Modulation of BMP Signaling by Noggin is Required for Induction of the Secondary (Nontylotrich) Hair Follicles

Vladimir A. Botchkarev, Natalia V. Botchkareva, Andrei A. Sharov, Keiko Funa,* Otmar Huber,† and Barbara A. Gilchrest

Department of Dermatology, Boston University School of Medicine, Boston, Massachusetts, U.S.A.; *Department of Anatomy and Cell Biology, University of Gothenburg, Gothenburg, Sweden; †Department of Clinical Chemistry and Pathobiochemistry, University Hospital Benjamin Franklin, Free University, Berlin, Germany

Increasing evidence suggests that morphogenesis of the distinct developmental structures derived from the same organ-committed epithelium is controlled by differential mechanisms. As was recently shown in mice with mutations in the downless (*dL*) gene, induction of primary or tylotrich hair follicles is strikingly dependent of signaling through the Tnf receptor homologue, Edar. Here, we show that dorsal skin of murine embryos with constitutive deletion of the BMP2/4 antagonist *noggin*, after transplantation into SCID mice, is characterized by the lack of induction of secondary hair follicles, and by the arrest of primary hair follicle development prior to hair shaft formation. The loss of *noggin* activity was associated with failure to express genes that specify hair follicle cell fates in the epidermis (Lef-1, β-catenin, Shh) and dermal papilla (p75 kDa neurotrophin receptor, alkaline phosphatase). This suggests that regulation of BMP2/4 signaling by noggin is essential for the induction of secondary hair follicles, as well as for advanced stages of development in primary hair follicles. *Key words: appendage/bone morphogenetic proteins/development/hair follicle/Lef-1/morphogenesis/Shh/skin/Wnt. J Invest Dermatol 118:3–10, 2002*

The hair follicle (HF) represents a hair shaft-producing mini-organ, which development is governed by the inductive interactions between epidermal keratinocytes committed to HF-specific differentiation and a population of mesenchymal cells selected to form the connective tissue compartment of developing HF – the dermal papilla (Hardy, 1992; Philpott and Paus, 1998; Fuchs *et al*, 2001). These interactions led to the construction of hair bulb, in which keratinocyte proliferate and differentiate into six distinct cell populations, forming the medulla, cortex, and cuticle of the hair shaft, as well as the cuticle, Huxley, and Henle layers of the inner root sheath. Inner root sheath separates the hair shaft from the outer root sheath, which forms the external concentric layer of epithelial cells in the HF (Sengel, 1976).

In mice, fur consists of a number of different types of HF characterized by differences in their morphology and time-course of induction during embryogenesis (Lyon *et al*, 1996; Vielkind and Hardy, 1996). Primary or tylotrich (guard) HF, which make up approximately 5–10% of all HF in mouse dorsal skin and are characterized by a large hair bulb, long straight hair, and two sebaceous glands, begin to be induced at embryonic day 14.5 (E14.5) (Vielkind and Hardy, 1996; Philpott and Paus, 1998; Paus and Cotsarelis, 1999). Induction of the secondary or nontylotrich (awl and zigzag) HF producing thinner and shorter hair shafts and having one sebaceous gland, occurs from E16.5 to postnatal day 0.5 (P0.5).

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Reprint requests to: Dr. V. A. Botchkarev, Department of Dermatology, Boston University School of Medicine, 609 Albany Street, Boston, MA 02118. Email: vladbotc@bu.edu

Several indications suggest that induction of primary and secondary HF may require different signaling pathways. As shown in mice with spontaneous mutation in the downless (*dL*) gene (which are characterized by lack of the primary HF and apparently normal development of the secondary HF), induction of primary HF is strikingly dependent of signaling through the Tnf receptor homologue, Edar (Headon and Overbeek, 1999).

The precise molecular mechanisms initiating the inductive process of the secondary HF, however, remain to be elucidated. The sites of induction of the secondary HF in embryonic epidermis show expression of Lef-1 transcription factor, β-catenin, and transforming growth factor-β receptor type II, implicating importance of the Wnt and transforming growth factor-β signaling pathways for these processes (Zhou *et al*, 1995; Paus *et al*, 1997; Botchkarev *et al*, 1999a; DasGupta and Fuchs, 1999; Paus *et al*, 1999). Indeed, Lef-1 and transforming growth factor-β2 knockout mice are characterized by the significantly reduced number of HF and retardation of HF development (van Genderen *et al*, 1994; Foitzik *et al*, 1999). Instead, Lef-1 overexpression leads to the ectopic HF formation in the oral epithelium, and overexpression of β-catenin, the upstream effector of Lef-1 in the Wnt signaling pathway, induces HF neogenesis in postnatal skin (Zhou *et al*, 1995; Gat *et al*, 1998).

Recent data have proven previous observations suggesting a fundamental role for Wnt/β-catenin/Lef-1 signaling pathway in hair development (reviewed in Fuchs *et al*, 2001; Cotsarelis and Millar, 2001). Wnt 10a and Wnt 10b are expressed in placodes at the onset of HF morphogenesis, and Wnt 5a is expressed in the developing dermal papilla (Reddy *et al*, 2001). Conditional disruption of β-catenin in epidermis leads to the failure in induction of both primary and secondary HF (Huelsken *et al*, 2001). Furthermore, overexpression of dominant-negative Lef-1

