Published in final edited form as:

Genesis. 2012 November ; 50(11): 783–800. doi:10.1002/dvg.22039.

A PAIR OF TRANSMEMBRANE RECEPTORS ESSENTIAL FOR THE RETENTION AND PIGMENTATION OF HAIR

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Abstract

Hair follicles are simple, accessible models for many developmental processes. Here, using mutant mice, we show that Bmpr2, a known receptor for bone morphogenetic proteins (Bmps), and Acvr2a, a known receptor for Bmps and activins, are individually redundant but together essential for multiple follicular traits. When Bmpr2/Acvr2a function is reduced in cutaneous epithelium, hair follicles undergo rapid cycles of hair generation and loss. Alopecia results from a failure to terminate hair development properly, as hair clubs never form, and follicular retraction is slowed. Hair regeneration is rapid due to premature activation of new hair-production programs. Hair shafts differentiate aberrantly due to impaired arrest of medullary-cell proliferation. When Bmpr2/Acvr2a function is reduced in melanocytes, gray hair develops, as melanosomes differentiate but fail to grow, resulting in organelle miniaturization. We conclude that Bmpr2 and Acvr2a normally play cell-type-specific, necessary roles in organelle biogenesis and the shutdown of developmental programs and cell division.

Keywords

anagen; catagen; telogen; terminal differentiation; melanosome; Bmp signaling

INTRODUCTION

Hair follicles are useful models for many aspects of mammalian morphogenesis, as throughout life they proceed through cycles of growth (anagen), regression (catagen), and rest (telogen), resulting in the periodic development of hair, repeated reshaping of follicular structure, and recapitulation of basic morphogenetic processes (recently reviewed in

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Schneider *et al.*, 2009). During anagen, a follicle elongates into the dermis and develops its hair-producing components: the hair bulb, from which the hair shaft and inner root sheath (IRS) grow, and a transient stalk, in which the IRS and hair complete their differentiation. Within the hair bulb, the hair matrix (a vigorously proliferating epithelial cell population) generates the precursor cells of the hair and IRS, and the developing hair shaft acquires its pigmentation from melanocytes, which intermingle with the bulbar epithelial cells and transfer melanin to the precursors of the hair medulla and cortex. Once an anagen follicle reaches full maturity, a hair is grown for a programmed period of time, after which cell proliferation ceases in the hair bulb, and the follicle eliminates its hair-producing components, principally through apoptosis. During this catagen phase, the follicle retracts to approximately one-third its maximum length and concomitantly develops an anchoring club at the base of the hair shaft. Upon finishing its involution, the follicle holds the completed hair in place and becomes comparatively quiescent. In due course, this telogen phase ends and a new anagen is initiated, leading to the formation of a new hair and the repetition of each phase of the cycle.

The transforming growth factor β (TGF β) superfamily is a set of secreted signaling proteins that regulates the development of virtually all mammalian tissues and whose members include the TGF β s, bone morphogenetic proteins (BMPs), and activins/inhibins (reviewed in Schmierer and Hill, 2007). Superfamily members produce most if not all of their effects by binding to two kinds of transmembrane receptors — type I and type II — both of which are serine/threonine kinases. To date, 7 type I receptors, 5 type II receptors, and at least 33 superfamily ligands have been identified in mammals (reviewed in Chang *et al.*, 2002). In the canonical mechanism of superfamily signaling, the formation of ligand-receptor complexes leads the type II receptor to phosphorylate the type I receptor, which then phosphorylates receptor-activated Smad transcription factors, resulting ultimately in Smad regulation of gene expression (reviewed in Schmierer and Hill, 2007). In a variety of circumstances, signals are also transduced by noncanonical mechanisms, which include the Smad-independent activation of mitogen-activated protein kinases, Rho GTPases, or phosphatidylinositol-3-kinase (reviewed in Derynck and Zhang, 2003).

