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# Disruption of Smad4 in Mouse Epidermis Leads to Depletion of Follicle Stem Cells

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**Follicle stem cells (SCs) residing in the bulge region of a hair follicle (HF) can give rise to multiple lineages during the hair cycle and wound healing. The activation and self-renewal of follicle SCs must be tightly regulated to maintain the HF and epidermal homeostasis. Here we show that, in young mice, disruption of epidermal *Smad4*, the common mediator of transforming growth factor-β (TGF-β) signaling, stimulated the activation of follicle SCs, leading to hyperplasia of interfollicular epidermis (IFE), HFs, and sebaceous glands (SGs). Increased proliferation of follicle SCs ultimately exhausted the SC niche, indicated by the loss of bromodeoxyuridine (BrdU) label–retaining cells (LRCs), loss of keratin 15 (K15), and CD34 expression. In addition, the colony-forming efficiency of *Smad4* mutant keratinocytes was significantly decreased. Increased nuclear localization of β-catenin and increased expression of c-Myc were correlated with the overactivation and depletion of follicle SCs. We concluded that Smad4 plays a pivotal role in follicle SC maintenance.**

## INTRODUCTION

Follicle stem cells (SCs) residing in the bulge of hair follicles (HFs) continuously self-renew and differentiate into interfollicular epidermis (IFE), HFs, and sebaceous glands (SGs) and are thus necessary for the maintenance of skin homeostasis and for hair regeneration (Blanpain and Fuchs, 2006). Follicle SCs derived cells can also be recruited to regenerate the various epithelial cell types of hairy skin after injury (Blanpain *et al.*, 2004; Morris *et al.*, 2004; Tumbar *et al.*, 2004; Levy *et al.*, 2005). The bulge area serves as a niche in which the self-renewal and activation of multipotent follicle SCs are tightly regulated. Although the Wnt/β-catenin, Sonic hedgehog, and Notch signaling pathways have been shown to regulate the activation and differentiation of follicle SCs, the molecular mechanism underlying the follicle SC maintenance remains poorly understood (Fuchs, 2007).

Bone morphogenetic protein (BMP) signaling has been shown to play essential roles in HF morphogenesis, as well as in regulating follicle SC activation and the differentiation of postnatal HFs. BMPs are differentially expressed in various HF cell lineages, with constitutive BMP type 1a receptor (BMPR1A) expression seen in all the HF epithelial cells including follicle SCs (Kobielak *et al.*, 2003). Ectopic overexpression of BMP4 or targeted disruption of the BMP antagonist *Noggin* results in disturbed HF induction and progressive hair loss. Overexpression of *Noggin* reveals that BMP signaling plays important roles in induction of the anagen phase (Botchkarev *et al.*, 1999), hair shaft differentiation (Kulessa *et al.*, 2000), and regulation of HF size (Sharov *et al.*, 2006). Blockade of BMP signaling by deletion of the *Bmpr1A* leads to aberrant de novo HF morphogenesis accompanied by hair matrix cell hyperplasia and the formation of follicular tumors (Andl *et al.*, 2004; Ming Kwan *et al.*, 2004). Recently, several lines of evidence have suggested that BMP signaling in the follicle SC niche inhibits follicle SC activation and HF anagen induction (Zhang *et al.*, 2006; Kobielak *et al.*, 2007). The dynamic expression of *Noggin* in follicle SCs during the HF cycling triggers the cyclic inactivation of BMP signaling which correlates with the activation of follicle SCs during the early anagen phase (Zhang *et al.*, 2006). A very recent study reveals that cyclic dermal BMP signaling regulates SC homeostasis during hair regeneration by the interorgan macroenvironment (Plikus *et al.*, 2008).

The Wnt/β-catenin pathway has been well characterized and shown to function in regulating follicle SC proliferation and fate determination. In the resting follicle, SCs are in a quiescent, Wnt-inhibited state (DasGupta and Fuchs, 1999; Tumbar *et al.*, 2004). Activation of Wnt/β-catenin signaling is critical for HF morphogenesis and plays a key role in driving follicle SCs along hair differentiation lineages (Merrill *et al.*, 2001; Zhang *et al.*, 2006). β-Catenin stabilization is essential for promoting the transition from quiescent follicle SCs into proliferating transit-amplifying cells (Lowry *et al.*, 2005). Recent studies suggest that BMP signaling could inhibit β-catenin stabilization in the follicle SC niche through regulation of PTEN/phosphatidylinositol 3′-kinase (PI3K)/Akt signaling pathway (Zhang *et al.*, 2006; Kobielak *et al.*, 2007).

BMP/transforming growth factor-β (TGF-β) signals transduce through transmembrane receptors and intracellular mediator Smads (Shi and Massague, 2003). Microarray profiling of bulge SCs has revealed that a variety of genes in the BMP and TGF-β pathways are preferentially expressed in the bulge relative to the proliferating basal cells of the epidermis (Blanpain *et al.*, 2004; Morris *et al.*, 2004; Tumbar *et al.*,

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Abbreviations used: BMP, bone morphogenetic protein; BMPR1A, BMP type 1a receptor; BrdU, bromodeoxyuridine; HF, hair follicle; IFE, interfollicular epidermis; K1, K14, and K15, keratin 1, 14, and 15, respectively; LRC, label-retaining cell; P20, postnatal day 20; SC, stem cell; SG, sebaceous gland; TGF-β, transforming growth factor-β.

2004). Overexpression of Smad2 results in disorganized epidermis, indicating an important role of Smad2 in regulating TGF-β–mediated epidermal homeostasis (Ito *et al.*, 2001). The up-regulated BMP/TGF-β signals in bulge SCs are correlated with nuclear localization of phospho-Smad1 and phospho-Smad2 together with activated target genes and/or Smad-interacting proteins (Tumbar *et al.*, 2004; Mou *et al.*, 2006), indicating a role for Smad-mediated BMP/TGF-β signals in maintaining the special quiescent nature of resting follicle SCs. However, the function of Smads in follicle SC maintenance remains largely undefined.

We and others have previously reported that targeted ablation of *Smad4* in the epidermis and outer root sheath of HFs results in progressive hair loss and skin tumor formation (Yang *et al.*, 2005; Qiao *et al.*, 2006). A recent study has shown that *Smad4* loss associated depletion of desmoglein-4 expression contributes to hair follicle degeneration in *Smad4* mutants (Owens *et al.*, 2008). In the current study, we reported that *Smad4* disruption in the epidermis leads to activation of follicle SCs, which caused hypertrophic of IFE, HFs, and SGs. This hypertrophic phenotype is transient, because activated SCs are eventually exhausted in the follicle. We further provide evidence that an increase in nuclear β-catenin and up-regulation of c-Myc may be responsible for the depletion of follicle SCs in *Smad4* mutant mice.

## MATERIALS AND METHODS

### *Mouse Strains and Genotyping*

Mouse genotyping of the keratinocyte specific *Smad4* gene knockout mice was performed as described in Yang *et al.* (2005). All animal studies were approved by the Review Board of Institute of Biotechnology.

### *Oil Red O and LacZ Staining*

Oil Red O staining was performed on frozen skin sections from *Smad4* mutants and control littermates aged 3, 6, and 12 mo. Slides were stained in 0.5% Oil Red O in 100% propylene glycol for 30 min and counterstained with Mayer's hematoxylin. Skin tissues of postnatal day 45 (P45) mouse were fixed in 4% paraformaldehyde for 2 h and then subjected to LacZ staining.

