Smad7-Induced β-Catenin Degradation Alters Epidermal Appendage Development

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Summary
To assess whether Smad signaling affects skin development, we generated transgenic mice in which a Smad antagonist, Smad7, was induced in keratinocytes, including epidermal stem cells. Smad7 transgene induction perturbed hair follicle morphogenesis and differentiation, but accelerated sebaceous gland morphogenesis. Further analysis revealed that independent of its role in anti-Smad signaling, Smad7 bound β-catenin and induced β-catenin degradation by recruiting an E3 ligase, Smurf2, to the Smad7/β-catenin complex. Consequently, Wnt/β-catenin signaling was suppressed in Smad7 transgenic hair follicles. Co-expression of the Smurf2 and Smad7 transgenes exacerbated Smad7-induced abnormalities in hair follicles and sebaceous glands. Conversely, when endogenous Smad7 was knocked down, keratinocytes exhibited increased β-catenin protein and enhanced Wnt signaling. Our data reveal a mechanism for Smad7 in antagonizing Wnt/β-catenin signaling, thereby shifting the skin differentiation program from forming hair follicles to sebaceous glands.

Introduction
Murine hair follicle morphogenesis starts at an embryonic stage and proceeds through the typical eight stages (Philpott and Paus, 1998). At approximately embryonic day 14.5 (E14.5), certain epidermal stem cells that reside in the basal layer of the epidermis begin to designate their fate toward hair follicle morphogenesis. During hair follicle development, some stem cells migrate downward along growing hair germ to form hair progenitor cells (matrix cells) in the hair bulb. The hair progenitor cells will further differentiate into the outer root sheath (ORS), the inner root sheath (IRS), and the hair shaft. As hair follicle morphogenesis is completed perinatally, sebaceous glands begin to develop from cells residing in a bulge area at the upper portion of the hair follicle. Postnatal hair follicles cycle through phases of growth (anagen), regression (catagen), and rest (telogen) during which sebaceous glands alter their size proportionally. The hair follicle bulge is more prominent postnatally and is a niche housing multipotent epidermal stem cells, which are responsible for the self-renewal of hair follicle cells, sebaceous gland cells, and postinjury interfollicular epidermal cells (Fuchs et al., 2004; Watt, 1998; Lavker et al., 2003; Levy et al., 2005). Hair follicle morphogenesis is tightly controlled by a variety of signal transduction pathways. For instance, Wnt signaling is essential for the initiation of hair follicle development (Andl et al., 2002; Huelsen et al., 2001; Gat et al., 1998), whereas hedgehog signaling is required for the down growth of the hair follicle and for sebaceous gland development (St Jacques et al., 1998; Chiang et al., 1999; Altaba, 1999; Allen et al., 2003; Mill et al., 2003).

Recent studies have shown that expression of Smads and certain Smad target genes is preferentially enriched in the epidermal stem cell population (Tumbar et al., 2004; Morris et al., 2004). It is unknown whether Smads directly affect or interact with other signal transduction pathways to regulate epidermal stem cell maintenance and function. To ablate Smad signaling in keratinocytes, we have previously generated transgenic mice in which a Smad antagonist, Smad7, is targeted by using the keratin 5 (K5) promoter (He et al., 2002). It has been reported that Smad7 blocks Smad signaling by inhibiting Smad phosphorylation and by recruiting a Smad ubiquitination-related factor (Smurf)2 to degrade the type I receptors of transforming growth factor β (TGFβ) and/or Smads (Massague et al., 2005). Smad7 is often overexpressed under pathological conditions, e.g., in intrinsically aged and photoaged human skin (Quan et al., 2002) and during skin carcinogenesis (He et al., 2001). We have shown that Smad7 transgene expression in keratinocytes inhibits Smad signaling from TGFβ/activin and bone morphogenetic protein (BMP), which results in multiple developmental defects in stratified epithelia, including decreased hair follicle size (He et al., 2002). These mice die perinatally due to epithelial hyperkeratosis in the upper digestive tract and severe thymic atrophy (He et al., 2002). To further examine the role of Smad7 overexpression at stages critical for stem cell fate decision and differentiation as well as in postnatal hair cycling, in the present study we generated transgenic mice, in which Smad7 transgene expression can be temporally induced in keratinocytes, including epidermal stem cells. The ability to control transgene expression at a pathologically relevant level and in an acute or a sustained manner further facilitated analysis of the underlying molecular mechanisms. Our analyses revealed a direct interaction between Smad7 and the Wnt/β-catenin pathway via recruitment of Smurf2 for β-catenin degradation, which affects hair follicle and sebaceous gland morphogenesis.

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Results

Smad7 Transgene Induction Perturbed Embryonic and Postnatal Hair Follicle Morphogenesis

We generated gene-switch-Smad7 mice by using the K5 promotor, in which Smad7 transgene expression can be induced by RU486 in the epidermis and hair follicles (Figure S1; see the Supplemental Data available with this article online). This inducible system consists of a transactivator line (GLp65) (Lu et al., 2004) and a target line (tata.Smad7). In general, Smad7 transgene expression levels were RU486 dose dependent and correlated with phenotype severity. The data presented here were from the doses of RU486 that induced Smad7 expression levels similar to those when endogenous Smad7 is overexpressed, e.g., in aged skin (Quan et al., 2002) and in cancer lesions (He et al., 2001).

