JCB Article

# Defining BMP functions in the hair follicle by conditional ablation of BMP receptor IA

**Krzysztof Kobielak, H. Amalia Pasolli, Laura Alonso, Lisa Polak, and Elaine Fuchs**

Howard Hughes Medical Institute and Laboratory of Mammalian Cell Biology and Development, The Rockefeller University, New York, NY 10021

Using conditional gene targeting in mice, we show that BMP receptor IA is essential for the differentiation of progenitor cells of the inner root sheath and hair shaft. Without BMPRIA activation, GATA-3 is down-regulated and its regulated control of IRS differentiation is compromised. In contrast, Lef1 is up-regulated, but its regulated control of hair differentiation is still blocked, and *BMPRIA*-null follicles fail to activate Lef1/β-catenin–regulated genes, including keratin genes. Wnt-mediated transcriptional activation can be restored by transfecting *BMPRIA*-null keratinocytes with a constitutively activated β-catenin. This places the block downstream from Lef1 expression but upstream from β-catenin stabilization. Because mice lacking the BMP inhibitor Noggin fail to express Lef1, our findings support a model, whereby a sequential inhibition and then activation of BMPRIA is necessary to define a band of hair progenitor cells, which possess enough Lef1 and stabilized β-catenin to activate the hair specific keratin genes and generate the hair shaft.

## Introduction

During embryonic development, a layer of multipotent stem cells gives rise to the epidermis and its appendages, including hair follicles. Hair follicle morphogenesis arises from a series of epithelial–mesenchymal cues, initiating an epithelial down-growth, which then proliferates and differentiates to form first the channel, or inner root sheath (IRS), and then the hair itself (Fig. 1). Postnatally, the mature hair shaft is composed of a core, or medulla, cloaked by a concentric ring of cortical cells, which in turn are surrounded by a layer of hair shaft cuticle (the hair surface). Beneath the skin surface, the hair is surrounded by an IRS composed of a cuticle, Huxley's layer and Henle's layer. IRS cuticle interlocks with hair cuticle to guide the hair, but near the skin surface, the IRS degenerates to release the shaft (Hardy, 1992). Above the base (bulb) of the follicle, the Henle's layer is encased by the companion cell layer and outer root sheath (ORS), a structure contiguous with and biochemically similar to the epidermal basal layer.

Both hair shaft and IRS form concomitantly through upward terminal differentiation of transiently amplifying progenitor (matrix) cells, which maintain contact with dermal papilla (DP), the follicular mesenchyme at the core of the hair bulb (Oliver and Jahoda, 1988). It is thought that a reservoir of stem cells, the bulge, resides at the base of the noncycling segment of the hair follicle and replenishes matrix cells as they differentiate (Cotsarelis et al., 1990). Contact with DP cells appears to be essential, although not necessarily sufficient, to convert stem cells to matrix cells (Taylor et al., 2000; Oshima et al., 2001).

The spatially defined differentiation programs of the hair follicle make it an excellent model to study the mechanisms that underlie stem cell lineage determination. A myriad of signal transduction pathways have been implicated in the process (for reviews see Fuchs et al., 2001; Panteleyev et al., 2001; Millar, 2002; Niemann and Watt, 2002; Alonso and Fuchs, 2003). *Sonic hedgehog* (*Shh*) is expressed in a subset of matrix cells and is thought to play a role in follicle proliferation (Oro and Higgins, 2003). Hair shaft differentiation is dependent on Wnt signaling, and alterations in this pathway generate a number of follicle abnormalities (Gat et al., 1998; Chan et al., 1999; Millar et al., 1999; Huelsken et al., 2001;

Address correspondence to Elaine Fuchs, Howard Hughes Medical Institute and Laboratory of Mammalian Cell Biology and Development, 1230 York Ave., Box 300, The Rockefeller University, New York, NY 10021-6399. Tel.: (212) 327-7953. Fax: (212) 327-7954.
email: fuchslb@rockefeller.edu

Key words: Smad; hair follicle differentiation; Wnt; bone morphogenetic proteins

Abbreviations used in this paper: DP, dermal papilla; DS, dermal sheath; IRS, inner root sheath; KO, knockout; ORS, outer root sheath; P, postnatal day; Shh, Sonic hedgehog WT, wild-type.

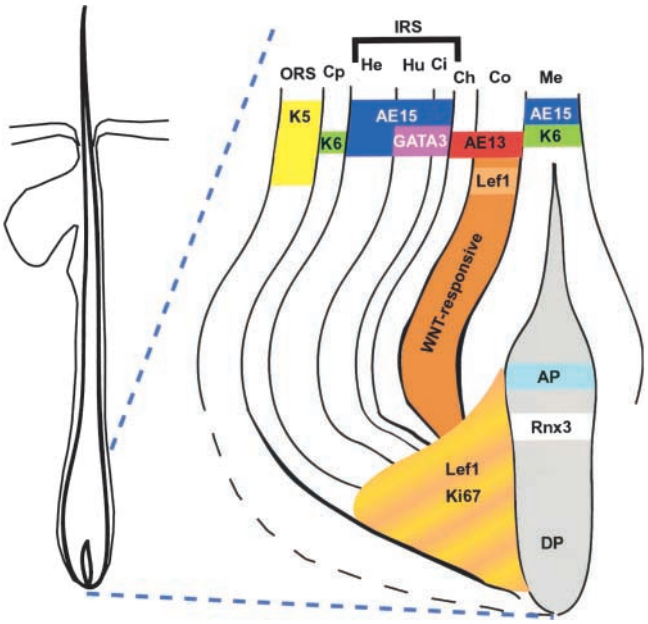


Figure 1. **Schematic of the hair follicle bulb.** The diagram depicts the distinct cell layers of the hair follicle: ORS, outer root sheath; Cp, companion cell layer; IRS, inner root sheath; He, Henle's layer; Hu, Huxley's layer; Ci, cuticle of IRS; Ch, cuticle of hair shaft; Co, cortex of hair shaft; Me, medulla; and DP, dermal papilla. The illustration also summarizes patterns based on expression of antibodies against known markers that distinguish the complex programs of differentiation. Antibodies were against the proteins indicated except for the following: K6, Ab specific for the Cp K6; AE13, Ab against hair-specific keratins that are expressed in the Co; and AE15, Ab against trichohyalin, found in all three IRS layers and the Me.

Andl et al., 2002). In mice, the Wnt reporter gene TOP-GAL is most active in the differentiating cells (cortex) of the developing hair shaft (DasGupta and Fuchs, 1999).

Canonical Wnt signaling results in the stabilization of β-catenin, which activates members of the Lef1/Tcf family of DNA-binding proteins (for review see Moon et al., 2002). For matrix progeny to respond to Wnt signaling (Reddy et al., 2001), they must express Lef1. Recently, it was shown that Lef1 expression in keratinocytes is dependent on Noggin, a DP-secreted inhibitor of BMP signaling (Botchkarev et al., 1999; Jamora et al., 2003). When Lef1-positive hair progenitor cells respond to Wnts, they activate hair-specific keratin genes, which possess Lef1 DNA-binding sites in their 5′ regulatory regions (Zhou et al., 1995; Merrill et al., 2001). Although Lef1–β-catenin complexes appear to be required for hair keratin gene expression, other transcription factors, including Foxn1 and Hoxc13, may also be involved (Prowse et al., 1999).

At first glance, the recently identified role for BMP inhibition in the hair follicle seems straightforward. However, Lef1 mRNA expression is highest in proliferating matrix cells, and there is an unexplained lag before Lef1 and β-catenin concentrate in the nucleus and cells progress toward hair differentiation (Zhou et al., 1995; DasGupta and Fuchs, 1999). Moreover, a positive role for BMPs has also been postulated in the follicle, where both proliferation and differentiation seem to be affected by BMP activation (Blessing et al., 1993; 1996). In addition, whereas Noggin has a positive effect on Lef1 expression, it has a negative effect in cortex, where ectopic expression of Noggin diminishes expression of a number of transcriptional regulators Msx1, Msx2, Foxn1, and Hoxc13 as well as blocking hair differentiation (Kulessa et al., 2000; Ma et al., 2003).

The difficulties in understanding BMP-mediated regulation in the follicle are compounded by the complex expression patterns involved. BMP2 and BMP4 are in epithelial hair progenitor cells; BMP4 and BMP7 are in the DP; BMP7 is in ORS; and BMP7, BMP8a, and BMP8b are in IRS (Zhao and Hogan, 1996; Takahashi and Ikeda, 1996; Kratochwil et al., 1996; Wilson et al., 1999). Although BMPs seem to be important for hair follicle development and differentiation, sifting through their potential functions has posed a substantial challenge.

In this paper, we use conditional knockout technology to explore the roles of BMP signaling in hair follicle differentiation. Given the potential for redundancy in BMP expression, we focused on the BMPRIA receptor, known to be expressed in hair follicle placodes (Botchkarev et al., 1999) and required for specification of limb ectoderm (Ahn et al., 2001; Soshnikova et al., 2003). By mating keratin 14 promoter-driven Cre mice (Vasioukhin et al., 1999) with *Bmpr1a* flox/flox mice (Mishina et al., 2002), we were able to drive Cre recombinase expression in the stem cell compartment of embryonic skin epithelium. Here, we identify and characterize the defects in hair morphogenesis that arise from a failure to undergo BMP signaling. We also evaluate how the loss of BMPRIA impacts on translation of Lef1/β-catenin–mediated gene expression in the developing follicles and in postnatal skin grafts. Our results provide strong support for the previously proposed role of inhibition of BMP signaling in producing and maintaining Lef1-positive matrix cells, and they also reveal a novel role for BMP receptor activation in priming the hair shaft progenitor cells to respond to Wnt signaling. The sequential inhibition and activation of BMPRIA are both essential for development of the hair shaft, and ironically, both steps precede Wnt signaling in the differentiation program.

## Results

### Conditional ablation of the *Bmpr1a* gene in skin epithelium

To introduce conditional mutations into the *Bmpr1a* locus, we mated *Bmpr1a flox/flox* (*fl/fl*) mice (Mishina et al., 2002) with our transgenic mice that express *Cre* under the control of the *K14* promoter (Vasioukhin et al., 1999). Offspring from matings of *Bmpr1a*(*fl*/+), K14Cre mice yielded litters of the expected numbers, genotype and Mendelian ratios. However, mice harboring two floxed alleles of *Bmpr1a* and K14-Cre were smaller in size (Fig. 2 A) and displayed aberrant whiskers (Fig. 2 B). Although the number of whiskers in knockout (KO) mice was nearly always less than their wild-type (WT) counterparts Fig. 2 B′), this varied, and some KO animals displayed no whiskers at all. As a measure of epidermal function, newborn KO animals and their WT counterparts were able to exclude blue dye, indicating that the epidermal barrier was intact (unpublished data). However, most KO animals died several days after birth, most likely due to