### *Histological Analysis and Immunofluorescence*

Dorsal skin was embedded in Cryomatrix (Thermo Scientific, Waltham, MA) and sectioned at 10 μm. Frozen sections were fixed in 4% paraformaldehyde, and immunofluorescence was performed as described in Yuan *et al.* (2008). Primary antibodies used in this study were K14 at 1:1000, K1 at 1:1000, involucrin at 1:1000 (Covance Laboratories, Madison, WI), Ki67 at 1:1000 (Abcam, Cambridge, MA), bromodeoxyuridine (BrdU) at 1:100 (Abcam), CD34 at 1:200 (eBioscience, San Diego, CA), K15 at 1:100 (Chemicon, Temecula, CA), active-β-catenin at 1:100 (Upstate Biotechnology, Lake Placid, NY), and p-Akt at 1:200 (Cell Signaling, Beverly, MA). Antigens were visualized with FITC or TRITIC-conjugated secondary antibodies (Zymed Laboratories, South San Francisco, CA). Nuclear DNA was stained with the DAPI reagent (Sigma, St. Louis, MO).

### *BrdU Label–retaining Analyses*

Ten-day-old mice were injected intraperitoneally with 50 μg/g body weight BrdU (Sigma) every 12 h for 48 h. Mice were killed at least 2 mo after the last injection. Whole mounts of mouse tail epidermis preparation and immunofluorescence were performed as previously described (Braun *et al.*, 2003). A laser scanning confocal microscope (Bio-Rad, Hercules, CA; Radiance 2100) was used to obtain fluorescence images.

### *Clonogenicity Assays and Immunofluorescence*

Keratinocytes from back skin of 6–8-wk-old mice were obtained as described previously (Frye *et al.*, 2003); although the *Smad4* mutant HFs resisted degeneration into catagen, we could peel off the epidermis and hypertrophic anagen HFs thoroughly from the dermis at ~2 mo old (Supplemental Figure S1). Cells, $1 \times 10^5$, were plated per 60-mm dish on mitomycin C–treated 3T3 feeder layer. After 32 d, colonies were photographed and stained with 0.5% rhodamine B. For immunofluorescence, cells were subsequently fixed in formaldehyde 4% and then immunostained and stained with DAPI for DNA before mounting as described in Sun *et al.* (2008).

### *Western Blot Analysis*

Tissue proteins were obtained from the extracts of epidermis of mice at P42 and Western blot performed as described in Yang *et al.* (2005). Antibody reaction was done with antibodies against active-β-catenin (Upstate), β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA), c-Myc (Santa Cruz), PTEN (Cell Signaling), p-Akt (Cell Signaling), GSK-3β (Cell Signaling), β1-integrin (Santa Cruz), and β-actin (Sigma).

### *Flow Cytometry*

Primary mouse keratinocytes were isolated from back skin of 72-d-old mice as described previously (Blanpain *et al.*, 2004). Cells were labeled with anti-α6-integrin antibody conjugated to phycoerithrin (eBioscience) and anti-CD34 conjugated to FITC (eBiosciences) for 45 min at room temperature with rotation. Cells were then analyzed using BD FACScalibur sorter and CellQuest FACS analysis system.

## RESULTS

### *The Hypertrophy Phenotypes in Smad4 Mutant Epidermis Are Transient*

In our previous study, we generated keratinocyte-specific *Smad4* knockout mice (*Smad4$^{co/co}$;K5-Cre*) using the K5-Cre transgenic mice (Yang *et al.*, 2005). The *Smad4$^{co/co}$;K5-Cre* mutant mice began to exhibit progressive hair loss after P20. By 12 mo of age, 70% of the mutant mice developed visible skin tumors (Yang *et al.*, 2005). We further observed that the majority of (22/25) tumors occurred in mice younger than 9 mo (Supplemental Figure S2). About 10% of mutant mice survived as long as 15 mo without any skin tumors.

We performed Oil Red O staining to analyze the histological changes in the epidermis of 3-, 6-, and 12-mo-old *Smad4$^{co/co}$;K5-Cre* and control mice (Figure 1). At 3 mo, the

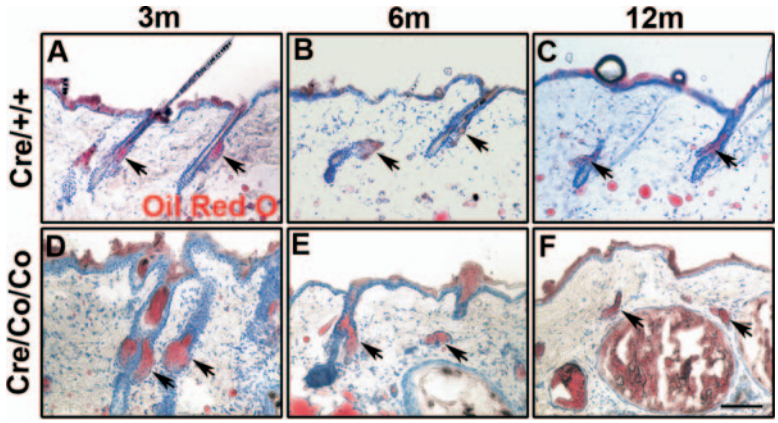


**Figure 1.** Hypertrophy phenotypes withered in aged *Smad4* mutant mice. (A–F) Histological analysis by Oil Red O staining performed on dorsal skin of 3- (A and D), 6- (B and E), and 12-mo-old (C and F) wild-type (Cre/+/+, A–C) and *Smad4* mutant mice (Cre/Co/Co, D–F). Bar, 50 μm.

*Smad4* mutant epidermis displayed significantly thickened IFE, hypertrophic HFs, and enlarged SGs compared with wide type littermates (Figure 1, A and D). Surprisingly, we found the cellularity of the IFE, HFs and SGs to be decreased in *Smad4* mutants at 6 mo of age compared with that at 3 mo (Figure 1, D and E). By 12 mo, the thickness of mutant epidermis was significantly reduced compared with controls, and all HF infundibula were found to have degenerated into cysts that contained a large amount of keratose debris (Figure 1, C and F).

We also performed immunofluorescence in *Smad4* mutant and control epidermis to determine the expression levels of keratin14 (K14, Figure 2, A–F), keratin1 (K1, Figure 2, G–L), and involucrin (Figure 2, M–R), which are markers, respectively, for the basal layer of the epidermis and the HF outer root sheaths, the spinous layers of the epidermis and inner root sheaths of HFs, and the granular layers of the epidermis. The results showed that IFE and HF hypertrophy diminished gradually in *Smad4* mutants (Figure 2, A–R; Supplemental Figure S3).

### *Loss of Smad4 Resulted in a Transient Increase in Epidermal Cellular Proliferation*

We next used Ki67 immunofluorescence to examine proliferation in the skin of mouse back (Figure 3, A–F) and tail (Figure 3, G–L) at 3, 6, and 12 mo, respectively. At 3 mo, there was a significantly increased proliferation in *Smad4* mutant epidermis (Figure 3D) compared with wild-type (Figure 3A). The number of Ki67-positive cells in *Smad4* mutants (Figure 3E) had decreased to control epidermal levels by 6 mo (Figure 3B). By 12 mo, the number of Ki67-positive cells in *Smad4* mutants was further declined (Figure 3F). Whole mount Ki67 labeling of mouse tail epidermis (Figure 3, G–L) confirmed the transient increased proliferation in *Smad4* mutant mice (Figure 3, J and M).

