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Embryonic hair follicle fate change by augmented β-catenin through Shh and Bmp signaling

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β-catenin signaling is one of the key factors regulating the fate of hair follicles (HFs). To elucidate the regulatory mechanism of embryonic HF fate determination during epidermal development/differentiation, we analyzed conditional mutant mice with keratinocytes expressing constitutively active β-catenin (K5-Cre Catnb$^{(ex3)fl/+}$). The mutant mice developed scaly skin with a thickened epidermis and showed impaired epidermal stratification. The hair shaft keratins were broadly expressed in the epidermis but there was no expression of the terminal differentiation markers K1 and loricrin. Hair placode markers (*Bmp2* and *Shh*) and follicular dermal condensate markers (noggin, patched 1 and *Pdgfra*) were expressed throughout the epidermis and the upper dermis, respectively. These results indicate that the embryonic epidermal keratinocytes have switched extensively to the HF fate. A series of genetic studies demonstrated that the epidermal switching to HF fate was suppressed by introducing the conditional mutation K5-Cre Catnb$^{(ex3)fl/+}$Shh$^{fl/-}$ (with additional mutation of Shh signaling) or K5-Cre Catnb$^{(ex3)fl/+}$BmprIA$^{fl/fl}$ (with additional mutation of Bmp signaling). These results demonstrate that Wnt/β-catenin signaling relayed through Shh and Bmp signals is the principal regulatory mechanism underlying the HF cell fate change. Assessment of *Bmp2* promoter activities suggested a putative regulation by β-catenin signaling relayed by Shh signaling towards Bmp2. We also found that Shh protein expression was increased and expanded in the epidermis of K5-Cre Catnb$^{(ex3)fl/+}$BmprIA$^{fl/fl}$ mice. These results indicate the presence of growth factor signal cross-talk involving β-catenin signaling, which regulates the HF fate.

KEY WORDS: Skin, Hair follicle (HF), Wnt, β-catenin, Bmp, Shh, Cell fate

INTRODUCTION

Recent studies have implicated members of the Wnt/β-catenin signaling pathway as vital regulators of the epithelial-mesenchymal interactions that specify the development of hair follicles (HFs) (Fuchs, 2007; Yu et al., 2008). The essential role of Wnt/β-catenin signaling during HF morphogenesis has been suggested by transgenic and knockout mouse studies (Andl et al., 2002; Gat et al., 1998; Huelsken et al., 2001; Lo Celso et al., 2004). Recent studies using embryos have revealed that embryonic HF fate change, HF differentiation and its excessive induction are induced by stabilized β-catenin (Narhi et al., 2008; Zhang et al., 2008).

Besides Wnt/β-catenin signaling, Bmp (bone morphogenetic protein) and Shh (sonic hedgehog) signaling have also been suggested to regulate HF formation. Bmp signaling has been suggested to regulate HF induction and the patterning of follicles within the skin by repressing the placode fate (Botchkarev et al., 1999; Jamora et al., 2003; Jiang et al., 1999; Noramly and Morgan, 1998; Rendl et al., 2008). Shh signaling regulates HF cell proliferation and morphogenesis (Chiang et al., 1999; St-Jacques et al., 1998). However, the mechanisms involved in the downstream effects of Wnt/β-catenin signaling to regulate HF fate are poorly understood.

To elucidate whether the embryonic HF fate change is regulated by several growth factor signaling pathways associated with Wnt/β-catenin signaling, a conditional cutaneous-specific recombination strategy was employed using a stabilized β-catenin allele, i.e. a β-catenin gene with exon 3 encoding serine and threonine residues flanked by LoxP sites [β-catenin flox(ex3); hereafter designated as Catnb$^{(ex3)fl/+}$]. Cre recombinase-mediated excision leads to the expression of a stabilized, constitutively active form of β-catenin (Harada et al., 1999). We observed that hair placodes and the dermal condensate expanded and that embryonic epidermal keratinocytes displayed an HF-like differentiation in K5-Cre Catnb$^{(ex3)fl/+}$ mutant mice. Intriguingly, those phenotypes were suppressed by introducing an additional conditional mutation: K5-Cre Catnb$^{(ex3)fl/+}$BmprIA$^{fl/fl}$ or K5-Cre Catnb$^{(ex3)fl/+}$Shh$^{fl/-}$. These results demonstrate that growth factor signal cross-talk under conditions of activated β-catenin are mediated through Shh and Bmp signaling, and are the principal mechanisms for regulating HF fate. The assessment of the Bmp(s) promoter activity and analysis of Shh protein expression also provided clues to understand the mechanisms of signal cross-talk during embryonic HF fate change.