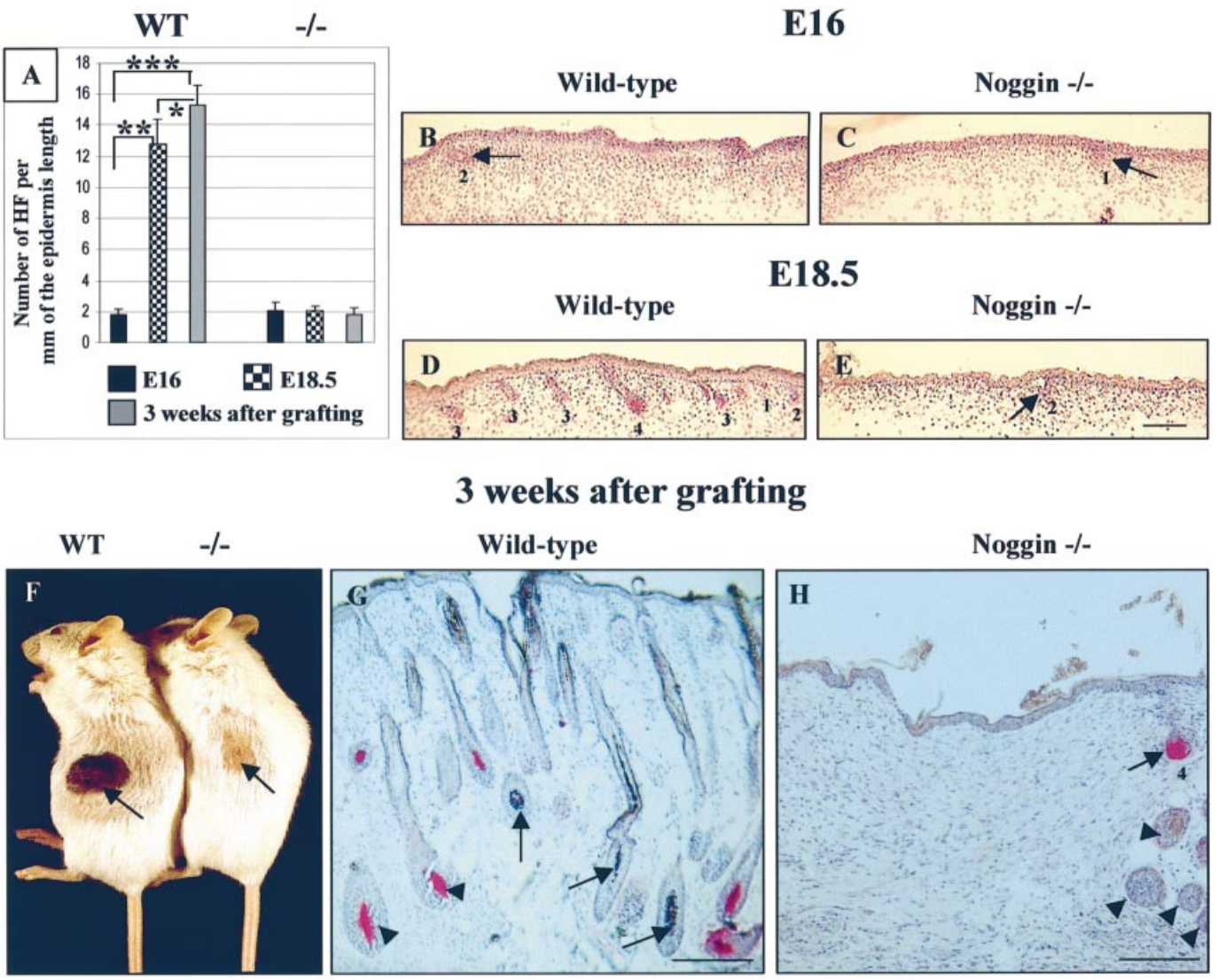

Figure 1. Alterations of the HF induction and development in the *noggin* null embryos and in the *noggin* null skin transplanted on to SCID mice. Dorsal skin of homozygous (–/–) *noggin* knockout embryos (n = 8) and corresponding wild-type embryos (n = 10) was studied at E16, E18.5, and 3 wk after transplantation on to 7–8 wk old SCID female mice (n = 8). Skin sections were processed for histoenzymatic detection of alkaline phosphatase to visualize HF dermal papilla. (*A*)Number of induced HF per mm of epidermal length in the wild-type and *noggin* null embryos and skin grafts (Student's t test, **$p < 0.01$, ***$p < 0.001$). (*B*, *C*) E16: wild-type (*B*)and *noggin* null skin (*C*)show presence of single primary HF (*arrows*). (*D*, *E*) E18.5: wild-type skin (*D*)shows numerous HF, whereas only single HF are seen in the *noggin* null skin (*E*, *arrow*). (*F–H*) Three weeks after transplantation: wild-type skin grafts show hair emerging through the epidermis (*F*, animal on left, *arrow*) and fully developed HF (*G*, *arrows*), whereas the *noggin* null skin grafts show no hairs (*F*, animal on the right, *arrow*) and arrest of development in single induced HF at stage 4 is seen (*H*, *arrow*). (*G*, *H*) HF of the albino host can be distinguished by their lack of black melanin granules and are indicated by *arrowheads*. (*B–E*, *H*) The stage of HF development for every HF is indicated by the Arabic numerals. *Scale bars*: 100 μm.

driven by K14 promoter leads to the suppression of hair differentiation and gives rise to sebocyte differentiation (Merrill *et al*, 2001).

Several indications suggest that during development, Wnt signaling could be modulated by members of the bone morphogenetic protein (BMP) superfamily (Chuong *et al*, 1996; Kratochwill *et al*, 1996; Dassule and McMahon, 1998; Noramly and Morgan, 1998; Baker *et al*, 1999; Noramly *et al*, 1999; Miyazaki *et al*, 2000). In turn, BMP2 and BMP4 represent important downstream components of β-catenin during hair placode formation (Huelsken *et al*, 2001). BMP fulfill multiple functions during development (Hogan, 1996, 1999). In particular, BMP2 and BMP4 inhibit the induction of ectodermal derivatives, such as neural tube, tooth, feather, and HF (Lamb *et al*, 1993; Neubuser *et al*, 1997; Jung *et al*, 1998; Botchkarev *et al*, 1999a), and BMP4 transgenic mice show a retardation of HF development (Blessing *et al*, 1993).