### *Smad4 Deletion Resulted in Overactivation of Follicle SCs*

The withering of the hypertrophy phenotypes after a transient increase in proliferation suggested that the follicle SCs may have been induced to exit from their compartment. To

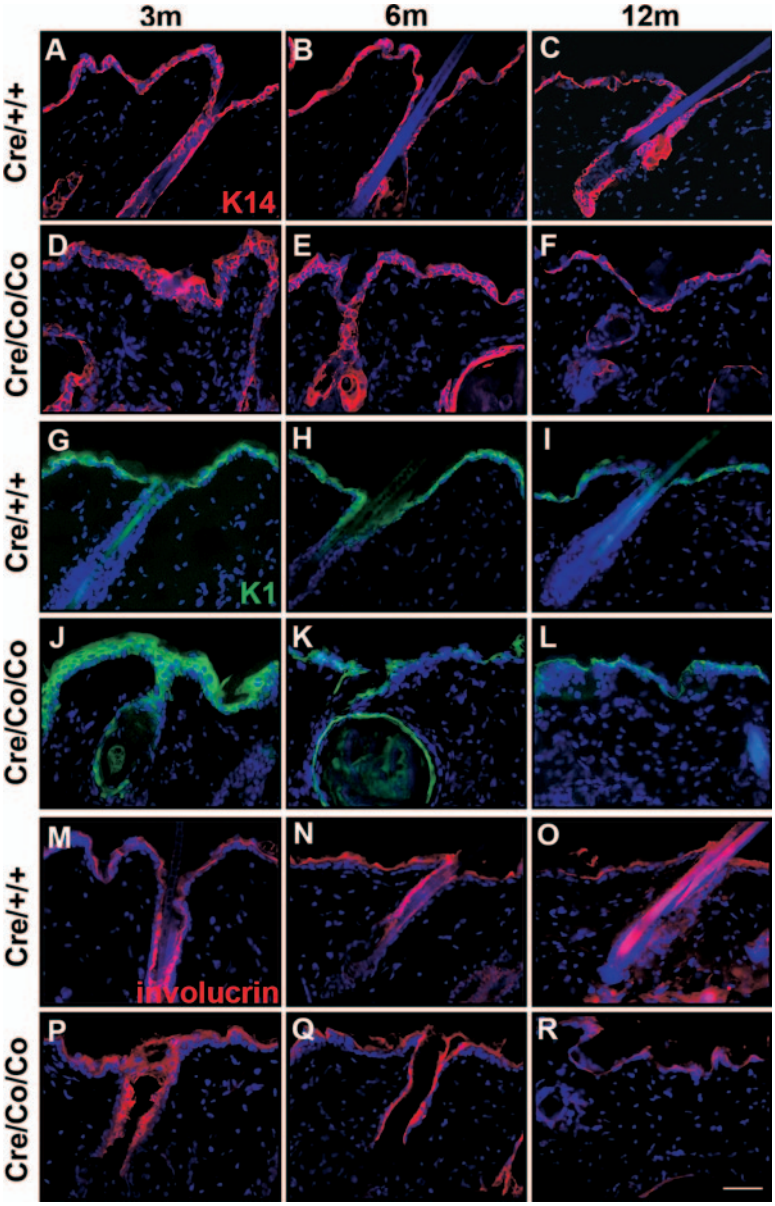


**Figure 2.** Dynamic changes of epidermis markers expression in postnatal *Smad4* mutant epidermis and HFs. Immunofluorescence staining with K14 (A–F), K1 (G–L), and involucrin (M–R) on dorsal skin of 3- (A, D, G, J, M, and P), 6- (B, E, H, K, N, and Q), and 12-mo-old (C, F, I, L, O, and R) wild-type (A–C, G–I, and M–O) and *Smad4* mutant mice (D–F, J–L, and P–R). Bar, 40 $\mu$m.

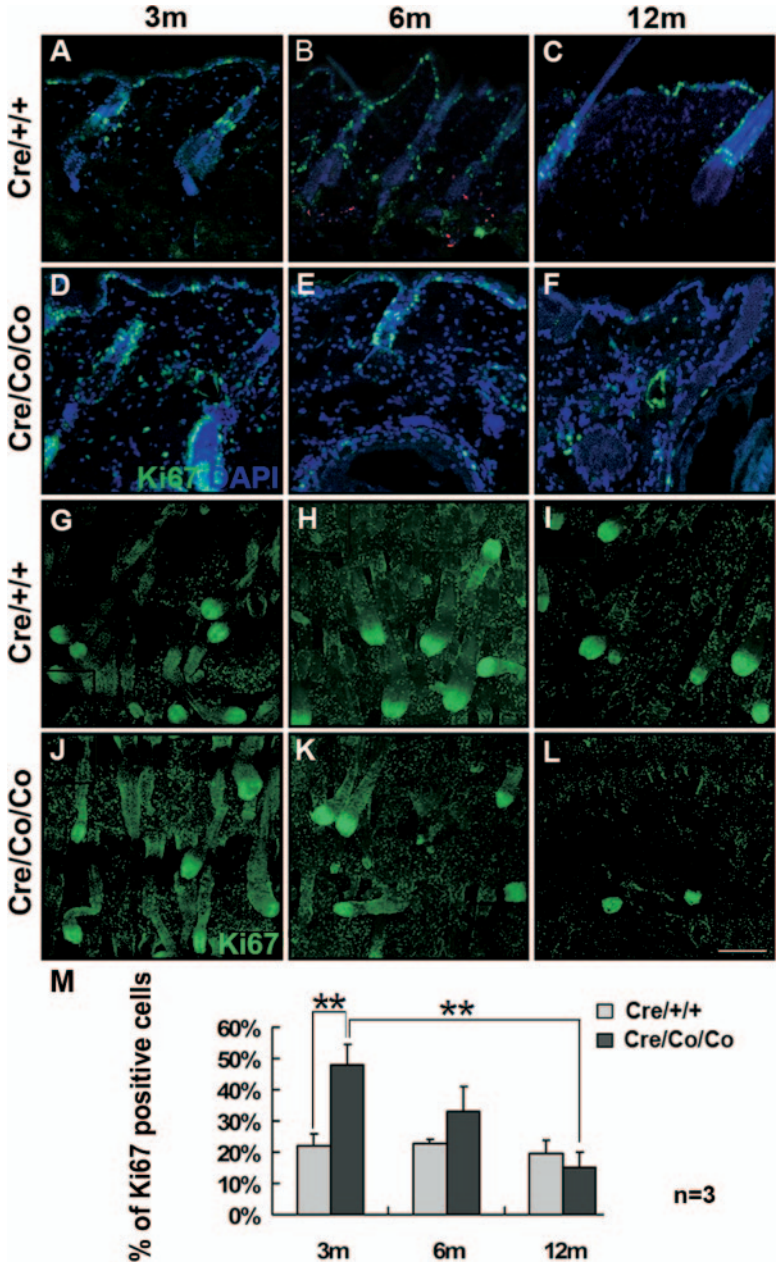


**Figure 3.** Loss of *Smad4* resulted in a transient increased proliferation in epidermis and HFs. (A–F) Ki67 immunofluorescence on mice dorsal skin sections at 3 (A and D), 6 (B and E), and 12 mo (C and F), respectively. In *Smad4* mutant epidermis there was an increase in the number of Ki67-positive keratinocytes at 3 mo (D) compared with that of wide-type mice (A), whereas it decreased gradually at 6 (E) and 12 mo (F). (G–L) Ki67 immunofluorescence of whole mount mouse tail epidermis at 3 (G and J), 6 (H and K), and 12 mo (I and L) confirmed the transient increased proliferation in *Smad4* mutants. (M) Percentage of Ki67-positive cells; n = 6 for each time point and genotype. **$p < 0.01$. Bar, (A–F) 50 $\mu$m; (G–L) 100 $\mu$m.

