Epithelial Bmpr1a regulates differentiation and proliferation in postnatal hair follicles and is essential for tooth development

Thomas Andl¹, Kyung Ahn³, Alladin Kairo¹, Emily Y. Chu¹, Lara Wine-Lee³, Seshamma T. Reddy¹, Nirvana J. Croft¹, Judith A. Cebra-Thomas¹, Daniel Metzger⁴, Pierre Chambon⁴, Karen M. Lyons⁵, Yuji Mishina⁶, John T. Seykora¹, E. Bryan Crenshaw III³ and Sarah E. Millar², *

¹Department of Dermatology, University of Pennsylvania Medical School, Philadelphia, PA 19104, USA
²Department of Cell and Developmental Biology, University of Pennsylvania Medical School, Philadelphia, PA 19104, USA
³Center for Childhood Communication, Abramson Research Center, The Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA
⁴Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, Collège de France, BP 163, 67404 Illkirch Cedex, France
⁵Departments of Molecular, Cell and Developmental Biology, Orthopaedic Surgery, and Biological Chemistry, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA
⁶Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, USA

*Author for correspondence (e-mail: millars@mail.med.upenn.edu)

Accepted 5 February 2004
Development 131, 2257-2268
Published by The Company of Biologists 2004
doi:10.1242/dev.01125

Summary
Bone morphogenetic protein (BMP) signaling is thought to perform multiple functions in the regulation of skin appendage morphogenesis and the postnatal growth of hair follicles. However, definitive genetic evidence for these roles has been lacking. Here, we show that Cre-mediated mutation of the gene encoding BMP receptor 1A in the surface epithelium and its derivatives causes arrest of tooth morphogenesis and lack of external hair. The hair shaft and hair follicle inner root sheath (IRS) fail to differentiate, and expression of the known transcriptional regulators of follicular differentiation Msx1, Msx2, Foxn1 and Gata3 is markedly downregulated or absent in mutant follicles. Lef1 expression is maintained, but nuclear β-catenin is absent from the epithelium of severely affected mutant follicles, indicating that activation of the WNT pathway lies downstream of BMPR1A signaling in postnatal follicles. Mutant hair follicles fail to undergo programmed regression, and instead continue to proliferate, producing follicular cysts and matricomas. These results provide definitive genetic evidence that epithelial Bmpr1a is required for completion of tooth morphogenesis, and regulates terminal differentiation and proliferation in postnatal hair follicles.

Key words: Hair follicle, Skin, Tooth, BMP, BMPR1A, BMPR1B, β-catenin, Gata3, Msx, Foxn1

Introduction
Postnatal hair growth relies on a precise balance between proliferation and differentiation of follicular epithelial cells. The base of the hair follicle contains relatively undifferentiated, pluripotent epithelial matrix cells that proliferate rapidly during periods of hair growth (anagen). The matrix is derived from long-lived epithelial stem cells located in the permanent, bulge region of the follicle (Cotsarelis et al., 1990; Oshima et al., 2001; Taylor et al., 2000). The stem cells proliferate transiently at anagen onset and otherwise remain quiescent (Wilson et al., 1994). The matrix gives rise to several different hair follicle lineages: the medulla, cortex and cuticle of the hair shaft; and the cuticle, Huxley’s layer and Henle’s layer of the IRS (Oshima et al., 2001).

Periods of hair growth are followed by a regression phase (catagen), when proliferation and differentiation of follicular epithelial cells cease and the lower two-thirds of the follicular epithelium degenerates (Lindner et al., 1997). Following catagen the follicles enter a quiescent phase (telogen). The onset of a new anagen phase is thought to involve signaling between the dermal component of the hair follicle, the dermal papilla and epithelial cells (Cotsarelis et al., 1990; Oliver and Jahoda, 1988).

Intercellular communication between the dermis and surface epithelium, and within the surface epithelium, is also crucial for the embryonic morphogenesis of hair follicles and other epithelial appendages, such as teeth and mammary glands, and several classes of secreted signaling molecules are implicated at early stages of appendage development (reviewed by Hardy, 1992; Jernvall and Thesleff, 2000; Millar, 2002; Veltmaat et al., 2003). Among these are BMPs, which function by binding type 1 (BMPR1A and BMPR1B) and type 2 (BMPR2) transmembrane serine/threonine kinase receptors, resulting in phosphorylation of the intracellular proteins SMADs 1, 5 and 8. Phosphorylated SMADs bind to SMAD4 and translocate to the nucleus, where they complex with transcription factors and regulate target gene transcription (Mishina, 2003). Ectopic expression experiments and analysis of mice lacking the BMP inhibitor noggin suggest that BMP signals act during embryogenesis to retard hair follicle development, and may...
control the spacing of hair and feather follicles (Botchkarev et al., 1999; Jung et al., 1998; Noramly and Morgan, 1998). Postnatally, ectopic expression studies suggest that BMP inhibition controls anagen onset (Botchkarev et al., 2001), and that BMP signaling regulates hair-shaft differentiation (Kulessa et al., 2000). Existing evidence suggests that BMPs play crucial roles at multiple stages of tooth morphogenesis (Jernvall and Thesleff, 2000), and BMP mRNAs are also expressed in developing mammary glands (Phippard et al., 1996).