During induction of the secondary HF, BMP2 and BMP receptor IA (BMPR-IA) are found in the hair placode, i.e., in the sites of Lef-1/β-catenin expression, whereas BMP4 and BMP antagonist *noggin* are expressed in cells of the mesenchymal condensation (Bitgood and McMahon, 1995; St-Jacques *et al*, 1998; Botchkarev *et al*, 1999a). In a previous study, we showed that in the E17.5 *noggin* knockout embryos, the number of induced HF is significantly decreased and HF development is retarded, compared with wild-type embryos (Botchkarev *et al*, 1999a). This was accompanied by downregulation of Lef-1 in the hair placodes (Botchkarev *et al*, 1999a) suggesting that neutralization of BMP2/BMP4 by its antagonist noggin stimulates HF induction, at least in part, via activation of Wnt pathway; however, as at E17.5 skin already contains both primary and secondary HF (Vielkind and Hardy, 1996; Philpott and Paus, 1998), in a previous study we were unable to identify precisely a role for BMP2/BMP4 and noggin in

the control of induction of the secondary HF (Botchkarev *et al*, 1999a).

In this study, we explore the roles for BMP2, BMP4, and their antagonist noggin in the control of induction of the secondary (nontylotrich) HF. Specifically, we compare the rate of HF induction between wild-type and *noggin* null embryos of different age (E16–E18.5), as well as between wild-type and *noggin* null skin transplanted on to SCID mice, and show that constitutive deletion of *noggin* leads to the complete inhibition of the secondary HF induction. This is associated with downregulation of the markers that specify HF cell fate in the epidermis (β-catenin, Lef-1, Shh) and dermis (alkaline phosphatase, p75 kDa neurotrophin receptor). This suggests BMP antagonist noggin as an essential factor stimulating induction of the secondary (nontylotrich) HF in mice.

MATERIALS AND METHODS

Animal models and tissue collection *Noggin* knockout mice generated as described previously (McMahon *et al*, 1998) were obtained from Dr A. P. McMahon's laboratory. Homozygous *noggin* knockout mice are not viable and die at E18.5 due to the neurologic and skeletal abnormalities (Brunet *et al*, 1998; McMahon *et al*, 1998). *Noggin* null (n = 8) and wild-type embryos (n = 10) were studied at E16 and E18.5. After harvesting, skin was quickly frozen in liquid nitrogen, and embedded, using a special technique for obtaining longitudinal cryosections through the HF from one defined site (Paus *et al*, 1999). For grafting experiments, dorsal skin of the E18.5 *noggin* homozygous knockout (n = 4) and wild-type mice (n = 4) was dissected and placed into sterile DEM medium (Gibco, Grand Island, NY), and then transplanted on to 7–8 wk old female SCID mice, as described previously (St-Jacques *et al*, 1998). Three weeks after grafting, transplants were harvested and embedded, as described above.

***In situ* hybridization and immunohistochemistry** *In situ* hybridization using digoxigenin-labeled riboprobes for BMP2, BMP4, Shh, and β-catenin mRNA was performed, as described previously (Bitgood and McMahon, 1995; St-Jacques *et al*, 1998; Botchkarev *et al*, 1999a). Immunohistochemical detection of BMP receptor-IA (BMPR-IA), β-catenin, plaroglobin, Lef-1, p75NTR, and Ki-67 was performed according to the previously described protocols (Botchkarev *et al*, 1999a, b; Botchkareva *et al*, 1999). Rabbit anti-sera for Lef-1 and BMPR-1A were generated, as described previously (ten Dijke *et al*, 1994; Huber *et al*, 1996), and rabbit anti-sera against murine Ki-67, β-catenin and plaroglobin were obtained from Dianova (Hamburg, Germany) and Sigma (St Louis, MO), respectively. Secondary goat anti-rabbit TRITC-conjugated IgG was obtained from Jackson Immuno-Research (West Grove, PA). p75NTR immunostaining was performed using rat monoclonal antibody against p75NTR (Chemicon International Inc., Temecula, CA), as described previously (Botchkareva *et al*, 1999; Botchkarev *et al*, 2000). Immunohistochemical detection of Shh was performed using mouse monoclonal anti-Shh antibody 5E1 (Ericson *et al*, 1996) (Developmental Studies Hybridoma Bank, University of Iowa, IA), which were conjugated with the biotinylated secondary goat anti-mouse IgG *in vitro* prior application on skin cryosections, as described previously (Eichmuller *et al*, 1996). Double immunovisualization of Ki-67 and TUNEL was performed using anti-serum against murine Ki-67 (Dianova) and ApopTag kit (Oncor, Gaithesburg, MD), as described before (St-Jacques *et al*, 1998; Botchkarev *et al*, 1999a). In all immunofluorescence procedures, nuclei were counterstained by TO-PRO-3 (Suzuki *et al*, 1997). Multicolor confocal microscope (Zeiss, Jena, Germany) and digital image analysis system (Pixera, San-Diego, CA) were used for analyses and preparation of images.

Quantitative histomorphometry The number of induced HF was assessed and calculated per mm of the epidermal length in dorsal skin of wild-type (n = 10) and *noggin* null (n = 8) embryos at E16, E18.5, and 3 wk after grafting on to SCID mice, as described previously (Botchkarev *et al*, 1999a). Fifty microscopic fields were assessed in every animal under ×200 magnification. The percentage of Ki-67 positive cells, interpreted as cycling cells, in the epidermis of wild-type and *noggin* null skin grafts was assessed as described previously (Botchkarev *et al*, 1999a, >c). Thickness of interfollicular epidermis was assessed in cryostat sections of skin grafts from *noggin* null (n = 4) and wild-type mice (n = 4), and at least 50 measurements for every group was performed using a digital image analysis system (Pixera). In order to identify the defined substages of HF morphogenesis as precisely as possible, histochemical detection of endogenous alkaline phosphatase activity was used, as this highlights the dermal papilla as a useful morphologic marker for staging HF development and cycling (Handjiski *et al*, 1994; Paus *et al*, 1999). All sections were analyzed at 100–200× magnification, mean and SEM were calculated from pooled data. Differences were judged as significant if the $p < 0.05$, as determined by the independent Student's t test for unpaired samples.