test this hypothesis, we first examined the expression pattern of Smad4 in the bulge. As shown in Figure 4A, Smad4 is normally expressed in the bulge SCs of HF at telogen (P45). $\beta$-Galactosidase staining done in the P45 *K5-Cre;ROSA26* double transgenic mice revealed Cre-mediated recombination in the bulge SCs at telogen (Figure 4B). No Smad4 expression was detected in P45 $Smad4^{Co/Co}$;*K5-Cre* mutant HFs (Figure 4C). These data indicated that the *Smad4* expression was successfully disrupted in the follicle SC compartment. We further investigated whether BrdU label–retaining cells (LRCs; Cotsarelis *et al.*, 1990) in the HF bulge were affected in *Smad4* mutants. Ten-day-old mice received an injection of BrdU every 12 h for a total of four injections. After a 60-d chase, keratinocytes in the bulge of wild-type tail epidermis retained the BrdU label (Figure 4D, bracket). Ki67 labeling was confined to the lower bulb region of the HF, indicating that most of the follicle SCs in wild-type tail epidermis was quiescent. In contrast, the LRC zones were expanded in *Smad4*-deleted mice (Figure 4E, bracket). Increased numbers of Ki67-positive LRCs were found in *Smad4* mutant tail HFs (Figure 4, E and G, arrows, 11.2 ± 5.0% in *Smad4* mutants (n = 16) vs. 2.9 ± 2.5% in controls, (n = 10), $p < 0.01$), indicating that more *Smad4* mutant LRCs were dividing.

### *Smad4 Deletion Led to Depletion of the Follicle SC Compartment*

Continued proliferation of LRCs eventually resulted in loss of the label after a 77-d chase, scarcely any LRCs were evident in the back skin of *Smad4* mutant mice (Figure 5B), whereas in wild-type back skin, many LRCs could be found in the bulge (Figure 5A, arrow).

To confirm that *Smad4* deletion led to depletion of the follicle SC compartment, we next examined the expression

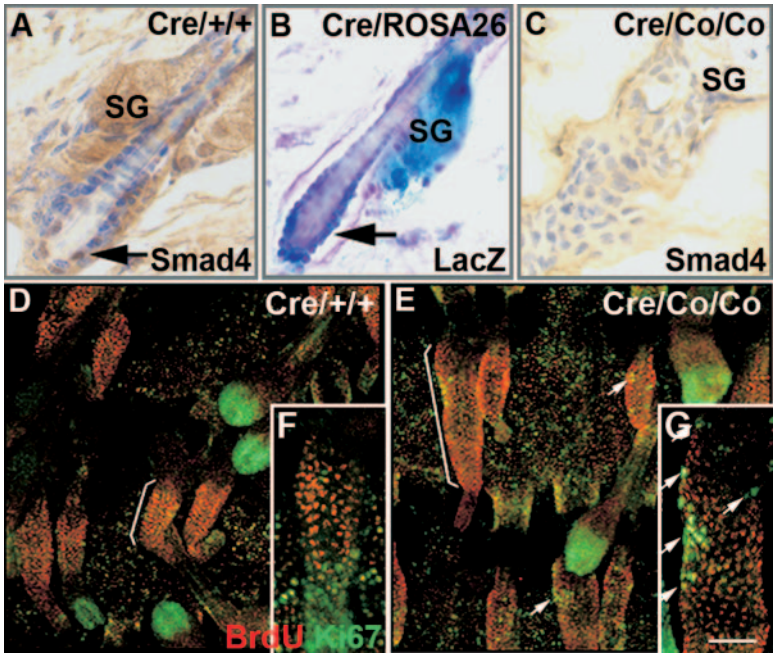


**Figure 4.** *Smad4* deletion result in overactivation of follicle SCs. Immunohistochemistry staining of Smad4 in HFs of wild-type (A) and *Smad4* mutants (C). (B) LacZ staining of a HF in a *K5-Cre;ROSA26* double transgenic mouse. (D–G) Costaining of BrdU and Ki67 were performed after a 60-d chase. LRCs concentrated in the bulge of wild-type tail epidermis (D and F, brackets), whereas Ki67-labeled cells were concentrated most in the bulb (D and F, arrows). In *Smad4* mutants, the LRC zones were expanded (G, brackets), and increased numbers of LRCs were colabeled with Ki67 (E and G, arrows). SG, sebaceous gland. Bar, (A–C) 25 $\mu$m; (D and E) 100 $\mu$m; (F and G) 40 $\mu$m.

of the HF bulge marker CD34. CD34 was detected in the bulge of P72 wild-type mice but not in that of their mutant littermates (Figure 5, C and D). The expression of another bulge marker, keratin 15 (K15), was also found to have been lost in the mutant tail epidermis (Figure 5, E and F). We subsequently examined the expression of K15 in mutant epidermis between P22 and P72. Comparable expression of K15 was detected in P22 and P29 mutants (Figure 5, G–J). The expression of K15 was significantly decreased at P40 (Figure 5, K and L) and was completely absent in mutants at