TGF β superfamily signaling plays key roles in various aspects of hair follicle development and cycling (reviewed in Li *et al.*, 2003). Tgf β 2 is required for the induction of hair follicle morphogenesis, whereas Tgf β 1 has an opposing effect and blocks hair follicle formation. Tgf β 1-3 all appear to play essential roles in the induction of catagen in hair follicles. Activin A, which is secreted by dermal-papilla cells, has an inhibitory effect on hair follicle morphogenesis and catagen entry, whereas follistatin, a secreted antagonist to activins and BMPs, is produced by hair follicle epithelial cells and blocks activin's effects (Nakamura *et al.*, 2003). BMPs, the largest subfamily of growth factors in the TGF β superfamily, control hair follicle morphogenesis at many different stages (reviewed in Botchkarev and Sharov, 2004). At the initiation stage, BMPs function as an inhibitory signal, while noggin, a secreted BMP antagonist, neutralizes BMP activity and thereby stimulates the induction of secondary hair follicles (Botchkarev *et al.*, 1999). In developing hair follicles, BMPs promote the differentiation of the inner root sheath and hair shaft (Andl *et al.*, 2004; Kobiela *et al.*, 2003; Ming Kwan *et al.*, 2004; Yuhki *et al.*, 2004) as well as cellular commitment to the sebaceous gland lineage (Guha *et al.*, 2004). During postnatal follicular cycling, Bmp2 and Bmp4 inhibit the induction of anagen and keep follicular stem cells quiescent, thus maintaining telogen (Plikus *et al.*, 2008).

The role of TGF β superfamily signaling in hair pigmentation and melanocyte biology is not well understood. TGF β 1 inhibits growth and melanogenesis of cultured melanocytes and also induces apoptosis of melanocytes in collagen gels (Alanko and Saksela, 2000). Similarly, activin induces growth inhibition and apoptosis in melanocyte cultures (Stove *et*

al., 2004). In quail neural crest cultures, BMP4 prevents the specification of melanoblasts (Jin *et al.*, 2001), the precursors of melanocytes, while BMP2 promotes melanosome differentiation and melanogenesis (Bilodeau *et al.*, 2001). In human melanocyte cultures, BMP4 induces cell division but inhibits melanogenesis (Yaar *et al.*, 2006), while BMP7 inhibits cell proliferation (Hsu *et al.*, 2008).

Numerous murine lines have been generated to either overproduce or delete members of the TGF β superfamily signaling pathway in cutaneous epithelium. Superfamily members whose activity was manipulated by mutation include: the ligands Bmp4 (Blessing *et al.*, 1993), Bmp6 (Blessing *et al.*, 1996), activin A (Nakamura *et al.*, 2003), and Tgf β 1 (Sellheyer *et al.*, 1993); the inhibitors noggin (Botchkarev *et al.*, 1999; Guha *et al.*, 2004; Kulesa *et al.*, 2000; Plikus *et al.*, 2004; Sharov *et al.*, 2005; 2006), follistatin (Nakamura *et al.*, 2003), and Smad7 (He *et al.*, 2002; Klopčic *et al.*, 2007); the type I receptors Bmpr1a (Andl *et al.*, 2004; Kobiela *et al.*, 2003; Ming Kwan *et al.*, 2004; Yuhki *et al.*, 2004) and Acvr1b (Qiu *et al.*, 2011); and the transcription factor Smad 4 (Owens *et al.*, 2008; Qiao *et al.*, 2006; Yang *et al.*, 2005). But to date, no study of type II receptor function in skin has been reported. Four type II receptors — Tgfbr2 (Tgfbeta receptor II), Bmpr2 (Bmp receptor, type II), Acvr2a (activin receptor IIa), and Acvr2b (activin receptor IIb) — have been detected in murine epidermis and hair follicles (He *et al.*, 2002). Tgfbr2 was found in the outer root sheath (ORS), the outermost epithelial cylinder of the hair follicle, while the other three type II receptors were present in both the ORS and matrix cells. In this study, we used knockout mice to analyze the cutaneous functions of two type II receptors — Bmpr2 and Acvr2a. We show that these receptors play overlapping but necessary roles in the differentiation of the hair shaft, development of the hair club, timing and length of each phase of the hair cycle, and generation of pigmentation.