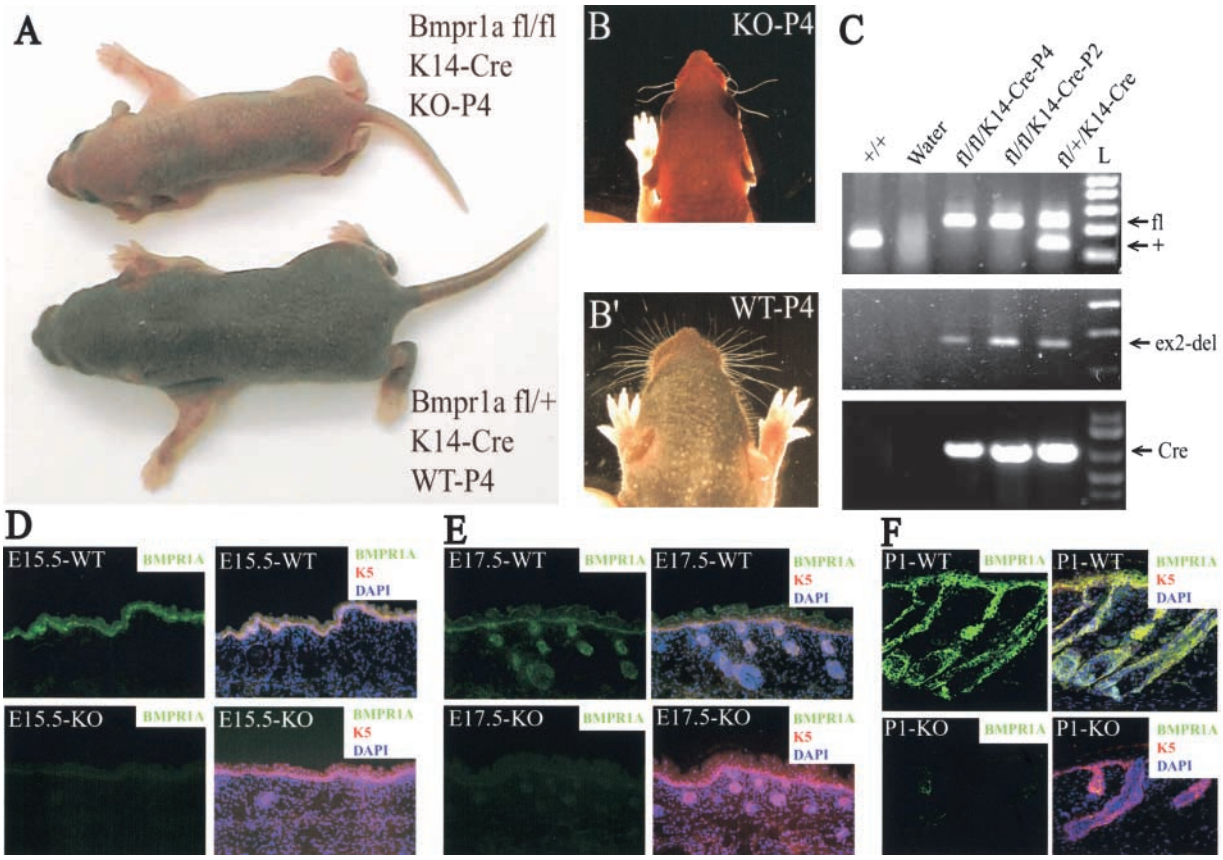


Figure 2. **Phenotype and genotype of K14-Cre, *Bmpr1a fl/fl* conditional knockout mice, which lack BMPRIA expression in skin epithelium.** (A and B) Phenotypes of neonatal KO and WT pups. Mice lacked whiskers if their skin epithelium was null for *Bmpr1a*; those with a few aberrant whiskers (shown) were mosaic. (C) PCR analysis of DNAs isolated from dispase-separated epidermises of mice harboring the genotypes indicated. Primer sets used were diagnostic for: (top) both the floxed and WT alleles; (middle) the Cre-mediated deletion of exon 2; (bottom) the presence of the K14-Cre transgene. (D–F) 8-μm frozen skin sections of WT or KO animals at the ages indicated were subjected to indirect immunofluorescence with anti-BMPRIA and anti-K5. Color codings are according to the fluor tag of the secondary antibodies or DAPI (blue).

defects in oral epithelium. Overall, neonatal mice conditionally targeted for disruption of *Bmpr1a* gene function could readily be sorted on the basis of phenotype (Fig. 2, A and B), and this was confirmed by genotype (Fig. 2 C).

As judged by immunofluorescence, the targeted ablation was nearly if not fully complete by E15.5. In contrast to WT skin, KO skin revealed few if any traces of residual BMPRIA protein in the developing epidermal basal layer (Fig. 2 D). By E17.5, BMPRIA-positive hair follicles were visible in WT backskin (Fig. 2 E), and by P1, WT follicles revealed BMPRIA-positive cells in most if not all differentiation stages (Fig. 2 F). Expression was highest in ORS, matrix, and precortex, in good agreement with Botchkarev et al. (1999). The absence of BMPRIA in embryonic skin verified the success of targeting, and importantly, revealed no unusual stability in *Bmpr1a* mRNA or protein that might interfere with our analyses.

The KO animals used for immunofluorescence exhibited few whiskers and no visible hair. In examining BMPRIA expression, we noticed a correlation between the extent of BMPRIA mosaicism and the number of whiskers and hair present. Furthermore, analyses revealed that the aberrant whiskers (Fig. 2 B) were genetically mosaic, whereas whiskers and hairs failed to form in complete absence of BMPRIA. For the present paper, we focused on conditional knockouts where the majority of the follicles displayed no traces of anti-BMPRIA staining.

## BMPRIA is essential for hair follicle morphogenesis

To assess whether follicular defects extended to backskin, and to probe more deeply into the nature of these defects, we next examined the histology of WT and neonatal *Bmpr1a*-null follicles at postnatal day two (P2). Toluidine blue–stained semithin sections revealed gross abnormalities in these follicles (Fig. 3). By this age, some WT backskin hairs began to display a well-differentiated structure, replete with ORS, IRS, and hair shaft (Fig. 3 A, HS). Most P2 KO follicles exhibited an ORS and bulbar like structure, but lacked a typical IRS and hair shaft (Fig. 3 B). Overall, follicles appeared immature, resembling those of late-stage embryonic skin, rather than postnatal skin. However, as KO follicles extended downward, they wove in and out of the plane of sectioning. In contrast, KO epidermis retained a differentiation program that was morphologically similar to WT.

By P4, differences in follicles were even more dramatic (Fig. 3, C and D). P4 KO follicles still showed no signs of a dark blue–stained cortex, a cross-striated medulla or an IRS,

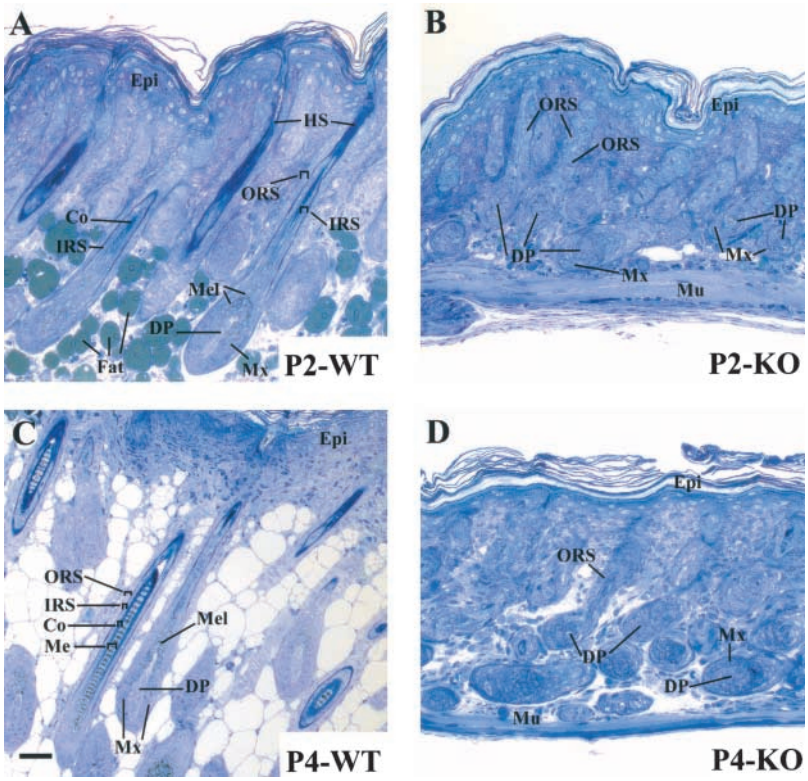


Figure 3. **Histological abnormalities in *Bmpr1a*-null follicles.** Matched P2 and P4 WT and *Bmpr1a*-null (KO) skin grafts were fixed, embedded in Epon, and sectioned (1 μm). Sections were stained with toluidine blue and subjected to light microscopic analysis. Mx, matrix; HS, hair shaft; Me, medulla; IRS, inner root sheath; ORS, outer root sheath; DP, dermal papilla; Co, cortex; epi, epidermis; Mu, muscle; and Mel, melanin granules (although skin epithelium does not make melanin granules, the Mx cells of the follicle and basal cells of the epidermis take up melanosomes from the cellular processes of the melanocytes). Note the lack of Me and paucity of IRS structures in the KO follicles. The paucity of fat in the dermis could be a reflection of malnutrition arising from oral complications. Bar, 20 μm, applies to all panels.

all well-developed in WT P4 counterparts (Fig. 1). In addition, the distinction between DP and matrix was less obvious than in WT follicles. Overall, aberrations in KO follicles did not seem to be due to a developmental lag, but rather a molecular defect in differentiation.

Ultrastructure of WT and *Bmpr1a*-null skin helped to define the extent of abnormalities (Fig. 4). WT P2 follicles sometimes displayed an immature core, as the medulla was still forming in younger follicles of this age. However, most P2 follicles did exhibit a keratinized cortex, packed with dense keratin filaments, and a well-developed IRS, whose appearance precedes the hair shaft (Fig. 4 A; Robins and Breathnach, 1970). At early stages of normal terminal differentiation, all IRS layers displayed trichohyalin granules (Th), which were larger and more abundant than in medulla. Progressively upward, the Henle's layer of IRS became keratinized, revealing it as an electron dense row of flat, vertical cells (Fig. 4 A). The companion cell layer abutted the Henle's layer.

In P2 *Bmpr1a*-null follicles, ORS appeared largely normal (Fig. 4 B). Internally to ORS was a group of cells, which possessed morphology and orientation similar to the companion cell layer. The follicle was encased by a dermal sheath (DS; Fig. 4, B and F, arrowheads), but the morphological distinction between surrounding mesenchymal cells and KO follicle cells was otherwise difficult to make. With the exception of occasional Henle cells, only a few P2 follicles (~1:100) displayed morphological signs of IRS cells (not depicted; and Fig. 3), and the number of such follicles correlated with the rare BMPRIA mosaic follicles.

At the base (bulb) of both WT and KO follicles, the relatively undifferentiated cells of the matrix were present (Fig. 4, C and D). The matrix displayed numerous mitoses, which are consistent with its proliferative status. In addition, a basal lamina of ECM separates the inner strand of DP, necessary for maintaining matrix as undifferentiated hair progenitors (Fuchs, et al., 2001; Millar, 2002). Otherwise, the difficulties faced earlier in distinguishing mesenchymal and epithelial cells persisted at the ultrastructural level. Because the keratin 14 promoter is only active in skin epithelial cells and not in DP (Vasioukhin et al., 1999), DP defects seemed to be indirectly due to BMPRIA loss.

Finally, the differences noted in P2 follicles were exaggerated by P4 (Fig. 4, E and F). At this stage, WT medulla was well developed, and its organized row of cells at the follicle center were packed with trichohyalin and melanin granules (Fig. 4 E, Mel). In contrast, the P4 KO follicle stem consisted predominantly of ORS and companion cell layer cells (Fig. 4 F). Although melanin granules were present in cells of KO follicles, the lack of medulla cells may account for the perturbations seen in their distribution.

## Biochemical abnormalities in IRS and hair shaft differentiation in BMPRIA-deficient follicles

To further characterize the abnormalities in *Bmpr1a*-null follicles, we turned toward immunofluorescence microscopy. In WT follicles, IRS and medulla label with an AE15 mAb, specific for trichohyalin (O'Guin et al., 1992). In contrast, AE13 marks the hair-specific keratins, the major structural proteins of hair (Lynch et al., 1986). In WT neonatal P1 skin, follicles are often positive for both AE15 and AE13 (Fig. 5, A and B). However, in P1 *Bmpr1a*-null skin, no AE15 or AE13 staining was observed (Fig. 5, C and D).