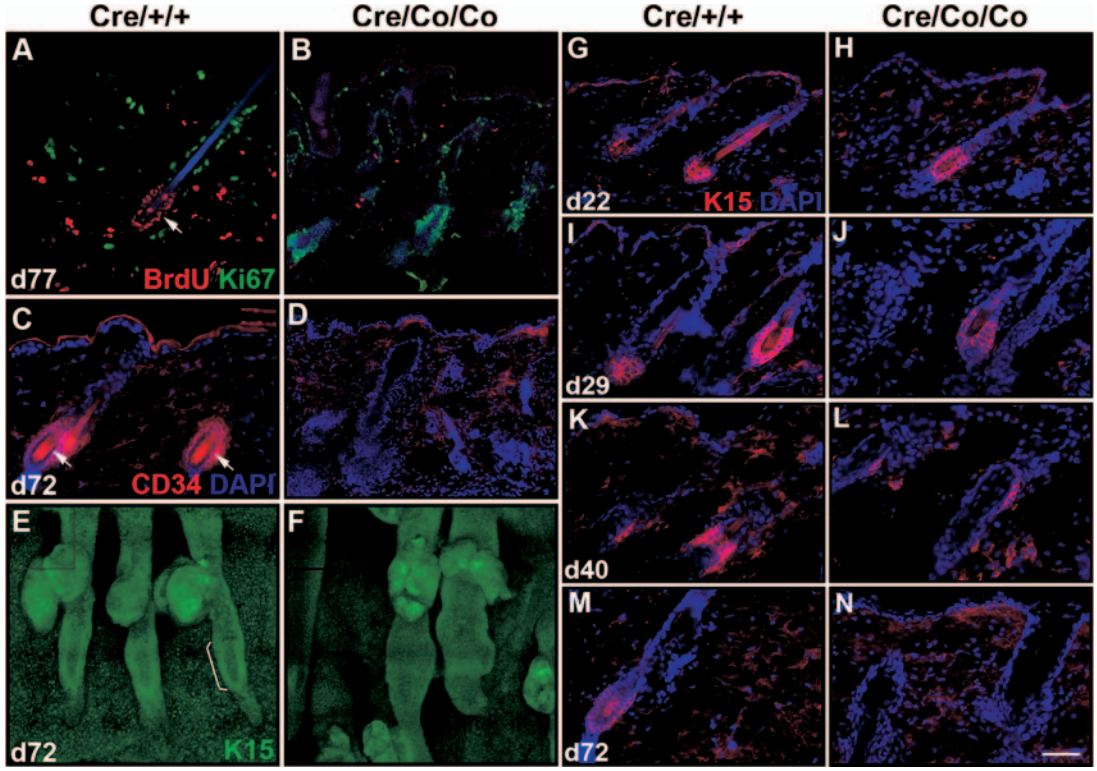


**Figure 5.** *Smad4* deletion led to depletion of the follicle SC compartment. (A and B) Costaining of BrdU and Ki67 after a 77-d chase. Note that LRCs were detected in the bulge of HFs of wild-type controls (A, bracket), but barely detected in *Smad4* mutants (B). (C–N) Immunofluorescence of CD34 and K15 marks the HF bulge of wild-type mice (C, E, G, I, K, and M), whereas in *Smad4* mutant mice they were gradually lost (D, F, H, J, L, and N). Bar, (A, C, G, and N) 35 $\mu$m; (B and D–F) 70 $\mu$m.

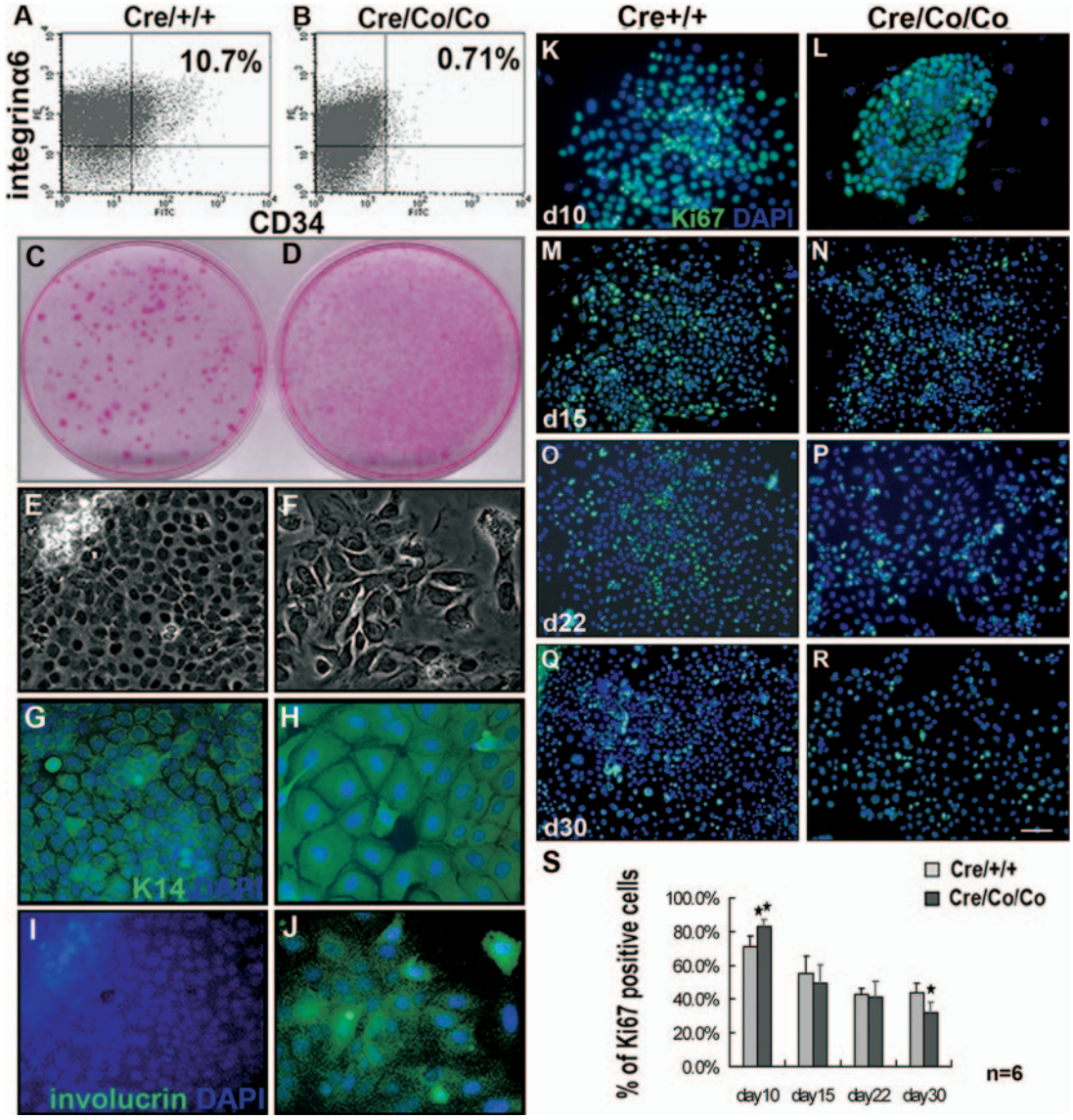


**Figure 6.** Significantly decreased colony-forming efficiency of *Smad4* mutant keratinocytes. (A and B) Flow cytometry of α6-integrin and CD34 double-labeled keratinocytes from dorsal epidermis of wild-type and *Smad4* mutant mice at P72. (C–J) Clonal forming assay with $1 \times 10^5$ total keratinocytes from 6- to 8-wk-old control (C, E, G, and I) and *Smad4* mutant mice (D, F, H, and J). Microscopic view of wild-type (E) and *Smad4* mutant (F) colonies. Immunofluorescence on wild-type and *Smad4* mutant keratinocytes in culture for K14 (G and H), involucrin (I and J), and Ki67 (K–R). Bar, (E–J) 20 μm; (K and L) 25 μm; (M–R) 50 μm.

P72 (Figure 5, M and N). The gradually diminishing expression of CD34 in the bulge is also illustrated in Supplemental Figure S4. The number of CD34-positive cells that expressed high levels of a6-integrin was significantly decreased in *Smad4* mutants at P72 (13.2 ± 5.5% in controls vs. 2.0 ± 1.3% in *Smad4* mutants, n = 4; Figure 6, A and B).

To further study the effect of *Smad4* inactivation on SC function, we carried out assays of clone formation. After a 32-d culture, wild-type SCs gave rise to large, round clones (Figure 6C) containing tightly arranged, small, round cells (Figure 6E). In contrast, *Smad4* mutant cells produced small, irregularly shaped, and fully differentiated colonies (Figure 6, D and F). Immunofluorescent detection of K14 revealed that clones derived from both wild-type and *Smad4* mutant keratinocytes were K14 positive (Figure 6, G and H). Notably, *Smad4* mutant, but not wild-type, keratinocytes expressed the terminal differentiation marker involucrin (Figure 6, I and J). Mutant keratinocytes contained a greater percentage of Ki67-positive cells at early stages of culture compared with controls (day 10, Figure 6, K, L, and S). A comparable percentage of Ki67-positive cells was detected in mutant and control keratinocytes cultured for 15 and 22 d (Figure 6, M–P and S). When cultured for 30 d, the majority of mutant keratinocytes were differentiated, and the percentage of proliferating cells was lower than controls (Figure 6, Q—S). These results suggested that the SC compartment might be reduced in *Smad4* mutant epidermis.

