

Inhibition of Bmp signaling affects growth and differentiation in the anagen hair follicle

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Growth and differentiation of postnatal hair follicles are controlled by reciprocal interactions between the dermal papilla and the surrounding epidermal hair precursors. The molecular nature of these interactions is largely unknown, but they are likely to involve several families of signaling molecules, including Fgfs, Wnts and Bmps. To analyze the function of Bmp signaling in postnatal hair development, we have generated transgenic mice expressing the Bmp inhibitor, Noggin, under the control of the proximal *Msx2* promoter, which drives expression in proliferating hair matrix cells and differentiating hair precursor cells. Differentiation of the hair shaft but not the inner root sheath is severely impaired in *Msx2*–Noggin transgenic mice. In addition to hair keratins, the expression of several transcription factors implicated in hair development, including *Foxn1* and *Hoxc13*, is severely reduced in the transgenic hair follicles. Proliferating cells, which are normally restricted to the hair matrix surrounding the dermal papilla, are found in the precortex and hair shaft region. These results identify Bmps as key regulators of the genetic program controlling hair shaft differentiation in postnatal hair follicles.

Keywords: Bmps/hair follicle/hair shaft differentiation/
Noggin

Introduction

Mammalian hair development is controlled by a series of reciprocal epithelial–mesenchymal interactions. The first visible sign of hair formation is the hair placode, a thickening of the embryonic ectoderm induced by the underlying mesoderm. A signal from the hair placode then causes the mesenchymal cells to condense. A second mesodermal signal in turn induces proliferation in the ectodermal placode, which starts to grow down into the mesenchyme. Eventually, the epithelial cells surround the mesodermal condensation, which forms the dermal papilla, a permanent structure at the base of the follicle thought to control growth and differentiation of the hair (Hardy, 1992).

Once the basic structure of the hair follicle is established, differentiation of the concentric layers of keratinocytes begins. The peripheral layer forms the outer root sheath (ORS), which is continuous with the basal layer of

the epidermis. Within the hair follicle, two new layers, the inner root sheath (IRS) and the hair shaft, develop from proliferating precursor cells in the matrix region surrounding the dermal papilla. As cells move distally, they stop dividing and differentiate according to their medio-lateral position within the follicle. Cells positioned next to the ORS form the IRS while centrally located precursors give rise to the hair shaft, consisting of the outer cuticle, the cortex and the central medulla.

Recent years have seen substantial progress in the identification of regulators of early hair and feather development, two initially very similar processes. Members of the Tnf, Fgf and Wnt families of signaling molecules are candidate inducers of epithelial appendages, while Bmps seem to regulate the spacing between them (Widelitz *et al.*, 1996; Gat *et al.*, 1998; Noramly and Morgan, 1998; Headon and Overbeek, 1999). Shh signaling induces epithelial proliferation and is required for the initial downgrowth of the hair follicle (Oro *et al.*, 1997; St-Jacques *et al.*, 1998; Chiang *et al.*, 1999). Although many of these molecules continue to be expressed in the postnatal hair follicle, their function at later stages is less well understood. Proper hair formation requires not only that the hair shaft and IRS are specified correctly, but also that proliferation and differentiation are coordinated along the proximo-distal axis. The proliferation of hair matrix cells is thought to be controlled in part by Fgf7, which is expressed in the dermal papilla and is a potent inducer of keratinocyte proliferation *in vitro* (Finch *et al.*, 1989). Wnt signaling seems to participate in the induction of hair shaft differentiation. The pathway is specifically activated in precortex cells at the base of the hair shaft, and binding sites for the transcription factor Lef1, which mediates transcriptional responses to Wnt signaling, are found in the promoter regions of many hair keratin genes (Zhou *et al.*, 1995; DasGupta and Fuchs, 1999). Finally, Bmp signaling has been implicated in the regulation of both proliferation and differentiation in the hair follicle. Several Bmp family members are expressed in the postnatal hair follicle. The two closely related genes, *Bmp2* and *Bmp4*, are both activated in hair shaft precursors, while expression of *Bmp4* is also found in the dermal papilla (Wilson *et al.*, 1999). Ectopic expression of *Bmp4* in the ORS inhibits proliferation in the hair matrix and activates hair keratin genes in the ORS (Blessing *et al.*, 1993). Expression of *Bmp7* has been reported in the IRS and ORS and the dermal papilla (Takahashi and Ikeda, 1996). *Bmp8a* and *b* are transcribed specifically in the IRS during the first postnatal hair cycle (Zhao and Hogan, 1996).

The identification of antagonists that bind Bmps extracellularly and prevent them from interacting with their Bmp receptors has provided tools to analyze the role of Bmp signaling in hair development. To inhibit Bmp signaling specifically in the postnatal hair follicle we have

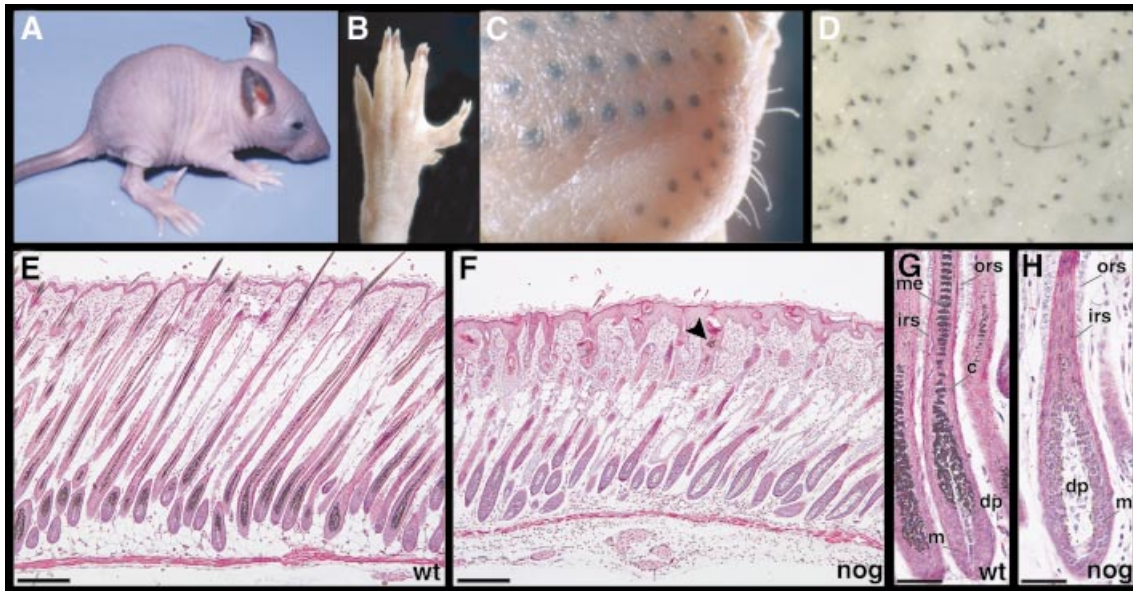


Fig. 1. *Msx2*-Noggin transgenic mice form hair follicles but lack external hairs. (A) Three-week-old *Msx2*-Noggin transgenic mouse of line #14. This line shows only mild limb abnormalities, with forelimbs often appearing normal and hindlimbs having an abnormal spreading of the first digit, small anterior outgrowths and malformed claws (B, left hind paw, 6 weeks). Vibrissae pads form in a normal pattern and can be recognized by melanin deposits (C, 6 weeks). Similarly, melanin-containing material is seen in the dorsal back skin (D, 6 weeks). Hematoxylin-eosin-stained sections of wild-type (E and G) and transgenic (F and H) P12 dorsal skin with hair follicles in anagen of the first hair cycle. Hair follicles in the transgenic animals are wavy, and deposits of keratinous material (arrowhead) are present beneath the epidermis. Dermal papillae in the transgenic follicles have a loose appearance and no cortex or medulla can be recognized in the hair shaft region. c, cortex; dp, dermal papilla; irs, inner root sheath; m, matrix; me, medulla; ors, outer root sheath. Scale bars: E, F, 50 μ m; G, H, 25 μ m.