Despite abundant data suggesting key roles for BMP signals in appendage development and in postnatal hair follicle differentiation and cycling, definitive genetic evidence from loss-of-function mutations in BMP or BMP receptor genes is lacking because of the early lethality of most of these mutants (Mishina, 2003). As the BMPR1A receptor is broadly expressed in the epidermis and hair follicles (Botchkarev, 2003), we investigated the requirement for epithelial Bmpr1a in these processes using several different mouse lines that express Cre recombinase (Lewandoski, 2001) in epidermal cells and their derivatives, in combination with a conditional allele of Bmpr1a (Ahn et al., 2001; Mishina et al., 2002). Tooth morphogenesis was arrested in the most severely affected conditional mutant mice, and postnatal development of hair follicles was strikingly defective. Hair follicle matrix cells failed to differentiate towards hair shaft or IRS, and expression of several known transcriptional regulators of hair shaft and IRS differentiation was decreased or absent. Mutant follicles failed to undergo catagen, instead continuing to proliferate and produce matricomas and follicular cysts. These results demonstrate that epithelial Bmpr1a plays essential roles in controlling differentiation and proliferation in postnatal hair follicles, as well as being required for completion of tooth morphogenesis.

Materials and methods

Generation and breeding of mouse lines, tamoxifen treatment and detection of floxed and recombined alleles

A K14-Cre transgene was made by inserting a modified Cre cDNA fragment PCR-amplified from the pACN vector (Bunting et al., 1999) into the BamHI site of a K14 expression vector (Andl et al., 2002). The transgene was microinjected into fertilized eggs from a B6SJLJF1/c57BL6J F1 cross (Jackson Laboratories). Transgenic founders were tested for Cre activity by breeding to ROSA26R mice (B6.129S4-Gt(Rosa)26Sortm1(Ber) (Soriano, 1999) (Jackson Laboratories). Detection of β-galactosidase in embryo wholemounts and 20 μm frozen sections of P1 tissue were as described previously (Phippard et al., 1999; Wang et al., 1997).

Mice homozygous for a floxed allele of Bmpr1a (Bmpr1a<sup>floxed<sup>P<sub>neo</sub></sup></sup>) were crossed to K14-Cre or K14-Cre-ERT2 (Indra et al., 2000) transgenic mice, and progeny heterozygous for both K14-Cre or K14-Cre-ERT2 and Bmpr1a<sup>floxed<sup>Cre<sub>ERT2</sub></sup></sup> were crossed to Bmpr1a<sup>floxed<sup>Cre<sub>ERT2</sub></sup></sup> homozygotes. Brm4-Cre line bcre-32 transgenic mice (Ahn et al., 2001) were used in combination with Bmpr1a<sup>floxed<sup>Cre<sub>ERT2</sub></sup></sup> and heterozygous Bmpr1a<sup>null</sup> mice (Bmpr1a<sup>null/heterozygous</sup>) (Mishina et al., 1999) mice. Bmpr1a<sup>null/heterozygous</sup> males homozygous for bcre-32 were crossed with females of genotype Bmpr1a<sup>null/heterozygous</sup>. In addition, we used mice carrying a null allele of Bmpr1b (Bmpr1b<sup>null</sup>) (Yi et al., 2001): Bmpr1b<sup>null/null</sup>, Bmpr1b<sup>heterozygous</sup> males homozygous for bcre-32 were crossed with Bmpr1b<sup>null/heterozygous</sup> females. PCR detection of wild-type, floxed and recombined Bmpr1a alleles was as described previously (Mishina et al., 2002). Newborn epidermis was isolated mechanically after incubation of skin overnight in 0.25% trypsin in PBS at 4°C. For tamoxifen induction, dorsal hair (if present) was clipped and 0.5 mg tamoxifen (Sigma) in 100 μl ethanol was applied to dorsal skin three times at 48 hour intervals. Controls were treated with vehicle only.

Histology, follicle density measurements, immunofluorescence, BrdU incorporation, TUNEL assays and in situ hybridization

Tissue samples were fixed in 4% paraformaldehyde, paraffin embedded and sectioned at 5 μm. Samples containing bone were decalcified with 4% formic acid, 4% hydrochloric acid for 4 hours after fixation. For histological analysis, sections were stained with Hematoxylin and Eosin. For hair follicle density measurements, 2-4 control animals and 2-4 experimental animals were used for each developmental stage. Follicles were counted at 10x magnification in 10 microscopic fields for each sample. Statistical significance was calculated using a two-tailed Student's t-test.