We induced Smad7 transgene expression in embryonic skin, beginning on E10.5, when a single epithelial layer begins transitioning into the stratified epidermis, or on E14.5, when hair follicle development is initiated. Skin samples were excised at birth (day one postpartum, P1) after daily RU486 treatments to verify sustained Smad7 transgene induction. Acute Smad7 transgene induction was assessed by topical RU486 application (20 μg/mouse) to the skin of P6 mice for 12 hr. Quantitative RT-PCR revealed an ~4-fold elevation in Smad7 expression in bigenic skin with either acute (12 hr, P6) or sustained (E14.5–P1) transgene induction compared to endogenous Smad7 expression levels in monogenic control skin (Figure 1A). Western analysis shows that the difference in Smad7 protein levels between Smad7 transgenic and control skin was greater than that in Smad7 mRNA levels (Figure 1B versus Figure 1A), which is likely a consequence of Smad7 protein accumulation, as Smad7 has a relatively long half-life (Liu et al., 2006). Consistently, the level of Smad7 protein upon sustained Smad7 transgene induction was higher than with acute Smad7 transgene induction (Figure 1B). In situ hybridization for Smad7 mRNA was performed 12 hr after the transgene was induced. In E15.5 control skin, endogenous Smad7 was detected in the epidermis, stromal cells, and the hair follicle placode, which is the first stage (Stage 1) of hair follicle development (Figure 1C). In E15.5 bigenic skin, the intensity of Smad7 staining had increased in all layers of the epidermis and was the strongest in hair follicle placodes (Figure 1C). In control P6 skin, endogenous Smad7 was detected primarily in the ORS of hair follicles (Figure 1D). At this stage, Smad7 was induced primarily in the epidermal basal layer and the ORS of hair follicles (Figure 1D).

When the Smad7 transgene was constitutively induced beginning either on E10.5 or on E14.5, hair follicle morphogenesis was delayed and hair follicle differentiation was perturbed in bigenic skin compared to control skin (Figure S2). In adults, we induced Smad7 transgene expression during the telogen-to-anagen transition of the hair follicles. The dorsal skin of bigenic and monogenic control mice were shaved on postnatal day 52 (P52), when the hair follicles enter into a long period of the telogen phase (Paus, 1998). A depilatory cream, Nair, was applied to the shaved skin to reintegrate the anagen phase (Paus et al., 1990). The depilated skin was then topically treated with 20 μg RU486 three times/week for 2 weeks. The skin samples at different time points were collected from the RU486-treated area by 3 mm punch biopsy. Histological analysis showed that P52 hair follicles in both bigenic and control mice were at the telogen phase (Figure 2A). On P58 (i.e., 6 days after Smad7 transgene induction), hair follicles in control mice grew downward and, in some areas, reached to the upper part of the subcutaneous layer of the skin (Figure 2A). In contrast, Smad7 skin exhibited rapid growth of sebaceous glands without hair follicle down growth (Figure 2A). Hair follicles in control P64 skin showed well-differentiated and mature anagen morphology, and most of them developed into the deep subcutaneous fat (Figure 2A). In contrast, hair follicles in P64 bigenic skin (i.e., 12 days after Smad7 transgene induction) grew barely below enlarged sebaceous glands (Figure 2A). Epidermal hyperplasia was also prominent in Smad7 transgenic skin (Figure 2A). When RU486 application was withdrawn on P66 to turn off Smad7 transgene expression, hair follicle regeneration gradually recovered (not shown). In contrast, continuous Smad7 transgene induction resulted in the blockade of hair follicle differentiation similar to that shown in Figure S2 (not shown), and eventually the hair follicles degenerated to cysts with hypertrophic sebaceous glands (Figure 2B). The epidermis was no longer hyperplastic, but was thinner than control epidermis (Figure 2B). At this stage, the skin became hairless in the

Figure 1. Smad7 Transgene Induction
(A) qRT-PCR showed Smad7 transgene induction 12 hr after a single RU486 (RU) application to P6 skin or daily injection of RU to pregnant females from gestation day 14.5 (E14.5) to P1. *, p < 0.01.
(B) Western analysis revealed increased Smad7 protein upon acute (RU 12h) and sustained (RU E14.5-P1) RU486 treatment.
(C) and (D) In situ hybridization with an antisense probe specific for Smad7 on (C) E15.5 and (D) P6 skin 12 hr after RU486 treatment. HF, hair follicle. The bar in the first panel of (C) represents 50 μm for both micrographs. The bar in each panel of (D) represents 50 μm.
focal area with sustained Smad7 transgene induction (not shown).

**Smad7 Overexpression Resulted in Premature Sebaceous Gland Development**

When we activated Smad7 transgene expression in skin beginning on E14.5, rudimentary sebaceous glands formed in association with the upper portion of P1 hair follicles in bigenic skin. These glands stained positively for adipophilin, an early sebaceous gland differentiation marker (Heid et al., 1998), whereas no adipophilin-positive cells were detected in control skin (not shown). To determine if accelerated sebaceous gland development in Smad7 transgenic skin represents a direct effect of Smad7 overexpression, we examined the time course of sebaceous gland formation after Smad7 transgene induction. RU486 (5 μg/mouse) was topically applied daily to the dorsal skin of control and bigenic neonates, beginning on P1. On P3, control skin did not exhibit adipophilin-positive cells, whereas rudimentary glands appeared in Smad7 hair follicles (Figure 2C). On P5, after 4 days of RU486 treatment, control skin showed more adipophilin-positive cells, but every bigenic hair follicle had developed sebaceous glands (Figure 2C). On P7, rudimentary sebaceous glands were forming in association with most of the hair follicles in control skin, whereas the sebaceous glands in bigenic follicles became further enlarged after 6 days of Smad7 induction (Figure 2C). We examined expression levels of sonic hedgehog (Shh) and Indian hedgehog (Ihh), two molecules required for sebaceous gland development (Allen et al., 2003; Niemann et al., 2003). In situ hybridization showed Shh expression in proliferative hair matrix cells of Smad7 hair follicles with a similar intensity to those in control hair follicles (not shown). Ihh expression levels were not significantly different from control levels at 12 hr after Smad7 induction, but they mildly increased after 10 days of Smad7 transgene induction (Figure 2D). Elevated Ihh protein was localized in the sebaceous glands of transgenic Smad7 skin (Figure 2E).