generated transgenic mice expressing Noggin, a known inhibitor of Bmp2, 4 and 7, under the control of 439 bp of the mouse *Msx2* promoter (Liu *et al.*, 1994; Zimmerman *et al.*, 1996). This promoter drives transgene expression in the hair bulb, including hair matrix cells and early differentiating precursors of the IRS and hair shaft. Transgenic mice show a range of hair abnormalities: from bare patches in the coat up to complete loss of all external hairs, including vibrissae. Hair follicles are formed normally, but differentiation of hair shaft cells is severely impaired. Expression of both hair keratins and a number of transcriptional regulators implicated in the control of hair differentiation is reduced or absent. In addition, proliferation is no longer restricted to the hair matrix, but is also found in the precortex and distal hair shaft region. Our results show that Bmps are a key component of the signaling network controlling hair development and are required to induce the genetic program regulating hair shaft differentiation in the anagen hair follicle.

Results

Msx2-Noggin transgenic mice lack all hair types including vibrissae

We have generated transgenic mice expressing *Xenopus Noggin* under the control of 439 bp of the mouse *Msx2* promoter. This promoter fragment has previously been reported to drive expression exclusively in the apical ectodermal ridge of the limb buds of E11.5 mouse embryos (Liu *et al.*, 1994). In agreement with this observation and with known functions of Bmp signaling in limb development, we find limb abnormalities in the

Msx2-Noggin transgenic animals, which will be reported separately (Figure 1B). In addition, the transgenic animals show a loss of body hairs and vibrissae. The hair defects observed in seven independent transgenic lines vary from bare patches up to the loss of >80% of the body hair and vibrissae. One transgenic line that has almost no external hairs and completely lacks vibrissae was chosen for further analysis (Figure 1A). Despite the lack of external hair, vibrissae pads are formed in their normal pattern and melanin is deposited in the dorsal skin, where hairs should normally have emerged (Figure 1C and D). Sections of the dorsal skin of 12-day-old pups show that approximately normal numbers of hair follicles are formed in *Msx2*-Noggin mice compared with wild-type littermates. However, the transgenic follicles have a wavy appearance and do not form a proper hair shaft. Although they show distinct anterior-posterior polarity, with a posteriorly located sebaceous gland, deeper portions of the follicles are often misaligned (Figure 1E and F). The transgenic dermal papillae often appear less compact, the hair shaft shows no distinct cortex and medulla, and melanin production seems to be reduced (Figure 1G and H).

To analyze the abnormal hair follicles in more detail, we used immunohistochemistry to identify the three main layers of the anagen hair follicle: the hair shaft, the IRS and the ORS. Three well characterized antibodies were used: MK5, recognizing keratin 5 expressed in the ORS and basal layer of the epidermis; AE15, specific for trichohyalin found in characteristic granules of the IRS and medulla; and AE13, detecting acidic hair keratins expressed in the cortex and cuticle of the hair shaft (Lynch *et al.*, 1986; O'Guin *et al.*, 1992). Keratin 5 in the ORS and trichohyalin in the IRS are expressed normally in

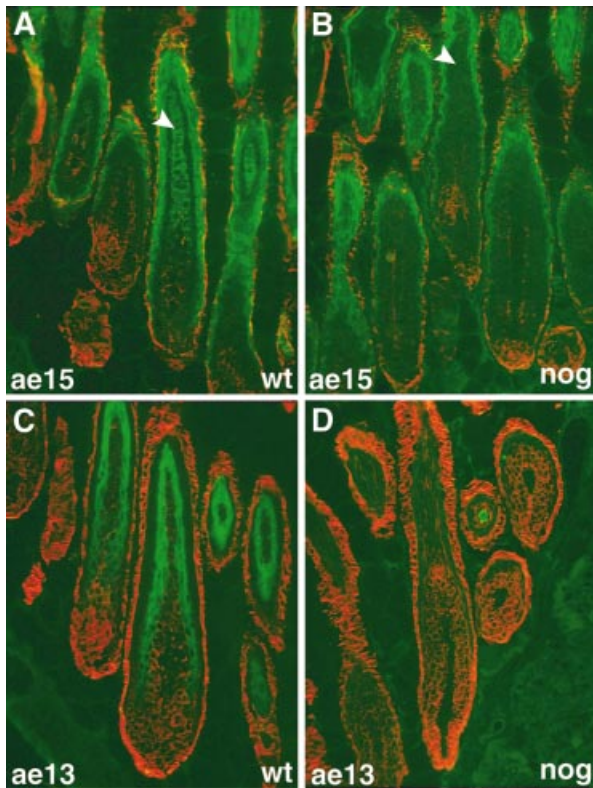


Fig. 2. Expression of keratin 5, trichohyalin and acidic hair keratins in transgenic and wild-type hair follicles. Frozen sections were prepared from wild-type (A and C) and transgenic (B and D) P12 dorsal skin and assayed by indirect immunofluorescence staining for the expression of keratin 5 (MK5, red) in the ORS, and trichohyalin (AE15, green) in the IRS and medulla (A and B), or acidic hair keratins (AE13, green) in the cortex and cuticle (C and D). Keratin 5 expression in the ORS is normal in *Msx2*-Noggin hair follicles. Trichohyalin is present in the IRS, but absent from the center of the transgenic hair follicles, where the medulla should have formed (arrowhead in B). Expression of acidic hair keratins in the cortex and cuticle is completely lost in the transgenic hair follicles (D).

the transgenic hair follicles. In contrast, trichohyalin expression in the central region of the hair follicles, corresponding to cells of the hair shaft medulla, is only seen in wild-type skin (Figure 2A and B). Expression of acidic hair keratins is also missing in the transgenic follicles, suggesting that hair shaft differentiation is severely impaired (Figure 2C and D). Although both the IRS and hair shaft cells are derived from proliferating cells in the matrix region, interference with Bmp signaling seems to inhibit the formation of the hair shaft selectively.

Expression of the *Msx2*-Noggin transgene in relation to *Bmp2* and *4*

To analyze where in the hair follicle ectopic Noggin interferes with Bmp signaling we determined *Noggin* transgene expression in relation to that of *Bmp2* and *Bmp4*, encoding the two Bmp family members that Noggin binds to with high affinity (Zimmerman *et al.*, 1996).