### *The Increased Nuclear Localization of β-Catenin and Increased c-Myc Expression in Smad4 Mutant Epidermis*

Stabilization of β-catenin is essential for promoting the transition from quiescent bulge SCs into transit-amplifying cells (Lowry *et al.*, 2005). Increased nuclear staining of active

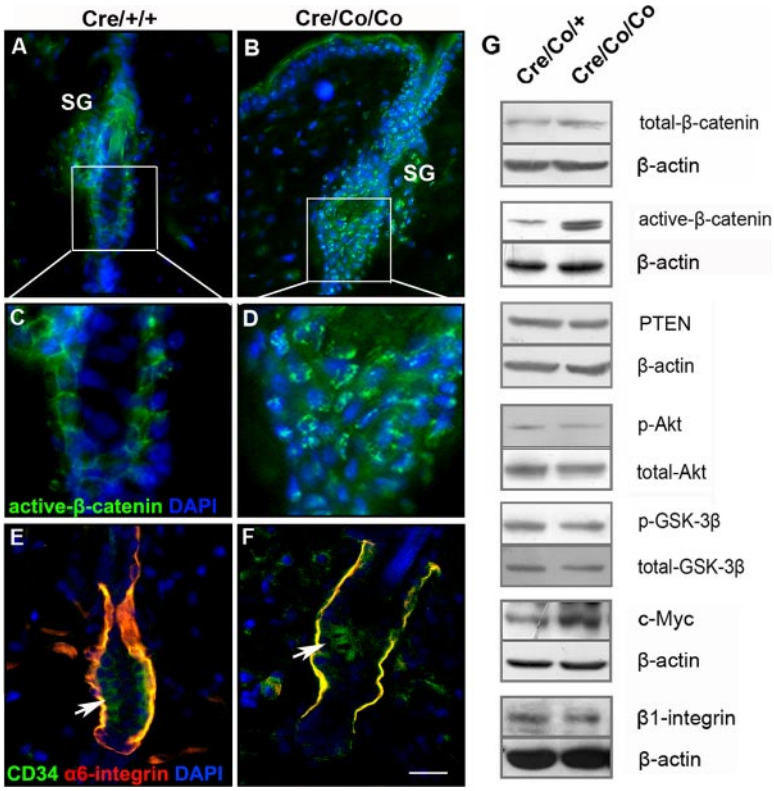


**Figure 7.** The increased nuclear localization of β-catenin and increased c-Myc expression in *Smad4* mutant epidermis. (A–D) The immunofluorescence of active-β-catenin in the HFs of the control (A and C) and *Smad4* mutant mice (B and D) at P42. (E and F) The immunofluorescence of α6-integrin and CD34 in HFs showed that α6-integrin was down-regulated in SC niche of *Smad4* mutants at P42. (G) The expression of total β-catenin, active-β-catenin, PTEN, p-Akt, p-GSK-3β, c-Myc, and β1-integrin in epidermis of P42 *Smad4* mutant and control mice were detected by Western blot analyses. Bar, (A and B) 35 μm; (C and D) 15 μm; (E and F) 25 μm.

β-catenin was observed in bulge keratinocytes of *Smad4* mutants at telogen (Figure 7, A–D). Western blots confirmed the increased level of active β-catenin in *Smad4* mutants, whereas total level β-catenin was not altered (Figure 7G). Pten, phospho-Akt (the active form of Akt), and phospho-GSK-3β were at normal levels in *Smad4* mutant epidermis compared with that in controls (Figure 7G). C-Myc has been reported as a downstream target of β-catenin/TCF–signaling pathways (He *et al.*, 1998), and both increased and decreased c-Myc levels have been shown to deplete epidermal SCs by modulating adhesive interactions with the local microenvironment (Waikel *et al.*, 2001; Braun *et al.*, 2003; Frye *et al.*, 2003; Zanet *et al.*, 2005). We found an increase in c-Myc expression in *Smad4* mutant epidermis, whereas β1-integrin expression was not altered (Figure 7G); in contrast, α6-integrin levels were diminished in *Smad4* mutant SC niche where CD34 was expressed (Figure 7, E and F).

## DISCUSSION

This study revealed an essential role for Smad4 in the regulation of follicle SCs. We have shown that disruption of *Smad4* in mouse epidermis, including bulge follicle SCs, results in persistent activation of follicle SCs, ultimately leading to their depletion. We further show that both β-catenin and c-Myc are up-regulated in *Smad4* mutants, independent of overactivation of the PI3K/AKT signaling pathway.

Many studies have indicated that TGF-β–signaling pathways, in particular BMP signals, play crucial roles in the regulation of follicle SC activation. Microarray profiling of follicle SCs has led to the identification of genes in the TGF-β pathways that are preferentially expressed in the bulge (Fuchs, 2007). Latent TGF-β–binding protein 1 and p-Smad2 expression in the bulge are confirmed by immunofluorescence (Morris *et al.*, 2004; Tumbar *et al.*, 2004). Recent studies revealed that cyclic dermal BMP signals control follicle SC activation during hair regeneration (Plikus *et al.*, 2008), whereas dynamic expression of Noggin, a BMP antagonist leads to activation of follicle SCs and initiation of the anagen phase (Zhang *et al.*, 2006). Conditional ablation of *BMPR1A* resulted in activation of epidermal SCs and loss of a quiescent niche (Zhang *et al.*, 2006; Kobielak *et al.*, 2007). In this study, we showed that Smad4 was expressed in bulge SCs and that targeted disruption of *Smad4* caused overactivation of follicle SCs, leading to significantly thickened IFE and hypertrophic HFs and SGs. The exit from the SC compartment was accelerated in young *Smad4* mutants, indicated by an expanded LRC zone and a transit increase in proliferation. Several lines of evidence suggested that conditional deletion of *Smad4* resulted in the depletion of follicle SCs. In relatively young *Smad4* mutant mice (P72), some keratinocytes in the expanded LRC zone were also marked by Ki67. Continued proliferation of LRCs eventually led to the loss of main features of follicle SCs, BrdU label retention, and K15 expression. The expression of CD34 which were important for bulge SC tumorigenesis (Trempus *et al.*, 2007) were also lost. Together, these data indicate that, in *Smad4* mutants, overactivated SCs generated more transient amplifying cells, at the expense of their self-renewal. As the population of SCs was exhausted, the number of transient amplifying cells was gradually decreased in Smad4 mutants. Consequently, the hypertrophic phenotypes in IFE, HFs, and SGs withered in aged *Smad4* mutant mice (6 and 12 mo) and there was scarcely any skin tumorigenesis in *Smad4* mutant older than 9 mo (Supplemental Figure S2). Additional evidence for follicle SC depletion came from the colony-forming experiments. *Smad4* mutant keratinocytes gave rise to abortive clones consisting of large, terminally differentiated keratinocytes after a 32-d culture. These findings suggest an important role of Smad4 in maintaining homeostasis of fol-

licle SCs. Surprisingly, wound healing experiments showed that 10-mo-old *Smad4* mutants were able to heal the wound at a rate comparable to that of controls (Supplemental Figure S6, G and H), although the reepithelialization rate was significantly reduced compared with that at 2 mo (Supplemental Figure S6, F, H, and J). This finding suggests that follicle SCs are not necessary for cutaneous wound healing, which is supported by a recent observation that an extended epidermal response heals cutaneous wounds in the absence of a follicular SC contribution (Langton *et al.*, 2008).