**Smad7 Transgene Induction Partially Blocked Smad Signaling**

To determine whether the current Smad7 transgene level is sufficient to block Smad signaling, we examined phospho-Smad2 (pSmad2) and pSmad1/5/8, which represent activation of TGF/β/activin and BMP, respectively, in the skin. In comparison with control skin, nuclear
pSmad2 staining was reduced in the epidermis and hair follicles of Smad7 transgenic skin, whereas nuclear staining for pSmad1/5/8 was reduced in the Smad7 epidermis but not the hair follicles (Figure S3). Additionally, we examined expression levels of several Smad target genes. Expression levels of p21 were reduced in Smad7 transgenic skin (Figure S4). In contrast, expression of c-myc and Lef1, two target genes involved in hair follicle/sebaceous gland development and differentiation (Arnold and Watt, 2001; Braun et al., 2003; Qiao et al., 2005), was not significantly altered in Smad7 transgenic skin (Figure S4). These results suggest that Smad7 transgene induction at the current level is insufficient to completely block Smad signaling in hair follicles.

**Smad7 Overexpression Resulted in the Downregulation of β-Catenin-Mediated Signaling**

To explore alternative mechanisms underlying Smad7-induced hair follicle/sebaceous gland abnormalities, we examined β-catenin expression and localization. We induced sustained Smad7 transgene expression beginning on E10.5 and excised the skin on E15.5 and P6. The β-catenin transcriptional level was not altered after Smad7 transgene induction (not shown). However, immunostaining revealed that the intensity of membrane staining of β-catenin was reduced in Smad7 transgenic epidermis and hair follicles compared to that in control skin (Figure 3A). By using conditions that partially destroy the membrane bound β-catenin but increase permeability to allow for better detection of nuclear β-catenin (Van Mater et al., 2003), we observed β-catenin in the nucleus of the germ cells of E15.5 control, but not Smad7, hair follicles. Dotted lines denote the epidermal-dermal boundaries. The bar in the lower panel represents 50 μm for both sections.

To determine whether reduced levels of β-catenin protein affect Wnt signaling, we bred Smad7 bigenic mice with TOPGAL reporter mice in which lacZ gene expression is induced by Wnt/β-catenin signaling (Das-Gupta et al., 2002). Skin samples were taken from E15.5 embryos that had received daily RU486 exposure since E10.5. Whole-mount β-galactosidase (β-GAL) staining on E15.5 embryos yielded numerous blue spots with a well-organized pattern in the trunk skin of TOPGAL+/Smad7− and TOPGAL+/Smad7+ embryos. The embryos were taken after 5 days of in utero RU486 treatment. No difference in staining intensity in the bone (yellow, open arrow) and cartilage (red, open arrow) was observed between the two genotypes. Representative areas from both embryos are enlarged to show positive staining in pelage hair follicles.

**Figure 3. Smad7 Transgenic Skin Exhibited a Reduction in β-Catenin Staining and in β-Catenin-Mediated Wnt Signaling**

(A) Immunohistochemistry of β-catenin on P6 control and Smad7 skin. The areas of the epidermis (Epi) and hair follicles (HF) marked by black boxes are enlarged to the right of the main panels. Hematoxylin was used as a counterstain. The bar represents 75 μm for both main panels.

(B) Immunofluorescence reveals nuclear staining for β-catenin in hair germ cells of E15.5 control, but not Smad7, hair follicles. Dotted lines denote the epidermal-dermal boundaries. The bar in the lower panel represents 50 μm for both sections.

(C) Whole-mount β-gal staining on E15.5 TOPGAL+/Smad7− and TOPGAL+/Smad7+ embryos. The embryos were taken after 5 days of in utero RU486 treatment. No difference in staining intensity in the bone (yellow, open arrow) and cartilage (red, open arrow) was observed between the two genotypes. Representative areas from both embryos are enlarged to show positive staining in pelage hair follicles.

(D) Microscopic appearance of the embryonic skin shown in (C). The dotted lines denote developing hair germs. Note that while the two Stage 1 (S1) hair follicles in the TOPGAL+/Smad7+ section did not exhibit positive β-gal staining, the staining intensity in bone tissue (red arrows) of the same section was similar to that in TOPGAL+/Smad7− bone tissue. The bar in the left panel represents 100 μm for both sections.

(E) β-gal staining on P6 skin with or without 5 days of Smad7 transgene induction (TOPGAL+/Smad7+ or TOPGAL+/Smad7−, respectively). The bar in the left panel represents 150 μm for both sections.
Western analysis for β-catenin since a GFP expression vector was included in all transfection experiments. Catenin level was detected with an anti-His antibody, and Smurf2 and Smad7 levels were detected with an anti-Flag antibody. A GFP antibody was used as a loading control since a GFP expression vector was included in all transfection experiments.