Bmp2 is expressed specifically in the precortex of the anagen hair follicle (Figure 3A). To look at the expression of *Noggin* and *Bmp4* in the hair follicle we took advantage of lacZ knock-in alleles of these two genes (Brunet *et al.*, 1998; Lawson *et al.*, 1999). The highest *Bmp4* expression, as monitored by β -galactosidase activity, is found in the dermal papilla. In differentiating keratinocytes, *Bmp4* shows a complementary expression pattern to *Bmp2*. *Bmp4* is most strongly expressed in the distal hair matrix region, in the medulla and in hair shaft cells adjacent to the IRS, which possibly represent precursors of the cuticle. Cells in the precortex express only low levels of *Bmp4*. Distally, β -galactosidase activity was detected in cells resembling the companion layer (Figure 6C). The lower third of the hair bulb, where progenitors of the IRS are located, is completely devoid of *Bmp4*^{lacZ} expression (Figure 3B and C). *Noggin*^{lacZ} is co-expressed with *Bmp4* in the dermal papilla, but in addition lacZ staining extends

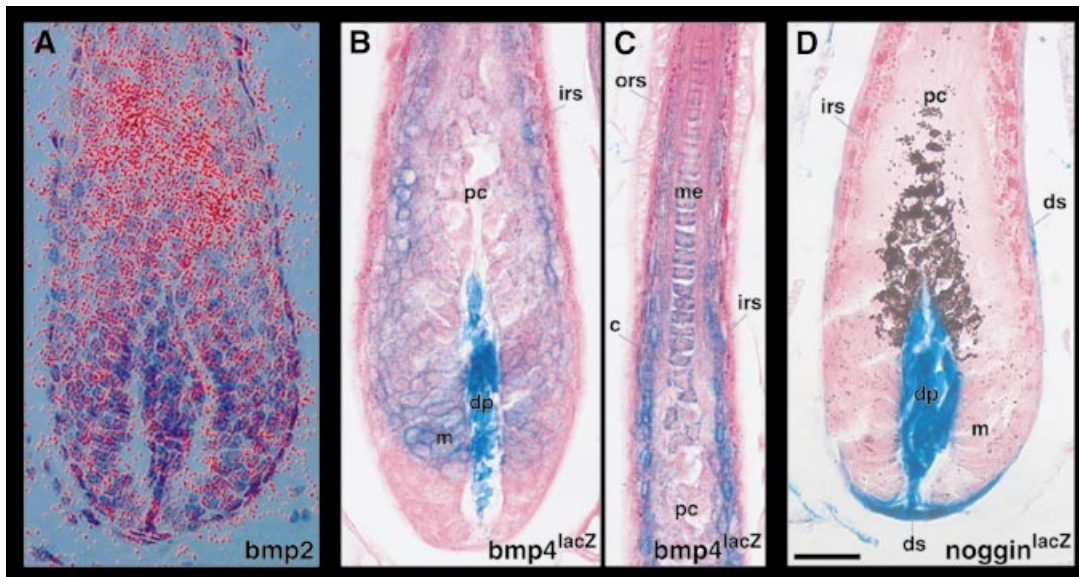


Fig. 3. Expression of *Bmp2*, *Bmp4* and *Noggin* in anagen hair follicles. A ³⁵S-labeled antisense riboprobe for *Bmp2* was hybridized to sections of dorsal skin of 12-day-old wild-type mice (A). Dorsal skin of albino *Bmp4*^{lacZ} (P4) and pigmented *Noggin*^{lacZ} (P12) heterozygous mice was processed for β -galactosidase activity, sectioned and counterstained with eosin (B–D). c, cortex; dp, dermal papilla; ds, dermal sheath; irs, inner root sheath; m, matrix; me, medulla; ors, outer root sheath; pc, precortex. Scale bar: 25 μ m.

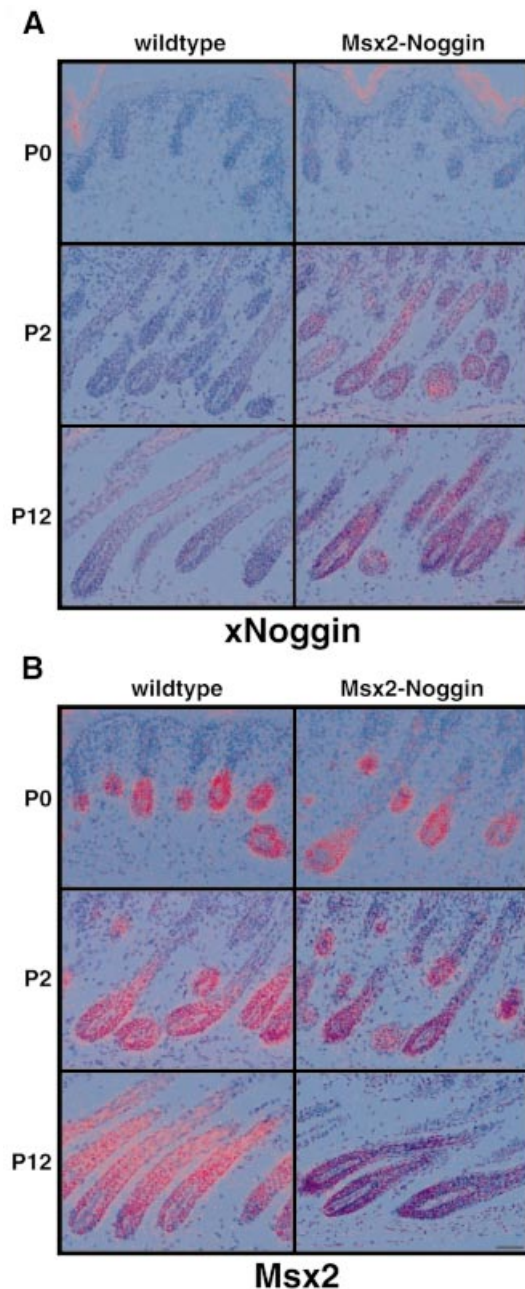


Fig. 4. Expression of the *Msx2*-*Noggin* transgene and the endogenous *Msx2* gene in wild-type and transgenic postnatal hair follicles. Sections of dorsal skin of newborn, P2 and P12 mice were probed with ^{35}S -labeled antisense riboprobes for *Xenopus* *Noggin* (A) and mouse *Msx2* (B). With the onset of detectable *Noggin* transgene expression at P2, expression of *Msx2* is reduced in transgenic compared with wild-type hair follicles. Scale bar: 50 μm .

into the cells of the dermal sheath located at the base of the hair follicle and surrounding the hair bulb (Figure 3D).

Expression of the *Msx2*-*Noggin* transgene is first detected at low levels in hair follicles of P2 pups and does not increase significantly until day 12. Expression is seen in epithelial cells of the hair bulb and in the precortex and hair shaft region, while the ORS and dermal papilla appear negative (Figure 4A). These data show that the *Msx2*-*Noggin* transgene is co-expressed with *Bmp2* and *4* in precursor cells of the hair shaft, the structure mostly

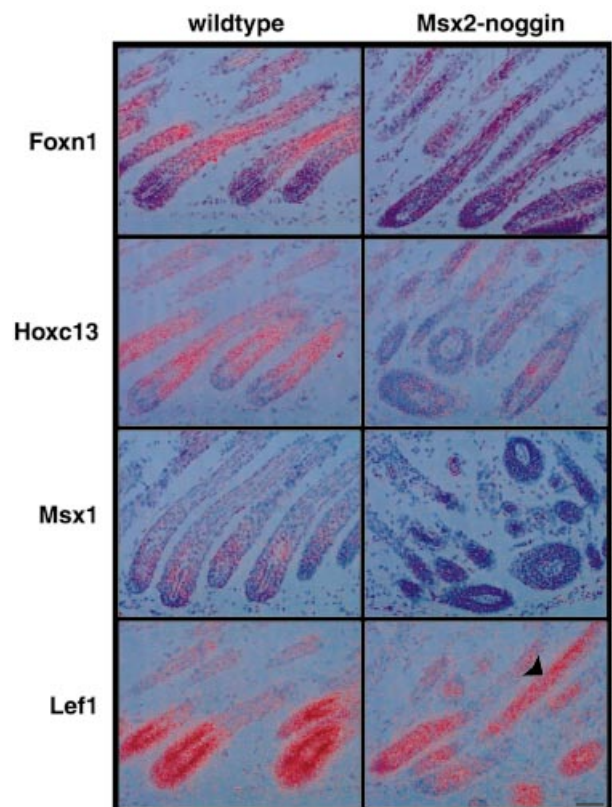


Fig. 5. Key regulators of hair formation are downregulated in *Msx2*-*Noggin* transgenic mice. Expression of *Foxn1*, *Hoxc13*, *Msx1* and *Lef1* in wild-type and transgenic hair follicles of P12 dorsal skin was analyzed by radioactive section *in situ* hybridization. Expression of *Foxn1*, *Hoxc13* and *Msx1* is strongly reduced in the transgenic hair follicles, while *Lef1* mRNA levels are almost normal but extend into the hair shaft (arrowhead). Scale bar: 50 μm .

affected in the transgenic animals, and is therefore likely to inhibit Bmp signaling in this region.