RESULTS

***Noggin* null skin is characterized by the lack of induction of the secondary HF** To explore the potential roles for noggin in the induction of secondary HF, we compared the number of induced HF in the dorsal skin of wild-type or *noggin* null embryos (McMahon *et al*, 1998) between E16 (i.e., when only primary HF are induced), and 3 wk after grafting of the E18.5 skin on to SCID mice (i.e., when morphogenesis in both primary and secondary HF is fully completed). The latter experiment was performed due to the loss of viability of the *noggin* null embryos between E18.5 and birth (McMahon *et al*, 1998).

At E16 (i.e., a time-point when only primary HF are induced), both wild-type and *noggin* null skin showed the equal numbers of HF at stages 1–2 of morphogenesis, which are characterized by epidermal thickening and downgrowth into the dermis (**Fig 1*A–C***); however, already at E18.5, due to massive induction of the secondary HF (Vielkind and Hardy, 1996; Philpott and Paus, 1998), the number of HF in wild-type skin was significantly increased ($p < 0.01$), compared with the E16 wild-type skin (**Fig 1*A***, ***B***, ***D***). In contrast, no significant differences in the number of HF was seen between *noggin* null skin at E16 and E18.5 (**Fig 1*A***, ***C***, ***E***). Wild-type skin at E18.5 showed numerous HF at different stages of morphogenesis. Some of the HF in wild-type skin were already at stage 5, characterized by the appearance of inner root sheath and melanin granules in proximal HF epithelium (Paus *et al*, 1999); however, only single stage 2–3 HF were seen at the E18.5 *noggin* null skin. Taken together, these observations suggest that no induction of secondary HF occurred in the *noggin* null skin between E16 and E18.5.

Three weeks after transplantation of the E18.5 skin on to the SCID mice, wild-type grafts showed fully developed HF and pigmented hairs emerging through the epidermis (**Fig 1*F***, ***G***). The number of these HF was significantly higher ($p < 0.05$) than in the E18.5 wild-type skin (**Fig 1*A***, ***D***, ***G***), indicating that some secondary HF in the wild-type grafts were induced postnatally; however, all skin transplants of *noggin* null embryos showed no hairs emerging through the epidermis (**Fig 1*F***). Microscopically, *noggin* null grafts contained only single HF, which morphogenesis was arrested at stage 4, prior to the hair shaft formation in developing hair bulb (**Fig 1*H***). Most importantly, HF number was not significantly different from that in the E16 or E18.5 *noggin* null skin (**Fig 1*A***, ***C***, ***E***, ***H***). Whereas the dermal papillae of these HF could be identified by alkaline phosphatase staining (Paus *et al*, 1999), no other condensations of alkaline phosphatase-positive cells were seen beneath the epidermis of *noggin* null skin (**Fig 1*H***).

When HF morphogenesis is completed, primary and secondary HF may only be distinguished by their differences in size of proximal hair bulb, thickness of hair shaft, and number of sebaceous glands (Philpott and Paus, 1998); however, morphogenesis of the induced HF in the *noggin* null skin was arrested prior to the hair shaft and sebaceous gland formation (**Fig 1*H***). Therefore, it appeared to be impossible to define morphologically whether HF induced in the *noggin* null grafts are primary or secondary HF.

The number of induced HF in the *noggin* null skin, however, did not increase significantly between E16 and E18.5, or by 3 wk after transplantation on to SCID mice (**Fig 1**). This strongly suggests that all HF in the *noggin* null grafts were induced before E16, and thus represent primary HF. Furthermore, the number of primary HF observed in the wild-type grafts (1.9 ± 0.3 per mm of epidermis length), was quite similar to the number of induced HF in the *noggin* null skin grafts (1.8 ± 0.4 per mm of epidermis length). This further suggests that the latter are the primary HF. In