Similar differences were seen in P8 follicles, a stage where WT follicles were nearing their height of growth and differ-

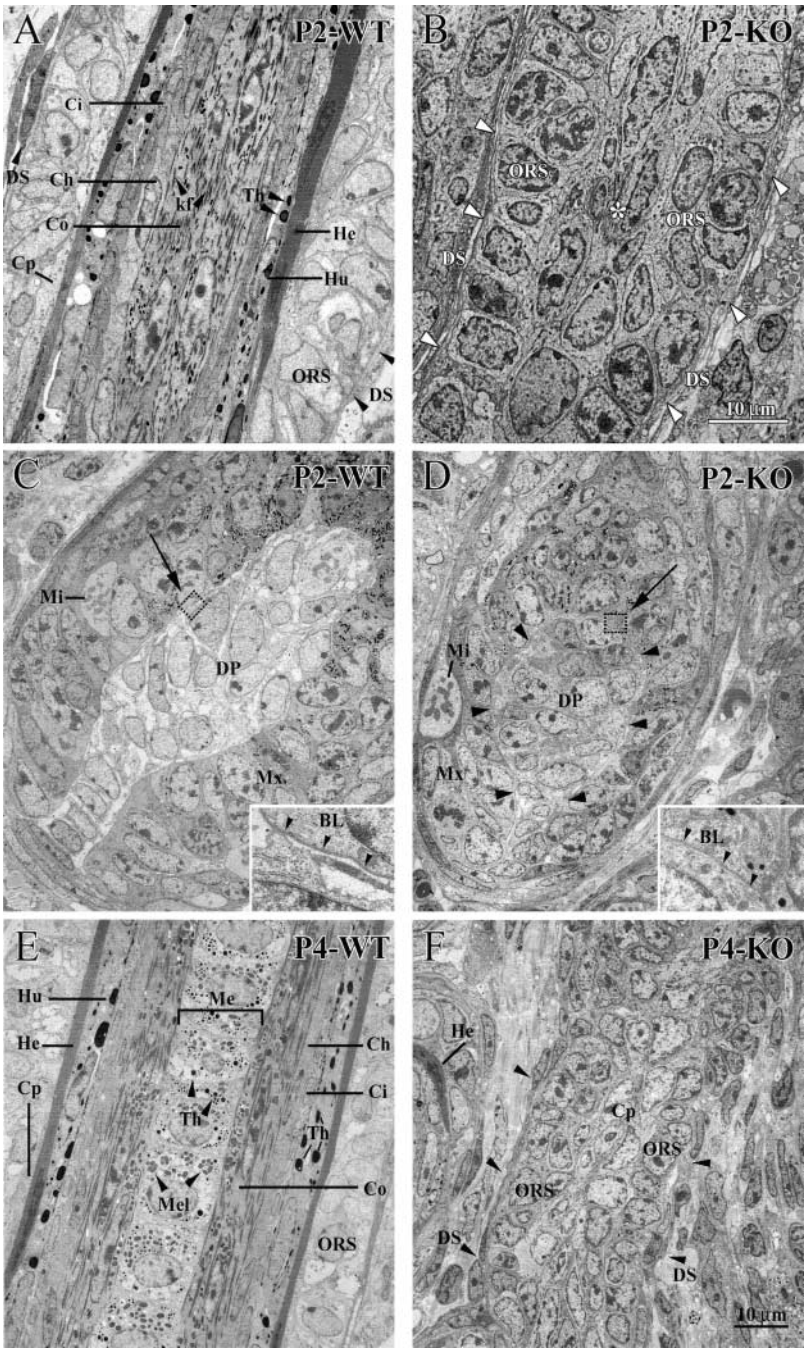


Figure 4. **Ultrastructural abnormalities in *Bmpr1a*-null follicles.** Backskins of WT and KO animals were processed for transmission EM. All follicles are oriented with the skin surface at the top. (A) Sagittal section of the mid-segment of a P2 WT follicle, depicting the thin Henle's layer (He) with uniform density due to keratinization. This layer is flanked externally by the companion layer (Cp) and ORS and finally the dermal sheath (DS; arrowheads). Internally, the Henle's layer (He) is flanked by the trichohyalin granule (Th)–rich Huxley's layer (Hu) of the IRS, and the thin IRS cuticle (Ci). The hair shaft is internal to the IRS, and at this stage most shafts are composed only of two major layers: the cortex (Co) rich with keratin filaments (kf), and the cuticle of the hair shaft (Ch). (B) Sagittal sections from *Bmpr1a*-null P2 follicles. View from the mid-region above the follicle bulb. Note mostly ORS cells. Note the lack of hair shaft or IRS structures. Arrowheads show DS and asterisk denotes Cp. (C and D) Sagittal sections of bulb from a WT and KO P2 follicle. Note mitosis (Mi) in Mx cell. Note also the central strand of DP, whose cytoplasmic density is less than the surrounding matrix (Mx) of WT follicles. Note that distinctions between DP and Mx are less obvious in KO follicle, despite their clear separation by an intact basal lamina (BL). Boxed areas are magnified in the insets. Arrowheads point to BL. (E and F) P4, sagittal sections of mid-segments of a P4 WT (E) and KO (F) follicle. Note that at this age, most WT follicles display a well-developed hair shaft composed of an inner core of medulla (Me) cells with trichohyalin granules (Th) and melanin granules (Mel; arrowheads); otherwise the morphology is largely similar to P2. In contrast, note that the P4 KO follicle is still undeveloped, and appears more analogous to its P2 state. Bar in B is valid for A and B; bar in F is valid for C–F.

entiation. By this age, all WT backskin follicles displayed AE15 and AE13 staining whenever sectioning was in the plane of these structures (Fig. 5, E and F). In contrast, >90% of P8 KO follicles lacked AE15 and AE13 reactivity (Fig. 5, G and H). The occasional KO backskin follicle that exhibited histological and biochemical signs of IRS and hair shaft production correlated with the low levels of *BMPRIA* mosaicism observed.

Antibodies against keratin 5 (K5) are specific for the epidermal basal layer and ORS of the normal hair follicle (Fig. 5, A and E; Byrne et al., 1994). In addition, certain keratin 6 (K6) proteins are specific for the companion cell layer, separating ORS and IRS (Fig. 5, B and F; Winter et al., 1998). Both of these markers were maintained in KO follicles (Fig. 5, C, D, G, and H). As judged by immunofluorescence, K6 expression appeared to be increased and expanded in KO versus WT follicles.

## Transcriptional changes resulting from a loss of BMP signaling

To probe further into the aberrations in IRS and hair differentiation, we examined the expression of the transcription factors known to regulate these programs. The transcription factor GATA-3 plays an essential role in the differentiation of IRS progenitor cells (Kaufman et al., 2003), whereas hair-specific keratin genes possess regulatory sequences for the Lef1 transcription factor (Zhou et al., 1995). GATA-3 was markedly diminished in KO follicles relative to their WT

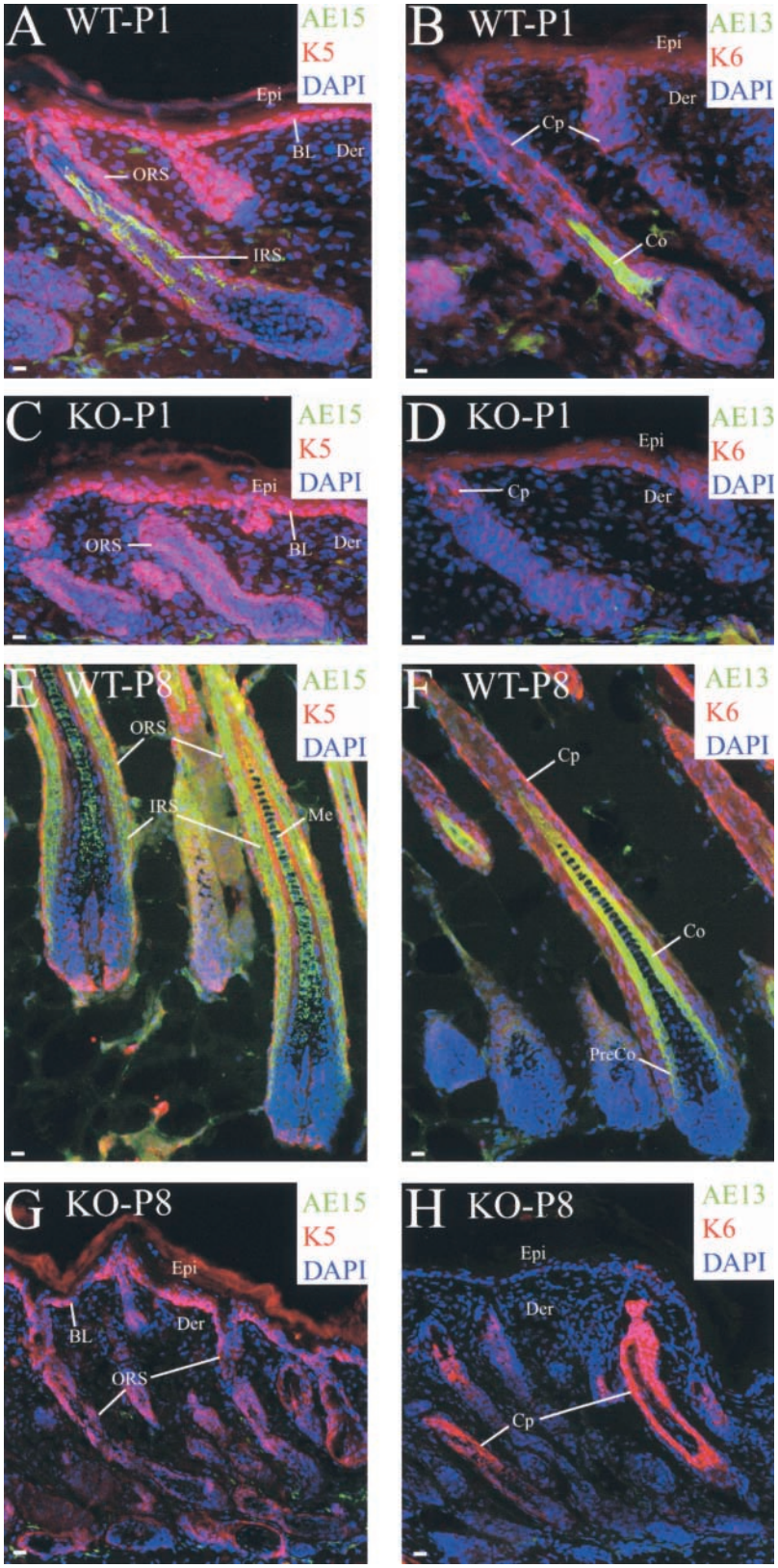


Figure 5. **Defects in IRS and hair shaft differentiation in *Bmpr1a*-null skin epithelium.** 8-μm frozen sections of P8 and P1 skins from *Bmpr1a*-null mice (KO) or their control littermates (WT) were subjected to indirect immunofluorescence using the antibodies indicated. Color coding is according to the secondary antibodies used: AE15, specific for all three layers of the IRS and medulla (Me); AE13, specific for the hair keratins of the cortex (Co) and precortex (Pre-Co); K6, specific for the K6 expressed in the companion layer (Cp); and DAPI, a fluorescent dye, which intercalates into DNA. Der, dermis; Epi, epidermis; BL, basal layer; ORS, outer root sheath; and K5, keratin 5. Bars, 10 μm.

counterparts (Fig. 6, A and B). In contrast, although KO follicles exhibited a loss of AE13 immunoreactivity, they retained Lef1 (Fig. 6, C and D). The result supported the hypothesis that Lef1 expression in skin keratinocytes may depend on inhibition of BMP signaling (Botchkarev et al., 1999; Jamora et al., 2003).