MATERIALS AND METHODS

Mouse mutant alleles

The Catnb$^{(ex3)fl/+}$, BmprIA$^{fl/fl}$, Shh$^{fl/fl}$ and Shh$^{f/-}$ alleles, and the keratin 5-Cre (K5-Cre) strain have been described previously (Chiang et al., 1996; Harada et al., 1999; Mishina et al., 2002; Tarutani et al., 1997) (Jackson Laboratories Stock #004293). The BAT-*lac*Z mouse containing a construct including the Tcf/Lef-binding sites has also been described (Nakaya et al., 2005). Sampling of dorsal skin specimens was performed

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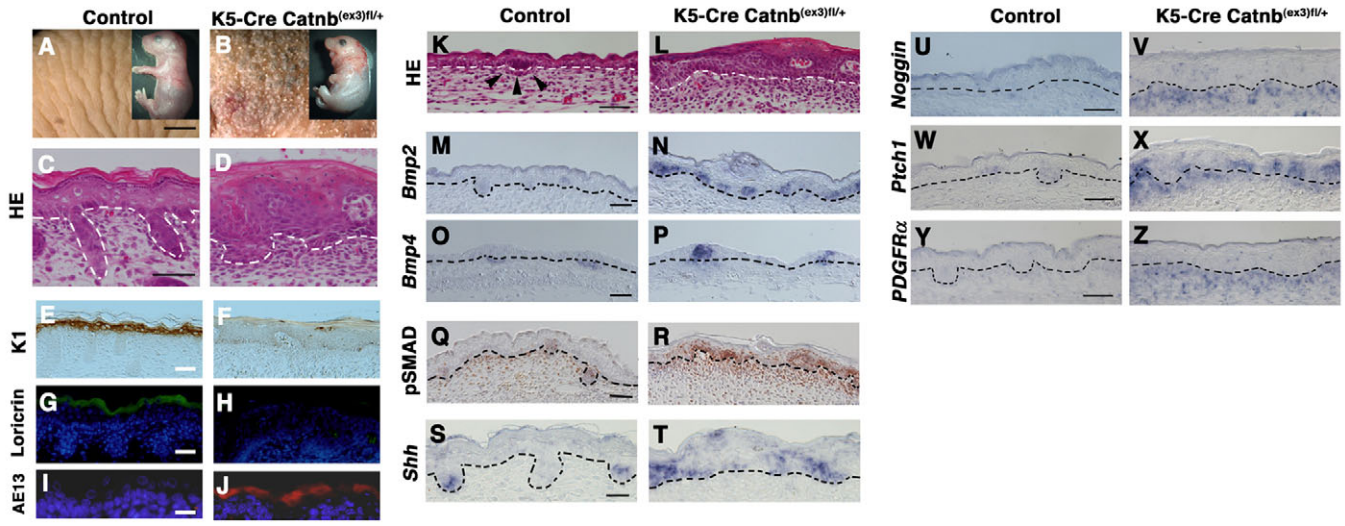

Fig. 1. Switching of embryonic epidermal keratinocytes to HF fate in K5-Cre Catnb$^{(ex3)fl/+}$ mutant skin. (**A**,**B**) Gross appearance of control and of K5-Cre Catnb$^{(ex3)fl/+}$ mutant skin at E18.5. (**C**,**D**) Histology of control and of K5-Cre Catnb$^{(ex3)fl/+}$ mutant skin at E18.5. (**E-H**) Epidermal differentiation marker expression: K1 (brown) and loricrin (green) at E18.5. (**I**,**J**) Immunostaining with AE13 antibody to detect hair shaft keratins (red) at E18.5. (**K**,**L**) Histological alteration of the K5-Cre Catnb$^{(ex3)fl/+}$ mutant dermis compared with control, showing the dermal condensate throughout the upper dermis. Arrowheads in K indicate dermal condensate. (**M**,**N**) *Bmp2* expression is broadly induced in K5-Cre Catnb$^{(ex3)fl/+}$ mutant epidermis at E16.5. (**O**,**P**) *Bmp4* expression is ectopically detected in the mutant epidermis at E15.0. (**Q**,**R**) The pSMAD level is prominently increased in the mutant epidermis and dermis compared with the control. (**S**,**T**) *Shh* expression is broadly detected in the mutant epidermis at E18.5. (**U-Z**) The dermal condensate markers noggin, *Ptch1* and *Pdgfra* are expressed throughout the upper dermis in K5-Cre Catnb$^{(ex3)fl/+}$ skin at E16.5. Dashed lines indicate the dermal-epithelial border. Scale bars: 1 mm for A,B; 50 μm for C-J,M-R; 25 μm for K,L,S-Z.