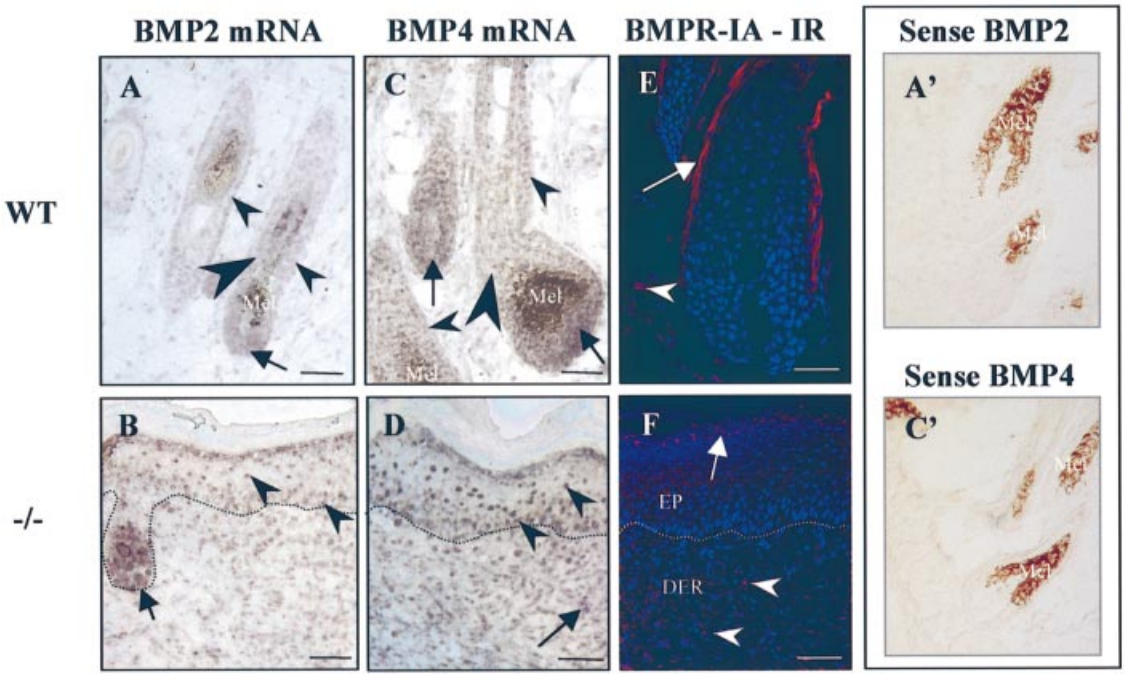

Figure 2. Expression of BMP2, BMP4, and BMPR-IA in wild-type and *noggin* null skin grafts. Skin sections of the wild-type and *noggin* knockout (–/–) transplants were analyzed for the expression of BMP2 and BMP4 by *in situ* hybridization, and BMPR-IA by immunohistochemistry. (*A*, *B*) BMP2 mRNA: expression in wild-type grafts is localized in the HF matrix and outer and inner root sheaths (*A*, *arrows*, small and large *arrowheads*, respectively), whereas in the *noggin* null skin increased expression is visible in the epidermis, dermis, and in single stage IV primary HF (*B*, small *arrowheads*, large *arrowhead*, and *arrow*, respectively). (*C*, *D*) BMP4 mRNA: expression in the HF matrix, outer and inner root sheaths of wild-type grafts (*C*, *arrows*, small and large *arrowheads*, respectively). Both primary (right) and secondary anagen HF (left) show essentially similar BMP4 expression (*C*). Prominent BMP4 expression is visible in the epidermis (*D*, *arrowheads*) and dermis (*D*, *arrow*) of *noggin* null grafts. (*E*, *F*) BMPR-IA immunoreactivity: positive immunostaining is visible in the proximal HF outer root sheath and in single dermal cells (*E*, *arrows* and *arrowhead*, respectively) of wild-type skin. Wear BMPR-IA immunoreactivity in the epidermis (*arrows*) and dermis (*arrowheads*) of *noggin* null skin (*F*). (*A′*, *C′*) Sense controls for BMP2 and BMP4 *in situ* hybridization: absence of specific signals in the HF matrix and outer and inner root sheaths of wild-type HF. (*B*, *D*, *F*) The dermoepidermal junction is indicated by the dotted line. Nuclei in (*E*, *F*) are counterstained by TO-PRO-3 (blue fluorescence). DER, dermis; EP, epidermis; Mel, melanin. *Scale bars*: (*A*, *C*) 100 μm; (*E*, *F*) 50 μm.

this case, the constitutive deletion of *noggin* results in failure of induction of secondary HF and arrest of the primary HF development at the stage prior to the hair shaft formation.

BMP2 and BMP4 are upregulated in the *noggin* null skin grafts To explore mechanisms that may underlie alterations in the HF development in *noggin* null skin, the expression of BMP2, BMP4, and BMPR-IA was compared between wild-type and *noggin* knockout skin grafts. In the wild-type skin grafts, BMP2 and BMP4 transcripts were absent in the epidermis and were selectively expressed in the HF matrix and outer and inner root sheath of both primary and secondary HF (**Fig 2*A***, ***C***). Sense controls for BMP2 and BMP4 showed absence of signals in the HF matrix and outer and inner root sheaths (**Fig 2*A′***, ***C′***). These data were consistent with expression patterns for BMP2 and BMP4 in postnatal HF published previously (Kulessa *et al*, 2000).

Noggin null skin grafts, however, were characterized by strong BMP2 and BMP4 expression in the basal and suprabasal layers of the epidermis and in the dermis (**Fig 2*B***, ***D***). In the *noggin* null grafts, single primary HF, arrested at stage 4, also showed prominent expression of BMP2 (**Fig 2*B***) and BMP4 (not shown). By immunohistochemistry, BMPR-IA was found in the proximal outer root sheath of wild-type HF, in single dermal cells (**Fig 2*E***), and in suprabasal layer of epidermis (not shown). In the *noggin* null skin, BMPR-IA was also seen in the suprabasal layer of epidermis and in single dermal cells (**Fig 2*F***).

Deletion of *noggin* is associated with downregulation of genes that specify HF cell fate in the epidermis and dermis To study further the mechanisms by which long-term deletion of *noggin* affects induction of secondary HF and advanced steps of the primary HF development, the expression of several molecules or markers (β-catenin, plakoglobin, Lef-1, Shh, p75 kDa neurotrophin receptor) implicated in the control of HF morphogenesis (van Genderen *et al*, 1994; Bitgood and McMahon, 1995; Zhou *et al*, 1995; Bierkamp *et al*, 1996; Gat *et al*, 1998; St-Jacques *et al*, 1998; Botchkarev *et al*, 1999a; Botchkareva *et al*, 1999; Paus *et al*, 1999), as well as the epidermal proliferation and apoptosis, were compared between wild-type and *noggin* knockout skin grafts.

In wild-type skin transplants, expression of β-catenin mRNA and β-catenin immunoreactivity was seen in the matrix, inner and outer root sheaths of fully developed HF (**Fig 3*A***, ***C***). Sense control for β-catenin *in situ* hybridization showed an absence of signal in the proximal HF epithelium (**Fig 3*A′***). Absence of β-catenin mRNA and protein was seen in the epidermis of *noggin* null skin (**Fig 3*B***, ***D***); however, plakoglobin immunoreactivity was markedly upregulated in the *noggin* null epidermis, compared with the wild-type skin (**Fig 3*E***, ***F***). Lef-1 protein was found in the precortical zone of the hair matrix in wild-type HF (**Fig 3*G***), and no Lef-1 immunoreactivity was seen in the epidermis of *noggin* knockout skin grafts (**Fig 3*H***).