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5 days, the precortex region of P6 hair follicles in RU486 was applied daily to neonatal bigenic skin for TOPGAL+/Smad7+ embryonic follicles exhibited little transgene induction did not block hair follicle initiation, whether reduced to the level of K14 protein (not shown). To determine whether reduced β-catenin protein in Smad7 transgenic keratinocytes was a result of β-catenin protein degradation, we examined β-catenin protein levels in cultured control and transgenic keratinocytes treated with and without MG132, a ubiquitin proteasome inhibitor. Keratinocytes were treated for 12 hr with 10 μM RU486 to induce Smad7 transgene expression (Smad7 transgenic) or with vehicle alone (control), and they were then treated for 4 hr with or without 20 μM MG132. In the absence of MG132, β-catenin protein was significantly reduced in Smad7 transgenic keratinocytes compared to control keratinocytes, but it was recovered upon MG132 treatment (Figure 4B). This result suggests that Smad7 induces β-catenin degradation via a ubiquitin proteasome pathway.

We then examined whether Smad7 protein is physically associated with β-catenin. Immunoprecipitation with either the Flag antibody, which recognizes the Flag-Smad7 transgene, or for β-catenin was used for immunoprecipitation, and a mouse IgG was used as a negative control in immunoprecipitation. The whole-cell lysate (WCL) from control keratinocytes treated with MG132 was used as a positive control for Western blotting with an antibody against β-catenin or Smad7. Smad7, in combinations as indicated. Western analysis was performed to detect ubiquitinated β-catenin by using an anti-HA antibody (upper) and to detect total levels of β-catenin in cells by using an anti-His antibody (bottom).

Figure 4. Smad7 Transgene Expression Triggered β-Catenin Degradation

(A) Western analysis of β-catenin in control and bigenic skin (Smad7) treated with RU486 either acutely (RU 12h) or chronically (RU 10d). E-cadherin is a loading control.

(B) Western analysis of β-catenin on cultured keratinocytes in the absence and presence of MG132. A K14 antibody was used as a loading control.

(C) Coimmunoprecipitation of Smad7 and β-catenin in lysates prepared from cultured gene-switch-Smad7 keratinocytes in the presence of MG132. The antibody for Flag, which recognizes the Flag-Smad7 transgene, or for β-catenin was used for immunoprecipitation, and a mouse IgG was used as a negative control in immunoprecipitation.

(D) Ubiquitin assay for β-catenin protein. HEK293 T cells were transfected with expression constructs for His-tagged β-catenin, Flag-Smurf2, Flag-Smurf2C716S, and Flag-Smad7, in combinations as indicated. Western analysis was performed to detect ubiquitinated β-catenin by using an anti-HA antibody (upper) and to detect total levels of β-catenin in cells by using an anti-His antibody (bottom).

transgene induction did not block hair follicle initiation, TOPGAL+/Smad7+ embryonic follicles exhibited little to no β-catenin staining in the placodes (Figure 3D). When RU486 was applied daily to neonatal bigenic skin for 5 days, the precortex region of P6 hair follicles in TOPGAL+/Smad7− control skin stained strongly with β-catenin (Figure 3E). However, β-GAL staining was diminished in hair follicles with Smad7 transgene induction (Figure 3E).

Smad7 Overexpression Triggered β-Catenin Degradation

We further assessed β-catenin protein levels in control and transgenic skin. As shown in Figure 4A, β-catenin protein was significantly reduced in the skin upon acute (12h) and sustained (10d) Smad7 induction. In contrast, the amount of E-cadherin, a protein associated with β-catenin in the adhesion complex, was not altered in Smad7 transgenic skin (Figure 4A) as when normalized to the level of K14 protein (not shown). To determine whether reduced β-catenin protein in Smad7 transgenic keratinocytes was a result of β-catenin protein degradation, we examined β-catenin protein levels in cultured control and transgenic keratinocytes treated with and without MG132, a ubiquitin proteasome inhibitor. Keratinocytes were treated for 12 hr with 10 μM RU486 to induce Smad7 transgene expression (Smad7 transgenic) or with vehicle alone (control), and they were then treated for 4 hr with or without 20 μM MG132. In the absence of MG132, β-catenin protein was significantly reduced in Smad7 transgenic keratinocytes compared to control keratinocytes, but it was recovered upon MG132 treatment (Figure 4B). This result suggests that Smad7 induces β-catenin degradation via a ubiquitin proteasome pathway.

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increased β-catenin ubiquitination compared to transfection with HA-ubiquitin alone, presumably due to the low level of endogenous Smurf2 (not shown). Transfection with Smurf2 (Figure 4D, lane 4) induced a higher level of β-catenin ubiquitination compared to Smad7 transfection alone, presumably due to a high endogenous Smad7 level (not shown). Cotransfection with Smurf2 and Smad7 resulted in the highest degree of β-catenin ubiquitination (Figure 4D, lane 5). Cotransfection of Smad7 and Smurf2C716A exhibited β-catenin ubiquitination at a level similar to that of transfection with Smad7 alone (Figure 4D, lane 6), suggesting that Smad7-mediated β-catenin ubiquitination requires the E3 ligase activity of Smurf2. Hence, β-catenin ubiquitination induced by Smad7 alone or by Smurf2 alone likely depended on the endogenous levels of each other. To further assess whether Smad7/Smurf2-induced β-catenin ubiquitination leads to β-catenin degradation, we examined β-catenin protein levels 48 hr after transfection of His-β-catenin to HEK293 T cells with Smad7 and/or Smurf2. Each of these conditions resulted in reduced β-catenin levels (Figure 4E). Collectively, these data suggest that Smurf2 is involved in Smad7-mediated β-catenin degradation through the ubiquitin proteasome pathway.