RESULTS

Type II Receptor Deficiency Causes Cyclic Alopecia

Germ line *Bmpr2* knockouts exhibit early embryonic death (Beppu *et al.*, 2000), preventing the study of Bmpr2 function in skin development. We thus employed a conditional knockout approach and ablated Bmpr2 from cutaneous epithelium using a floxed *Bmpr2* allele (*Bmpr2^{fllox}*, Beppu *et al.*, 2005) and *K14-Cre* transgene (Li *et al.*, 2001). The floxed *Bmpr2* allele contains *loxP* sites flanking exons 4 and 5, which encode the receptor's transmembrane domain and part of its kinase domain. Accordingly, Cre-mediated recombination deletes a key portion of *Bmpr2* and concomitantly yields a frameshift in the coding sequence, inactivating the receptor and generating a deletion/frameshift identical to that of the germ line *Bmpr2* knockout. The *K14-Cre* transgene produces Cre in the progenitor cells of the epidermis and hair follicles (Li *et al.*, 2001) and thus should ablate Bmpr2 throughout the cutaneous epithelium.

Surprisingly, *K14-Cre;Bmpr2^{fllox/fllox}* mice were healthy, fertile, and normal to the naked eye. This absence of overt skin defects did not appear to result from a failure to ablate *Bmpr2*, as the transgene-derived Cre rendered the undeleted (floxed) *Bmpr2* allele undetectable in cutaneous epithelial cells, converting the overwhelming majority of *Bmpr2^{fllox}* to the null allele (Fig. 1a shows a representative example of the recombination in skin cells carrying the *K14-Cre* transgene and *Bmpr2^{fllox}* allele). Likewise, *K14-Cre;Bmpr2^{fllox/-}* mice, which carry one copy of a germ line *Bmpr2* null allele (*Bmpr2^{-/-}*; Beppu *et al.*, 2000), were identical to *K14-Cre;Bmpr2^{fllox/fllox}* mice (data not shown), consistent with the results shown in Fig. 1a.

As other type II receptors may compensate for the loss of Bmpr2, we generated type II receptor double mutants in which the conditional *Bmpr2* deficiency was combined with

germ line *Acvr2a* deficiency (the *Acvr2a*⁻ allele; Song *et al.*, 1999). The *Acvr2a*⁻ allele (Fig. S1) contains a deletion in the sequence encoding the receptor's kinase domain. This mutation, when homozygous, decreases the viability of a minority of animals but has not been previously associated with skin abnormalities. *K14-Cre;Bmpr2*^{fllox/fllox};*Acvr2a*^{-/-} mice died around the time of birth with open eyes and a substantial impairment of hair-follicle morphogenesis. Whereas normal skin was already producing coat hair fibers and vibrissa, double-knockout skin was not generating either of these hair types, as coat follicles typically lacked hair bulbs (Fig. 1b–c), and vibrissa follicles, though possessing hair bulbs, were short (exhibiting less downgrowth into the dermis) and generally lacked terminally differentiating structures (such as the inner root sheath and hair shaft; Fig 1d–e) and terminal differentiation markers (such as trichohyalin; data not shown). Thus, the double-knockout hair follicles appeared to be slowed or arrested in their development. Death did not appear to result from a skin defect, such as a barrier defect, as the epidermis was intact and possessed all standard layers.