Shh mRNA was seen in the clusters of epithelial cells located unilaterally in the matrix of wild-type HF (**Fig 3*I***), which was consistent with expression patterns of Shh in the HF published previously (Gat *et al*, 1998). Sense control for Shh *in situ* hybridization showed an absence of signal in the proximal HF epithelium (**Fig 3*I′***); however, no Shh message was seen either in the epidermis nor in the primary HF of *noggin* null skin (**Fig 3*J***). Lack of Shh protein in the epidermis of *noggin* null skin grafts was confirmed by immunostaining with anti-Shh antibody (data not

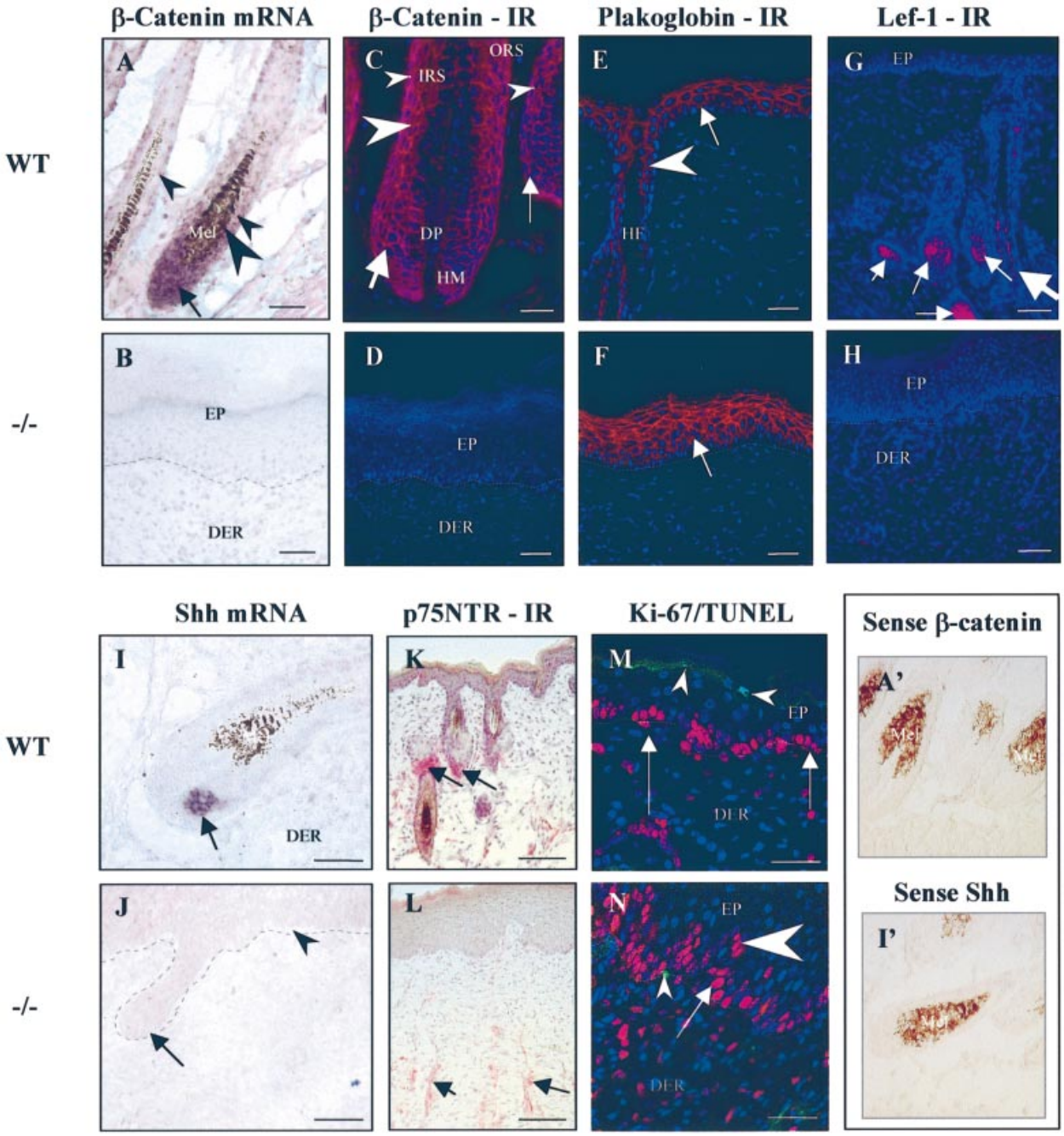

Figure 3. Expression of markers that specify HF cell fate in the wild-type and *noggin* null skin grafts. Skin sections of the wild-type and *noggin* knockout (–/–) transplants were analyzed for the expression of β-catenin and Shh by *in situ* hybridization, or β-catenin, plakoglobin, Lef-1, and p75NTR by immunohistochemistry. In addition, double immunovisualization of the proliferative marker Ki-67 and TUNEL was performed. (*A*, *B*) β-catenin mRNA: in wild-type skin grafts, expression is visible in the HF matrix and outer and inner root sheaths (*A*, *arrows*, small and large *arrowheads*, respectively), whereas no expression is seen in the *noggin* null skin (*B*). (*C*, *D*) β-catenin immunoreactivity: in wild-type HF, expression is visible in the matrix and outer and inner root sheaths (*C*, *arrows*, small and large *arrowheads*, respectively), whereas no expression is seen in the *noggin* null skin grafts (*D*). (*E*, *F*) Plakoglobin immunoreactivity: in wild-type skin, immunostaining is visible in the epidermis and distal HF epithelium (*E*, *arrow* and *arrowhead*, respectively). Increase of immunostaining in the epidermis of *noggin* null graft (*F*, *arrow*). (*G*, *H*) Lef-1 immunoreactivity: in wild-type skin grafts, expression is visible only in the HF matrix (*G*, *arrows*), whereas no expression is seen in the *noggin* null skin (*H*). (*I*, *J*) Shh mRNA: in wild-type skin grafts, a unilateral pattern of expression is visible only in the HF matrix (*I*, *arrow*), whereas no expression is seen in the epidermis and primary HF in *noggin* null skin (*J*). *K*, *L*) p75NTR immunoreactivity: in wild-type skin, p75NTR is expressed in nerve fibers around HF isthmus (*K*, *arrows*), whereas in the *noggin* null skin grafts expression is only visible in the vertically oriented nerves (*L*, *arrows*) and no p75NTR is seen in dermal cells beneath epidermis. (*M*, *N*) Ki-67 (pink fluorescence)/TUNEL (green fluorescence). Increase of Ki-67-positive cells (*N*, *arrows*), and appearance of proliferating cells in suprabasal epidermal layer (*N*, large *arrowhead*) indicating alterations in the keratinocyte proliferation/differentiation transition in the epidermis of *noggin* null skin grafts, compared with wild-type grafts (*M*, *arrows*). TUNEL-positive cells are indicated by small *arrowheads*. (*A′*, *I′*) Sense controls for β-catenin and Shh *in situ* hybridization: absence of specific signals in the HF matrix and outer and inner root sheaths of wild-type HF. (*B*, *D*, *F*, *H*, *J*, *M*, *N*) The dermoepidermal junction is indicated by the dotted line. Nuclei in (*C–H*, *M*, *N*) are counterstained by TO-PRO-3 (blue fluorescence). DER, dermis; DP, dermal papilla; EP, epidermis; HF, hair follicle; HM, hair matrix; IRS, inner root sheath; Mel, melanin; ORS, outer root sheath. *Scale bars*: (*A*, *J*, *K*, *L*) 100 μm; (*B–F*, *H*, *J*) 50 μm; (*M*, *N*) 25 μm.