Alkaline phosphatase staining for identification of dermal papillae was as described previously (Handjiski et al., 1994). For immunofluorescence staining, sections were microwave pretreated and incubated with primary antibodies against phospho-SMAD1, phospho-SMAD5 and phospho-SMAD5 (polyclonal rabbit serum, Cell Signaling Technology: 1:50), type I hair keratin (mouse monoclonal antibody clone AE13) (Lynch et al., 1986) (1:10), trichohyalin (mouse monoclonal antibody clone AE15; 1:6) (O’Guin et al., 1992), mouse keratin 6 (polyclonal rabbit serum, Covance; 1:300), GATA3 (mouse monoclonal antibody, Santa Cruz HCG3-31; 1:100) and cytokeratin K17 (polyclonal rabbit serum; 1:500) (McCowan and Coulombe, 1998). For detection of nuclear β-catenin, antibody clone 15B8 (Sigma; 1:1000) was used in combination with the MOM kit (Vector Laboratories). Polyclonal rabbit serum (ten Dijke et al., 1994) (1:100) was used for detection of BMPRIA in 8 μm cryosections. Sections were counterstained with 2 μg/ml Hoechst 33258 (Sigma).

For proliferation assays, mice were injected with BrdU (50 μg BrdU/ml body weight) and sacrificed after 1 hour. Detection of BrdU incorporation with anti-BrdU antibody (Roche) was as previously described (Andl et al., 2002). The In Situ Cell Death Detection Kit (Roche Diagnostics) was used for apoptosis assays.

In situ hybridization with 35S-labeled probes, and whole-mount and paraffin section in situ hybridization with digoxigenin-labeled probes were as described previously (Decimo et al., 1995; Grachtchouk et al., 2003; Reddy et al., 2001). The following PCR products containing a T7 promoter were used as templates for sense and antisense probe synthesis: Foxn1 (Accession Number NM_008238, nucleotides 1720-2339), and Cre (Accession Number A169416, nucleotides 2533-3151). Mx, Lef1, Wnt10b and Shh probes were as described previously (Andl et al., 2002; Jowett et al., 1993).

Results

BMP signaling is active in postnatal hair follicles

Cells responding to BMP signals can be identified by the presence of phosphorylated SMAD1, SMAD5 and SMAD8 in the nucleus (von Bubnoff and Cho, 2001). To determine the sites of BMP signalling activity in postnatal hair follicles, we used an antibody to phospho-SMAD1/5/8 at different stages of the embryonic and first postnatal hair growth cycles. In early anagen [postnatal day (P) 5], nuclear phospho-SMAD was detected in the companion layer of the outer root sheath (Fig. 1A,B). By full anagen (P13), phospho-SMAD staining was present in precursor cells of the medulla, cortex and cuticle of the hair shaft, in a subset of IRS cells, and in the companion layer (Fig. 1C,D). Phospho-SMAD staining faded at the
Bmpr1a regulates hair and tooth development

**Generation of mice lacking Bmpr1a in the epidermis and its derivatives**

Bmpr1a-deficient mice die early in gestation (Mishina et al., 1995). To determine the requirement for Bmpr1a in development of the epidermis and its appendages, we generated three lines of transgenic mice that constitutively express Cre recombinase in epidermal cells under the control of a well-characterized keratin 14 (K14) promoter (Wang et al., 1997). In addition, we made use of bcre-32 mice that carry a Brn4-Cre transgene that directs Cre expression to the neural tube, and also shows ectopic Cre expression in limb epithelium and in ventral epidermis (Ahn et al., 2001). Lastly we used tamoxifen-inducible K14-Cre-ERT2 mice, which express a Cre recombinase-estrogen receptor fusion protein under the control of the K14 promoter (Indra et al., 2000). The K14-Cre and bcre-32 transgenics were crossed to a ROSA26R reporter strain that carries a lacZ gene whose expression is activated by Cre-mediated excision of an inhibitory sequence, allowing identification of cells that express Cre and their descendents by staining for β-galactosidase (Soriano, 1999). β-galactosidase expression in K14-Cre mice was assayed at E14.5 and P1, and was observed specifically in epidermis, hair follicles, oral epithelium and developing teeth, with very low levels in the esophagus and forestomach, as previously described (Byrne et al., 1994; Vassar and Fuchs, 1991; Vassar et al., 1989; Wang et al., 1997) (Fig. 2A-C and data not shown). β-galactosidase activity in the suprabasal epidermis and in all hair follicle epithelial cells reflects derivation of these cells from basal epidermis and K14-expressing follicular stem cells, and inheritance of the recombined ROSA26R allele (Vasioukhin et al., 1999). All three lines showed similar Cre activity at this level of analysis. Strong Cre activity was observed in ventral epidermis of bcre-32 embryos by E15 (Fig. 2D).

Each of the Cre-expressing lines was mated to mice carrying a conditional allele of Bmpr1a, Bmpr1acl, in which exon 2 of the Bmpr1a gene is flanked by loxP sites (Ahn et al., 2001; Mishina et al., 2002). Mice homozygous for Bmpr1acl show no apparent phenotype (Ahn et al., 2001; Mishina et al., 2002). However, global expression of Cre recombinase in Bmpr1acl homozygotes produces a phenotype identical to the null mutant phenotype, indicating that deletion of exon 2 produces a null allele (Mishina et al., 2002).