Given the early lethality of the *Bmpr2*/*Acvr2a* deficiency, we tested the effect of combining a lack of *Bmpr2* (*K14-Cre;Bmpr2*^{fllox/fllox}) with a reduced dose of functional *Acvr2a* (*Acvr2a*^{+/-}). The resulting *K14-Cre;Bmpr2*^{fllox/fllox};*Acvr2a*^{+/-} mice were healthy and fertile but exhibited fully penetrant, cyclical defects in the hair coat. Following birth, a dense coat developed, but a subset of hair fibers appeared thinner than those of controls (mice with *Bmpr2*^{fllox/fllox} or *Bmpr2*^{fllox/fllox};*Acvr2a*^{+/-} genotypes but lacking *K14-Cre*). Most strikingly, starting from approximately postnatal day 14 (P14), *K14-Cre;Bmpr2*^{fllox/fllox};*Acvr2a*^{+/-} mice underwent the total, progressive loss of coat hair. Hair loss began on the face and then proceeded towards the tail so that the mice were completely bald by about three weeks of age (Fig. 1g). Shortly following the loss of all coat hair, a new hair coat formed in full once more, and this coat was quickly lost again after reaching its maximum length. These cycles of hair re-growth and loss were repeated throughout life and thus constituted a new example of 'cyclic alopecia' (Ma *et al.*, 2003), a phenotype previously observed in *Msx2* (Ma *et al.*, 2003) and *Sfn* (*Er*) mutants (Hunsicker, 1960; Herron *et al.*, 2005; Li *et al.*, 2005). Adult *K14-Cre;Bmpr2*^{fllox/fllox};*Acvr2a*^{+/-} mice exhibited hair growth and loss simultaneously, resulting in the appearance of stripes of coat hair and bald skin (Fig. 1h). On these striped adults, the patterns of hair growth resembled the simplified patterns described for *Krt14-Nog* transgenic mice (Plikus *et al.*, 2008), whose hair follicles are grouped into larger, fewer domains of synchronized activity and which produce the Bmp inhibitor noggin from the keratin 14 promoter. Concomitant with their hair defects, the *K14-Cre;Bmpr2*^{fllox/fllox};*Acvr2a*^{+/-} mice displayed excessive nail growth, which became apparent after two months of age (Fig. 1f). Thus, in contrast to the *Bmpr2* and *Acvr2a* single mutants, *Bmpr2* and *Acvr2a* double mutants developed prominent skin and nail abnormalities, with the *K14-Cre;Bmpr2*^{fllox/fllox};*Acvr2a*^{+/-} mice exhibiting a particularly conspicuous oscillation between coat formation and loss. The alopecia appeared to arise at a specific point in the hair cycle, as hair was lost from juvenile mice in a head-to-tail progression, and hair follicles develop and initially cycle in a similar head-to-tail sequence. Likewise, the regionalized hair loss in adult mice was consistent with a defect specific to one hair-cycle point, as adults normally exhibit a more regionalized synchronicity of follicular cycling. The emergence of the alopecia and nail abnormalities in *K14-Cre;Bmpr2*^{fllox/fllox};*Acvr2a*^{+/-} mice suggests that the *Acvr2a*⁻ mutation, though typically recessive, reduces total *Acvr2a* activity when heterozygous. Overall, the results suggest that the *Bmpr2* and *Acvr2a* receptors perform redundant functions but that these functions are essential for the development and maintenance of normal hair and nails.

Type II Receptors and the Hair Cycle

To determine the causes of the hair defects of the *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice, the histology of back skin was examined at different stages of follicular hair production, regression, and quiescence. During the earlier stages of follicular morphogenesis (e.g., P0), the skin of *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice was indistinguishable from that of normal (control) littermates and developed a normal number of hair follicles (data not shown). These hair follicles subsequently reached maturity and produced hair (Fig. 2a–b) but exhibited defects in hair shaft differentiation (see below). In many double-mutant hair follicles, hair production terminated prematurely, and catagen started early, as narrowing, amelanotic hair bulbs (signs of catagen) were observed as early as P15, a time when controls continued to grow hair (Fig. 2c–d). By P16–17, all double mutant hair follicles entered catagen, but their progression through this phase of the hair cycle was slow. Whereas normal hair follicles began catagen at P16–18 and completed their involution in two days, double-mutant hair follicles were still regressing at P20 (Fig. 2e–f), as they possessed extended epithelial strands (the involuting remnants of the outer root sheath and hair bulb). These slowly regressing epithelial strands often appeared abnormally curled (Fig. 2f), and concomitant with the impaired follicular involution, hair shafts disappeared from the skin. At this time point, normal skin, in contrast, exhibited firmly anchored hair shafts in fully retracted follicles (Fig. 2e). Thus, the alopecia of the *Bmpr2/Acvr2a* double mutants resulted from a disruption of catagen, showing that *Bmpr2/Acvr2a* function is necessary for proper catagen timing and progression.