shown). p75 kDa neurotrophin receptor (p75NTR), an early marker of the HF dermal papilla (Holbrook and Minami, 1991; Botchkareva *et al*, 1999), was only seen in cutaneous nerves located deeply in the dermis of *noggin* null skin, and no p75NTR-positive mesenchymal cell condensations were seen beneath the epidermis (**Fig 3*K***); however, in wild-type grafts, p75NTR was found in skin nerves around the HF isthmus (**Fig 3*L***).

Interestingly, in 75% of *noggin* null skin grafts, the proliferation rate in the epidermis, as determined by Ki-67 expression, was significantly higher (49.1 ± 8.4%, $p < 0.01$) than in wild-type transplants (37.6 ± 5.8%), whereas number of apoptotic TUNEL-positive cells in skin was not altered (**Fig 3*M*, *N***). In those skin grafts, which showed increased keratinocyte proliferation, the epidermis was significantly thicker (96.4 ± 8.8 μm), than in wild-type control (51.7 ± 6.9 μm; $p < 0.01$); however, in the 25% of the *noggin* null grafts, epidermal thickness (53.5 ± 5.2 μm) and proliferation rate (38.2 ± 6.9%) did not show significant differences to wild-type grafts (**Fig 1*G*, *H***). Furthermore, *noggin* null grafts that showed increased thickness and proliferation in the epidermis, and those grafts of *noggin* mutants that displayed apparently normal proliferation rate and thickness, both were characterized by the essentially similar HF phenotype, described above (**Figs 1*H*, 2*B***, and **3*J***). This suggests that alterations in the HF development and in epidermal homeostasis observed in the *noggin* mutants most likely occur independently and are controlled by different mechanisms.

DISCUSSION

Here, we demonstrate that modulation of BMP signaling by the BMP antagonist noggin is required for the induction of the secondary (nontylotrich) HF, representing about 90–95% of the HF in mouse fur. We show that long-term excess of BMP in the *noggin* knockout skin transplanted on to SCID mice results in the inhibition of induction in this HF type (**Fig 1**). Our data also demonstrate that induction of the primary (tylotrich) HF is not affected by BMP, which is consistent with the previous report that showed requirement of the tumor necrosis factor receptor homologue Edar for the induction of this HF subtype (Headon and Overbeek, 1999).

During early steps of development, the expression patterns for Edar, BMP2, BMP4, BMPR-IA, noggin, and Lef-1 in both HF types appears to be similar (Bitgood and McMahon, 1995; Zhou *et al*, 1995; Botchkarev *et al*, 1999a; Headon and Overbeek, 1999). Furthermore, we found no differences in the expression of BMPR-IA, noggin, β-catenin, Lef-1, and Shh between primary and secondary HF in the postnatal skin (Botchkarev *et al*, 2001). Thus, the molecular basis of the differential response of primary *vs* secondary HF to BMP stimulation remains to be dissected. One as yet intended possibility is that Edar signaling may abrogate the inhibitory activity of BMP on primary HF induction. Recently, it was demonstrated that β-catenin represent an important downstream component of Edar signaling during primary HF induction (Huelsken *et al*, 2001). The other molecular targets of Edar signaling in the HF are unknown, however, and a search for Edar downstream components in tooth enamel knots shows that Lef-1, Msx-2, Shh, FGF4, and Wnt10b genes do not appear to be targets for Edar regulation (Pispa *et al*, 1999; Laurikkala *et al*, 2001).