This hypothesis is supported by the analysis of endogenous *Msx2* gene expression in the transgenic hair follicles. Like the *Msx2*-*Noggin* transgene, *Msx2* itself is expressed in the hair matrix and in differentiating precursors of the hair shaft and IRS (Figure 4B) (Jiang *et al.*, 1999). We see a strong downregulation of *Msx2* expression in the transgenic follicles, suggesting that Bmp signaling normally controls endogenous *Msx2* gene expression in hair follicles. This could explain the low expression level of the *Msx2*-*Noggin* transgene, which might negatively regulate its own transcription.

***Bmp* signaling controls expression of transcriptional regulators of hair differentiation**

The loss of keratin and trichohyalin expression in the hair shaft region of transgenic follicles suggests an important function for Bmp signaling in the control of hair shaft differentiation, but leaves open the question of where in the genetic network Bmps act. Bmp signaling might directly control the expression of terminal differentiation markers like keratins, or act further upstream in the regulatory hierarchy. A number of transcriptional regulators have been implicated in the control of hair follicle development. Among these, *Hoxc13* and the forkhead transcription factor *Foxn1* (formerly *Whn*, Winged helix

nude) are of interest for this study because loss of their function severely impairs hair shaft differentiation without interfering with hair follicle formation (Nehls *et al.*, 1994; Godwin and Capecchi, 1998). Within the normal anagen hair follicle, *Foxn1* is expressed in non-proliferating precursor cells of the hair shaft and IRS, but in the transgenic follicles expression is completely lost (Figure 5) (Lee *et al.*, 1999). Similarly, *Hoxc13* expression in differentiating precursor cells in anagen hair follicles is strongly reduced in transgenic animals (Figure 5). *Msx1* and 2, encoding two closely related homeobox transcription factors, are co-expressed in the developing hair follicle and have redundant functions in the induction of hair follicle formation, which is severely impaired in their absence (Satokata *et al.*, 2000). We find that expression of *Msx1*, like that of *Msx2*, is strongly reduced in the hair bulb of transgenic hair follicles (Figure 5). The HMG box transcription factor *Lef1* is thought to regulate keratin genes directly and has been implicated as a possible Bmp target in tooth and hair development (Kratowil *et al.*, 1996; Botchkarev *et al.*, 1999). *Lef1* mRNA is expressed in precursors of the hair shaft in both the hair matrix and the precortex (DasGupta and Fuchs, 1999). In hair follicles of the *Msx2*-Noggin mutant mice we see only a minor reduction of *Lef1* expression in the hair bulb, but the expression extends further distally than normal (Figure 5).

These results place Bmp signaling upstream of a number of transcriptional regulators implicated in the control of hair development. The strong reduction in the expression of *Msx1*, *Msx2*, *Foxn1* and *Hoxc13* suggests that they are part of a genetic program activated by Bmps. In contrast, *Lef1* expression is maintained independently of Bmp signaling. However, the distal expansion of the *Lef1* expression domain in transgenic hair follicles raises the possibility that Bmps directly or indirectly inhibit *Lef1* transcription during normal differentiation.

Expression of *Bmp4* in the hair matrix is inhibited by Bmp signaling

The dermal papilla is thought to be the source of signals regulating growth and differentiation of the hair matrix cells. *Bmp4* expressed in the dermal papilla might therefore be the primary signal inducing differentiation and *Bmp4* expression in the surrounding keratinocytes. To determine whether *Bmp4* expression in the hair matrix requires Bmp signaling we analyzed the expression of the *Bmp4^{lacZ}* allele in mice carrying the *Msx2*-Noggin transgene. Surprisingly, expression of *Bmp4^{lacZ}* is greatly increased in the presence of ectopic Noggin, suggesting that the endogenous *Bmp4* gene is normally repressed by Bmp signaling (Figure 6A and B). Laterally, intense β -galactosidase staining extends all the way up to the ORS in the transgenic hair follicles, in contrast to wild-type follicles where *Bmp4^{lacZ}* expression is absent in the IRS. The proximal *Bmp4^{lacZ}*-negative cell population present at the base of the wild-type hair follicles is reduced or absent in most transgenic follicles (Figure 6A and B). In addition, expression of *Bmp4^{lacZ}* is expanded distally along the entire hair shaft (Figure 6C and D).

These results show that epithelial expression of *Bmp4* is normally repressed rather than activated by Bmp signaling. This argues against any model in which *Bmp4* produced in the dermal papilla is required for activation of

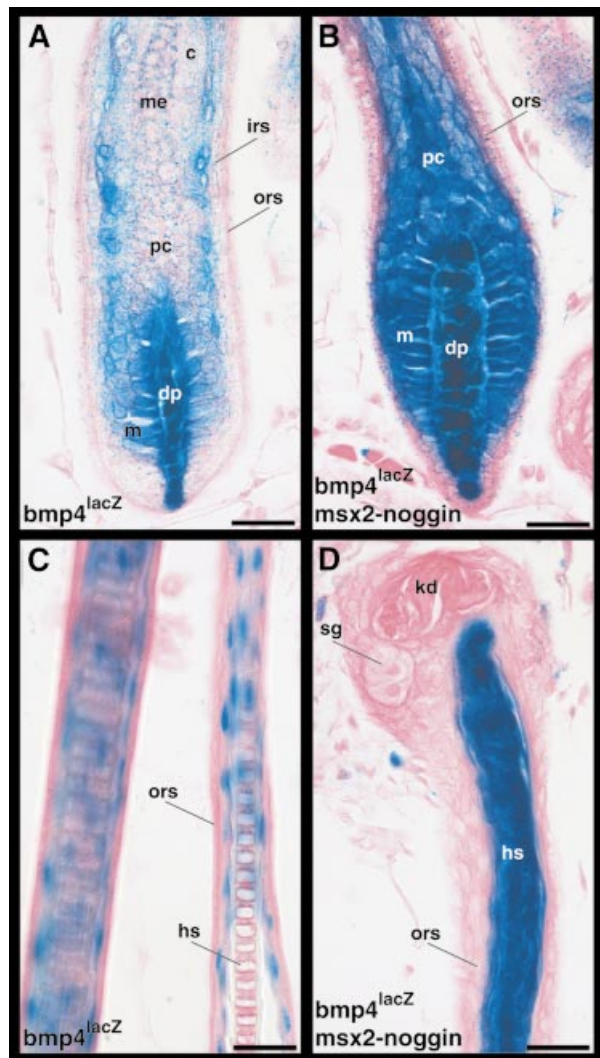


Fig. 6. Negative feedback control of *Bmp4* expression in epithelial cells. Dorsal skin samples of P12 albino *Msx2*-Noggin transgenic and non-transgenic mice carrying the *Bmp4^{lacZ}* allele were processed for β -galactosidase activity, sectioned and counterstained with eosin. *Bmp4^{lacZ}* expression is greatly enhanced and expanded distally in the *Msx2*-Noggin transgenic (B and D) compared with non-transgenic (A and C) hair follicles. Note the lack of lacZ-negative cells at the unusually narrow base and in the IRS region of the transgenic hair follicles (B). The lacZ-positive cells in distal wild-type hair follicles resemble cells of the companion layer (C). Because of the strong β -galactosidase staining in the distal hair shaft, it is difficult to determine whether this layer is present in transgenic hair follicles or not (D). c, cortex; dp, dermal papilla; hs, hair shaft; irs, inner root sheath; kd, keratinous deposit; m, matrix; me, medulla; ors, outer root sheath; pc, precortex; sg, sebaceous gland. Scale bar: 25 μ m.