between embryonic day (E) 10.5 and E18.5. All animal experiments were approved by the Animal Study Committee of the Kumamoto University School of Medicine.

Histology, immunohistochemistry and X-gal staining analysis

The gross skin phenotype images were captured using the VHX system (Keyence). Embryonic dorsal skin specimens were fixed overnight in 4% paraformaldehyde (PFA)/PBS, dehydrated in methanol and embedded in paraffin. Serial sections (6 μm) were prepared for Hematoxylin and Eosin (HE) staining and immunohistochemistry.

Antibodies used were: keratin 1 (Covance PRB-165P), AE13 (AbCam), loricrin (Covance PRB-145P), β-catenin (BD Bioscience), Ki67 (Novo Castra), Shh (Santa Cruz H-160) and pSmad1/5/8 (Cell Signaling) (Ahn et al., 2001). Secondary antibodies were conjugated to Alexa Fluor 488 or 546 IgGs (Molecular Probes/Invitrogen). X-gal staining was performed as described previously (Haraguchi et al., 2007).

In situ hybridization for gene expression analysis

In situ hybridization analysis was performed on 8-μm paraffin sections of embryonic back skin (Suzuki et al., 2008) with probes for *Bmp2*, *Shh*, *Lef1*, *Dkk1*, *Msx2* and *Pdgfra* (kindly provided by B. L. Hogan, C. Shukunami, H. Clevers, U. Rüther, Y. Liu and P. Soriano, respectively), *Ptch1* (Goodrich et al., 1996), *Bmp4* (Jones et al., 1991) and noggin (McMahon et al., 1998). The *Wnt10b* probe was generated by PCR using the following primers (F, 5′-GCG GGT CTC CTG TTC TTG GC-3′; R, 5′-AGA GGC GGC TGG TCT TGT TG-3′).

Promoter assay with luciferase reporter activity

Bmp2 and *Bmp4* promoter reporter constructs contain murine gene fragments of 1725 bp (–410 to +1315) and 1828 bp (–1402 to +426), respectively, in the reporter gene plasmid pGL3 basic (Invitrogen); numbers are relative to the transcriptional start site. HaCaT cells were plated into 24-well plates at 2×10^5 cells per well in DMEM/10% FBS 24 hours prior to transfection. The reporter plasmids were co-transfected with a control vector or with pcDNA3.1-His-mouse Gli2-delN2 (N-terminally truncated Gli2 as a strong activator for hedgehog signaling) (Sasaki et al., 1999), using the TransFast Transfection Reagent (Promega), and luciferase activity was measured using a Dual Luciferase Assay Kit (Promega) (Nishida et al., 2008).

RESULTS AND DISCUSSION

Augmented β-catenin switches embryonic epidermal keratinocytes to the HF fate

To examine whether embryonic HF fate is determined through signaling pathways regulated by β-catenin, conditional epidermal modulation of β-catenin signaling was employed. Keratin 5-Cre (K5-Cre)-mediated recombination and the expression kinetics of the constitutively active β-catenin in the developing skin epidermis are shown in Fig. S1 in the supplementary material.