Bmpr1acl heterozygotes carrying Cre transgenes, and Bmpr1aclcl mice lacking a Cre transgene, were indistinguishable from non-transgenic mice that were wild type for Bmpr1a. Bmpr1aclcl homozygotes carrying K14-Cre transgenes displayed phenotypes of graded severity depending on the Cre line used. This observation suggested subtle differences in the level of activity of Cre recombinase in the three lines, as all of the mice were maintained on a similar,
mixed strain background. K14-Cre43; Bmpr1aclcl mice died within 24 hours of birth, and displayed severe limb defects and open eyes (Fig. 3A). The limb defects reflected the known requirement of epithelial Bmpr1a for maintenance of the apical ectodermal ridge (Ahn et al., 2001). K14-Cre52; Bmpr1aclcl mice usually died by P4. They had grossly normal limbs and eyes, were severely runted, and lacked external hair and teeth (Fig. 3C,D,E). K14-Cre40; Bmpr1aclcl mice showed the weakest phenotype of the constitutive K14-Cre lines and occasionally survived for several months. These mice were runted, and showed greatly decreased external hair (Fig. 3F-H), K14-Cre-ERT2; Bmpr1aclcl mice that had not been treated with tamoxifen survived for at least 6 months. They displayed a sparse hair phenotype that became more severe with age (Fig. 3J). Surviving K14-Cre40; Bmpr1aclcl and K14-Cre-ERT2; Bmpr1aclcl mice had abnormal growths under their nails (Fig. 3I). Surviving K14-Cre40; Bmpr1aclcl and K14-Cre-ERT2; Bmpr1aclcl mice caused these animals to become sickly, requiring euthanasia 7 days after cessation of treatment. This may have been due to trans-cutaneous absorption of tamoxifen and induction of Bmpr1acl recombination in internal stratified epithelia. Homozygous bcre-32 transgenic mice that were also heterozygous for a null allele of Bmpr1a (Bmpr1amut) (Mishina et al., 1995) were crossed to Bmpr1aclcl mice to produce progeny of genotype bcre-32; Bmpr1 aclnull, as well as bcre-32; Bmpr1 aclcl control mice. bcre-32; Bmpr1 aclnull mice survived for up to 17 days and showed absence of external hair in the mid-ventrum, corresponding to the region of Cre activity in ventral epidermis (Fig. 3B). The lethality of bcre-32; Bmpr1 aclnull mice was due to hydrocephalus and CNS defects (data not shown). K14-Cre43; Bmpr1 aclnull and K14-Cre52; Bmpr1 aclnull mice had oral abnormalities, resulting from defective tooth development (see below), and had difficulty suckling. Decreased lifespan of K14-Cre40; Bmpr1 aclnull mice may have been due to loss of Bmpr1a in internal epithelia.

Absence of BMPR1A protein from K14-Cre43; Bmpr1 aclnull newborn epidermis and hair follicle epithelium was confirmed by immunofluorescence with anti-BMPR1A antibody (Fig. 3K,L). In addition, PCR of genomic DNA, using primers specific for the floxed, recombined and wild-type alleles (Mishina et al., 2002), demonstrated that the Bmpr1 acl allele was efficiently recombined in the epidermis of K14-Cre43; Bmpr1 aclnull mice to produce a non-functional allele (Mishina et al., 2002) (Fig. 3M). Skin-specific recombination of the Bmpr1 acl allele in uninduced K14-Cre-ERT2; Bmpr1 aclnull mice was demonstrated by PCR of genomic DNA extracted from skin and other organs (Fig. 3M). This result indicated that Cre-ERT2 is active at a low level in the epidermis of K14-Cre-ERT2 mice, even in the absence of tamoxifen. Leakiness of Cre-ERT2 activity was not observed in previous studies (Indra et al., 2000; Li et al., 2000), and may have been detected because of the strain background on which our mice were maintained, or because of particular susceptibility of the Bmpr1 acl locus to recombination at even very low levels of Cre activity.

**Accelerated hair follicle morphogenesis in epithelial Bmpr1a mutants**

Development of primary hair follicles in mouse embryos is initiated at approximately E14, and secondary hair follicles develop in several waves between approximately E16 and birth. To determine whether deletion of epithelial Bmpr1a affects hair follicle morphogenesis, we examined the histology of K14-Cre; Bmpr1 aclnull dorsal and ventral skin, and bcre-32; Bmpr1 aclnull mid-ventral skin, between E16.5 and birth. In addition, anti-K17 antibody was used to clearly reveal the location of developing hair follicles (Fig. 4C,D). For all the mutants analyzed, newborn skin appeared similar to littermate controls (Fig. 4E-H and data not shown). Measurements of follicle density in newborn dorsal K14-Cre43; Bmpr1 aclnull and littermate control skin revealed no significant difference (average of 12±3 versus 12±2 follicles per mm; P=0.766). At E16.5 and later embryonic stages, mutant follicles were histologically similar to controls (Fig. 4A,B and data not shown). However, we found that the follicle density in E16.5 K14-Cre43; Bmpr1 aclnull dorsal skin was significantly greater than in control dorsal skin (7±2 versus 5±2 follicles per mm; P=0.0002). As the sizes of mutant and control individuals were similar at these stages, these results suggested that follicular morphogenesis was accelerated in Bmpr1a mutants, but that the total number of follicles that developed by birth was not affected by loss of epithelial Bmpr1a.