**Smurf2-Mediated β-Catenin Degradation**

**Exacerbated Smad7-Mediated Skin Defects**

To determine whether Smad7-mediated β-catenin degradation depends on its role in blocking TGFβ/BMP signaling, we examined pSmad2 and pSmad1/5/8 in a K5.Smurf2 transgenic line in which the Smad7 transgene expression level was only 2-fold higher than that of endogenous Smad7 (Figure 4A); these cells still exhibited hair follicle defects. This transgenic line was developed as previously described (He et al., 2002), except that the founder had a low transgene expression level and thus survived to adulthood. In K5.Smurf2 skin, Smad7 transgene expression was insufficient to reduce pSmad2 and pSmad1/5/8 (Figure 5B). The numbers of cells positive for nuclear pSmad2 were 175 ± 12/mm epidermis and 115 ± 9/hair follicle in control skin, and 190 ± 15/mm epidermis and 109 ± 11/hair follicle in Smad7 skin (n = 5, p > 0.05). The numbers of cells positive for nuclear pSmad1/5/8 were 190 ± 13/mm epidermis and 25 ± 5/hair follicle in control skin and 180 ± 16/mm epidermis and 24 ± 3/hair follicle in Smad7 skin (n = 5, p > 0.05). However, when this K5.Smurf2 line was bred with TOPGAL mice, Wnt reporter activity was completely abolished in the anagen hair follicles of TOPGAL/K5.Smurf2 skin (Figure 5C). Thus, Smad7-induced β-catenin degradation and the subsequent Wnt signaling blockade appeared to be independent of its role in blocking TGFβ/BMP signaling.

To determine whether Smurf2 expression alone is sufficient to induce skin abnormalities similar to those in Smad7 transgenic skin, we generated Smurf2 transgenic mice by using the K5 promoter (K5.Smurf2). K5.Smurf2 skin did not show obvious abnormalities (Figures 6A–6D) or β-catenin degradation (Figure 6E). Since the level of endogenous Smad7 is very low in normal skin, we suspected that Smurf2 activation requires a Smad7 level higher than the endogenous Smad7 level. We bred K5.Smurf2 mice with the above-described K5.Smurf2 transgenic line. In neonates, the morphology of K5.Smurf2 skin was indistinguishable from nontransgenic control littermates, i.e., most hair follicles in control and K5.Smurf2 skin are in Stages 3–5 (Figure 6A). The majority of K5.Smurf7 hair follicles were in Stages 2 and 3 (Figure 6A). However, most hair follicles in mouse skin coexpressing the Smurf2 and Smad7 transgenes (Smad7/Smurf2) were in Stages 1 and 2 (Figure 6A). Staining with adipophilin revealed an absence of sebocytes in P1 control and K5.Smurf2 skin (Figure 6B). In contrast, a number of sporadic sebocytes were detected in P1 K5.Sm7 skin, and a greater number of sebocytes were observed in Smad7/Smurf2 skin (Figure 6B). The synergistic effect of Smad7 and Smurf2 was more obvious in adult skin. At P25, the skin of control and K5.Smurf2 littermates appeared completely normal. K5.Smurf7 littermates began to show patchy hair loss at this age (Figure 6C), and K5.Smurf7/K5.Smurf2 littermates exhibited nearly complete hair loss (Figure 6C).
Histological analysis of skin samples revealed that hair follicles in control and K5.Smurf2 skin were in the telogen (resting) phase (Figure 6D). In contrast, K5.Smad7 skin exhibited aberrant hair follicle cycling, i.e., a mix of anagen, catagen, and telogen phases (Figure 6D). Sebaceous glands of similar size among hair follicles at different phases (Figure 6D). K5.Smad7/K5.Smurf2 littermates exhibited degenerated hair follicles, resulting in papillary cysts and canals (Figure 6D). Sebaceous glands were more hypertrophic in K5.Smad7/K5.Smurf2 skin than those in K5.Smad7 skin (Figure 6D). These data provide in vivo evidence that the effect of Smurf2 on hair follicle/sebaceous gland morphogenesis is Smad7 dependent. Correlating with the severity of the skin phenotypes, levels of β-catenin protein were reduced ~50% and ~80% in K5.Smad7 and K5.Smad7/K5.Smurf2 skin, respectively (Figure 6E).

To further confirm that Smurfl2 was recruited by Smad7 to the β-catenin complex and therefore induced β-catenin degradation, we performed immunoprecipitation assays with an antibody against β-catenin, followed by Western analysis with an antibody against the Flag tag fused to the Smad7 and the Smurfl2 transgenes. Keratinocytes were isolated from control, K5.Smad7, K5.Smurfl2, and K5.Smad7/K5.Smurfl2 skin and treated with 20 μM MG132 for 4 hr. The amount of Smad7 that coprecipitated with β-catenin from lysates of Smad7 transgenic keratinocytes was comparable to the amount from lysates of Smad7/Smurfl2 keratinocytes (Figure 6F). The Smurfl2 protein was coprecipitated with β-catenin in lysates from Smad7/Smurfl2 keratinocytes, but it was barely detectable in Smurfl2 transgenic keratinocytes, even though these cells contained an amount of Smurfl2 protein similar to that in Smad7/Smurfl2 transgenic cells (Figure 6F). This result suggests that Smurfl2 did not bind independently to β-catenin, but that it was recruited by Smad7 to the β-catenin complex.