Upon completing regression, the double-mutant hair follicles quickly initiated anagen, and following a brief telogen, normal follicles did the same, leading to the production of new hair shafts in control and *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice (Fig. 3a–b). This anagen concomitantly caused the double-mutant epidermis to become thicker than that of control animals (Fig. 3a–b, insets). Similar to the first period of hair production, this second period of hair production was shorter in double mutants than in controls (Fig. 3c–d), as the visibly-detectable component of this anagen averaged 10.7 days (± 0.7 ; $n = 9$) in *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice versus 12.4 days (± 0.9 ; $n = 9$) in normal mice. Thus, the decrease in *Bmpr2/Acvr2a* function shifted the anagen/catagen transition, leading to the premature end of anagen and induction of catagen.

Coat follicles normally exhibit a long second telogen, and while subsequent telogens vary in length, they are substantially longer than other phases of the hair cycle (Plikus *et al.*, 2008 and references therein). In contrast, the coat follicles of *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice always displayed short telogens, as new hair was produced soon after the preceding hair fibers were lost. For example, at P50 the coat follicles of normal mice were still in the second telogen, while those of *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice had left this telogen and uniformly re-entered anagen (Fig. 3e–f). Thus, the reduction of *Bmpr2/Acvr2a* function caused hair follicles to enter anagen precociously, which in turn led to a greater number of hair cycles.

Type II Receptors and the Development of the Hair Shaft and Club

As *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice always generate a grossly abnormal hair coat, the histology of their back skin was examined during hair production. The double mutants possessed all of the follicular compartments found in normal controls (including sebaceous glands with no obvious defects) but generated abnormal medullas (Fig. 4a–b), the central and typically thickest cylinder of murine hair shafts. Whereas medullary cells normally form one or more well-organized columns and arrange their pigment into a ladder-like pattern, the medullary cells of the double mutants appeared disorganized, and their ladder-like pattern of pigmentation was lost (Fig. 4a–b). In *K14-Cre;Bmpr2^{flox/flox}* mice, the hair medulla

occasionally appeared disorganized too, though these defects were less severe than those of the *Bmpr2*/*Acvr2a* double mutants (Fig. 4c). To assess the mutations' effects on cell differentiation, skin sections were stained by immunofluorescence for trichohyalin, a marker of the hair medulla and IRS (Fietz *et al.*, 1993). In the IRS, trichohyalin appeared unaffected by the *Bmpr2* and *Acvr2a* mutations (Fig. 4d–f), suggesting that IRS cells differentiated appropriately in the single (*K14-Cre;Bmpr2^{flox/flox}*) and double (*K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}*) mutants. In contrast, medullary trichohyalin was clearly reduced in the *K14-Cre;Bmpr2^{flox/flox}* mice and greatly diminished in the *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice (Fig. 4d–f), suggesting that medullary cells fail to differentiate properly in both mutants. As the abnormalities in trichohyalin levels and medulla structure were more severe in the double mutant than in the single mutant, the results suggest that *Bmpr2* and *Acvr2a* make additive or synergistic contributions to the differentiation of the hair medulla.