Our findings may not completely concur with the reports that induced conditional inactivation of *BMPR1A* leads to overproduction of HF stem/progenitor cells (Zhang *et al.*, 2006) or expansion of early progenitors of bulge cells (Kobielak *et al.*, 2007). The deletion of *Smad4* would be expected to disrupt the cellular responses to both TGF-$\beta$ and BMP signals, resulting in a discrepancy between Smad4 and BMPR1A in epithelial differentiation as well as follicle SC homeostasis maintenance. In our previous study, we found that *Smad4* mutants showed defective programmed regression of HFs, which is in line with the observations that loss of TGF-$\beta$1 results in failure of catagen induction (Foitzik *et al.*, 2000; Yang *et al.*, 2005). Moreover, the *Smad4* mutant mice exhibit failure in hair cycling and skin tumor formation, which resemble the phenotypes of *BMPR1A*-deficient mice (Andl *et al.*, 2004; Ming Kwan *et al.*, 2004; Yang *et al.*, 2005; Zhang *et al.*, 2006). Deletion of Smad4 may intercept BMP in repressing follicle SCs activation; however, we could not exclude the possibility that Smad4-mediated TGF-$\beta$ signaling is also required for the maintenance of the follicle SCs. How BMP signals are orchestrated with other TGF-$\beta$ signals or antagonists to regulate the activation, proliferation, and maintenance of follicle SCs needs to be further investigated.

We have provided evidence showing that overactivation of $\beta$-catenin might contribute to the depletion of follicle SCs in *Smad4* mutants. Transient increased levels of $\beta$-catenin or transient activation of $\beta$-catenin leads to accelerated transition from the resting to the growth phase of the hair cycle and new follicle formation (Van Mater *et al.*, 2003; Lo Celso *et al.*, 2004; Silva-Vargas *et al.*, 2005). Activated $\beta$-catenin is usually restricted to in the bulge region when follicle SCs are activated during the early anagen phase (DasGupta and Fuchs, 1999; Zhang *et al.*, 2006). Our previous study showed that cyclin D1 and c-Myc, the direct targets of Wnt/$\beta$-catenin signaling, are both significantly up-regulated in *Smad4* mutants (Yang *et al.*, 2005). In this study, we further showed that increased nuclear staining of $\beta$-catenin in the bulge region was correlated with the overactivation of follicle SCs. A recent study has provided genetic evidence showing that cyclic dermal BMP signaling prevents the activation of bulge SCs, a process usually involving periodic $\beta$-catenin activity (Plikus *et al.*, 2008). Consistently, enhanced $\beta$-catenin stabilization is presented in *BMPR1A*-deficient mice and is accompanied by the activation of the PI3K/AKT pathway (Zhang *et al.*, 2006; Kobielak *et al.*, 2007). However, no alteration of Pten, p-Akt and p-GSK-3$\beta$ were detected in *Smad4* mutants, suggesting that Smad4 functions differently from BMPR1A in maintaining follicle SCs homeostasis.

Balanced c-Myc expression appears to be crucial for the maintenance of follicle SCs, because its overexpression in transgenic mice depletes follicle SCs and drives them to terminal differentiation (Arnold and Watt, 2001; Waikel *et al.*, 2001). Conditional loss of endogenous *c-Myc* also leads to a loss of epidermal SCs and precocious differentiation of basal epidermal cells (Zanet *et al.*, 2005). Previous studies have shown that expression of c-Myc could be activated by $\beta$-catenin in colorectal cancer cells (He *et al.*, 1998). In this study, we showed that up-regulation of c-Myc was correlated with increased nuclear localization of $\beta$-catenin in *Smad4* mutants. The consistently up-regulated c-Myc led to transient epidermal hyperproliferation and skin tumor formation (Yang *et al.*, 2005) and eventually caused the depletion of follicle SCs. Up-regulation of c-Myc in *Smad4* mutants could also be due to blocked cellular responsiveness to TGF-$\beta$, because in vitro studies have demonstrated that TGF-$\beta$ inhibits cell growth through transcriptional repression of c-Myc (Frederick *et al.*, 2004).

Previous studies have suggested that c-Myc activation depletes the epidermal SC niche by reducing adhesive interactions with the local microenvironment (Frye *et al.*, 2003). In this study, we showed that c-Myc activation was associated with down-regulation of $\alpha$6-integrin expression, suggesting a potential role for c-Myc–induced suppression of adhesion in the stimulation of follicle SCs to differentiate. A c-Myc binding site has been found about 350–360 base pairs upstream of the transcription start site of human $\alpha$6-integrin promoter (Nishida *et al.*, 1997). Coexpression of H-Ras and c-Myc in a hematopoietic cell line significantly inhibits the expression of $\alpha$6-integrin and leads to a loss of adhesiveness to laminin (Nagashima *et al.*, 2001). Notably, sustained activation of c-Myc together with down-regulation of $\alpha$6-integrin expression leads to apoptosis and differentiation of human embryonic SCs (Sumi *et al.*, 2007).

In summary, we have shown that targeted ablation of *Smad4* in epidermis leads increased $\beta$-catenin nuclear localization independent of overactivation of the PI3K/Akt pathway. It also leads to c-Myc activation, which reduces $\alpha$6-integrin expression and results in the depletion of follicle SCs. These data suggest a critical role for Smad4 in the normal maintenance of follicle SCs and potentially in related disorders.

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## REFERENCES

Andl, T., *et al.* (2004). Epithelial Bmpr1a regulates differentiation and proliferation in postnatal hair follicles and is essential for tooth development. Development *131*, 2257–2268.

Arnold, I., and Watt, F. M. (2001). c-Myc activation in transgenic mouse epidermis results in mobilization of stem cells and differentiation of their progeny. Curr. Biol. *11*, 558–568.

Blanpain, C., and Fuchs, E. (2006). Epidermal stem cells of the skin. Annu. Rev. Cell Dev. Biol. *22*, 339–373.

Blanpain, C., Lowry, W. E., Geoghegan, A., Polak, L., and Fuchs, E. (2004). Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. Cell *118*, 635–648.

Botchkarev, V. A., *et al.* (1999). Noggin is a mesenchymally derived stimulator of hair-follicle induction. Nat. Cell Biol. *1*, 158–164.

Braun, K. M., Niemann, C., Jensen, U. B., Sundberg, J. P., Silva-Vargas, V., and Watt, F. M. (2003). Manipulation of stem cell proliferation and lineage commitment: visualisation of label-retaining cells in wholemounts of mouse epidermis. Development *130*, 5241–5255.

Cotsarelis, G., Sun, T. T., and Lavker, R. M. (1990). Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell *61*, 1329–1337.

DasGupta, R., and Fuchs, E. (1999). Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. Development *126*, 4557–4568.

Foitzik, K., *et al.* (2000). Control of murine hair follicle regression (catagen) by TGF-beta1 in vivo. FASEB J. *14*, 752–760.

Frederick, J. P., Liberati, N. T., Waddell, D. S., Shi, Y., and Wang, X. F. (2004). Transforming growth factor beta-mediated transcriptional repression of c-myc is dependent on direct binding of Smad3 to a novel repressive Smad binding element. Mol. Cell. Biol. *24*, 2546–2559.

Frye, M., Gardner, C., Li, E. R., Arnold, I., and Watt, F. M. (2003). Evidence that Myc activation depletes the epidermal stem cell compartment by modulating adhesive interactions with the local microenvironment. Development *130*, 2793–2808.