Endogenous Smad7 Was Involved in β-Catenin Degradation and Wnt Signaling Inhibition

In aged skin, which characteristically exhibits reduced hair follicles and enlarged sebaceous glands, Smad7 is overexpressed (Quan et al., 2002). Therefore, we assessed whether Smad7 overexpression in aged skin reduced β-catenin protein levels. Skin samples with telogen hair follicles from wild-type B6D2 mice (the same strain as the transgenic skin) at 2 months (“young”) and 2 years (“aged”) of age were examined. The aged mice exhibited typical aging-related, but not dermatitis-related, sparse hairs. Histology reveals that, similar to
the pathological alterations in 7-month-old Smad7 transgenic skin shown in Figure 2B, aged skin, compared to young skin, exhibited numerous enlarged sebaceous glands and degenerated hair follicles (Figure 7A). In comparison with young mouse skin, aged mouse skin exhibited a 6-fold increase in the Smad7 mRNA level (Figure 7B), which was similar to that of Smad7 transgene expression (Figure 1A). Western analysis further confirmed the increase of Smad7 protein in aged skin (Figure 7C) to be at a level similar to that in Smad7 transgenic protein shown Figure 1B. The level of β-catenin protein was significantly lower in aged skin than in young skin (Figure 7C). To further determine whether endogenous Smad7 is involved in β-catenin degradation, we transfected wild-type primary mouse keratinocytes with Smad7 siRNA and examined β-catenin protein levels. Western analysis showed that Smad7 siRNA significantly reduced the endogenous Smad7 level by over 70% (Figure 7D). Conversely, the β-catenin protein level was increased by ~80%–100% in Smad7 siRNA-transfected cells compared to mock-transfected cells (Figure 7D). These data suggest that endogenous Smad7 is involved in β-catenin degradation. To assess whether endogenous Smad7-mediated β-catenin degradation affects Wnt signaling, we transfected Smad7 siRNA to hair follicle cells isolated from TOPGAL reporter mice. Similar to that seen in vivo (Figure 3E), β-GAL staining was positive in precortex hair follicle cells (Figure 7E). In mock-transfected TOPGAL follicle cells, ~20% of the hair follicles contained β-GAL-positive cells (Figure 7E). In contrast, in Smad7 siRNA-transfected TOPGAL follicle cells, ~80% of hair follicles possessed β-GAL-positive cells (Figure 7E). These data suggest that endogenous Smad7 inhibits Wnt signaling.

Discussion

β-Catenin Degradation Independent of Its Effect on Blocking TGFβ/BMP Signaling

Previously, we have identified a role for Smad7 in blocking TGFβ/activin and BMP signaling in keratinocytes, which contributes greatly to epidermal hyperproliferation and delayed hair follicle development (He et al., 2002). In the current study, when the Smad7 transgene was expressed at a level lower than in the previous study, the hyperproliferative effect was not as severe, whereas the effect on hair follicle morphogenesis was just as severe. Consistently, under the current levels of
Smad7 transgene expression, which either partially inhibited or did not inhibit TGFβ/BMP signaling, Smad7 still induced β-catenin degradation and Wnt inhibition. Therefore, Smad7-induced β-catenin degradation appeared to be independent of its effect on inhibiting TGFβ signaling.

Consistent with a previous report (Edlund et al., 2005), we confirmed a direct association between Smad7 and β-catenin. We further found that Smad7 recruited Smurf2 to mediate β-catenin ubiquitination and subsequent degradation. Similar to the manner in which Smurf2 ubiquitini- nates other substrates (Kavask et al., 2000; Izzii and Attisano, 2004; Ogunjimi et al., 2005; Lin et al., 2000; Zhang et al., 2001), Smurf2 did not directly bind to β-catenin, but it was recruited by Smad7. Once Smurf2 is recruited to the Smad7/β-catenin complex, the E3 ligase activity of Smurf2 is required for β-catenin ubiquitination and degradation. This is evidenced by the fact that the enzyme-deficient Smurf2 mutant did not cooperate with Smad7 for β-catenin degradation. Our data also suggest that endogenous Smad7 may use this mechanism to degrade β-catenin, since endogenous Smad7 levels inversely correlated with β-catenin levels between aged and young skin and Smad7 siRNA-treated keratinocytes exhibited increased β-catenin protein levels. However, it remains to be determined if once Smad7 expression reaches the level sufficient to inhibit TGFβ/BMP signaling, it induces additional β-catenin degradation mechanisms, which are a result of blocking the TGFβ/BMP target genes that regulate either β-catenin stabilization or Wnt signaling. In either case, both the biochemical activity and functions of Smurf2 in keratinocytes appeared to depend on Smad7 levels, as Smurf2 transgenic skin did not exhibit any abnormalities in skin development, but exacerbated the abnormalities in Smad7 transgenic skin.

Smad7-Increased β-Catenin Degradation Contributes to Wnt Signaling Inhibition in Physiological and Pathological Conditions

Although β-catenin protein is expressed at a high level in keratinocytes in both interfollicular epidermis and hair follicles, Wnt signaling activity can only be detected in certain cell populations of hair follicles (Kobiela et al., 2003; Lowry et al., 2005; Van Mater et al., 2003), and it is crucial for development and differentiation of hair follicles, but not the epidermis (Huelsken et al., 2001). In the current study, we show that as a result of Smad7-initiated β-catenin degradation, Wnt/β-catenin signaling in hair follicles was reduced. The degree of β-catenin degradation correlated with the severity of hair follicle defects. Furthermore, the aged mouse skin, which expressed endogenous Smad7 at a level similar to that in gene-switch-Smad7 skin, also showed significant reduction of β-catenin protein and histopathological changes strikingly similar to the skin with sustained Smad7 transgene induction. Therefore, under a pathological condition, Smad7-mediated β-catenin degradation contributed, at least in part, to the defect of hair follicle morphogenesis.