K5-Cre Catnb$^{(ex3)fl/+}$ mutant mice displayed scaly skin with pillar-shaped comedo-like white spots in the embryonic epidermis (Fig. 1B). Histological analyses of the mutant embryos demonstrated a thickened epidermis without the granular layers at E18.5 (Fig. 1C,D; the kinetics of the morphological alterations are shown in Fig. S2 in the supplementary material). In addition, the mutant skin also showed abnormal epidermal differentiation and denser cell layers in the upper dermis (Fig. 1D). Interestingly, the mutant epidermis showed follicular keratinization with morphological trichilemma-type structures (see Fig. S3 in the supplementary material). To determine the degree of such structural changes, we analyzed the expression of terminal differentiation markers [K1 (Krt1 – Mouse Genome Informatics) and loricrin] and hair shaft keratins that are specifically recognized by the AE13 antibody (Lynch et al., 1986). Expression of K1 and loricrin was dramatically reduced in K5-Cre Catnb$^{(ex3)fl/+}$ skin at E18.5 (Fig. 1E-H). By contrast, hair shaft keratins were expressed broadly and strongly in the K5-Cre Catnb$^{(ex3)fl/+}$ mutant epidermis at E18.5, suggesting that augmented β-catenin signaling induces HF-like differentiation (Fig. 1E-J; the expression kinetics are shown in Fig. S4 in the supplementary material).

Embryonic HF morphogenesis is governed by epithelial-mesenchymal interactions between keratinocytes in the hair placode and fibroblasts in the mesenchymal condensate (Hardy, 1992; Oro and Scott, 1998; Sengel, 1976). Signals from the hair placode induced the underlying mesenchymal cells to condense (dermal

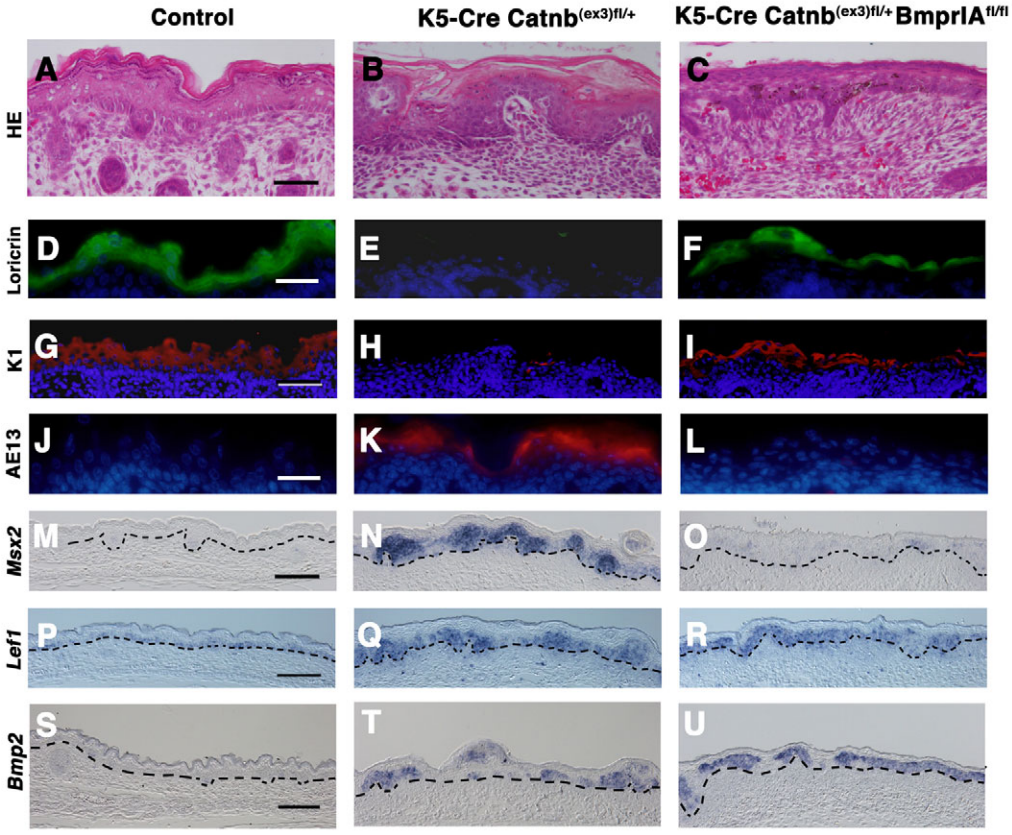

Fig. 2. The loss of Bmp signaling in the epidermis restores the K5-Cre Catnb$^{(ex3)fl/+}$ mutant HF-like differentiation.
(**A-C**) Histological analysis of control, K5-Cre Catnb$^{(ex3)fl/+}$ and double mutant skin at E18.5. (**D-L**) The restoration of loricrin (green) and K1 (red) expression, and the suppression of expression of hair shaft keratins recognized by AE13 antibody (red), in double mutant skin at E18.5. (**M-R**) In situ hybridization for *Msx2* and *Lef1* expression at E16.5. (**S-U**) Upon induction, expression of hair placode marker gene *Bmp2* remained in the K5-Cre Catnb$^{(ex3)fl/+}$ BmprIA$^{fl/fl}$ mutant epidermis. Dashed lines indicate the dermal-epithelial border. Scale bars: 50 μm for A-L; 100 μm for M-U.