To assess the effects of *Bmpr2* and *Acvr2a* on hair type and other macrostructural features of the hair shaft, hair was collected from the backs of control and receptor-mutant mice with a forceps. Hair fibers were harvested at P20, at which point the *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice were in late catagen and had lost the coat hair from the anterior halves of their bodies but still possessed hair on the posterior halves. Murine hair coats normally contain four hair types — guard, awl, auchene, and zigzag — which differ in length, shape (kinks), or the number of columns of medullary cells. In particular, guard hairs are greatest in length, auchene and zigzag hairs possess kinks, and zigzag hairs have single (rather than multiple) columns of medullary cells. These four hair types were recognizable in the coats of *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice, as the hairs exhibited defining differences in relative length and number of kinks (Fig. 5a–b). Nonetheless, the double-mutant hairs displayed several macrostructural abnormalities. In double-mutant hair coats, the nontylotrich hairs, i.e., the awl, auchene, and zigzag hairs (which develop later than guard hairs), were substantially shorter than normal (Fig. 5a–b), consistent with their skins' premature entry into catagen, and the normally straightest hair types — guard and awl — were curved instead (Fig. 5a–b). Additionally, the double-mutant awl and auchene hairs lacked multiple columns of medullary cells and possessed instead a single column (Fig. 5c–d). As such, these awl/auchene hairs had zigzag-like medullas and widths equivalent to normal zigzag fibers, making them substantially thinner than normal awl/auchene hairs (which together normally comprise ~25% of the coat; Sundberg and Hogan, 1994). Consistent with the similar widths of nontylotrich hair shafts, the nontylotrich hair follicles exhibited no differences in the size of the dermal papillae or other follicular structures. In all double-mutant hair types, the medullary cells failed to form well-defined ladders of pigment bands, as the medullary pigment appeared amorphously clumped. The hair fibers of *Bmpr2* single mutants (*K14-Cre;Bmpr2^{flox/flox}* mice) at times displayed a similar clumping and disorganization of pigment in regions of the hair medulla (Fig. 5e, which shows a zigzag hair). Thus, *Bmpr2* and *Acvr2a* are necessary for the development of hair shafts of proper length and structure.

The final component of the hair to develop is the club, which forms from keratinized epithelial cells, envelops the base of the shaft, and interdigitates with the surrounding outer root sheath to hold the hair in place. Whereas control mice generated well-formed clubs, no club structures were observed in hair from *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice (Fig. 5f–g). Instead, the double-mutant hair shafts possessed ends of variable length that were tapered, thin, and wavy. Identical observations were made when hair was collected from the double mutants with a comb rather than a forceps. Gentle combing easily dislodged double-mutant hair during late catagen, and these comb-collected hair fibers had the same tapered ends as those gathered with a forceps. Thus, hair shafts failed to acquire hair clubs in the *Bmpr2*/*Acvr2a* double mutants, showing that *Bmpr2*/*Acvr2a* function is essential for club

differentiation and/or stability. As the club is necessary for the retention of a completed hair, the absence of the club is presumably the principal cause of the total hair loss of the double mutants.

Type II Receptors and Follicular Epithelial Cell Proliferation

As catagen includes the cessation of cell proliferation and the involution of the hair follicle via apoptosis, we examined the *Bmpr2*/*Acvr2a* mutants for defects in these processes. TUNEL assays did not reveal any clear differences between control and *K14-Cre;Bmpr2^{fllox/flox};Acvr2a^{+/-}* skin in apoptosis, as similar patterns of cell death were detected during catagen (data not shown). Thus, the *Bmpr2* and *Acvr2a* mutations did not appear to disrupt the distribution of apoptotic cells.