Bmp4 expression in the differentiating keratinocytes. Instead, this negative autoregulatory feedback loop is likely to buffer Bmp activity in the epithelial cells. Negative autoregulation of *Bmp4* expression is not restricted to hair keratinocytes, but has also been observed in the perichondrium during chondrogenesis and it may therefore stabilize Bmp activity in additional developmental processes (Pathi *et al.*, 1999).

Proximal restriction of proliferation is lost in Noggin transgenic hair follicles

Proliferation and differentiation in the anagen hair follicle have to be coordinated to ensure the growth of a

structurally intact hair. *Bmp4* suppresses proliferation in the hair matrix when expressed ectopically in the ORS (Blessing *et al.*, 1993). Therefore, we used BrdU labeling to study the proliferation of precursor cells in the hair bulb of wild-type and *Noggin* transgenic anagen hair follicles.

In wild-type mice, proliferating cells are confined to the proximal half of the hair bulb (Figure 7A and B). In contrast, no such restriction is seen in transgenic follicles, where proliferating cells are found throughout the hair bulb, in the precortex and farther distal in the hair shaft (Figure 7C and D). Interestingly, the total number of proliferating cells within the P12 transgenic hair bulbs including the precortex does not increase significantly compared with the number of cycling cells in the wild-type hair matrix [32.5 ± 4.6 in wild-type versus 39.0 ± 9.3 in transgenic hair follicles ($n = 29$), not counting cells in the distal hair shaft] and there are actually fewer proliferating cells at the base of the transgenic follicles than normal.

To determine whether there is a correlation between proliferation and *Bmp4* expression we performed BrdU labeling in wild-type and *Noggin* transgenic animals carrying the *Bmp4^{lacZ}* allele, and simultaneously stained for β -galactosidase expression and BrdU incorporation. The *Bmp4*-negative cell population at the base of the hair follicle always contains a high percentage of proliferating cells. A considerable number of BrdU-positive cells can also be found among the *Bmp4*-positive hair matrix cells, but the extent of proliferation in this cell population shows a greater variability (Figure 7E and F). In the transgenic hair follicles, most proliferating cells express *Bmp4*. In the rare case that a significant population of *Bmp4*-negative cells is present proximally, proliferation in these cells seems rather limited (Figure 7G and H).

In summary, our data show that Bmp signaling causes the complete inhibition of proliferation in the precortex, but is compatible with extensive proliferation in the matrix region.

Influence of Bmp signaling on the expression of known growth factors for keratinocytes

The dermal papilla is thought to be the source of growth promoting signals for the surrounding hair matrix cells. Part of this activity may be mediated by Fgf7, a potent inducer of keratinocyte proliferation (Finch *et al.*, 1989). To see whether the altered proliferation in the transgenic hair follicles correlates with changes in the Fgf growth signal, we determined the expression of *Fgf7* in anagen hair follicles, but found no difference between wild-type and transgenic mice (Figure 7I and J). Similarly, expression of *Fgf10*, the only other Fgf known to be expressed in the dermal papilla, is unchanged (data not shown) (Hamada *et al.*, 1999). The receptor for Fgf7 and 10, *Fgfr2*, is expressed in the hair matrix and its expression has been reported to be activated by Bmp signaling in embryonic avian skin during feather bud formation (Rosenquist and Martin, 1996; Noramly and Morgan, 1998). The level of *Fgfr2* mRNA is elevated in the transgenic hair follicles and the expression extends farther distal into the hair shaft than normal (Figure 7K and L). This suggests that Bmp signaling negatively regulates *Fgfr2* expression in the mouse hair follicle, opposite to its function in the chicken epidermis (Noramly and Morgan, 1998).

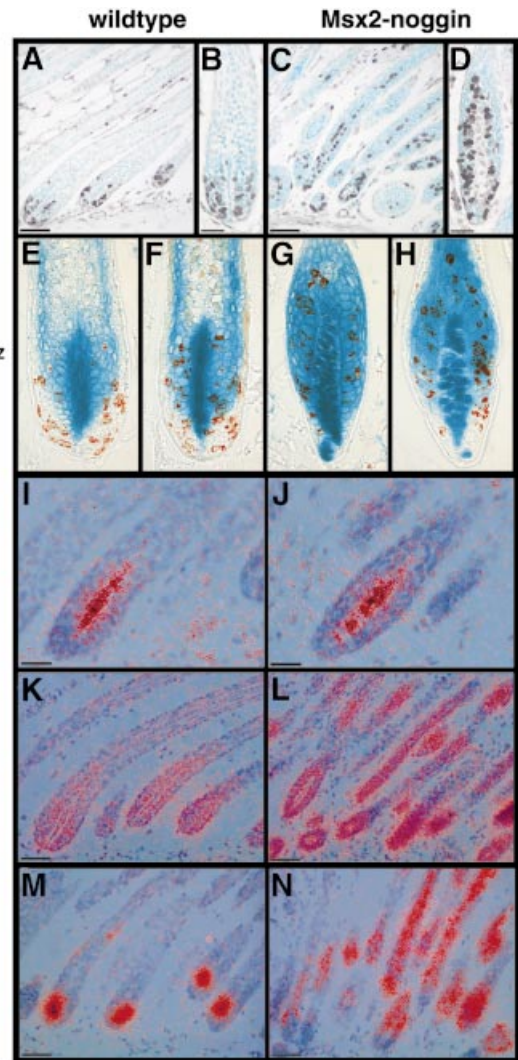


Fig. 7. Altered proliferation in *Msx2*-*Noggin* transgenic hair follicles. Sections of dorsal skin of P12 wild-type and transgenic animals injected with BrdU 4 h before being sacrificed were analyzed for BrdU incorporation by immunohistochemistry. Skin samples from animals carrying the *Bmp4^{lacZ}* allele were also assayed for β -galactosidase activity. Proliferating cells in wild-type follicles are restricted to the hair matrix (A and B), but are distributed throughout the hair bulb, the precortex and the hair shaft in the transgenic skin (C and D). *Bmp4^{lacZ}* expression and proliferation overlap in the distal hair matrix of normal hair follicles (E and F). Proliferating cells are found throughout the *lacZ*-positive hair bulb of transgenic hair follicles (G). *LacZ*-negative cells at the base of transgenic hair follicles show little proliferation compared with *lacZ*-negative proximal cells in normal follicles (compare H with E and F). Expression of *Fgf7*, *Fgfr2* and *Shh* was determined by radioactive *in situ* hybridization. No change is seen in the expression of *Fgf7* in the dermal papilla (I and J), while *Fgfr2* expression in the transgenic hair follicles is increased and extends further into the hair shaft than normal (K and L). *Shh* expression, which is normally restricted to a small group of cells in the anterior or posterior hair matrix, is found throughout the distal hair bulb and in the hair shaft region of transgenic follicles (M and N). Scale bars: A, C, K–M, 50 μ m; B, D, I, J, 25 μ m.