condensate; Fig. 1K; arrowheads). K5-Cre Catnb$^{(ex3)fl/+}$ mutant skin showed such dermal condensates throughout the upper dermis at E16.5 (Fig. 1L, the kinetics of these morphological changes are shown in Fig. S2 in the supplementary material). To further analyze the basis of the excessive induction of HFs, the expression of hair placode markers (Bmps and *Shh*) and dermal condensate markers [noggin, patched 1 (*Ptch1*) and *Pdgfra*] was examined. *Bmp2* and *Bmp4* are expressed in the hair placode and in the underlying mesenchymal condensate, respectively, in control skin (Fig. 1M,O). *Bmp2* expression was increased broadly in the mutant epidermis at E16.5 (Fig. 1N). *Bmp4* expression was localized ectopically in the mutant epidermis at E15.0 with expanded expression in later stages (Fig. 1P; data not shown). To investigate the extent of Bmp signaling, the pSMAD levels were analyzed and were significantly increased in the mutant epidermis and in the underlying mesenchyme compared with the control at E16.5 (Fig. 1Q,R; see also Fig. S5 in the supplementary material). *Shh* expression was also broadly detected in the mutant epidermis at E18.5 (Fig. 1T). The induced expression of *Bmp2*, *Bmp4*, pSMAD, *Shh* and *Wnt10b* (another early placode marker) was already observed at E11.5 (see Figs S5, S6 in the supplementary material). Dermal condensate markers were expressed throughout the upper dermis in K5-Cre Catnb$^{(ex3)fl/+}$ mutant mice at E16.5 (Fig. 1U-Z). These results suggest that augmented β-catenin signaling induces the excessive HF induction and HF-like differentiation, leading to an HF fate.

Suppression of HF-like differentiation by the conditional mutation of K5-Cre Catnb$^{(ex3)fl/+}$BmprIA$^{fl/fl}$

To investigate the potential effect of the increased Bmp signaling in K5-Cre Catnb$^{(ex3)fl/+}$ mutant mice, a conditional double mutant (K5-Cre Catnb$^{(ex3)fl/+}$BmprIA$^{fl/fl}$) was examined. BmprIA (Bmpr1a – Mouse Genome Informatics) is a type I Bmp receptor and its signaling is essential for hair shaft differentiation (Yuhki et al., 2004). The HF-like epidermal differentiation observed in K5-Cre Catnb$^{(ex3)fl/+}$ mutant mice was suppressed by introduction of the double mutation at E18.5 (Fig. 2A-L). Loricrin and K1 expression were restored (Fig. 2E,F,H,I). In addition, the augmented AE13 epitope reactivity observed in K5-Cre Catnb$^{(ex3)fl/+}$ mutants was suppressed in the double mutants, confirming the dramatic suppression of HF-like differentiation (Fig. 2K,L). *Msx2* is one of the downstream target genes of Bmp signaling and regulates the expression of *Foxn1*, which controls the transcription of hair keratin genes (Ma et al., 2003; Meier et al., 1999). The expression of *Msx2* was dramatically upregulated in K5-Cre Catnb$^{(ex3)fl/+}$ mutant epidermis, whereas its expression suppressed in the double mutants at E16.5 (Fig. 2N,O). The Wnt/β-catenin pathway transcriptional effector Lef1 regulates differentiation of the hair shaft (Merrill et al., 2001). Its increased expression was maintained in the double mutant epidermis at E16.5 (Fig. 2Q,R). We also found that the region with the induced hair placode marker gene expression, which includes that of *Bmp2*, remained in the K5-Cre Catnb$^{(ex3)fl/+}$BmprIA$^{fl/fl}$ mutant epidermis (Fig. 2T,U; data not shown). These results indicate that the pathway in which β-catenin is relayed by Bmp signaling plays a principal role in inducing HF-like differentiation, but not in the excessive induction of HFs (Fig. 4H).