Given the fact that endogenous Smad7 is expressed at a very low level in keratinocytes (He et al., 2001), we were surprised to find a marked increase in β-catenin protein and enhanced Wnt signaling after knocking down endogenous Smad7 in keratinocytes. Since Wnt signaling is dominant during hair follicle initiation, endogenous Smad7-mediated β-catenin degradation and Wnt inhibition should not be sufficient to perturb hair follicle morphogenesis. However, this may represent a negative feedback mechanism to prevent de novo hair follicle formation. Therefore, our study prompts future studies on the contribution of endogenous Smad7 in hair follicle homeostasis by keratinocyte-specific Smad7 ablation.

Accelerated Sebaceous Gland Development Induced by Smad7 Overexpression Represents a Shifted Balance between β-Catenin/Wnt Signaling and Hedgehog Signaling

It has been shown that reduced Wnt signaling and/or enhanced hedgehog signaling stimulates committed stem cell progenitors to differentiate toward the sebocyte lineage (Labbe et al., 2000; Niemann et al., 2003; Allen et al., 2003; Takeda et al., 2006). In early stages of hair follicle development (e.g., embryonic stages), activation of both Wnt/β-catenin and hedgehog signaling is crucial, and a positive regulatory loop between these two pathways has been suggested (Watt, 2004). Conversely, reduced Wnt signaling subsequently results in reduced hedgehog signaling, which perturbs the development of hair follicles and sebaceous glands (Watt, 2004; Kalderon, 2002; Huelsken et al., 2001). The requirement for a proper balance between Wnt/β-catenin signaling and hedgehog signaling on stem cell fate decision and differentiation is also evidenced by the normal hair follicle developmental process, in which sebaceous gland development in mice does not occur until after birth, when Wnt/β-catenin signaling begins to decline and hedgehog signaling continues (Kobiela et al., 2003; Watt, 2004). In the present study, we observed that while Wnt signaling was directly reduced in Smad7 transgenic skin, expression of Shh was still present in proliferative hair matrix cells of Smad7 hair follicles (not shown). An upregulation of Ihh was observed in sebocytes of Smad7 transgenic skin after constitutive Smad7 transgene induction. This result indicates that although Ihh is not likely a direct target of Smad7, activation of hedgehog signaling still occurs, at least in sebaceous glands. Therefore, suppression of Wnt/β-catenin signaling along with relatively intact hedgehog signaling in Smad7 transgenic keratinocytes appeared to promote committed stem cell progenitors to differentiate toward the sebocyte lineage.

In summary, our study identified an alternative β-catenin degradation mechanism mediated by Smad7 and subsequent Smurf2 recruitment. Our data suggest that the maintenance of a low Smad7 level in the skin is essential to permit the balanced signaling between Wnt/β-catenin and hedgehog pathways for normal hair follicle and sebaceous gland development and regeneration. Hence, overexpression of Smad7 under a pathological condition perturbs such a balance. Given the significant variations of endogenous Smad7 levels in β-catenin-positive cells during development, a high frequency of Smad7 overexpression in pathological conditions, and multiple functions of β-catenin in Wnt signaling and adhesion, our study prompts future investigations regarding the impact of the interaction between Smad7 and
β-catenin under different developmental/pathological conditions.

Experimental Procedures

Generation of Transgenic Mice Overexpressing Smad7 and/or Smurf2

The gene-switch-Smad7 transgenic mice were generated by using the gene-switch transgenic system as we previously described (Lu et al., 2004). The activator (GLP65) line was generated previously (Lu et al., 2004). The target line (tata.Smad7) was generated by inserting a full-length mouse Smad7 cDNA with a 5′ Flag epitope into a minimal tata promoter with GAL4 binding sites (Lu et al., 2004). The generation of K5.Smurf2 mice was described in our previous study (He et al., 2002). The offspring showed the same phenotypes throughout generations. To generate K5.Smurf2 transgenic mice, the human Smurf2 cDNA with a Flag epitope (Lin et al., 2000) was inserted into the K5 promoter and microinjected into the pronuclei of mouse embryos obtained from ICR female mice mated to B6D2F1 male mice. PCR genotyping was performed with primers specific for a fragment between 468 bp and 950 bp in the full-length human Smurf2 cDNA (forward: 5′-CCTCAGTTGTTGCTGTTCTCT-3′, reverse: 5′-TGTTAGTCTGAGGAGGATAT-3′).

In Situ Hybridization

In situ hybridization for Smad7 and Lef1 was performed by using digoxigenin-11-dUTP-labeled probes as we have previously described (He et al., 2001; Qiao et al., 2005). The cDNA fragment was first amplified by reverse transcriptase PCR with PRCs with specific for a mouse Smad7 (accession number: NM_008543, forward: 5′-GCTGACGGATCTGCTGCTCA-3′, reverse: 5′-CCAGGCTCCAGAAGAATTGTG-3′) cDNA sequence and then subcloned into the pEGM-T easy vector (Promega, Madison, WI). The plasmid was further linearized and purified to create templates for the synthesis of in situ hybridization probes.

Histology, Immunofluorescence, and Immunohistochemistry

Skin samples were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Immunofluorescence (IF) or immunohistochemistry (IHC) was performed on OCT-embedded frozen sections or paraffin-embedded sections as previously described (Wang et al., 1997). The antibodies used included: K14 (CRP, Denver, PA), AE15 and AE13 (gifts from Dr. Tung-Tien Sun), adipophilin (Fitzgerald, Concord, MA), β-catenin (Sigma, St. Louis, MO), Smad7 (He et al., 2002), Ihh (Santa Cruz Biotechnology, Santa Cruz, CA), pSmad2 and pSmad1/5/8 (Cell Signaling Technology, Danvers, MA). IHC or IF was performed to detect membrane bound β-catenin as previously described (Han et al., 2005). To detect nuclear β-catenin, IF was performed by using the same β-catenin antibody, and the sections were counterstained with 0.1% Triton X-100 for 4 hr before nuclear β-catenin staining was performed (Van Mater et al., 2003), conditions that partially destroy membrane-associate epitopes but increase permeability to the nucleus.