Another signaling molecule implicated in the control of proliferation in the hair follicle is *Shh*, which is required for the downgrowth of the hair placode into the underlying mesenchyme (St-Jacques *et al.*, 1998; Chiang *et al.*, 1999). *Shh* expression is normally found at the tip of the growing hair placode and becomes restricted to a small cell

population in the anterior or posterior hair matrix in the mature anagen hair follicle (Figure 7M) (Gat *et al.*, 1998). In *Noggin* transgenic follicles, *Shh* is expressed throughout the distal hair bulb and in the hair shaft region corresponding to the domain of ectopic cell proliferation (Figure 7N).

In conclusion, the analysis of growth factor expression in the transgenic hair follicles suggests that alterations in Fgf signaling are not responsible for the loss of proximal hair matrix cells or ectopic distal proliferation, while expanded *Shh* expression may induce proliferation in the precortex and hair shaft region.

Discussion

A number of studies in mouse and chicken have identified Bmps as negative regulators of embryonic hair follicle and feather formation. In contrast, this work suggests a positive function for Bmp signaling in postnatal hair development. Using the Bmp inhibitor *Noggin* we have shown that Bmp signaling is required to induce differentiation of hair shaft progenitors, which is coupled to the loss of their proliferative capacity and thereby restricts proliferation to the hair matrix.

***Bmp4* expression as a marker of hair shaft progenitors in the hair matrix**

It is generally assumed that lineage commitment of progenitor cells in the hair matrix is position dependent, but the timing, spatial organization and factors involved in this process remain largely unknown. While the layers of the hair follicle are eventually organized in a medio-lateral fashion, they probably originate from cells in different proximo-distal positions along the boundary with the dermal papilla (Epstein and Maibach, 1969). According to this model, precursors of the outer layers, belonging to the IRS, are located near the base of the dermal papilla, while precursors of the central hair shaft occupy more distal positions. Epithelial expression of *Bmp4* in the hair bulb is restricted to the distal matrix, while IRS cells and proximal matrix cells are *Bmp4* negative. *Bmp4*-positive and -negative hair matrix cells therefore coincide with the putative hair shaft and IRS precursor domains, respectively. To our knowledge, *Bmp4* expression is the first marker that links the medio-lateral to the putative proximal–distal lineage border.

Functions of Bmp signaling in hair shaft and IRS differentiation

Inhibition of Bmp signaling prevents the induction of hair shaft differentiation, as shown by the absence of

trichohyalin in the medulla and acidic hair keratins in the cortex and cuticle of *Noggin* transgenic hair follicles. In addition, the expression of a number of transcriptional regulators of keratinocyte differentiation, like *Hoxc13*, *Foxn1*, *Msx1* and 2, is strongly reduced, suggesting that Bmps act upstream of a complex genetic program rather than directly activating terminal hair shaft differentiation markers. Expression of *Lef1*, which has also been implicated in hair shaft differentiation, does not seem to require Bmp signaling, suggesting that Bmps do not control the entire hair shaft differentiation program. The transcription factor *Lef1*, which mediates nuclear responses to Wnt signaling, is activated in the precortex, where it is thought to control the transcription of hair shaft-specific genes directly (Zhou *et al.*, 1995; DasGupta and Fuchs, 1999). This suggests that *Lef1* plays a positive role in hair formation and cooperates with Bmps to induce hair shaft differentiation. This contrasts with the antagonistic interaction between Bmp signaling and *Lef1* during early stages of hair follicle development, where the inhibitory activity of Bmps is thought to be mediated in part by their repression of *Lef1* expression (Botchkarev *et al.*, 1999).

Cells in the precortex and hair shaft of transgenic hair follicles continue to proliferate and to express genes normally downregulated during differentiation, like *Lef1*, *Fgfr2* and *Bmp4*, suggesting that these cells remain hair shaft precursors. Since transgenic *Noggin* expression may not completely suppress Bmp activity, we cannot tell whether hair shaft progenitors are independent of Bmp signaling or whether they require a low level of Bmp activity, insufficient to induce differentiation. Previous studies have shown that *Bmp4* overexpression in the ORS both inhibits proliferation and induces ectopic expression of hair keratins, suggesting that an increase in Bmp activity in the precortex is normally sufficient to inhibit proliferation and induce differentiation (Blessing *et al.*, 1993).

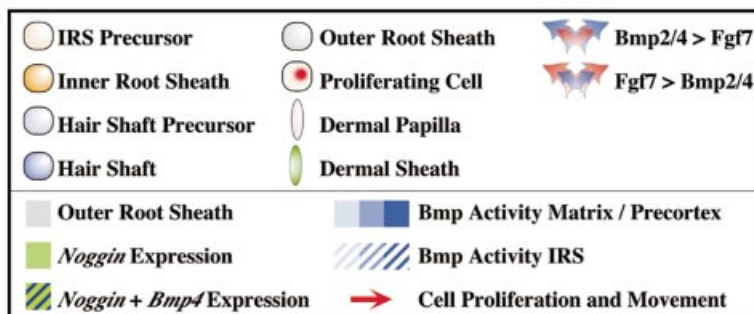
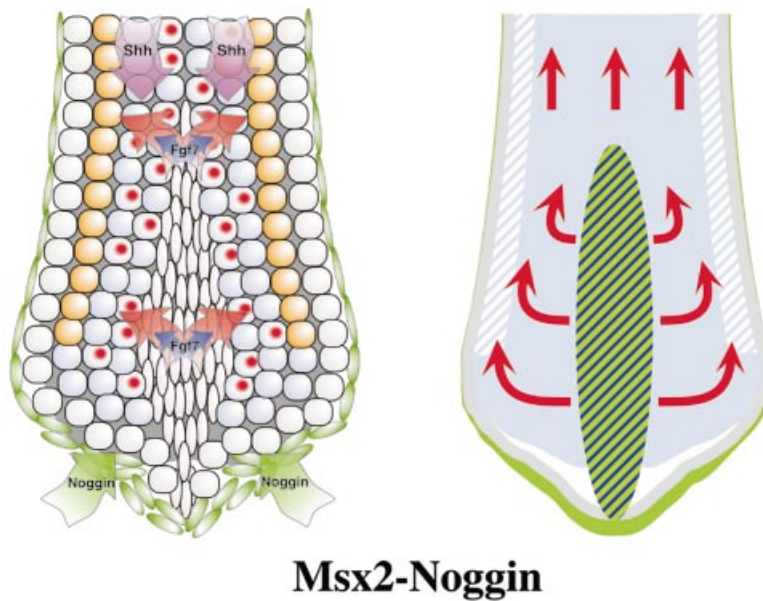
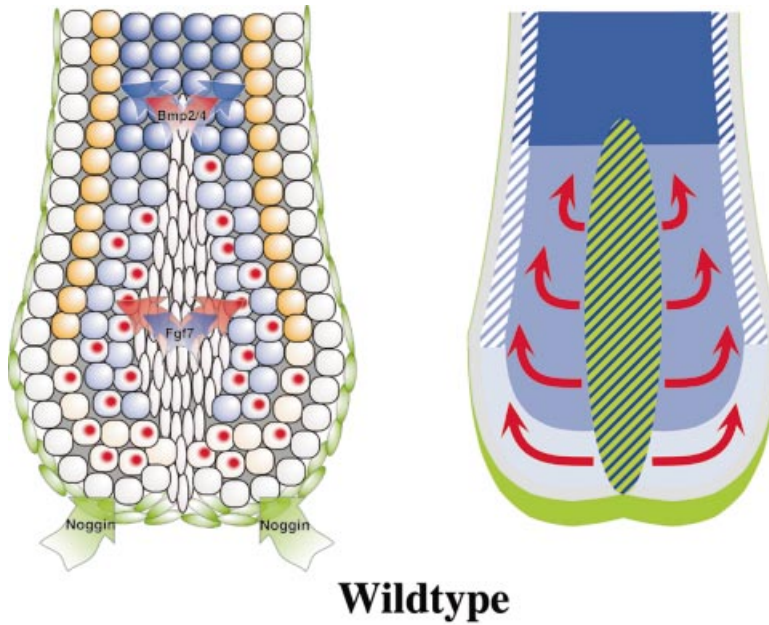
In contrast to the hair shaft, the IRS seems to differentiate normally in the *Msx2*–*Noggin* transgenic hair follicles, although *Bmp4*-negative IRS cells are missing. This absence may be caused by an expansion of the normal *Bmp4* expression domain into the IRS, which would leave the origin of the IRS cells unchanged. However, the narrow base of the transgenic hair follicles suggests a loss of the proximal *Bmp4*-negative cells and consequently their putative descendants in the IRS. In this case, the outer layer of the hair shaft precursors would be respecified to form the IRS, suggesting that it is the final medio-lateral position of the precursors rather than their origin that determines the lineage decision. Fate mapping within the hair follicle will be required to distinguish between these two possibilities.