To determine whether Bmpr1b might act redundantly with Bmpr1b in hair follicle patterning, we examined the mid-ventral skin of bcre-32; Bmpr1 aclnull mice that were also homozygous for a targeted deletion of Bmpr1b (Bmpr1bmut) (Yi et al., 2000) at E17.5. At this stage the histology and follicle density of bcre-32; Bmpr1 aclnull, Bmpr1 bnull aclnull ventral skin was indistinguishable from bcre-32; Bmpr1 aclnull bnull ventral skin; however, the average follicle density was significantly greater than in control littermate ventral skin (2±1 versus 1±1 per mm; P=0.0074). At birth, ventral skin from bcre-32; Bmpr1 aclnull, Bmpr1 bnull aclnull, bcre-32; Bmpr1 aclnull and control mice had a similar histology, and follicle densities did not differ
Bmpr1a regulates hair and tooth development significantly (Fig. 4G-I). These data provide further evidence for accelerated follicular morphogenesis in Bmpr1a mutants, and indicate that epithelial Bmpr1a and Bmpr1b do not act redundantly in embryonic hair follicle development. To further solidify this conclusion, surviving K14-Cre; Bmpr1a cl/cl adult females were able to nurse pups, indicating that functional mammary glands developed. Histological analysis revealed that mammary buds and glands were present in severely affected K14-Cre; Bmpr1a cl/cl females at E13.5 and birth, respectively, and whole-mount in situ hybridization with a probe for the mammary bud marker Wnt10b revealed a normal pattern of bud development at E13.5 (Fig. 4J-O). However, determining whether these mutants have subtle defects in mammary morphogenesis will require further analysis.

By contrast, tooth development was strikingly affected in K14-Cre; Bmpr1a cl/cl mice. Analysis of the histology of the oral cavity of K14-Cre43; Bmpr1a cl/cl newborns revealed a complete absence of incisor or molar tooth structures (Fig. 4T,U). A dental lamina was present in K14-Cre43; Bmpr1a cl/cl embryos at E13.5, and tooth bud formation was initiated. However, the tooth buds were significantly smaller and less well developed in mutant embryos than in controls (Fig. 4P,Q), and by E16.5 molar and incisor tooth structures had regressed (Fig. 4R,S and data not shown), indicating failure of tooth development after the bud stage.

Epithelial Bmpr1a is required for normal postnatal development of hair follicles

At later postnatal stages, dorsal and ventral skin of K14-Cre; Bmpr1a cl/cl and K14-Cre40; Bmpr1a cl/cl mice, and mid-ventral skin of bcre-32; Bmpr1a cl/cl mice, showed striking defects in hair follicle morphology. By P8, control hair follicles were in mid-anagen, with large hair bulbs that had descended deep into the fat layer of the skin, and well-differentiated hair shafts that had undergone terminal differentiation and lacked nuclei. By
contrast, mutant mice displayed hair follicles that were smaller, and were wavy and misaligned. The mutant follicles had not descended as deeply into the dermis as control follicles, and, perhaps in part as a consequence of this, the fat layer appeared less expanded than normal. Reduction of the fat layer probably also resulted from difficulties in feeding caused by tooth defects in some mutants. The hair follicles had misshapen, expanded dermal papillae, and the majority of follicles failed to produce hair shafts. Instead, nuclei were present in the centers of the abnormal follicles (Fig. 5A-D and data not shown).

**Bmpr1a is required for BMP pathway activity in postnatal hair follicle epithelium**

To determine the effect of Cre-mediated deletion of Bmpr1a on activity of the BMP signaling pathway, we used anti-phospho-SMAD1/5/8 antibody to localize phospho-SMADs in K14-Cre40; Bmpr1a<sup>cl/cl</sup> skin at P8. We observed greatly reduced staining for phospho-SMAD in the abnormal mutant hair follicle epithelium (Fig. 5E,F). Occasional staining was observed in the suprabasal epidermis, consistent with a report of Bmpr1b expression in these cells (Botchkarev et al., 1999). Weak phospho-SMAD staining was present in the dermal papillae of mutant follicles, suggesting that BMP signaling is activated in dermal papillae as an indirect response to lack of epithelial Bmpr1a.

**Epithelial Bmpr1a is required for hair shaft and IRS differentiation**

To investigate whether the phenotype of mutant hair follicles...
Bmpr1a regulates hair and tooth development

resulted from abnormal follicular differentiation we examined the expression of several differentiation markers. Staining with AE13, an antibody to type I low-sulfur hair-shaft cortex keratins (Lynch et al., 1986), was absent in the majority of mutant follicles (Fig. 5G,H). Staining with AE15, which recognizes proteins in the medulla of the hair shaft and in the IRS (O’Guin et al., 1992), was greatly reduced in the majority of follicles, and was absent from the most distorted follicles, indicating absence of the hair-shaft medulla and defective differentiation of the IRS (Fig. 5I,J). By contrast, the outer root sheath markers, keratins (K) 17 and 6, were expressed in mutant follicles, and weak K6 expression was observed in mutant epidermis (yellow arrows; E) but is much reduced in mutant follicle epithelium (F). Weak staining is present in mutant dermal papillae and in a few epithelial cells close to the epidermis (yellow arrows). (G-L) Immunofluorescence of P8 dorsal skin from K14-Cre40; Bmpr1a<sup>fl/fl</sup> control littermate (G,I,K) and K14-Cre40; Bmpr1a<sup>fl/fl</sup> mutant (H,J,L) using AE13 (G,H), AE15 (L) and anti-keratin 17 (K,L) antibodies. (M,N) Immunofluorescence of P8 mid-ventral skin from bcre-32; Bmpr1a<sup>fl/fl</sup> control littermate (M) and bcre-32; Bmpr1a<sup>fl/fl</sup> mutant (N) using anti-keratin 6 antibody. Yellow arrows indicate the hair shaft (G), IRS and medulla (I), and outer root sheath (K,N). White arrow indicates epidermal staining (N). Immunofluorescence signals are red in E-L and green in M,N. Nuclei are counterstained with Hoechst 33258. Scale bars: in D applies to A-D; in F applies to E,F; in N applies to G-N.