To measure cell proliferation in the skin, BrdU labeling was performed at different ages. During periods of hair production, *K14-Cre;Bmpr2^{fllox/flox};Acvr2a^{+/-}* mice exhibited normal cell proliferation in most compartments of the skin, as multiplying cells were abundant in the hair matrix and scattered in the outer root sheath and epidermis (Fig. 6a–b and data not shown). However, the double mutants also possessed a compartment with aberrant cell division, the hair medulla, as columns of proliferating epithelial cells rose from the hair matrix along the central axis of the hair shaft, one-third or more of the distance to the skin's surface (Fig. 6b). Normally, this region of the hair is comprised exclusively of cells undergoing terminal differentiation and lacking the ability to divide. In some hair follicles of *Bmpr2* single mutants (i.e., *K14-Cre;Bmpr2^{fllox/flox}* mice), the differentiating medulla contained proliferating cells as well (Fig. 6c), though such cells were fewer in number than those of the double mutants. Thus, a lack of type II receptor function led to a failure of pre-medullary cells to withdraw from the cell cycle. During catagen, *K14-Cre;Bmpr2^{fllox/flox};Acvr2a^{+/-}* skin did not exhibit cell proliferation in an abnormal location but instead displayed an increase in proliferating cells in the 'permanent' segment of the ORS (the superficial ORS segment that survives catagen; Fig. 6d–e). Likewise, at the onset of anagen, *K14-Cre;Bmpr2^{fllox/flox};Acvr2a^{+/-}* mice exhibited substantially more proliferating cells in the epidermis than controls (Fig. 6f–g), consistent with the thick epidermis observed at this time in the double mutant (Fig. 3b). Thus, a lack of type II receptor function led to localized increases in cell proliferation, suggesting that *Bmpr2* and *Acvr2a* normally promote the cessation of growth. This growth-inhibitory function appears necessary for the differentiation of the hair medulla.

Type II Receptor Inactivation Causes Gray Hair

BMPR2 (Yaar *et al.*, 2006) and *ACVR2A* (Stove *et al.*, 2004) are expressed in human melanocytes, and this expression is conserved in murine melanocytes, as these cells possessed the *Bmpr2* and *Acvr2a* transcripts and proteins (Fig. 7a). To determine the roles of type II receptors in pigment cells, mice were generated in which *Bmpr2* was specifically ablated from the melanocyte lineage and *Acvr2a* was rendered wild-type (*Acvr2a^{+/-}*), reduced in dosage (*Acvr2a^{+/-}*), or functionally lacking (*Acvr2a^{-/-}*). To delete *Bmpr2* from melanocytes, the *Bmpr2^{fllox}* allele was converted to a null allele using *Tyr::Cre* (Fig. 7b), an X-linked transgene that produces Cre from the promoter for the melanogenic enzyme tyrosinase (Delmas *et al.*, 2003).

Mice exhibited normal coat color as long as the melanocyte lineage possessed at least one fully functional allele of either *Bmpr2* or *Acvr2a*. But when their genotype was *Tyr::Cre;Bmpr2^{fllox/flox};Acvr2a^{-/-}*, a dramatic loss of pigmentation was observed, as gray hair developed during the first period of hair production. Male pups developed uniformly gray coats (Fig. 7c), while female pups, which were hemizygous for *Tyr::Cre*, produced gray hair mosaically (not shown), consistent with the mosaic inactivation of the *Tyr::Cre*-carrying

X chromosome. Thus, *Bmpr2* and *Acvr2a* are redundant individually but together essential for the development of pigmented hair.

To determine the status of melanocytes in the gray-haired mice, skin sections were stained using tyramide-based tyrosinase assay (TTA; Han *et al.*, 2002), a sensitive technique that detects active tyrosinase *in situ* and thereby identifies pigment cells, including melanoblasts and other relatively undifferentiated melanocyte progenitors. As shown in Fig. 7d–e, uniformly gray-haired mice exhibited the same TTA staining pattern as their normally pigmented littermates, suggesting that melanocytes were normal in location and approximately normal in number in gray-haired animals. Immunofluorescent staining for the melanocyte markers *Tyrp1* and *Mitf* was likewise similar in normal and gray-haired skin samples, again consistent with a normal distribution of melanocytes in gray-haired skin (data not shown). Despite this normal melanocyte distribution but consistent with the hair coat's hypopigmentation, gray-haired skin displayed substantially less melanin in the hair follicles than normal skin (Fig. 7f–g, which shows the same hair follicles as Fig. 7d–e). The decrease in melanin was evident in the hair bulb, where melanocytes form a conical cluster, as well as in the hair shaft. Thus, the lack of pigmentation did not result from a lack of melanocytes but from a failure of melanocytes to produce or accumulate melanin.

