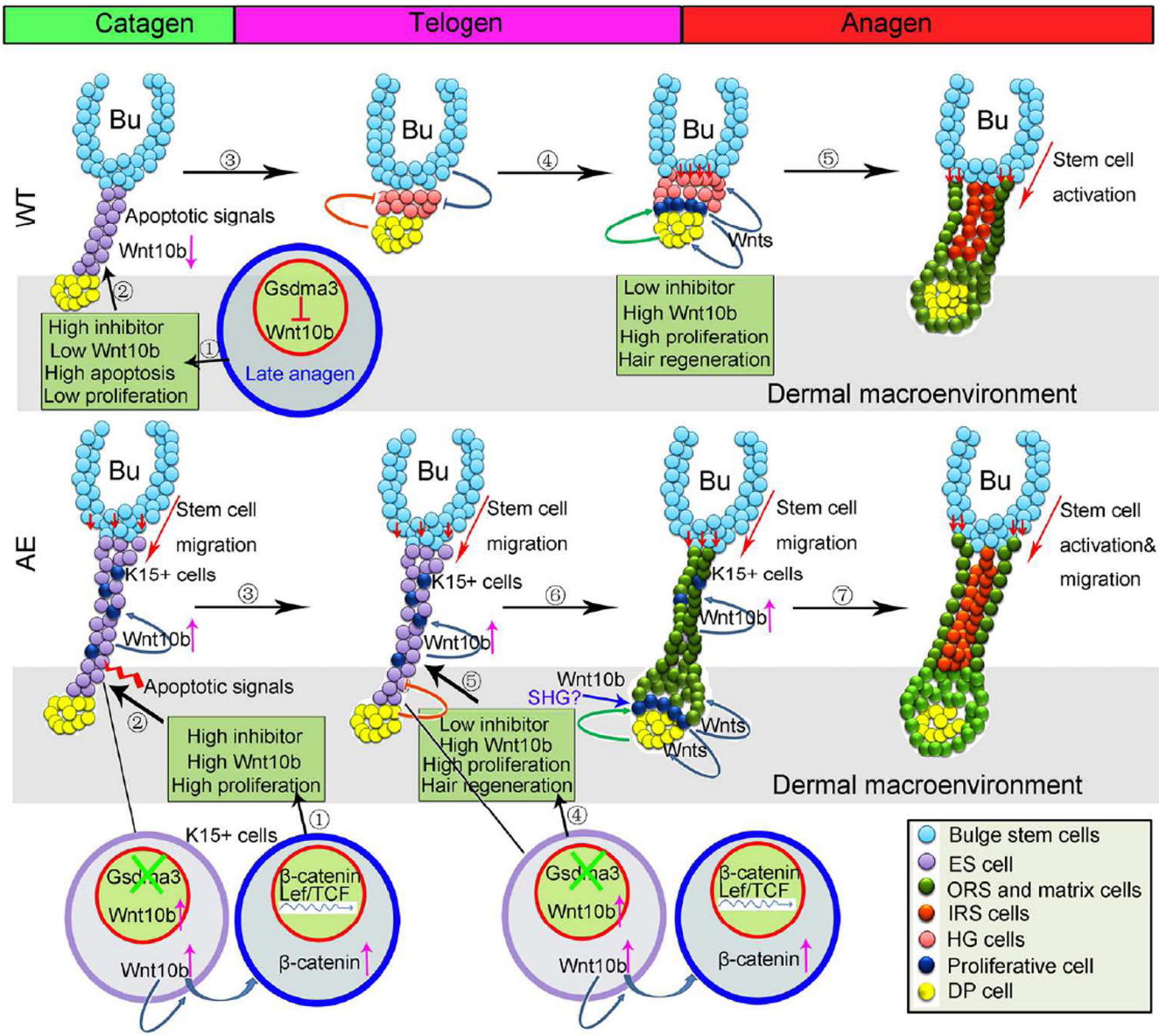

Figure 6. Schematic of hair cycle after Gsdma3 mutation

At mid-catagen phase of WT hair follicles, Gsdma3 inhibits *Wnt10b* which leads to low proliferation. Combined with high inhibitors and high apoptosis, the hair follicles transit from catagen to telogen. However, after *Gsdma3* mutation, *Wnt10b* is not inhibited leading to high proliferation. Moreover, low apoptosis and high *Wnt10b*-promotes hair stem cell emigration from the bulge region to keep the ESLS, resulting in a blocked catagen-telogen transition. At anagen onset of WT hair follicles, low inhibitor and high activator promote anagen phase entry. But after *Gsdma3* mutation, low inhibitors and high Wnt10b will force catagen stage follicles to directly enter anagen phase. The ESLS may become different lineages including SHG, ORS and IRS.