**Fig. 5.** Effects of epidermal-specific deletion of Bmpr1a on the histology of postnatal anagen hair follicles, localization of phospho-SMAD1/5/8, and expression of hair follicle differentiation markers. (A,B) Histology of dorsal skin from K14-Cre40; Bmpr1a<sup>fl/fl</sup> control littermate (A) and K14-Cre40; Bmpr1a<sup>fl/fl</sup> mutant (B) at P8. Nuclei in the center of a mutant follicle are indicated by an arrow (B). (C,D) Histology of mid-ventral skin from bcre-32; Bmpr1a<sup>fl/fl</sup> control littermate (C) and bcre-32; Bmpr1a<sup>fl/fl</sup> mutant (D) at P8. (E,F) Anti-phospho-SMAD1/5/8 immunofluorescence of P8 dorsal skin from K14-Cre40; Bmpr1a<sup>fl/fl</sup> control littermate (E) and K14-Cre40; Bmpr1a<sup>fl/fl</sup> mutant (F). Staining is present in nuclei of IRS and hair-shaft precursors in control follicles (yellow arrows; E) but is much reduced in mutant follicle epithelium (F). Weak staining is present in mutant dermal papillae and in a few epithelial cells close to the epidermis (yellow arrows). (G-L) Immunofluorescence of P8 dorsal skin from K14-Cre40; Bmpr1a<sup>fl/fl</sup> control littermate (G,I,K) and K14-Cre40; Bmpr1a<sup>fl/fl</sup> mutant (H,J,L) using AE13 (G,H), AE15 (L) and anti-keratin 17 (K,L) antibodies. (M,N) Immunofluorescence of P8 mid-ventral skin from bcre-32; Bmpr1a<sup>fl/fl</sup> control littermate (M) and bcre-32; Bmpr1a<sup>fl/fl</sup> mutant (N) using anti-keratin 6 antibody. Yellow arrows indicate the hair shaft (G), IRS and medulla (I), and outer root sheath (K,N). White arrow indicates epidermal staining (N). Immunofluorescence signals are red in E-L and green in M,N. Nuclei are counterstained with Hoechst 33258. Scale bars: in D applies to A-D; in F applies to E,F; in N applies to G-N.

Transcriptional regulators of IRS and hair-shaft differentiation show reduced or absent expression in mutant follicles

Msx2 function is required for normal expression levels of Foxn1, which controls the transcription of several hair keratin genes (Ma et al., 2003; Meier et al., 1999; Schlake and Boehm, 2001). Expression of Msx2, the related gene Mso1, and Foxn1 was dramatically reduced in mutant skin (Fig. 6A-D,I,J). Differentiation of the IRS is regulated by the transcription factor GATA3 (Kaufman et al., 2003), and GATA3 expression was similarly reduced or absent in mutant follicles (Fig. 6G,H). Expression of the WNT pathway transcriptional effector Lef1 was maintained in mutant follicles (Fig. 6K,L); however, nuclear localization of β-catenin, which complexes with LEF1 to activate WNT target transcription, was absent from the epithelium of the most affected follicles (Fig. 6M,N). Nuclear LEF1 and β-catenin localize to hair-shaft precursor cells and are thought to regulate differentiation of the hair shaft (DasGupta and Fuchs, 1999; Merrill et al., 2001). Taken together, therefore, these results indicate that BMPR1A is required for cells exiting the matrix to begin to differentiate to
form hair shaft and IRS. Consistent with this role for BMP1A, Shh, which regulates proliferation in hair follicles (Chiang et al., 1999; Oro and Higgins, 2003; St-Jacques et al., 1998), was expressed in mutant follicles (Fig. 6E,F).

Interestingly, elevated levels of nuclear localized β-catenin were detected in the abnormal, expanded dermal papillae of mutant follicles (Fig. 6N and data not shown). Together with elevated phospho- SMAD1/5/8 in mutant dermal papillae (Fig. 5F), these data suggest that BMP signaling may be capable of activating the WNT pathway in dermal as well as epithelial cells of postnatal follicles.