Suppression of excessive HF induction by the conditional mutation of K5-Cre Catnb$^{(ex3)fl/+}$Shh$^{fl/-}$

One of the prominent phenotypes caused by augmented β-catenin is aberrant HF patterning, the excessive hair placode induction with the underlying dermal condensate (Fig. 1) (Narhi et al., 2008; Zhang et al., 2008). The excessive induction of HFs was not suppressed in K5-Cre Cantb$^{(ex3)fl/+}$BmprIA$^{fl/fl}$ mutant skin (Fig. 2T,U).

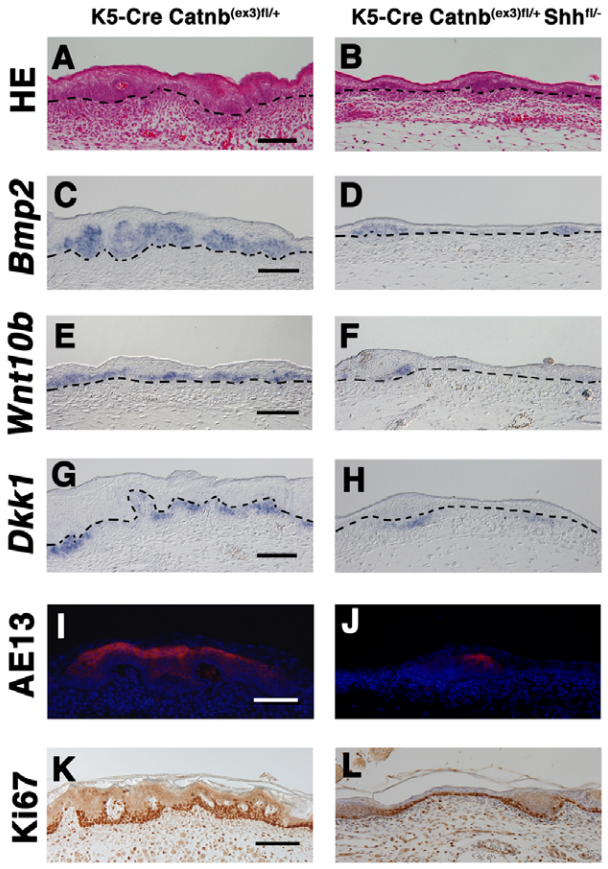

Fig. 3. The involvement of Shh signaling as a crucial downstream effector of β-catenin signaling for the excessive HF induction. (**A**,**B**) Suppression of excessive HF induction in the double conditional mutant K5-Cre Catnb$^{(ex3)fl/+}$Shh$^{fl/-}$ at E16.5. (**C-J**) Suppression of induced *Bmp2*, *Wnt10b* and *Dkk1* expression, and of AE13 antibody staining (red), in the double mutant skin at E16.5. (**K**,**L**) Cell proliferation analysis using Ki67 antibody at E18.5. Cell proliferation is increased in K5-Cre Catnb$^{(ex3)fl/+}$ mutant (K) and is suppressed in K5-Cre Cantb$^{(ex3)fl/+}$Shh$^{fl/-}$ double mutant (L) epidermis. Dashed lines indicate the dermal-epithelial border. Scale bars: 50 μm for A-L.