Fig. 8. Model for the role of *Bmp2/4*, *Fgf7* and *Noggin* in the regulation of proliferation and differentiation of keratinocytes in wild-type and transgenic hair follicles. The two representations of the hair bulb predict the prevailing influence of growth or differentiation factors (left) and the distribution of Bmp activity (right). In the left representations, only the prevailing signaling activity is printed in the arrows. In the right representations, the intensity of the blue color indicates the level of Bmp activity (low, medium or high). The red arrows symbolize the presence of proliferating cells and their movement. According to the model, high Bmp activity in the precortex of wild-type hair follicles suppresses proliferation and induces differentiation, while moderate Bmp activity in the distal hair matrix is compatible with proliferation induced by *Fgf7* from the dermal papilla. Both the lack of epithelial *Bmp4* expression and *Noggin* released from the proximal dermal sheath are likely to create a zone of low Bmp activity at the base of the hair follicle. In the transgenic hair follicle, ectopic *Noggin* inhibits Bmp signaling in the distal hair matrix and precortex, preventing hair shaft differentiation. Consequently, *Shh* expression is not localized to the hair matrix and activates ectopic proliferation in the precortex and hair shaft region. The proximal *Bmp4*-negative cell population is absent and the IRS in the transgenic hair follicle is possibly derived from lateral hair shaft precursor cells located adjacent to the ORS. Further details of the model are described in the text.

Control of proliferation in the anagen hair follicle

In the normal anagen hair follicle, proliferating cells are only found in the hair matrix. Upon suppression of Bmp

activity by ectopic Noggin, cycling cells also appear in the precortex and hair shaft, suggesting that differentiation signals play a key role in restricting proliferation to the



hair matrix (Figure 7). Our results suggest two mechanisms by which Bmps may prevent distal proliferation. One is the restriction of growth factor expression to the hair matrix, as seen for *Shh*, expression of which spreads distally in the absence of Bmp-induced differentiation (Figure 7M and N). A mitogenic function for *Shh* in the hair shaft region is supported by earlier findings that *Shh* is required for the downgrowth of the hair placode into the dermis (St-Jacques *et al.*, 1998; Chiang *et al.*, 1999). The second mechanism is the direct antagonism between growth and differentiation signals. *Fgf7* is a potent mitogen for keratinocytes, but neither the expression of *Fgf7* in the dermal papilla nor that of its receptor, *Fgfr2*, in the surrounding hair matrix and precortex is significantly affected by Bmp signaling (Finch *et al.*, 1989) (Figure 7I–K). This implies that it is the relative local strength of proliferation and differentiation signals that determines the behavior of cells in the hair matrix and precortex. Growth signals, including Fgfs, prevail in the hair matrix, while differentiation signals are stronger in the precortex, where an increase in Bmp signaling may be sufficient to tip the balance toward differentiation (Figure 8).

The proximal, highly proliferative *Bmp4*-negative cell population is missing in transgenic hair follicles, raising the possibility that Bmp signaling is directly or indirectly involved in promoting survival and/or proliferation of proximal hair matrix cells. Interestingly, *Foxn1*, which is strongly downregulated in the transgenic hair follicles, has been shown to control a feedback signal in the epidermis, where it induces proliferation in the basal layer when overexpressed in the adjacent suprabasal cells (Prowse *et al.*, 1999). *Foxn1* may therefore similarly upregulate the production of growth or survival factors for proximal matrix cells in differentiating hair keratinocytes.

Model for three zones of Bmp activity in the anagen hair follicle

Previous transgenic experiments, and the present study, show that Bmp signaling suppresses proliferation in the hair matrix and is required for the induction of hair shaft differentiation in the precortex (Blessing *et al.*, 1993). These findings suggest that Bmp activity is low among the proliferating cells in the proximal half of the hair bulb and high in differentiating cells in the precortex. How do the complex expression patterns of *Bmp2*, *Bmp4* and *Noggin* contribute to the establishment of this Bmp activity gradient?

The highest *Bmp4* expression is found in the dermal papilla, but it shows no apparent proximo-distal gradation. Similarly, *Noggin* is expressed evenly throughout the dermal papilla and cannot account by itself for the formation of a Bmp activity gradient. Graded Bmp activity could be generated if inactive Bmp–Noggin complexes are released from the dermal papilla and are then activated specifically in the precortex by a tolloid-like protease. A sea urchin metalloprotease capable of antagonizing *Noggin* function has recently been identified (Wardle *et al.*, 1999). Alternatively, *Noggin* might simply reduce the overall amount of active *Bmp4* released from the dermal papilla, thereby generating a moderate, but evenly distributed Bmp signal. In this case, the epithelial expression of Bmps together with *Noggin* released from the

proximal dermal sheath is likely to generate the putative Bmp activity gradient.

Outside the dermal papilla, *Noggin* is normally expressed in the proximal dermal sheath, *Bmp4* in hair shaft precursors in the distal hair matrix and *Bmp2* in differentiating hair shaft cells in the precortex. We hypothesize that these sources of Bmps and *Noggin*, together with *Bmp4* released from the dermal papilla, create three zones of Bmp activity, which allow proliferation proximally and induce differentiation distally (Figure 8).

According to this model, *Noggin* produced in the proximal dermal sheath inhibits *Bmp4* released from the dermal papilla and distal hair matrix cells to create a proximal zone of low Bmp activity. The importance of a signal from the proximal dermal sheath for the growth of hair follicles has been shown in hair reconstitution experiments in which the dermal papilla surrounded by ORS cells has to contact dermal sheath cells to induce the formation of a new hair follicle (Jahoda and Reynolds, 1996). *Noggin* could be part of this signal. In the distal hair matrix, Bmp activity is buffered by the inhibition of epithelial *Bmp4* expression by Bmp signaling. The existence of such a feedback control is suggested by the increased *Bmp4^{lacZ}* expression in *Noggin* transgenic hair follicles (Figure 6). Hair shaft precursors in this region are therefore likely to receive an evenly distributed Bmp signal, which we think is moderate since it still allows extensive proliferation. In the precortex, locally expressed *Bmp2*, together with *Bmp4* released from the dermal papilla, could generate a high level of Bmp activity that cannot be balanced by the feedback reduction of *Bmp4* expression, leading to an increase in Bmp signaling that triggers differentiation. The sharp fall in *Bmp4^{lacZ}* expression in the precortex is consistent with high Bmp activity in this region.