Finally, we tested for possible secondary abnormalities in DP expression. Despite a relatively uncondensed DP, both Lef1 and the mesenchymal transcriptional regulator Runx3 were expressed in both WT and KO DP (Fig. 6, C, D, G, and H). WT and KO follicle bulbs also exhibited a line of labeling with antibodies against β4 integrin, a

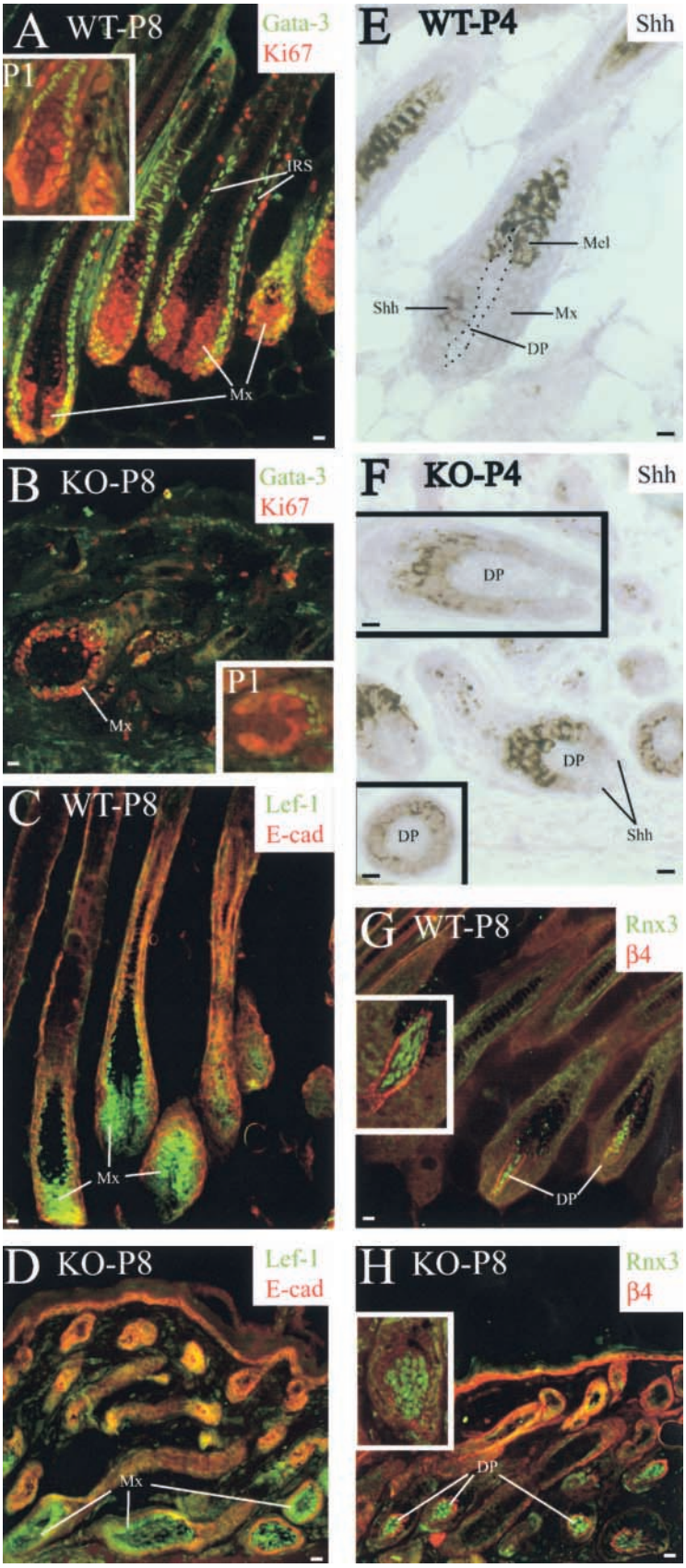


Figure 6. **Defects in transcriptional regulators of the IRS and hair shaft and epithelial–mesenchymal alterations in *Bmpr1a*-null skin epithelium.** 8-μm frozen sections of P8 or P4 skins from *Bmpr1a*-null mice (KO) or control littermates (WT) were subjected to either indirect immunofluorescence using the antibodies indicated (color coding according to the secondary antibodies) or in situ hybridization. Antibodies/probes were: GATA-3, a differentiation regulator of Hu and cuticle layers of the IRS; Ki67, a marker of cycling (proliferating) cells; Lef1, specific for Mx, pre-Co, Co, and DP; E-cad, specific for E-cadherin, present throughout skin epithelium, but reduced in Mx and its progeny; a digoxygenin-labeled cRNA probe for *Sonic hedgehog* (*Shh*), thought to be a proliferation regulator in skin; Rnx3, Runx3, a transcriptional regulator found in DP; β4 integrin, specific for hemidesmosomes attached to an underlying basement membrane; and DAPI, a fluorescent dye, which intercalates into DNA. DP, dermal papilla; Mx, matrix; IRS, inner root sheath; and Mel, melanin granules. Bars: (A–D, G, and H) 10 μm; (E, F, and inset) 20 μm.

component of hemidesmosomes which help to adhere matrix cells to the basement membrane at the DP boundary (Fig. 4, G and H). With this said, other DP aberrations are noted below.

To examine proliferation status of KO follicle cells, we used an antibody against Ki67, a proliferating nuclear antigen that is active in cycling cells. This antibody strongly stains the proliferating matrix cells of WT P8 follicles (Fig. 6

A). Importantly, the antibody also labeled matrix in KO follicles, although the pattern of labeling was different from that in WT (Fig. 6 compare B with A). In addition, as judged by in situ hybridization, expression of *Sonic hedgehog* (*Shh*) was still seen in KO follicle bulbs. However, rather than the highly restricted, asymmetric pattern typical of *Shh* in WT bulbs (Fig. 6 E), *Shh* expression was expanded and expressed symmetrically in matrix cells near the DP boundary (Fig. 6 F). When together, with our ultrastructural studies, these data suggested that matrix cells were not growth arrested, but rather disorganized and blocked in their differentiation. For the IRS, the block appeared to be at the level of elevated GATA-3 expression, although it is not clear whether the decrease was a cause or consequence of BMP signaling; for the hair shaft, the block appeared to be past the level of Lef1 expression but before the Wnt-mediated activation of hair-specific keratin genes. Further experiments addressing this issue are provided below.

### Skin grafting reveals defects in postnatal development of *Bmpr1a*-null hair follicles

*Bmpr1a*-null mice were typically frail, and only mosaic animals survived beyond several days after birth. Consequently, to assess the long-term consequences of ablating BMPRIA expression in skin epithelium, we grafted full thickness neonatal skin from *Bmpr1a*-null and WT littermate mice onto the hairless backs of immunocompromised, recipient *nu/nu* mice. To confirm the uniformity of targeting within the skin selected for grafting, we used KO skin that exhibited spots of organized melanin granules, but no signs of body hairs (Fig. 7, A and A′). At 24 d after engraftment, hairs were evident only on WT grafts (Fig. 7 B). Histologically, these follicles appeared normal (unpublished data). In contrast, follicles formed in KO grafts were aberrant (Fig. 7, C and C′). In some cases, odd follicle-like structures had grown downward but displayed only wisps of IRS cells (Fig. 7 C, left). These follicles lacked a hair shaft and IRS, but possessed a well-developed sebaceous gland. In most cases, cystlike structures developed (Fig. 7, C and C′). At the light microscopy level, the cells within the cysts appeared morphologically similar to those of embryonic follicles, but ultrastructural analyses revealed their peculiar organization (Fig. 7 D). The cysts typically contained invaginations of mesenchymal cells, which were separated from the epithelial cells by a basal lamina (Fig. 7 D, inset). Each cyst was also surrounded by a basal lamina, on the other side of which was a dermal sheath of mesenchymal cells (Fig. 7 D). At the central core, some cyst cells possessed melanin granules (unpublished data). Their presence was interesting, as these granules are taken up by early stage, differentiating hair cells. With this possible

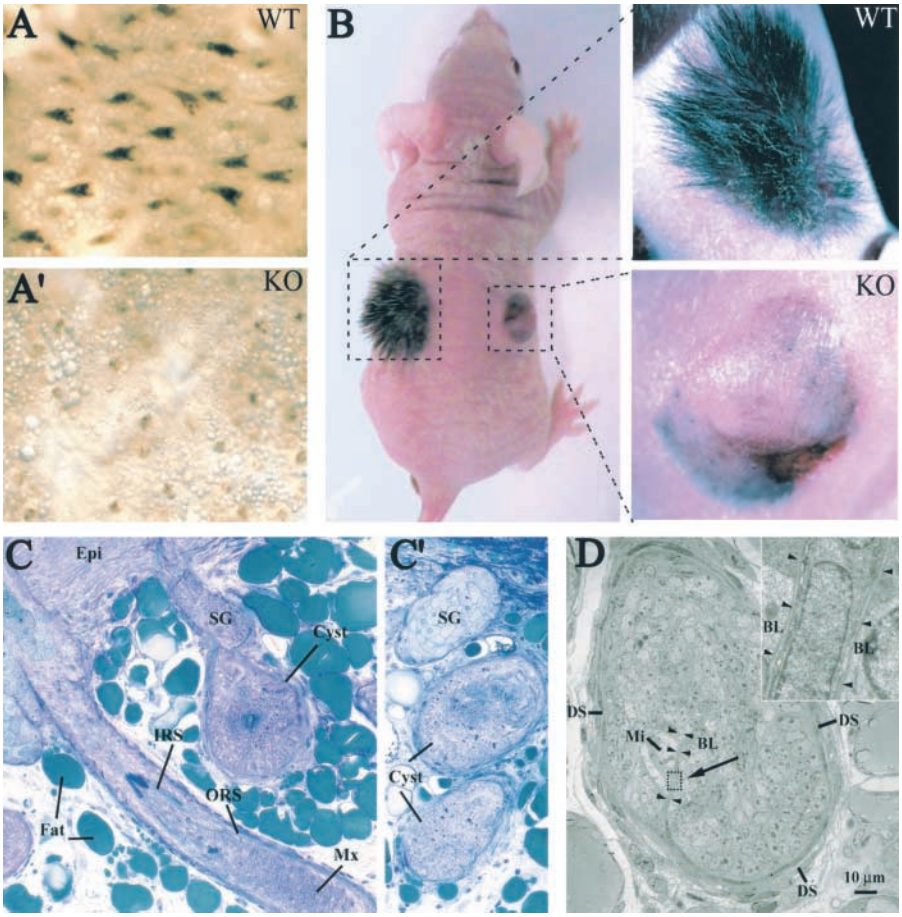


Figure 7. ***Bmpr1a*-null skin grafts develop strikingly abnormal hairs.** Full-thickness skins from WT and *Bmpr1a*-null (KO) P2 C57/Bl6 mice were grafted onto the left and right sides, respectively, of *nu/nu* mice. (A and A′) Skin of WT animals displayed black hairs, which protruded from the skin surface. In contrast, black melanin granule spots, indicative of underlying, developing hair placodes (pigmented) were visible, but no hairs were detected. Sectioning and staining of adjacent regions confirmed the paucity of BMPRIA in KO skin. (B) After engraftment, hairs (black) appeared in WT grafts but appeared absent in *Bmpr1a*-null grafts. By 24 d (shown), pelage hairs were only seen on WT grafts; KO grafted skin displayed a taut, shiny surface; the grayish tone was due to melanin granules from melanocytes present in the full thickness grafts. (C) Skins from 24-d grafts were fixed in glutaraldehyde, embedded in epon, and sectioned (1 μm). Sections were stained with toluidine blue. Note that normal follicles, replete with IRS and medulla, were seen in WT grafted skin (not depicted). Here, we focus on the cystlike follicular structures, prevalent in KO grafted skin. Some cysts emanated from the epidermis, indicating that these structures reflected aberrant folliculogenesis, rather than transverse sections of follicles. Note the presence of larger follicle, rare in KO skin. Sg, sebaceous gland; Epi, epidermis; ORS, outer root sheath; IRS, inner root sheath; and Mx, matrix. (D) Ultrastructural analyses of KO cyst. Note mitoses (Mi) in cysts, and also invaginations of DP-like cells (arrowheads). These pockets of dermal cells are surrounded by a basal lamina (BL; inset), which extends to and encases the cyst surface. DS, dermal sheath. The arrow indicates the area, which is magnified in the inset.

exception, cyst cells appeared to be a mixture of matrix, ORS, and companion cell layer cells. They displayed numerous mitoses, reflective of a proliferative state, but they also exhibited some apoptotic figures, in contrast to their WT counterparts.