Fuchs, E. (2007). Scratching the surface of skin development. Nature *445*, 834–842.

He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998). Identification of c-MYC as a target of the APC pathway. Science *281*, 1509–1512.

Ito, Y. *et al*. (2001). Over expression of Smad2 reveals its concerted action with Smad4 in regulating TGF-beta-mediated epidermal homeostasis. Dev. Biol. *236*, 181–194.

Kobielak, K., Pasolli, H. A., Alonso, L., Polak, L., and Fuchs, E. (2003). Defining BMP functions in the hair follicle by conditional ablation of BMP receptor IA. J. Cell Biol. *163*, 609–623.

Kobielak, K., Stokes, N., de la Cruz, J., Polak, L., and Fuchs, E. (2007). Loss of a quiescent niche but not follicle stem cells in the absence of bone morphogenetic protein signaling. Proc. Natl. Acad. Sci. USA *104*, 10063–10068.

Kulessa, H., Turk, G., and Hogan, B. L. (2000). Inhibition of Bmp signaling affects growth and differentiation in the anagen hair follicle. EMBO J. *19*, 6664–6674.

Langton, A. K., Herrick, S. E., and Headon, D. J. (2008). An extended epidermal response heals cutaneous wounds in the absence of a hair follicle stem cell contribution. J. Invest. Dermatol. *128*, 1311–1318.

Levy, V., Lindon, C., Harfe, B. D., and Morgan, B. A. (2005). Distinct stem cell populations regenerate the follicle and interfollicular epidermis. Dev. Cell *9*, 855–861.

Lo Celso, C., Prowse, D. M., and Watt, F. M. (2004). Transient activation of beta-catenin signalling in adult mouse epidermis is sufficient to induce new hair follicles but continuous activation is required to maintain hair follicle tumours. Development *131*, 1787–1799.

Lowry, W. E., Blanpain, C., Nowak, J. A., Guasch, G., Lewis, L., and Fuchs, E. (2005). Defining the impact of beta-catenin/Tcf transactivation on epithelial stem cells. Genes Dev. *19*, 1596–1611.

Merrill, B. J., Gat, U., DasGupta, R., and Fuchs, E. (2001). Tcf3 and Lef1 regulate lineage differentiation of multipotent stem cells in skin. Genes Dev. *15*, 1688–1705.

Ming Kwan, K., Li, A. G., Wang, X. J., Wurst, W., and Behringer, R. R. (2004). Essential roles of BMPR-IA signaling in differentiation and growth of hair follicles and in skin tumorigenesis. Genesis *39*, 10–25.

Morris, R. J., Liu, Y., Marles, L., Yang, Z., Trempus, C., Li, S., Lin, J. S., Sawicki, J. A., and Cotsarelis, G. (2004). Capturing and profiling adult hair follicle stem cells. Nat. Biotechnol. *22*, 411–417.

Mou, C., Jackson, B., Schneider, P., Overbeek, P. A., and Headon, D. J. (2006). Generation of the primary hair follicle pattern. Proc. Natl. Acad. Sci. USA *103*, 9075–9080.

Nagashima, G., Asai, J., Suzuki, R., and Fujimoto, T. (2001). Different distribution of c-myc and MIB-1 positive cells in malignant meningiomas with reference to TGFs, PDGF, and PgR expression. Brain Tumor Pathol. *18*, 1–5.

Nishida, K., Kitazawa, R., Mizuno, K., Maeda, S., and Kitazawa, S. (1997). Identification of regulatory elements of human alpha 6 integrin subunit gene. Biochem. Biophys. Res. Commun. *241*, 258–263.

Owens, P., Bazzi, H., Engelking, E., Han, G., Christiano, A. M., and Wang, X. J. (2008). Smad4-dependent desmoglein-4 expression contributes to hair follicle integrity. Dev. Biol.

Plikus, M. V., Mayer, J. A., de la Cruz, D., Baker, R. E., Maini, P. K., Maxson, R., and Chuong, C. M. (2008). Cyclic dermal BMP signalling regulates stem cell activation during hair regeneration. Nature *451*, 340–344.

Qiao, W., Li, A. G., Owens, P., Xu, X., Wang, X. J., and Deng, C. X. (2006). Hair follicle defects and squamous cell carcinoma formation in Smad4 conditional knockout mouse skin. Oncogene *25*, 207–217.

Sharov, A. A., Sharova, T. Y., Mardaryev, A. N., Tommasi di Vignano, A., Atoyan, R., Weiner, L., Yang, S., Brissette, J. L., Dotto, G. P., and Botchkarev, V. A. (2006). Bone morphogenetic protein signaling regulates the size of hair follicles and modulates the expression of cell cycle-associated genes. Proc Natl. Acad. Sci. USA *103*, 18166–18171.

Shi, Y., and Massague, J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell *113*, 685–700.

Silva-Vargas, V., Lo Celso, C., Giangreco, A., Ofstad, T., Prowse, D. M., Braun, K. M., and Watt, F. M. (2005). Beta-catenin and Hedgehog signal strength can specify number and location of hair follicles in adult epidermis without recruitment of bulge stem cells. Dev. Cell *9*, 121–131.

Sumi, T., Tsuneyoshi, N., Nakatsuji, N., and Suemori, H. (2007). Apoptosis and differentiation of human embryonic stem cells induced by sustained activation of c-Myc. Oncogene *26*, 5564–5576.

Sun, Q., *et al.* (2008). Transforming growth factor-beta-regulated miR-24 promotes skeletal muscle differentiation. Nucleic Acids Res. *36*, 2690–2699.

Trempus, C. S., *et al.* (2007). CD34 expression by hair follicle stem cells is required for skin tumor development in mice. Cancer Res. *67*, 4173–4181.

Tumbar, T., Guasch, G., Greco, V., Blanpain, C., Lowry, W. E., Rendl, M., and Fuchs, E. (2004). Defining the epithelial stem cell niche in skin. Science *303*, 359–363.

Van Mater, D., Kolligs, F. T., Dlugosz, A. A., and Fearon, E. R. (2003). Transient activation of beta-catenin signaling in cutaneous keratinocytes is sufficient to trigger the active growth phase of the hair cycle in mice. Genes Dev. *17*, 1219–1224.

Waikel, R. L., Kawachi, Y., Waikel, P. A., Wang, X. J., and Roop, D. R. (2001). Deregulated expression of c-Myc depletes epidermal stem cells. Nat. Genet. *28*, 165–168.

Yang, L., *et al.* (2005). Targeted disruption of Smad4 in mouse epidermis results in failure of hair follicle cycling and formation of skin tumors. Cancer Res. *65*, 8671–8678.

Yuan, C., *et al.* (2008). The up-regulation of 14-3-3 proteins in Smad4 deficient epidermis and hair follicles at catagen. Proteomics *8*, 2230–2243.

Zanet, J., Pibre, S., Jacquet, C., Ramirez, A., de Alboran, I. M., and Gandarillas, A. (2005). Endogenous Myc controls mammalian epidermal cell size, hyperproliferation, endoreplication and stem cell amplification. J. Cell Sci. *118*, 1693–1704.

Zhang, J., He, X. C., Tong, W. G., Johnson, T., Wiedemann, L. M., Mishina, Y., Feng, J. Q., and Li, L. (2006). Bone morphogenetic protein signaling inhibits hair follicle anagen induction by restricting epithelial stem/progenitor cell activation and expansion. Stem Cells *24*, 2826–2839.