RNA Extraction and Analyses

Total RNA was isolated from skin samples with RNazol B (Tel-Test, Friendswood, TX). cDNA synthesis was performed by using Taqman Assays-on-Demand probes (Applied Biosystems, Foster City, CA) as previously described (Lu et al., 2004). Results from 3–5 samples of each genotype are shown.

Western Analysis

Protein extraction was performed as previously described (He et al., 2002). The primary antibodies used included Smad7 (He et al., 2002), E-cadherin and β-catenin (BD Biosciences, San Jose, CA), Flag (Sigma), and K14 (Fitzgerald). Gray-scale images were obtained and quantified with Odyssey v.1.2 software (LI-COR Biosciences, Lincoln, NE).

Epidermal Keratinocyte and Hair Follicle Cell Culture

Primary epidermal keratinocytes were isolated from neonatal mouse skin with different genotypes as previously described (Wang et al., 1997). The cells were cultured in defined keratinocyte serum-free medium (DK-SFM) (Invitrogen) to maintain keratinocyte proliferation.

When cells reached nearly 80% confluence, they were treated with RU486 at a final concentration of 10−7 M to induce transgene expression. The cells were harvested 12 hr after RU486 treatment for protein extraction. Isolation and culture of murine hair follicle cells were performed by following an established protocol with minor modifications (Rogers et al., 1987). Briefly, neonatal mouse dermis separated from the epidermis was digested with 0.35% type I collagenase (Worthington Biochemical, Lakewood, NJ) at 37°C for 30 min, filtered through a cell strainer (BD Biosciences, San Jose, CA), and then centrifuged at 800 rpm for 5 min. The pellet was resuspended in 1.25 mM Ca2+ EMEM (Cambrex, Walkersville, MD) and then centrifuged at 300 rpm for 5 min. Subsequently, the pellet was resuspended in 8 ml 1.25 mM Ca2+ EMEM/9% Ficoll (1:1) and overlaid on 5 ml 9% Ficoll for centrifugation at 400 rpm. The precipitated hair follicles were then washed in 1.25 mM Ca2+ EMEM on type I collagen-coated plates. The medium was then replaced with 1.25 mM Ca2+ EMEM 24 hr later.

β-gal Staining

β-gal staining for whole mount, tissue sections, and cells was performed with a β-gal staining kit (Invitrogen, Calsbad, CA), by following the manufacturer’s instructions.

Immunoprecipitation

Primary keratinocytes were cultured as described above. When cells reached subconfluence, RU486 was added at a final concentration of 10−7 M to induce Smad7 overexpression. A total of 12 hr later, the cells were treated with or without MG132 at a final concentration of 20 μM for 4 hr prior to harvesting. Subsequently, the cells were lysed in 1× CST cell lysis buffer (Cell Signaling Technology) containing freshly added protease inhibitors that included AEBSF, aprotinin, bestatin, E-64, leupeptin, and pepstatin A (Sigma). For immunoprecipitation, 2 μg of a β-catenin antibody or a Flag antibody was added to 500 μg whole-cell lysate, and 2 μg mouse IgG (Santa Cruz) was used as a negative control. Protein precipitation and subsequent Western blotting were carried out as previously described (Lin et al., 2000).

Assay of Ubiquitination and Degradation of Exogenous β-Catenin

HEK293 T cells were transfected with expression plasmids for His-tagged β-catenin, Flag-Smurf2, Flag-Smad7, and HA-ubiquitin, as specified in Figure 5D. A total of 48 hr after transfection, cells were treated with MG132 at a final concentration of 20 μM for 4 hr prior to harvesting. His-tagged β-catenin was immobilized on Ni-NTA beads (Qiagen, Valencia, CA) and then eluted in elution buffer (Lin et al., 2000). Eluted proteins were subjected to Western blotting analysis with anti-HA antibody (Roche Diagnostics, Indianapolis, IN) to detect the β-catenin-ubiquitin conjugation. For the degradation assay, HEK293 T cells were transfected with expression plasmids for His-tagged β-catenin, Flag-Smurf2, and Flag-Smad7. A total of 48 hr after transfection, protein was extracted from transfected cells and subjected to Western blotting. The His-β-catenin level was detected with an anti-His antibody (Santa Cruz) and Smurfl2 and Smad7 levels were detected with an anti-Flag antibody.

Smad7 Knockdown

Smad7 siRNA oligonucleotides were designed to recognize mouse Smad7 (sense: 5′-GAGGGCTGTGGTCTGTAAGA-3′, antisense: 5′-TTCACGAAGACACAGCCTC-3′) and were synthesized (Ambion, Austin, TX). The annealed double-strand Smad7 siRNA was then transfected to cultured keratinocytes and hair follicle cells at a final concentration of 50 nM by using FuGENE 6 (Roche Diagnostics, Alameda, CA), following the manufacturer’s instructions. Mock transfections were used as negative controls. The cells were harvested 72 hr posttransfection for further analyses.

Supplemental Data

Supplemental data include four figures and are available at http://www.developmentalcell.com/cgi/content/full/11/3/301/DC1/.

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