At the immunohistochemistry level, some rare follicle-like structures were seen in KO skin (Fig. 8 A, center structure). These possessed sebaceous glands and ORS, which were positive for K5; they also displayed a cystlike structure at the base (Fig. 8 A, example shown). However, the stem of these rare structures were hollow, with no signs of AE15 or AE13 staining (Fig. 8, A and B). Most cysts expressed K5 toward their periphery and K6 at their centers. Many of the more peripheral cells were also positive for Ki67, confirming the proliferative state of the cysts (Fig. 8 C). Antibodies against GATA-3 weakly labeled a small subset of cells within most cysts (Fig. 8 C, inset). In contrast, many cyst cells labeled with antibodies against Lef1 (Fig. 8 D).

In situ hybridization revealed *Shh* expression, but in an unusual pattern, paralleling the number of invaginations of mesenchymal cells seen within each cyst (Fig. 8 E). Closer inspection revealed that *Shh* was restricted to a subset of cells maintaining close contact with invaginating DP-like, mesenchymal cells. Even at this stage, the follicle-like structures from grafts continued to express Wnts, as shown here by the marked expression of mRNAs for Wnt10b, a major Wnt of the hair follicle (Fig. 8 F; Reddy et al., 2001). Wnt3, another Wnt prominent in the hair follicle, has recently been shown to be expressed in the developing limb epithelium of *Bmpr1a*-null animals (Soshnikova et al., 2003). Thus, expression of both Shh and Wnt ligands seemed to persist in the aberrant KO follicles.

The corresponding epithelial invaginations surrounding the mesenchymal inlets of cysts were distinguished by anti-β4 staining (Fig. 8 G). In addition, a ring of anti-β4 staining marked the epithelial boundaries of cysts, which were

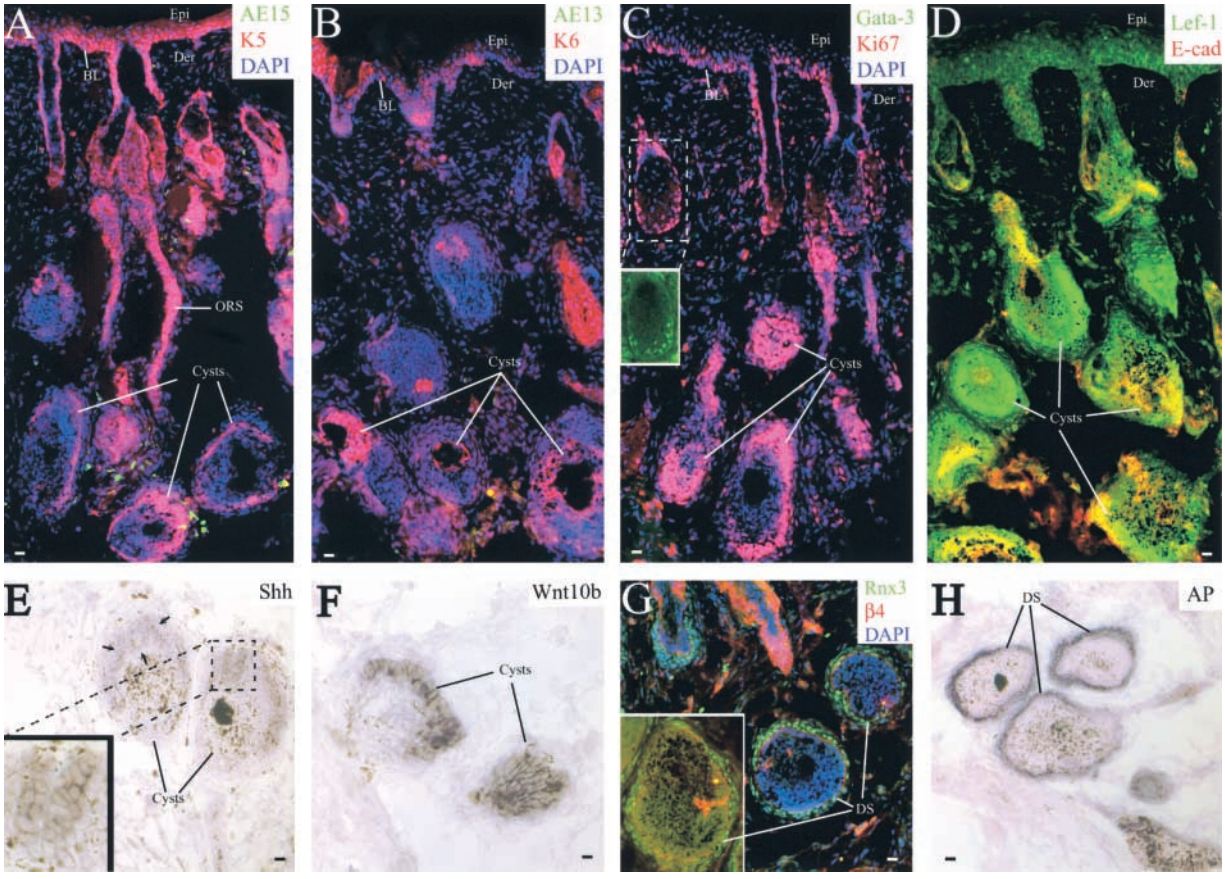


Figure 8. **Biochemical analyses of cysts generated in grafted *Bmpr1a*-null skin epithelium.** 8-μm frozen sections of grafted P2 skins (24-d grafts) of *Bmpr1a*-null mice (KO) or their control littermates (WT) were subjected to indirect immunofluorescence or in situ hybridizations as indicated. Color coding is according to the secondary antibodies used: AE15, specific for IRS and Me; AE13, specific for hair keratins of the cortex; GATA-3 specific for the Huxley's and cuticle layers of the IRS (boxed area shows only the green channel to reveal a low level of GATA-3 expression in some cyst cells); Ki67, a marker of cycling (proliferating) cells; K6, specific for the K6 expressed in the Cp; Lef1, specific for the matrix, precortex, cortex, and DP (note its aberrant up-regulation in epidermis lacking BMPRIA); E-cad, specific for E-cadherin, present throughout skin epithelium, but reduced in the matrix; Runx3 and alkaline phosphatase (AP), present in DP cells; β4 integrin, specific for hemidesmosomes attached to an underlying basement membrane; DAPI, a fluorescent dye, which intercalates into DNA; *Shh*, a cRNA probe against sonic hedgehog (arrows denote *Shh* expression; boxed region is magnified to see expression); and *Wnt10b*, a cRNA probe against this Wnt, expressed in the follicle bulb of WT skin (Reddy et al., 2001). Note that larger brown grains are melanin granules. The DAPI channel was removed for the inset shown in G, to better visualize the internal β4-positive cells, which mark dermal invaginations. Epi, epidermis; Der, dermis; BL, basal layer; DS, dermal sheath; ORS, outer root sheath; and K5, keratin 5. Bars, 10 μm.

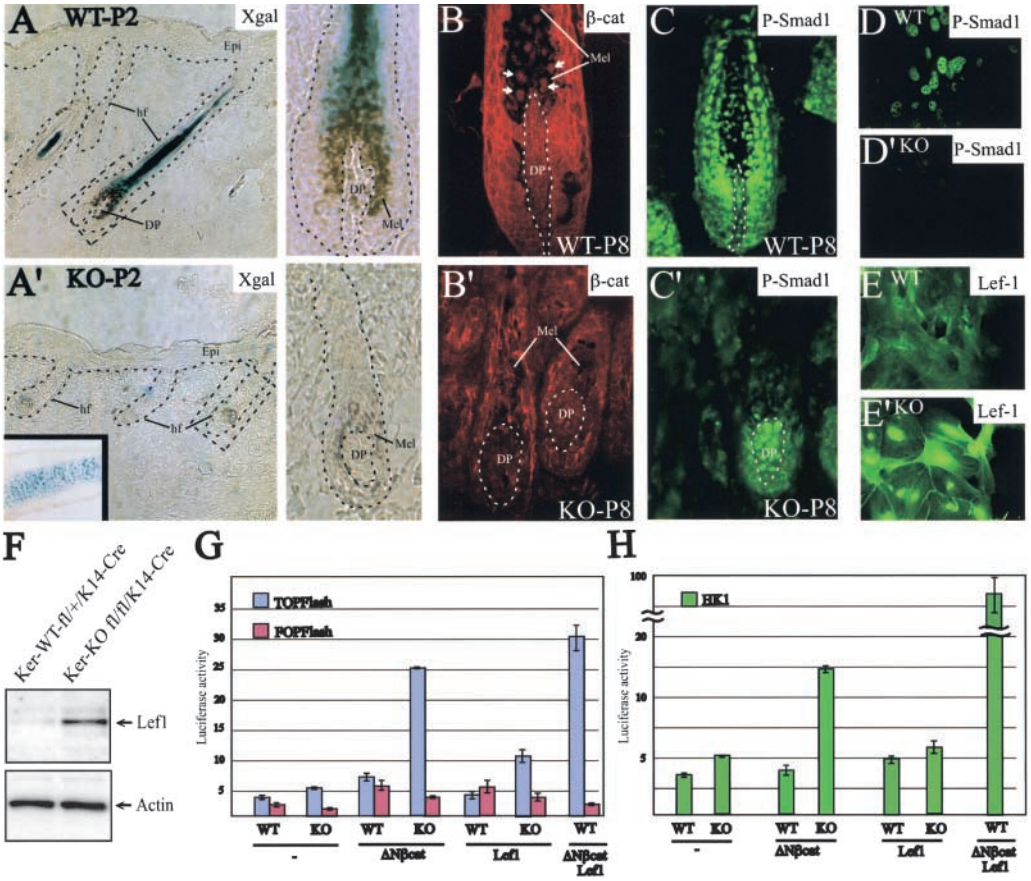


Figure 9. **Failure to activate Wnt-responsive genes and accumulate nuclear β-catenin when BMPRIA signaling is blocked.** (A and A′) TOPGAL activity in postnatal follicles. Note activity in WT follicles, absent in *Bmpr1a*-null follicles. Inset shows control TOPGAL activity in cartilage, verifying TOPGAL activation in non-K14Cre expressing cells. Mel, melanin granules. (B and B′) Nuclear β-catenin in postnatal (P8) WT follicles (double arrows), absent in KO follicles. (C and C′) Immunoreactivity against nuclear phospho-Smad1 (induced upon BMPRIA activation) in developing IRS/hair in WT but not KO. (D–E′) Immunoreactivity of primary cultured keratinocytes to either antiphospho-Smad1 or anti-Lef1, as indicated. (F) Anti-Lef1 and anti–β-actin (control) immunoblot of lysates from WT and KO cells. (G and H) Effect of *Bmpr1a* ablation on activity of Wnt-regulated promoters. Two Wnt-responsive luciferase reporter genes were used for the work: TOPFlash (Korinek et al., 1997) and the murine hair keratin promoter equivalent, HK1Flash (Zhou et al., 1995; Merrill et al., 2001). The TOP promoter with a mutation in the TCF/Lef1 binding site (FOPFlash) was used as a control for binding specificity. Constructs were transiently transfected ± K14-ΔNβ-catenin (Gat et al., 1998) ± K14-Lef1 into keratinocytes, and 48 h after transfection, cells were lysed and processed for luciferase activity. CMV-renilla was used to control for transfection efficiency, and the results are the average of three independent experiments performed in triplicate, with the standard mean of deviation provided. Luciferase activities are given in arbitrary units relative to control. ≈, represents a gap in the scale of the histogram.

encased by a basal lamina. On the opposite side were several layers of mesenchymal cells. They differed from a normal DS in that they were positive for Runx3 (Fig. 8 G) and alkaline phosphatase activity (Fig. 8 H). Curiously, both Runx3 and alkaline phosphatase are found in dermal condensates including DP (Handjiski et al., 1994; Yamashiro et al., 2002).