Targets of Bmp signaling in the hair follicle

The expression of four genes, *Foxn1*, *Hoxc13*, *Msx1* and *Msx2*, is strongly reduced or absent in *Msx2*–*Noggin* transgenic hair follicles, suggesting that they lie in a genetic pathway directly controlled by Bmp signaling. Can the loss of any of these genes account for the phenotype of the *Msx2*–*Noggin* transgenic mice?

Foxn1 mutant nude mice develop a normal number of hair follicles, but show incomplete differentiation of the hair shafts, which form a discernible hair cortex and medulla but rarely penetrate the skin. Although *Foxn1*, like Bmps, is thought to influence both the proliferation and differentiation of hair keratinocytes, the nude hair phenotype is much weaker than that of the *Msx2*–*Noggin* transgenic mice (Kopf-Maier *et al.*, 1990; Brissette *et al.*, 1996). The loss of *Foxn1* expression in the *Noggin* transgenic hair follicles may therefore contribute to the observed phenotype, but does not fully explain it.

Hoxc13 mutant mice develop hair follicles with hair shafts that do not protrude through the skin (Godwin and Capecchi, 1998). The morphological abnormalities and the molecular nature of the defect in *Hoxc13* mutant hair follicles have not been characterized extensively, so it is difficult to say how they compare to the *Msx2*–*Noggin* phenotype. Nevertheless, the co-expression of *Bmp4* and *Hoxc13* is very striking. It is not only seen in the hair

follicle, but extends to other keratinized structures like the nails and the filiform papillae of the tongue (our unpublished observation), suggesting that Bmps and *Hoxc13* form part of a more general genetic program directing 'hard' keratin expression.

Msx1 and *Msx2* have been proposed as Bmp target genes in a number of tissues, suggesting that they mediate a common rather than a tissue-specific function of Bmp signaling (Vainio *et al.*, 1993; Furuta *et al.*, 1997). Overexpression of *Msx2* in the hair matrix reduces proliferation and induces premature differentiation, consistent with it mediating part of the growth regulatory functions of Bmps (Jiang *et al.*, 1999). Gene inactivation of *Msx1* and *2* has shown that they function redundantly during hair development (Satokata *et al.*, 2000). The requirement of *Msx* function for hair follicle induction raises the possibility that Bmp activity is already necessary at early stages of hair follicle development to maintain expression of *Msx1* and *2*.

None of the Bmp-regulated transcription factors identified here clearly mediates the entire function of Bmp signaling in the hair follicle revealed by this study. This implies that it is the coordinated regulation of multiple factors by Bmps that controls hair shaft differentiation rather than the activation of a single key regulator.

Materials and methods

Generation of *Msx2*-*Noggin* transgenic mice

The *Msx2* transgene was constructed by cloning 439 bp of the *Msx2* promoter described in Liu *et al.* (1994) in front of the rabbit β -globin second intron and poly(A) signal, and inserting the *xNoggin* coding sequence from pNoggin Δ 5' (Smith and Harland, 1992) after the intron. The transgene was released from the vector backbone and injected into one-cell (B6D2)_{F2} embryos. Transgenic founders usually showed a weaker phenotype than their offspring and were verified by PCR using an *xNoggin*-specific primer pair (5' GCCAACATTATCTGCACATC, 3' CTACTTTCACATAGCGAGGC). To facilitate the molecular analyses of the skin sections, line #14 was crossed into the CD1 albino background. Owing to low fertility of female transgenic animals, the line was maintained by backcrossing transgenic males with CD1 females. The phenotype of the transgenic animals did not change in the different genetic backgrounds.

Immunohistochemistry

Frozen sections (10 μ m) of wild-type and transgenic P12 dorsal anterior skin were fixed for 30 min at room temperature in 4% paraformaldehyde and analyzed by indirect immunofluorescence. The primary antibodies used were rabbit anti-keratin 5 (MK5; BabCo, Richmond, CA) and the mouse monoclonal antibodies anti-trychohyalin (AE15; O'Guin *et al.*, 1992) and anti-acidic hair keratin (AE13; Lynch *et al.*, 1986). Cy3-coupled donkey anti-rabbit IgG and fluorescein-coupled donkey anti-mouse IgG secondary antibodies (Jackson Laboratory, West Grove, PA) were used. Slides were mounted with antifade (Biomedica, Foster City, CA) and analyzed using a Zeiss Axioplan 2 microscope.

In situ hybridization

Dorsal anterior skin samples were fixed overnight in 4% paraformaldehyde, embedded in paraffin and sectioned at 5 μ m. ³⁵S-labeled *in situ* probes were generated from the following templates using the transcription kit from Roche Molecular Biochemicals (Indianapolis, IN): pNoggin Δ 5' (Smith and Harland, 1992); pBSwhn containing 650 bp (1223–1873) of the *Foxn1* cDNA (H.Kulesa, unpublished); pTZ19 λ 2.2 (*Msx1*) (Hill *et al.*, 1989); pHox8 (*Msx2*) (Monaghan *et al.*, 1991); pKGFr-1 containing a cDNA fragment corresponding to amino acids 548–703 of the Fgfr2 (gift of Dr A.McMahon, Harvard University, Boston, MA); pKGF (*Fgf7*) (Mason *et al.*, 1994); pGL1-3' (*Lef1*) (Travis *et al.*, 1991); pmBmp-2a (*Bmp2*) (Lyons *et al.*, 1989).

β -galactosidase assay

Dorsal anterior skin of *Bmp4*^{lacZ} and *Noggin*^{lacZ} heterozygous mice was fixed for 2 h at 4°C in 4% paraformaldehyde, washed twice in phosphate-buffered saline (PBS) and incubated for 20 h at 37°C in X-Gal staining solution containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl₂, 1 mg/ml X-Gal and 0.02% NP-40 in PBS. After staining, the tissues were washed twice in PBS and fixed overnight in 4% paraformaldehyde at 4°C. The skin biopsies were dehydrated using isopropanol as the final step instead of xylene to avoid leaching of the blue precipitate, embedded in paraffin, and sectioned at 7 μ m.

BrdU labeling

Mice were injected with 50 μ g of BrdU and 10 μ g of FdU per gram body weight in PBS and killed 4 h later. Dorsal anterior skin samples were fixed overnight in 4% paraformaldehyde, embedded in paraffin and sectioned at 5 μ m. The sections were digested with 20 μ g/ml proteinase K for 15 min at 37°C and blocked by incubation with 10% sheep serum for 30 min at room temperature. BrdU incorporation was detected by immunostaining using the cell proliferation kit from Amersham (Amersham UK) as recommended by the manufacturer. BrdU-positive nuclei in the hair bulb region of longitudinally sectioned follicles from four sibling pairs were counted. The misalignment of the transgenic hair follicles led to a greater variability in the plane of section through the hair bulb, causing the high standard variation.

For the simultaneous analyses of BrdU incorporation and *Bmp4*^{lacZ} expression, skin samples of BrdU-injected mice were treated as described above for the β -galactosidase assay, but sectioned at 5 μ m. Sections were blocked with 10% goat serum, and a Cy3-coupled goat anti-mouse IgG secondary antibody (Jackson Laboratory, West Grove, PA) was used for the immunodetection of BrdU.

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