Shh controls cell proliferation and formation of the dermal papilla (Fuchs, 2007; Millar, 2002; Schmidt-Ullrich and Paus, 2005). Its overexpression leads to the induction of dermal condensate during feather formation (Ting-Berreth and Chuong, 1996) and its inhibition impairs dermal papilla formation (Nanba et al., 2003). Shh has been suggested to be regulated by the β-catenin signaling pathway (Huelsken et al., 2001; Zhang et al., 2008). Indeed, expression of *Shh* was increased in the skin of K5-Cre Cantb$^{(ex3)fl/+}$ mice (Fig. 1T). To elucidate whether the excessive induction of HFs is mediated by the Shh signaling pathway associated with augmented β-catenin signaling, we analyzed K5-Cre Cantb$^{(ex3)fl/+}$Shh$^{fl/-}$ double mutant skin. Shh signaling was indeed decreased in K5-Cre Cantb$^{(ex3)fl/+}$Shh$^{fl/-}$ skin based on reduced *Ptch1* expression at E14.5 (see Fig. S7 in the supplementary material). The number of hair placodes was increased in the K5-Cre Catnb$^{(ex3)fl/+}$ epidermis, spreading from the early-induced hair placodes (Fig. S2 in the supplementary material; data not shown). The excessive induction of HFs was suppressed in the double mutant skin based on reduced hair placode marker gene expression (*Bmp2* and *Wnt10b*) and reduced *Dkk1* expression at E16.5 (Fig. 3A-H). The expression of *Dkk1* is elevated in the dermis at sites of placode development in normal embryos (Andl et al., 2002). *Dkk1* expression was strongly induced in the K5-Cre Catnb$^{(ex3)fl/+}$ dermis, but its expression was significantly decreased in K5-Cre Cantb$^{(ex3)fl/+}$Shh$^{fl/-}$ skin throughout the upper dermis at E16.5 (Fig. 3H). The increased expression of dermal condensate markers (noggin and *Pdgfra*) was also suppressed in K5-Cre Cantb$^{(ex3)fl/+}$Shh$^{fl/-}$ skin (data not shown). Furthermore, the induction of HF-like differentiation was suppressed in the K5-Cre Cantb$^{(ex3)fl/+}$Shh$^{fl/-}$ mutant based on the reduced immunostaining observed for AE13 at E16.5 (Fig. 3I,J). We also observed decreased epidermal cell proliferation in K5-Cre Cantb$^{(ex3)fl/+}$Shh$^{fl/-}$ mutants compared with K5-Cre Catnb$^{(ex3)fl/+}$ mice at E18.5 (Fig. 3K,L). These results suggested that Shh signaling is a crucial downstream pathway of β-catenin signaling for the excessive induction of HFs with increased cell proliferation (Fig. 4H).

Wnt/β-catenin signaling may also be one of the genetic upstream pathways of Bmp during embryonic HF development (Huelsken et al., 2001; Narhi et al., 2008). The intensity of pSMAD staining in K5-Cre Cantb$^{(ex3)fl/+}$ mutant skin was suppressed in both the epidermis and the mesenchyme of K5-Cre Cantb$^{(ex3)fl/+}$Shh$^{fl/-}$ mutant skin at E16.5 (Fig. 4C, brackets). As for the regulatory mechanisms controlling Bmp expression, we found several candidate Lef/Tcf-binding sites in the 1.8-kb *Bmp4* promoter and several GLI-binding sites in the 1.7-kb *Bmp2* promoter using rVISTA bioinformatics analysis (Fig. 4D, yellow boxes; data not shown). Transient promoter assays showed that the *Bmp4* promoter was not regulated through stabilized β-catenin signaling under the current experimental conditions (data not shown), but revealed an increase of *Bmp2* promoter activity caused by Gli2 in vitro (Fig. 4D). In fact, the current double mutant analyses on K5-Cre Catnb$^{(ex3)fl/+}$Shh$^{fl/-}$ skin showed suppression of the increased *Bmp2* expression, suggesting that the regulation of Bmp signaling through Shh signaling is an essential molecular mechanism for the HF fate change (Fig. 3C,D). Increased *Bmp2* expression, the intensity of pSMAD staining and AE13 immunostaining remained in early-induced HFs of the K5-Cre Catnb$^{(ex3)fl/+}$Shh$^{fl/-}$ epidermis (Fig. 3; Fig. 4C, outside of the brackets). It has been shown that Shh signaling is not required for the initiation of HF formation and that HF differentiation is not inhibited in Shh mutant skin (Chiang et al., 1999; St-Jacques et al., 1998). Our current study indicates that Shh signaling is required for the expansion of hair follicle fate by augmented β-catenin signaling, although it is not required for either the initial specification of hair placodes or the differentiation of early-induced HFs.

The regulation of HF space has been considered to be controlled by diffusible molecules that either promote or repress follicular fate (Jiang et al., 2004; Mikkola and Millar, 2006; Millar, 2002). Previously, it was shown that Shh is one of the placode activators, while Bmps are generally regarded as being placode inhibitors that mediate lateral inhibition, which is known as the reaction-diffusion mechanism (Jung et al., 1998). Studies on chick embryonic skin suggested that Shh induces the expression of Bmps, whereas Bmps suppress *Shh* expression during feather development (Harris et al., 2005; Jung et al., 1998). We further analyzed the expression of Shh protein in K5-Cre Cantb$^{(ex3)fl/+}$BmprIA$^{fl/fl}$ skin. Interestingly, Shh protein expression was both increased and expanded in K5-Cre Cantb$^{(ex3)fl/+}$BmprIA$^{fl/fl}$ mutant epidermis at E16.5 (Fig. 4E-G).