Thus, in the absence of BMPRIA, follicles appeared to develop, but the ORS could not sustain its normal architecture in the absence of an IRS and hair shaft. The bulbar structures continued to grow, but reorganized into cystlike structures. Interestingly, changes in the biochemistry of the KO cyst cells appeared to impact indirectly on the organization/specification of both epithelium and mesenchyme.

Finally, GATA-3, which is expressed in WT epidermis (Kaufman et al., 2003), was down-regulated in KO epidermis as it was in the KO IRS progenitors (Fig. 8 C). In addition, normally expressed in embryonic epidermis, Lef1 was induced in postnatal KO epidermis, albeit at reduced levels relative to KO cysts (Fig. 8 D). Some epidermal regions in grafted skin were also thicker than WT, and exhibited K6 expression and increased Ki67 labeling, reflective of hyperproliferative epidermis (Fig. 8, B and C). For the purposes of the present paper, we focused on understanding the role of BMPRIA on hair follicles, although clear epidermal abnormalities were noted.

### Dissecting the molecular defects underlying the block in hair differentiation: a failure of *Bmpr1a*-null follicles to activate Lef1/β-catenin gene expression

To further explore the molecular defects underlying BMPRIA-related defects in hair shaft differentiation, we tested the ability of Bmpr1a-null matrix cells to activate Lef1/β-catenin–regulated genes. For these studies, we generated

mice conditionally null for *Bmpr1a* and transgenic for *TOPGAL*, driving β-galactosidase under the control of the Wnt-responsive TOP promoter. In vivo, *TOPGAL* is faithfully expressed in skin, wherever Lef1 and nuclear β-catenin are present, e.g., as in precortical and cortical cells (DasGupta and Fuchs, 1999). Genotyping was conducted to confirm that the desired genetics were successful (unpublished data).

For analyses, we took frozen sections of neonatal skin (test) and cartilage (control) and subjected them to X-gal assays. As shown in Fig. 9 A, WT follicles scored positive for TOPGAL activity in the cells known to respond to Wnt signaling. In contrast, follicles from *Bmpr1a*-null, *TOPGAL*-positive mice did not (Fig. 9 A′). That TOPGAL was present in KO animals was confirmed by its presence in tissues where the K14-Cre recombinase was not, e.g., in cartilage (Fig. 9 A′, inset).

In WT skin, *TOPGAL* expression correlated well with cells that exhibit nuclear Lef1 as well as nuclear β-catenin (Fig. 9 B). In KO skin, even though Lef1 was expressed, nuclear β-catenin was not detected in the corresponding region of developing follicles (Fig. 9 B′). These findings suggested that the defect resided in a failure of BMPRIA-deficient follicles to progress to the stage where they respond to Wnts, stabilize β-catenin and activate Lef1, and their downstream targets, including the hair keratin genes.

That BMPRIA signaling normally occurs at this time was verified by immunostaining with antibodies against phospho-Smad1, the transcriptional regulator, which is phosphorylated and activated upon ligand engagement of BMPRIA (for review see Massague, 1998). In WT follicles, these antibodies stained nuclei in the developing IRS, as well as in some layers of the developing hair (Fig. 9 C). In contrast, no nuclear staining was detected in either WT ORS, or in the hair bulb of KO follicles, although it was still detected in the DP, where BMP receptors were not targeted for ablation (Fig. 9 C′). These findings suggest that BMP signaling normally occurs in both the developing IRS and hair, and both are compromised in KO follicles.

To explore these differences in greater detail, we cultured primary keratinocytes isolated from skins of WT and KO animals. PCR was used to confirm the genotype of the cell populations. Two independently derived cultures were prepared for each genotype, and analogous behavior was observed within a particular genotype.

Because keratinocytes produce and secrete high levels of BMP2 and BMP4 (Jamora et al., 2003), we first verified that loss of BMPRIA was sufficient to prevent BMP signaling in these cultures. As shown in Fig. 9 D, WT keratinocytes displayed prominent nuclear anti–P-Smad1 staining. In contrast, KO keratinocytes exhibited little if any staining (Fig. 9 D′).

Previously, we showed that cultured neonatal keratinocytes require conditioned medium containing the BMP inhibitor Noggin in order to induce the expression of Lef1 mRNA and protein (Jamora et al., 2003). In the absence of added Noggin, WT keratinocytes displayed little if any nuclear staining of anti-Lef1 (Fig. 9 E). In contrast, KO keratinocytes displayed strong nuclear anti-Lef1 staining (Fig. 9 E′). Western blot analyses confirmed a marked increase in Lef1 expression in the absence of BMPRIA (Fig. 9 F). Overall, these data were consistent with a block in BMP signaling in response to the loss of BMPRIA. As importantly, the data demonstrated convincingly that the action of Noggin-conditioned medium on Lef1 expression observed previously in keratinocytes (Jamora et al., 2003), is mediated through effects on BMP receptor activation.

To test whether the Lef1 produced by *Bmpr1a*-null keratinocytes was able to activate Wnt-regulated genes, we conducted in vitro studies using two well-established Wnt-responsive promoters: TOP, containing multimerized Lef1 DNA-binding sites (Korinek et al., 1997); and HK1, one of the hair-specific keratin promoters containing functional Lef1 DNA-binding sites (Zhou et al., 1995; Merrill et al., 2001). As a control, we used the FOP promoter, harboring mutations in the Lef1 binding sites of TOP. All promoters were used to drive expression of luciferase (Flash).

Under normal media conditions, both cultures exhibited low activation of *TOPFlash* and *HK1Flash*, relative to *FOPFlash* (Fig. 9, G and H). In the presence of an $NH_2$-terminally truncated, stable β-catenin, WT keratinocytes still showed minimal activation of either *TOPFlash* or *HK1Flash*. In contrast, KO cells displayed a greater than fivefold upregulation of *TOPFlash* and *HK1Flash* in response to transfected ΔNβ-catenin (Fig. 9, G and H). The results obtained with *Bmpr1a*-null keratinocytes were similar to those which we had observed with WT keratinocytes treated with Noggin (Jamora et al., 2003). Together, these data demonstrated that (a) *Bmpr1a*-null keratinocytes are deficient in Smad activation; (b) these KO keratinocytes produce Lef1, whereas WT keratinocytes require Noggin treatment for activation; and (c) Wnt-responsive promoter activity in *Bmpr1a*-null keratinocytes can be achieved by transfection with a constitutively stabilized β-catenin.

## Discussion

### A surprising role of BMPRIA in IRS development

The first obvious morphological defect in K14-Cre, *Bmpr1a fl/fl* skin was the failure of the IRS to develop. Our studies also show clearly that BMPRIA signaling occurs in the IRS, and in the absence of the receptor, developmental defects in the IRS arise under conditions where the ORS appears relatively unperturbed and the hair shaft has not yet developed. Thus, it is curious that ectopic expression of an Msx2-Noggin transgene in the epithelial cells of the hair bulb of >P2-aged mice resulted in primarily hair shaft defects without IRS abnormalities (Kulessa et al., 2000). One explanation is that differential expression of BMP4 in the IRS but not the hair shaft spares the IRS from Msx2-noggin action. In this regard, BMP signaling molecules have been reported to have relatively short-range effects (Nikaido et al., 1999).

Precisely how IRS is affected is not yet clear. Our results suggest an inability of *Bmpr1a*-null skin to sustain elevated levels of GATA-3, the transcription factor that we recently implicated in IRS differentiation (Kaufman et al., 2003). Intriguingly, links between GATA factors and BMP signaling have been reported in other cell types (Patient and McGhee, 2002). Thus, in cardiomyocytes, Noggin suppresses expression of GATA-4, required for terminal differentiation (Monzen et al., 1999), whereas conversely in embryonic

blood, BMP4 induces GATA-2 (Xu et al., 1999). In addition, GATA factors have recently been shown to induce and/or sustain the expression of BMPs (Nemer and Nemer, 2003; Peterkin et al., 2003), thereby providing a potential feedback loop to promote terminal differentiation. When considered with our present findings on BMPRIA, the IRS lineage becomes a new candidate for a cell type whose differentiation may be regulated by BMP-GATA circuitry.

### New insights into the importance of both inhibition and promotion of BMP signaling during hair shaft development

For more than a decade, BMPs and BMP antagonists have been implicated in hair follicle morphogenesis (for reviews see Botchkarev, 2003; Alonso and Fuchs, 2003). During embryonic development, increased BMP signaling inhibits the initiation of follicle morphogenesis, whereas noggin activity stimulates the process (Noramly and Morgan, 1998; Botchkarev et al., 1999; Botchkarev, 2003). Postnatally, noggin still seems to block hair shaft differentiation and hair keratin expression (Botchkarev et al., 1999; Kulessa et al., 2000; Jamora et al., 2003). The fact that hair progenitor cells form in the absence of BMPRIA provides compelling evidence that BMP signaling is not required for hair progenitor formation. However, the defect in hair formation in these follicles suggests that BMP signaling is necessary for the differentiation of these progenitors.

BMPRIA is the only BMP receptor known to be expressed in the follicle (Botchkarev et al., 1999), and the pattern of phospho-Smad1 antibody staining was consistent with the activation of this receptor not only in IRS but also in some cells of the developing hair. With this said, because the IRS develops before the hair shaft and is required for its proper differentiation (Kaufman et al., 2003), we cannot be unequivocally certain that the defects in hair differentiation and hair keratin gene expression seemed to be stronger than in the GATA-3 knockout, leading us to surmise that at least some of the defects in matrix to cortical conversion are attributable directly to the absence of BMPRIA activation in this lineage.

Although phospho-Smads have not been implicated in hair keratin gene expression, several other DNA-binding proteins are critical, including Lef1, which functions as a positive regulator upon Wnt signaling of the precortical cells (Zhou et al., 1995; DasGupta and Fuchs, 1999; Merrill et al., 2001), Hoxc13, a homeobox gene expressed in matrix, precortex, cortex, and cuticle (Jave-Suarez et al., 2002); and Foxn1, a forkhead/winged-helix transcription factor (Schlake et al., 2000). When defective, all of these genes yield marked hair follicle phenotypes (Nehls et al., 1994; van Genderen et al., 1994; Segre et al., 1995; Godwin and Capecchi, 1998).

Foxn1 and Hoxc13 are down-regulated in Msx2-noggin transgenic follicles (Kulessa et al., 2000). A priori, because Lef1 is up-regulated, both in the presence of noggin (Kulessa et al., 2000; Jamora et al., 2003) and in the absence of BMPR1 (this paper), Foxn1 and Hoxc13 might seem more likely candidates for BMP control. In fact, we have found that Foxn1 is down-regulated in our *Bmpr1a*-null skin (unpublished data). With this said, Hoxc13- and Foxn1- null mutations both permit development of cortex and medulla, whereas Lef1-null mutations act considerably earlier in hair follicle morphogenesis (van Genderen et al., 1994).