In a previous study, we showed that retardation of HF development in E17.5 *noggin* knockout mice is associated with alterations of Lef-1 expression in the hair placode and p75NTR in the HF mesenchyme, whereas expression of BMP2 and BMP4 was not altered (Botchkarev *et al*, 1999a). Here, we show that long-term deletion of *noggin* leads to strong upregulation of BMP2 and BMP4 in the epidermis and dermis of *noggin* null skin transplants (**Fig 2**). These results are consistent with the ability of BMP and activins to regulate their own expression via autocrine stimulation of the corresponding receptors, as published previously (Eramaa *et al*, 1995; Di Simone *et al*, 1999; Pereira *et al*, 2000). On the other hand, we show that long-term excess of BMP in the *noggin* null skin grafts results in marked downregulation of other markers that specify HF cell fate in the epidermis and dermis (β-catenin, Shh, alkaline phosphatase; **Figs 1** and **3**).

Interestingly, marked downregulation of β-catenin mRNA, and protein in the epidermis of *noggin* null skin grafts (**Fig 3**) was not accompanied by alterations of cell adhesion. β-catenin represents an important component of the cadherin/cytoskeleton complex maintaining adherens junctions (reviewed in Steinberg and McNutt, 1999). It was also recently shown, however, that β-catenin knockout embryos do not show alterations in cell adhesion, probably due to the replacement of β-catenin in cadherin/cytoskeleton complex by plakoglobin, the closest relative of β-catenin in vertebrates (Huelsken *et al*, 2000). The *in vitro* data also suggest that plakoglobin can mediate interaction between E-cadherin and α-catenin (Huelsken *et al*, 1994). As plakoglobin protein was upregulated in the epidermis of *noggin* null skin grafts compared with wild-type control (**Fig 3**), we suppose that plakoglobin may substitute for loss of β-catenin and maintain cell adhesion in the *noggin* null epidermis.

In contrast to our previous findings, which had suggested a lack of alterations in epidermal proliferation in the E17.5 *noggin* null skin (Botchkarev *et al*, 1999a), we have demonstrated that 75% of *noggin* null skin grafts showed increased epidermal proliferation and thickness, compared with wild-type skin (**Fig 3**). Three weeks after transplantation, a process of wound healing in both *noggin* null and wild-type skin grafts was completed, and all transplants showed lack of inflammatory infiltration (**Fig 1**). Therefore, we assume that influence of pro-inflammatory cytokines on epidermal proliferation in skin grafts 3 wk after transplantation is likely to be minimized. To explain the differences in epidermal proliferation between *noggin* null and wild-type skin grafts, one should take into consideration that the effects of BMP on many developmental structures strongly depend on the morphogenetic stage, concentration of the locally secreted BMP antagonists, and expression of the corresponding receptors (Kratochwill *et al*, 1996; Neubuser *et al*, 1997; Chuong, 1998; Jung *et al*, 1998; Noramly and Morgan, 1998; Noramly *et al*, 1999; Perrimon and McMahon, 1999). We speculate that, in contrast to embryonic skin, epidermal keratinocytes in postnatal skin may show more heterogeneous sensitivity to BMP or to the other factor(s), whose secretion or activity is affected by the excess of BMP in *noggin* null skin grafts.

We also demonstrate here that in the *noggin* null skin grafts, long-term excess of BMP leads to the retardation and arrest of the primary HF development at stage 4, i.e., prior to the onset of hair shaft formation (**Figs 1–3**). It would be interesting to test whether the administration of noggin or other BMP2/4 antagonists (chordin, gremlin, etc.) would rescue hair phenotype observed in the *noggin* null grafts. It was recently shown that transgenic mice overexpressing *noggin* driven by the Msx-2 promoter in the hair matrix show lack of external hairs due to the alterations in the proliferation/differentiation transition of matrix keratinocytes and hair shaft synthesis (Kulessa *et al*, 2000). Taken together, these two sets of data suggest that the genetic program for hair shaft production is tightly controlled by the balance of noggin and BMP in the HF and that either excess or limitation of BMP signaling in the HF may disturb initiation and maintenance of the hair shaft-specific differentiation in the hair matrix keratinocytes.

Interestingly, Msx-2-driven overexpression of *noggin* is also associated with upregulation and ectopic expression of Lef-1 in the HF (Kulessa *et al*, 2000). As shown previously, BMP2, BMP4, and Lef-1 are expressed in the precortical cells of the HF matrix, and Lef-1 binding sites are present on selected hair keratin genes (Lyons *et al*, 1990; Zhou *et al*, 1995; Dale and Wardle, 1999; Kulessa *et al*, 2000). Here, we show that β-catenin, an upstream effector of Lef-1, is also expressed in the HF matrix in wild-type neonatal skin (**Figs 2** and **3**). Furthermore, similar to our results in *noggin* null skin grafts, the HF morphogenesis in Lef-1 knockout mice is also arrested at the stage just prior to hair shaft formation (van Genderen *et al*, 1994). Therefore, these data suggest a cross-talk between BMP and Wnt/β-catenin/Lef-1 signaling pathways in the control of hair

shaft-specific differentiation in the HF, and are consistent with results obtained in other models that showed that BMP and Wnt signaling play antagonistic roles during development (Baker *et al*, 1999).

Taken together, our data suggest that during HF development the Wnt/β-catenin/Lef-1 signaling pathway represents an important target for BMP regulation. Selective requirement of BMP antagonist *noggin* for the induction of secondary HF supports a concept that Edar and Lef-1 signaling pathways at certain stages of development are running and regulated independently of each other (Headon and Overbeek, 1999; Tucker *et al*, 2000). Furthermore, differential signaling requirements during the formation of primary and secondary HF suggest parallels between morphogenesis of distinct developmental structures derived from the same organ-committed epithelium. Similarly, morphogenesis of different types of teeth (mandibular molars/incisors and maxillary molars) requires strikingly differential dependence on activin βA and the activity of a number of transcriptional regulators, including *Dlx1/Dlx2* (Thomas *et al*, 1997; Ferguson *et al*, 1998).

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