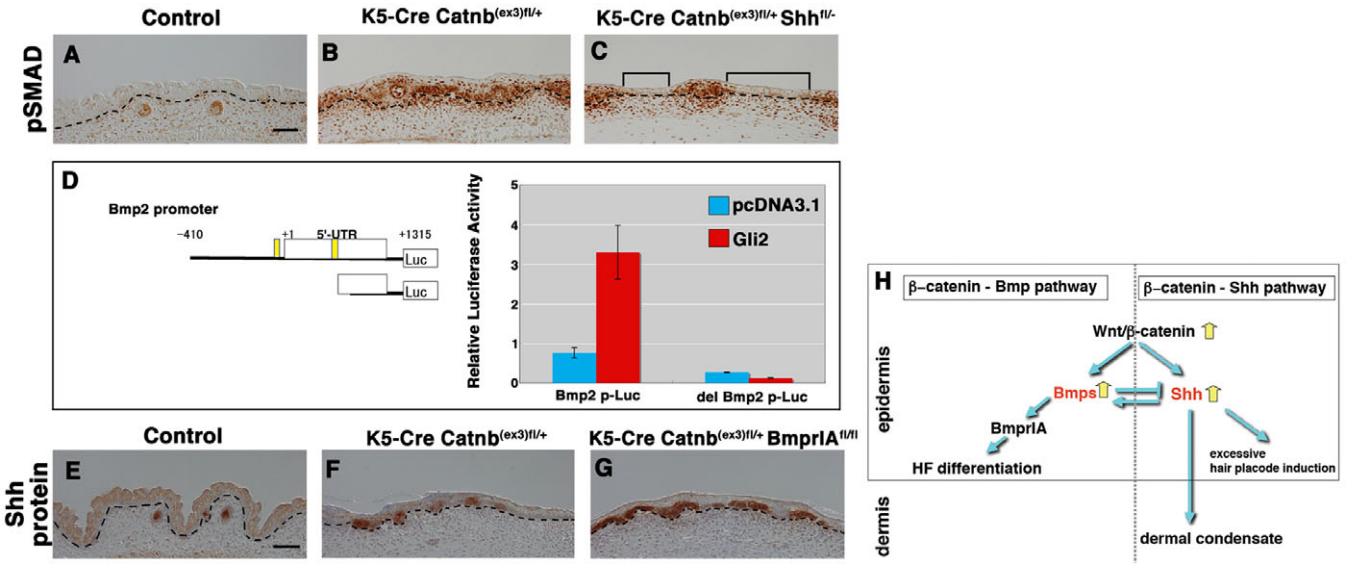

Fig. 4. A possible regulatory mechanism between Shh and Bmp signaling that underlies Wnt/β-catenin signaling pathway. (**A-C**) The intensity of pSMAD staining in K5-Cre Cantb$^{(ex3)fl/+}$ is suppressed both in the epidermis and the mesenchyme of K5-Cre Cantb$^{(ex3)fl/+}$Shh$^{fl/-}$ skin at E16.5 (C, brackets). Such intense pSMAD staining remains in early-induced HFs (outside of the brackets). (**D**) Activation of the *Bmp2* promoter (Bmp2 p-Luc) by introducing the activated Gli2 expression vector; the activation is diminished by deleting the two putative GLI-binding sites (yellow boxes; del Bmp2 p-Luc). (**E-G**) Shh protein expression is increased and expanded in K5-Cre Cantb$^{(ex3)fl/+}$BmprIA$^{fl/fl}$ mutant epidermis at E16.5. (**H**) Schematic of the growth factor network regulating HF fate change. Scale bars: 50 μm for A-C,E-G.

Taken together, the current results are in agreement with the reaction-diffusion mechanism, via the cross-talk between the activator (Shh signaling) and the inhibitor (Bmp signaling) implicated in the periodic patterning of HFs (Fig. 4H) (Jiang et al., 2004; Jung et al., 1998).

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/3/367/DC1

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