Because the *TOPGAL* transgene is only responsive to Lef1/β-catenin and not to Foxn1 or Hoxc13, the absence of its activity in the postnatal follicles of *Bmpr1a*-null mice was informative. Lef1 expressed by KO keratinocytes was clearly functional, as evidenced by the ability of a constitutively stabilized ΔNβ-catenin to rescue TOP as well as hair keratin promoter activity in *Bmpr1a*-null keratinocytes. In addition, at least some Wnts were still expressed by *Bmpr1a*-null skin. Thus, although other explanations are possible, a defect in Wnt responsiveness could explain the failure of hair keratin

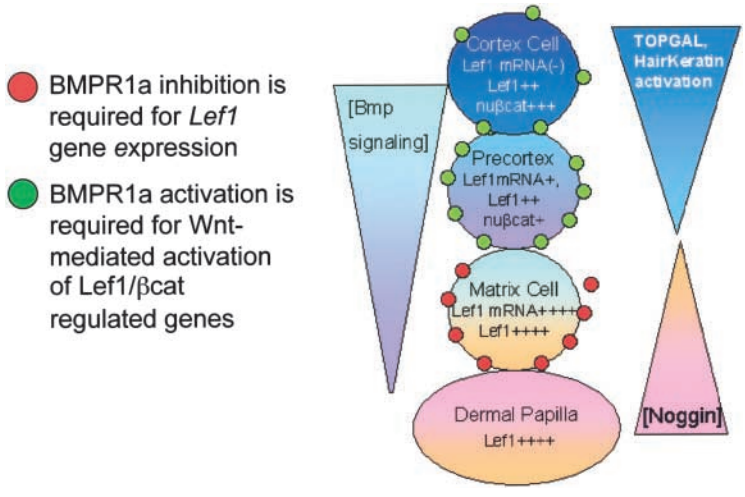


Figure 10. **Model for how opposing signaling through BMPR1A controls Lef1/βcat signaling in stem cell lineage differentiation of the hair shaft.** According to the model, in the postnatal follicle, epithelial stem cells are activated through interactions with DP to generate matrix cells (Cotsarelis et al., 1990). DP cells produce a noggin signal, which inhibits BMP signaling, leading to elevated Lef1 mRNA expression and proliferation, maintaining the cells in an undifferentiated state (Zhou et al., 1995; Botchkarev et al., 1999; Jamora et al., 2003). Despite the expression of Lef1 and some Wnts, hair cell progenitors do not activate Lef1/β-catenin when they are inhibited from BMPRIa activation (this paper). Whether the inhibition of BMP signaling prevents Wnt receptor expression (Soshnikova et al., 2003), prevents nuclear β-catenin from being stabilized, or blocks some earlier step in differentiation is unknown. However, matrix cells exposed to Noggin begin to express BMP4 (Kulessa et al., 2000), and in combination with their upward movement, the cells are likely to activate their BMPRIa receptor. The predicted outcome is attenuation of Lef1 mRNA expression, but a window of differentiation (the precortex) may exist where cells have stabilized nuclear β-catenin and still have sufficient Lef1 protein to activate Wnt-mediated genes. Natural Wnt target genes include the hair-specific keratin genes and Foxn1, another transcriptional regulator of hair keratin gene transcription (Zhou et al., 1995; DasGupta and Fuchs, 1999; Merrill et al., 2001; Andl et al., 2002; Balciunaite et al., 2002). Wnt/Lef1-mediated active chromatin may further act to stabilize Lef1 to enable target genes to remain active in the cortex after Lef1 mRNA is down-regulated.

genes to be activated in *Bmpr1a*-null skin. Interestingly, in thymus, Foxn1 expression was recently shown to be dependent on Wnt signaling (Balciunaite et al., 2002), providing a possible explanation as to why Foxn1 expression was also down-regulated in the *Bmpr1a*-null skin.

Our results are particularly intriguing in light of recent works by Soshnikova et al. (2003), who targeted conditional ablation of the BMPRIA receptor in the apical ectodermal ridge. Not only was limb development severely impaired in these animals, but in addition, Wnt responsiveness was markedly impaired. Experiments using gain or loss of function mutations in β-catenin revealed that β-catenin stabilization acts downstream of BMPRIA receptor activation in the AER (Soshnikova et al., 2003). Although BMP signaling need not always occur upstream from Wnt signaling (Barrow et al., 2003), our findings suggest that it appears to act earlier than Wnts in hair shaft differentiation, in a fashion more similar to the AER.

In the context of our prior studies on hair shaft lineage determination, our findings suggest a new twist on the interplay between Wnts and BMP signaling. Ironically, in the postnatal hair follicle, inhibition and activation of BMP signaling both seem to be required to prime cells in order to execute Wnt signaling and activate its differentiation program. Fig. 10 provides a model, which may explain this paradox. In the hair shaft lineage, DP cells secrete noggin, which may inhibit BMPRIA activation in matrix. This inhibition may then allow matrix cells to produce elevated levels of Lef1 mRNA (Zhou et al., 1995; Botchkarev et al., 1999; Jamora et al., 2003) along with the other transcription factors such as Msx1/2 (Kulessa et al., 2000). However, without BMP signaling, the matrix cells may be prevented from activating Lef1/β-catenin and differentiating.

Interestingly, exposing cells to noggin results in BMP4 up-regulation (Kulessa et al., 2000). Thus, in time, matrix cells may balance BMP inhibitors with BMP activators, thereby activating BMPRIA. This process could also be accelerated as maturing matrix cells sever their connection with DP and move upward. As long as the hair progenitor cells have stockpiled sufficient Lef1, they should subsequently be able to respond to Wnt signaling, and activate the hair keratin genes (Merrill et al., 2001), *Foxn1* (Balciunaite et al., 2002), the Wnt reporter gene *TOPGAL* (DasGupta and Fuchs, 1999), and presumably other genes, which then drive the cells into terminal differentiation. Although additional studies will be necessary to test this model, our current results take us one important step closer to understanding the intricacies that underlie the complex spatiotemporal patterns of hair follicle growth and differentiation.

## Materials and methods

### Mice

The generation and characterization of the *Bmpr1a fl/fl* mice has been described previously (Mishina et al., 2002). The mice were mated with previously generated K14-Cre mice (Vasioukhin et al., 1999) to generate mice homozygous for the loss of BMPRIA function in skin epithelium. K14-Cre is active by E9 of skin development, and is effective at quantitative ablation by E15. These mice were also mated on the background of TOPGAL transgenic mice, driving expression of β-galactosidase only under conditions where cells are responsive to Wnt signaling and already express a member of the Lef1/Tcf family of DNA-binding proteins (DasGupta and Fuchs, 1999).

### Histology and EM

Tissues for immunofluorescence and hematoxylin and eosin staining were embedded and frozen immediately on dry ice. For transmission EM, tissues were fixed in 2% glutaraldehyde, 4% formaldehyde, and 2 mM $CaCl_2$ in 0.05 M sodium cacodylate buffer for >1 h at room temperature and processed for Epon embedding as described previously (Kaufman et al., 2003). Samples were visualized using a transmission electron microscope (Tecnai G2).

### In situ analysis

Digoxygenin probe synthesis was performed according to the manufacturer's instructions (Roche). In situ hybridizations on 10-μm frozen sections were performed as described previously (Schaeren-Wiemers and Gerfin-Moser, 1993).

### X-gal staining and immunofluorescence

OCT sections of indicated thickness were briefly fixed (30 s) in 0.1% glutaraldehyde, washed three times in PBS, and incubated in X-gal staining solution for 1–2 h at 37°C. Standard procedures were used for immunofluorescence staining. In short, OCT sections of indicated thickness were fixed for 10 min in 4% PFA in PBS and washed four times for 5 min in PBS. When staining with mouse mAbs, we used the reagents and protocol from the MOM Basic kit (Vector Laboratories). In other cases, the following block/diluent was used 2.5% NDS, 2.5% NGS, 1% BSA, 2% gelatin, and 1% Triton X-100 in PBS. The following primary antibodies were used at the indicated concentrations: AE13 (mouse, 1:50–1:100) (Lynch et al., 1986); AE15 (mouse, 1:10) (O'Guin et al., 1992); K5 (gp, 1:250) (Fuchs laboratory); GATA-3 (mouse, 1:100) and BMPRIA (1:100) (Santa Cruz Biotechnology, Inc.); β-galactosidase (rabbit, 1:400; mouse, 1:100) (Harlan; Sigma-Aldrich); Lef-1 (rabbit, 1:250) (Fuchs laboratory); K6 (rabbit, 1:500) (Fuchs laboratory); and Ki67 (rabbit, 1:1000) (NovoCastra Laboratories Ltd.). Relevant FITC or TxR-conjugated donkey or goat antibodies (1:100) (Jackson Laboratories) were used for detection of primary antibodies. For phospho-Smad1, we used the TSA™ Plus Fluorescing System from Perkin Elmer Life Sciences.

### Skin grafting and cell culture

Neonatal *Bmpr1a* conditionally null animals were identified by their lack of whisker follicles, and were killed along with their WT littermates. Full-thickness skins were removed from the torsos of wild-type and null embryos, spread on a sterile plastic dish, and stored briefly at 4°C. During this time, each skin graft recipient site was prepared by removal of a patch of full-thickness skin on an anesthesized female *nu/nu CD-1* mouse. Donor skin was placed on the graft bed and secured by sterile gauze and cloth bandages. Each showed a consistent phenotype dependent on the presence or absence of BMPRIA in the donor skin. Primary keratinocytes were cultured as described previously (Jamora et al., 2003).

We thank Yuji Mishina (National Institutes of Health, Bethesda, MD) for the *Bmpr1a* floxed mice and for careful reading of our manuscript before submission. We thank T.T. Sun (New York University School of Medicine, New York, NY) for the gift of AE13 and AE15 antibodies; Y. Groner (Weizmann Institute, Rehovot, Israel) for the Runx3 antibodies; and C. Tabin (Harvard Medical School, Boston, MA) for the *Shh* probe.

E. Fuchs is an HHMI Investigator. This research was funded by a grant from National Institutes of Health.

Submitted: 5 September 2003
Accepted: 1 October 2003

## References

Ahn, K., Y. Mishina, M.C. Hanks, R.R. Behringer, and E.B. Crenshaw, III. 2001. BMPR-IA signaling is required for the formation of the apical ectodermal ridge and dorsal-ventral patterning of the limb. *Development.* 128:4449–4461.

Alonso, L., and E. Fuchs. 2003. Stem cells in the skin: waste not, Wnt not. *Genes Dev.* 17:1189–1200.

Andl, T., S.T. Reddy, T. Gaddapara, and S.E. Millar. 2002. WNT signals are required for the initiation of hair follicle development. *Dev. Cell.* 2:643–653.

Balciunaite, G., M.P. Keller, E. Balciunaite, L. Piali, S. Zuklys, Y.D. Mathieu, J.

Gill, R. Boyd, D.J. Sussman, and G.A. Hollander. 2002. Wnt glycoproteins regulate the expression of Foxn1, the gene defective in nude mice. *Nat. Immunol.* 3:1102–1108.

Barrow, J.R., K.R. Thomas, O. Boussadia-Zahui, R. Moore, R. Kemler, M.R. Capecchi, and A.P. McMahon. 2003. Ectodermal Wnt3/beta-catenin signaling is required for the establishment and maintenance of the apical ectodermal ridge. *Genes Dev.* 17:394–409.

Blessing, M., L.B. Nanney, L.E. King, C.M. Jones, and B.L. Hogan. 1993. Transgenic mice as a model to study the role of TGF-beta-related molecules in hair follicles. *Genes Dev.* 7:204–215.