**Mutant hair follicles fail to undergo programmed regression**

To determine whether hair follicles cycle normally in the skin of surviving mutant mice, we examined the histology of dorsal and ventral skin from K14-Cre40; Bmpr1a<sup>cl/c</sup> mice at P12, P21 and P34, and ventral skin from bcre-32; Bmpr1a<sup>cl/c</sup> mice at P17, compared with control littersmates. At P12, control hair follicles were in a fully developed anagen stage, with large hair bulbs and differentiated, pigmented hair shafts (Fig. 7A). Mutant follicles had not descended further into the dermis than they had at P8, and were still wavy and misangled (Fig. 7B). At P17, control follicles were entering catagen (Fig. 7C). By contrast, mutant hair follicles did not regress; instead they persisted in an abnormal anagen phase (Fig. 7D). At P21, control follicles were in the telogen, resting phase, whereas mutant follicles were in an abnormal, anagen-like state (Fig. 7E,F). The dermal papillae of mutant follicles, revealed by staining for alkaline phosphatase, were expanded compared with those of control littermate follicles (Fig. 7E′,F′). Anti-BrdU immunofluorescence revealed high levels of incorporation in the mutant hair follicle bulbs and outer root sheaths at P21 (Fig. 7F′); by contrast, little proliferation was detected in control littermate skin at this stage (Fig. 7E′). By P34, control follicles had re-entered anagen and high levels of proliferation were detected in the follicle bulbs (Fig. 7G,G′). At this stage some less distorted mutant follicles were entering catagen. However, the majority of mutant follicles had still not undergone regression, and cells in the hair bulb and outer root sheath continued to proliferate (Fig. 7H,H′). The mutant epidermis also showed more proliferation than controls (Fig. 7G′,H′).

**Long term consequences of epithelial Bmpr1a deficiency: development of matricomas and follicular cysts**

To assess the longer term consequences of loss of epithelial Bmpr1a on the skin we made use of K14-Cre-ERT2; Bmpr1a<sup>cl/c</sup> mice that fortuitously displayed a mild, progressive phenotype due to leakiness of uninduced epidermal Cre-ERT2. This presumably caused a low level of continuous recombination of the Bmpr1a<sup>cl/c</sup> allele in the basal epidermis and hair follicle stem cells, leading to progressive accumulation of cells that lacked functional BMP1A (Vasioukhin et al., 1999). At 5 months of age, most control hair follicles were in telogen (not shown). By contrast, skin from K14-Cre-ERT2; Bmpr1a<sup>cl/c</sup> mice and surviving K14-Cre40; Bmpr1a<sup>cl/c</sup> mice contained distorted wavy hair follicles maintained in an abnormal proliferative anagen-like state. In addition, large numbers of benign proliferations that appeared to derive from hair follicles were present, as well as follicular cysts containing dead cells, and, in some cases, massive accumulations of pigment (Fig. 8A,B,D,F, and data not shown). Pigment was extruded to the epidermis via pigmented casts resulting in the appearance of pigment depositions on the skin surface (Fig. 3H,I). Tamoxifen...
Bmpr1a regulates hair and tooth development

Bmpr1a regulates hair and tooth development.

Discussion

We show that Cre-mediated loss of epithelial Bmpr1a results in striking defects in postnatal hair follicle differentiation, as well as in an early arrest of tooth morphogenesis. Our results indicate that BMPR1A is required for differentiation of both the hair follicle IRS and the hair shaft, suggesting that it plays an early and key role in facilitating the differentiation of matrix cell derivatives.

We observed accelerated embryonic hair follicle development in Bmpr1a mutants, consistent with observations that loss of the BMP inhibitor noggin inhibits hair follicle morphogenesis and with data indicating that BMP signals express the epidermal differentiation marker K1, indicating that they did not differentiate towards epidermis (Fig. 8I,K,M,N,P,Q,S,T). The lower portions of the abnormal follicles and the benign follicular epithelial proliferations did not express keratins, except for very low levels of K17 (Fig. 8J,L,M,O,P,R,S,U). The lack of follicular differentiation within these epithelial proliferations, together with the absence of epithelial nuclear β-catenin (data not shown), suggested that these tumors comprised undifferentiated matrix cells.

Follicular tumors comprising such cells are histologically similar to human matricomas rather than pilomatricomas, which typically exhibit prominent hair shaft-like differentiation (Ackerman et al., 2000; Chan et al., 1999). Nuclear phospho-SMADS 1/5/8 were not detected in the dermis surrounding control follicles (not shown), but were present at high levels in the condensed dermis surrounding the proliferations (Fig. 8V), indicating activation of BMP signaling in the dermis. This observation is consistent with previous descriptions of a negative-feedback loop that regulates BMP activity in hair follicles (Botchkarev et al., 2002; Kulessa et al., 2000). A thickened epithelium, unusual expansion of the mesenchyme, and abnormal growth of cartilage and bone were observed under the nails of these mice (Fig. 8W,X), suggesting that loss of BMPR1A from nail epithelium may also induce elevated BMP signaling in the mesenchyme.

treatment, to induce higher levels of activity of Cre-ERT2, caused accelerated formation of follicular cysts (Fig. 8C).