Blessing, M., P. Schirmacher, and S. Kaiser. 1996. Overexpression of bone morphogenetic protein-6 (BMP-6) in the epidermis of transgenic mice: inhibition or stimulation of proliferation depending on the pattern of transgene expression and formation of psoriatic lesions. *J. Cell Biol.* 135:227–239.

Botchkarev, V.A., 2003. Bone morphogenetic proteins and their antagonists in skin and hair follicle biology. *J. Invest. Dermatol.* 120:36–47.

Botchkarev, V.A., N.V. Botchkareva, W. Roth, M. Nakamura, L.H. Chen, W. Herzog, G. Lindner, J.A. McMahon, C. Peters, R. Lauster, et al. 1999. Noggin is a mesenchymally derived stimulator of hair-follicle induction. *Nat. Cell Biol.* 1:158–164.

Byrne, C., M. Tainsky, and E. Fuchs. 1994. Programming gene expression in developing epidermis. *Development.* 120:2369–2383.

Chan, E.F., U. Gat, J.M. McNiff, and E. Fuchs. 1999. A common human skin tumour is caused by activating mutations in beta-catenin. *Nat. Genet.* 21:410–413.

Cotsarelis, G., T.T. Sun, and R.M. Lavker. 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 E. Fuchs. 1999. Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. *Development.* 126:4557–4568.

Fuchs, E., B.J. Merrill, C. Jamora, and R. DasGupta. 2001. At the roots of a never-ending cycle. *Dev. Cell.* 1:13–25.

Gat, U., R. DasGupta, L. Degenstein, and E. Fuchs. 1998. De novo hair follicle morphogenesis and hair tumors in mice expressing a truncated beta-catenin in skin. *Cell.* 95:605–614.

Godwin, A.R., and M.R. Capecchi. 1998. Hoxc13 mutant mice lack external hair. *Genes Dev.* 12:11–20.

Handjiski, B.K., S. Eichmuller, U. Hofmann, B.M. Czarnetzki, and R. Paus. 1994. *Br. J. Dermatol.* 131:303–310.

Hardy, M.H. 1992. The secret life of the hair follicle. *Trends Genet.* 8:55–61.

Huelsken, J., R. Vogel, B. Erdmann, G. Cotsarelis, and W. Birchmeier. 2001. beta-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. *Cell.* 105:533–545.

Jamora, C., R. DasGupta, P. Kocieniewski, and E. Fuchs. 2003. Links between signal transduction, transcription and adhesion in epithelial bud development. *Nature.* 422:317–322.

Jave-Suarez, L.F., H. Winter, L. Langbein, M.A. Rogers, and J. Schweizer. 2002. HOXC13 is involved in the regulation of human hair keratin gene expression. *J. Biol. Chem.* 277:3718–3726.

Kaufman, C.K., P. Zhou, H.A. Pasolli, M. Rendl, D. Bolotin, K.C. Lim, X. Dai, M.L. Alegre, and E. Fuchs. 2003. GATA-3: an unexpected regulator of cell lineage determination in skin. *Genes Dev.* 17:2356–2369.

Korinek, V., N. Barker, P.J. Morin, D. van Wichen, R. de Weger, K.W. Kinzler, B. Vogelstein, and H. Clevers. 1997. Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC−/− colon carcinoma. *Science.* 275: 1784–1787.

Kratochwil, K., M. Dull, I. Farinas, J. Galceran, and R. Grosschedl. 1996. Lef1 expression is activated by BMP-4 and regulates inductive tissue interactions in tooth and hair development. *Genes Dev.* 10:1382–1394.

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

Lynch, M.H., W.M. Oguin, C. Hardy, L. Mak, and T.T. Sun. 1986. Acidic and basic hair/nail ("hard") keratins: their colocalization in upper cortical and cuticle cells of the human hair follicle and their relationship to "soft" keratins. *J. Cell Biol.* 103:2593–2606.

Ma, L., J. Liu, T. Wu, M. Plikus, T.X. Jiang, Q. Bi, Y.H. Liu, S. Muller-Rover, H. Peters, J.P. Sundberg, et al. 2003. "Cyclic alopecia" in Msx2 mutants: defects in hair cycling and hair shaft differentiation. *Development.* 130:379–389.

Massague, J. 1998. TGF-beta signal transduction. *Annu. Rev. Biochem.* 67:753–791.

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

Millar, S.E. 2002. Molecular mechanisms regulating hair follicle development. *J. Invest. Dermatol.* 118:216–225.

Millar, S.E., K. Willert, P.C. Salinas, H. Roelink, R. Nusse, D.J. Sussman, and G.S. Barsh. 1999. WNT signaling in the control of hair growth and structure. *Dev. Biol.* 207:133–149.

Mishina, Y., M.C. Hanks, S. Miura, M.D. Tallquist, and R.R. Behringer. 2002. Generation of BMPr/Alk3 conditional knockout mice. *Genesis.* 32:69–72.

Monzen, K., I. Shiojima, Y. Hiroi, S. Kudoh, T. Oka, E. Takimoto, D. Hayashi, T. Hosoda, A. Habara-Ohkubo, T. Nakaoka, et al. 1999. Bone morphogenetic proteins induce cardiomyocyte differentiation through the mitogen-activated protein kinase kinase kinase TAK1 and cardiac transcription factors Csx/Nkx-2.5 and GATA-4. *Mol. Cell. Biol.* 19:7096–7105.

Moon, R.T., B. Bowerman, M. Boutros, and N. Perrimon. 2002. The promise and perils of Wnt signaling through beta-catenin. *Science.* 296:1644–1646.

Nehls, M., D. Pfeifer, M. Schorpp, H. Hedrich, and T. Boehm. 1994. New member of the winged-helix protein family disrupted in mouse and rat nude mutations. *Nature.* 372:103–107.

Nemer, G., and M. Nemer. 2003. Transcriptional activation of BMP-4 and regulation of mammalian organogenesis by GATA-4 and -6. *Dev. Biol.* 254:131–148.

Niemann, C., and F.M. Watt. 2002. Designer skin: lineage commitment in postnatal epidermis. *Trends Cell Biol.* 12:185–192.

Nikaido, M., M. Tada, H. Takeda, A. Kuroiwa, and N. Ueno. 1999. In vivo analysis using variants of zebrafish BMPR-IA: range of action and involvement of BMP in ectoderm patterning. *Development.* 126:181–190.

Noramly, S., and B.A. Morgan. 1998. BMPs mediate lateral inhibition at successive stages in feather tract development. *Development.* 125:3775–3787.

O'Guin, W.M., T.T. Sun, and M. Manabe. 1992. Interaction of trichohyalin with intermediate filaments: three immunologically defined stages of trichohyalin maturation. *J. Invest. Dermatol.* 98:24–32.

Oliver, R.F., and C.A. Jahoda. 1988. Dermal-epidermal interactions. *Clin. Dermatol.* 6:74–82.

Oro, A.E., and K. Higgins. 2003. Hair cycle regulation of Hedgehog signal reception. *Dev. Biol.* 255:238–248.

Oshima, H., A. Rochat, C. Kedzia, K. Kobayashi, and Y. Barrandon. 2001. Morphogenesis and renewal of hair follicles from adult multipotent stem cells. *Cell.* 104:233–245.

Panteleyev, A.A., C.A. Jahoda, and A.M. Christiano. 2001. Hair follicle predetermination. *J. Cell Sci.* 114:3419–3431.

Patient, R.K., and J.D. McGhee. 2002. The GATA family (vertebrates and invertebrates). *Curr. Opin. Genet. Dev.* 12:416–422.

Peterkin, T., A. Gibson, and R. Patient. 2003. GATA-6 maintains BMP-4 and Nkx2 expression during cardiomyocyte precursor maturation. *EMBO J.* 22: 4260–4273.

Prowse, D.M., D. Lee, L. Weiner, N. Jiang, C.M. Magro, H.P. Baden, and J.L. Brissette. 1999. Ectopic expression of the nude gene induces hyperproliferation and defects in differentiation: implications for the self-renewal of cutaneous epithelia. *Dev. Biol.* 212:54–67.

Reddy, S., T. Andl, A. Bagasra, M.M. Lu, D.J. Epstein, E.E. Morrisey, and S.E. Millar. 2001. Characterization of Wnt gene expression in developing and postnatal hair follicles and identification of Wnt5a as a target of Sonic hedgehog in hair follicle morphogenesis. *Mech. Dev.* 107:69–82.

Robins, E.J., and A.S. Breathnach. 1970. Fine structure of bulbar end of human foetal hair follicle at stage of differentiation of inner root sheath. *J. Anat.* 107:131-146.

Schaeren-Wiemers, N., and A. Gerfin-Moser. 1993. A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridization using digoxigenin-labelled cRNA probes. *Histochemistry.* 100:431–440.

Schlake, T., M. Schorpp, and T. Boehm. 2000. Formation of regulator/target gene relationships during evolution. *Gene.* 256:29–34.

Segre, J.A., J.L. Nemhauser, B.A. Taylor, J.H. Nadeau, and E.S. Lander. 1995. Positional cloning of the nude locus: genetic, physical, and transcription maps of the region and mutations in the mouse and rat. *Genomics.* 28:549–559.

Soshnikova, N., D. Zechner, J. Huelsken, Y. Mishina, R.R. Behringer, M.M. Taketo, E.B. Crenshaw, III, and W. Birchmeier. 2003. Genetic interaction between Wnt/beta-catenin and BMP receptor signaling during formation of the AER and the dorsal-ventral axis in the limb. *Genes Dev.* 17:1963–1968.

Takahashi, H., and T. Ikeda. 1996. Transcripts for two members of the transforming growth factor-beta superfamily BMP-3 and BMP-7 are expressed in developing rat embryos. *Dev. Dyn.* 207:439–449.

Taylor, G., M.S. Lehrer, P.J. Jensen, T.T. Sun, R.M. Lavker. 2000. Involvement of follicular stem cells in forming not only the follicle but also the epidermis. *Cell*. 102:451–461.

van Genderen, C., R.M. Okamura, I. Farinas, R.G. Quo, T.G. Parslow, L. Bruhn, and R. Grosschedl. 1994. Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in LEF-1-deficient mice. *Genes Dev.* 8:2691–2703.

Vasioukhin, V., L. Degenstein, B. Wise, and E. Fuchs. 1999. The magical touch: genome targeting in epidermal stem cells induced by tamoxifen application to mouse skin. *Proc. Natl. Acad. Sci. USA.* 96:8551–8556.

Wilson, N., P.I. Hynd, and B.C. Powell. 1999. The role of BMP-2 and BMP-4 in follicle initiation and the murine hair cycle. *Exp. Dermatol.* 8:367–368.

Winter, H., L. Langbein, S. Praetzel, M. Jacobs, M.A. Rogers, I.M. Leigh, N. Tidman, and J. Schweizer. 1998. A novel human type II cytokeratin, K6hf, specifically expressed in the companion layer of the hair follicle. *J. Invest. Dermatol.* 111:955–962.

Xu, R.H., K.T. Ault, J. Kim, M.J. Park, Y.S. Hwang, Y. Peng, D. Sredni, and H. Kung. 1999. Opposite effects of FGF and BMP-4 on embryonic blood formation: roles of PV.1 and GATA-2. *Dev. Biol.* 208:352–361.

Yamashiro, T., T. Aberg, D. Levanon, Y. Groner, and I. Thesleff. 2002. Expression of Runx1, -2, and -3 during tooth, palate and craniofacial bone development. *Gene Expr. Patterns.* 2:109–112.

Zhao, G.Q., and B.L. Hogan. 1996. Evidence that mouse BMP8a (Op2) and BMP8b are duplicated genes that play a role in spermatogenesis and placental development. *Mech. Dev.* 57:159–168.

Zhou, P., C. Byrne, J. Jacobs, and E. Fuchs. 1995. Lymphoid enhancer factor plays a major role in hair follicle morphogenesis and gene expression. *Genes Dev.* 9:700–713.