To further characterize the abnormal hair follicles, cysts and benign proliferations, we examined their patterns of BrdU incorporation and keratin gene expression. High levels of proliferation were observed in the outer layer of abnormal follicle epithelium, in cyst walls, and in the outer layer of follicle-derived proliferations (Fig. 8G-I). The upper regions of abnormal follicles and the cysts expressed K14, K17 and K6, characteristic of hair follicle outer root sheaths, but did not...
suppress feather follicle fate (Botchkarev et al., 2002; Jung et al., 1998; Noramly and Morgan, 1998). In contrast to this relatively mild embryonic hair follicle phenotype, tooth morphogenesis was highly abnormal in K14-Cre43; Bmpr1a cl/c mice. A dental lamina was formed, and tooth bud development was initiated; however, at E13.5 these structures were smaller and less developed than in control littermate embryos, and were resorbed by E16.5. These observations are consistent with a previously proposed role for BMP signaling from the mesenchyme to the epithelium that is thought to regulate transition from the bud to the cap stage of tooth development (Jernvall and Thesleff, 2000). Our finding that deletion of Bmpr1a causes severe defects in postnatal differentiation of the hair shaft and IRS is consistent with upregulation of BMP pathway genes during anagen (Botchkarev et al., 2001; Kulessa et al., 2000), and with our observation that nuclear phospho-SMAD accumulates in hair shaft and IRS precursor cells. Similar defects in hair shaft and IRS differentiation resulting from K14-Cre-mediated excision of Bmpr1a were reported by Kobielak et al. while this manuscript was under revision (Kobielak et al., 2003), and transgenic mice expressing the BMP inhibitor noggin under the control of an Msx2 promoter also show defective hair-shaft differentiation (Kulessa et al., 2000). IRS differentiation is relatively normal in Msx2-noggin mice, perhaps due in part to the restricted domain of Msx2 promoter activity and to the fact that noggin downregulates the Msx2 promoter and so is not expressed at high levels.

In Bmpr1a mutant hair follicles, we observe absent or severely decreased expression of the hair-shaft regulatory genes Foxn1, Mxl1 and Mxs2. This suggests that BMPR1A signaling may not activate hair-shaft keratin gene expression directly, but instead by inducing expression of Mxs genes, which in turn activate Foxn1 (Ma et al., 2003). In addition, we find that expression of GATA3, a transcription factor that regulates differentiation of matrix cells into IRS (Kaufman et al., 2003), requires epithelial BMPR1A, a result that was also obtained by Kobielak et al. (Kobielak et al., 2003).

Although hair-shaft keratins and several transcription factors associated with terminal differentiation are strongly downregulated or not expressed in Bmpr1a mutants, Lef1...
expression was maintained in the matrix of mutant follicles. Despite expression of Lef1, nuclear translocation of b-catenin, indicating activation of the WNT pathway, was not observed in the epithelium of the most affected mutant follicles, suggesting that WNT pathway activation lies downstream of epithelial BMPR1A signaling. The recent identification of Fossn1 as a direct WNT target gene (Balcuinaite et al., 2002) suggests that BMPR1A might regulate Fossn1 expression by dual mechanisms: activation of WNT signaling and induction of Mx6 expression.

In wild-type hair follicles, matrix cells cease proliferating as they rise up in the follicle beyond the level of the dermal papilla, and begin to express transcription factors that regulate differentiation of the hair shaft and IRS. In the absence of epithelial Bmpr1a, post-mitotic cells are observed in the upper follicle bulb (Fig. 7F’; H’; Fig. 8G), but these fail to express regulatory factors or to differentiate towards either hair shaft or IRS. Thus Bmpr1a is not required for exit from the highly proliferative matrix compartment, but regulates the next step: differentiation.

Interestingly, the more severely affected epithelial Bmpr1a-deficient hair follicles do not undergo programmed regression, but instead continue to proliferate, eventually forming follicular cysts and matricomas. As nuclear localized phospho-SMAD is dramatically decreased at the anagen-catagen transition, it is unlikely that epithelial BMPR1A plays a direct role in initiating catagen onset. Instead, continued proliferation of mutant follicles may indicate that, in the absence of terminal differentiation, the factors that normally signal the end of anagen are not produced. Alternatively, as we find that BMP signaling is active in the hair follicle bulge during the resting phase, and ectopic noggin induces anagen onset (Botchkarev et al., 2001), it is possible that epithelial BMPR1A signaling normally acts to suppress the proliferation of stem cells. Proliferation of these cells in the absence of epithelial BMPR1A could potentially feed expansion of the matrix, over-riding the signals that would normally induce catagen, and, in the absence of follicular differentiation, leading to the development of matricomas.

We thank Richard Behringer for sharing unpublished data; George Cotsarelis and Ed Morrisey for helpful discussions; Elaine Fuchs for the K14 promoter; Andrew McMahon for the Shh plasmid; Pierre Coulombe for anti-K17; Henry Sun for AE13 and AE15 antibodies; Peter ten Dijke for anti-BMPR1A; Philippe Soriano for the ROSA26R mice; Dorothy Campbell, Annette Sheldon and Mark Chapman for histology; and Jean Richa for transgenic mouse production. This work was supported by NIH grants AR47709 (S.E.M.), DC03917 and NS39159 (E.B.C.).

References


Li, M., Indra, A. K., Warot, X., Brocard, J., Messaddeq, N., Kato, S.,
Research article