To identify the defects underlying this melanin deficiency, skin from normally pigmented and gray-haired mice was examined by transmission electron microscopy. The melanin deficiency was thus traced to an abnormality in the development of melanosomes, the melanocyte organelles responsible for melanin synthesis. As shown in Fig. 7h–i, the melanocytes of gray-haired skin produced 'miniaturized' melanosomes, i.e., melanosomes substantially smaller in size than those developed by normal melanocytes. This reduction in melanosome size was not accompanied by a substantial reduction in melanosome number or pigment-making ability, as the miniaturized melanosomes were numerous and filled with pigment, appearing about as heavily melanized as normal melanosomes. Thus, in gray-haired skin, the principal melanogenic defect appeared to be an impairment of melanosome growth, leading to the development of small melanosomes and the production/accumulation of less melanin per melanosome. Like the melanosomes of normal skin, the miniaturized melanosomes were transferred to epithelial cells of the hair shaft (Fig. 7j–k); but given each melanosome's reduced melanin content, the hair shafts received substantially less pigment in total, resulting in their hypopigmentation and gray color. Thus, the lack of *Bmpr2* and *Acvr2a* led to a failure of melanosomes to grow to their normal size, which in turn resulted in the production of small quantities of melanin. These results show that *Bmpr2* and *Acvr2a* are necessary for proper melanosome biogenesis.

DISCUSSION

Previous studies performed *in vitro* have suggested that the Bmp type II receptor binds Bmps exclusively whereas the activin type II receptors bind activins and Bmps (Attisano *et al.*, 1992; Ebisawa *et al.*, 1999; Hoodless *et al.*, 1996; Liu *et al.*, 1995; Mathews and Vale, 1991; Mathews *et al.*, 1992; Nishitoh *et al.* 1996; Nohno *et al.*, 1995; Rosenzweig *et al.*, 1995; Yamashita *et al.*, 1995). Similarly, studies performed in cell culture have shown that *Bmpr2* and *Acvr2a* can transduce Bmp signals and that each receptor can partially compensate for the loss of the other (Lavery *et al.*, 2008; Yu *et al.*, 2005). Our results extend these findings, as the present study demonstrates for the first time that *Bmpr2* and *Acvr2a* perform compensatory functions in animals and that this functional overlap occurs in multiple cell lineages, including epithelial and pigment cell lineages. In cutaneous epithelial cells, the effects of *Bmpr2* and *Acvr2a* appear largely additive, as the defects of *Bmpr2*-deficient epithelia become progressively more severe as *Acvr2a* decreases (i.e., as genotypes change from *Acvr2a*^{+/+} to *Acvr2a*^{+/-} to *Acvr2a*^{-/-}). In contrast, melanocytes generate a

normal coat color until they are deficient in both *Bmpr2* and *Acvr2a*, at which point they exhibit substantial defects in melanosome development and largely fail to pigment the coat. Thus, type II receptor function is necessary for proper epithelial and melanocytic morphogenesis, and the precise level of receptor function required — and the threshold separating normal from abnormal development — appears to be cell-type or trait specific.

Based on several lines of evidence, we think it likely that the skin defects of the *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice result primarily from a lack of Bmp signaling. Firstly, some of these defects, such as those of the hair medulla, are observed in milder form in *K14-Cre;Bmpr2^{flox/flox}* mice, whose principal signaling abnormality is presumably a reduction in Bmp signaling. Secondly, *K14-Cre;Bmpr2^{flox/+};Acvr2a^{-/-}* mice (generated by us in this study) do not display any of the defects found in *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice, such as the abnormalities in follicular differentiation, cycling, club formation, or cell proliferation (data not shown). Thus, these processes seem to require the signaling of ligands that primarily use *Bmpr2*. Finally, when the Bmp type I receptor *Bmpr1a* is ablated from murine cutaneous epithelium, hair shafts fail to differentiate (Andl *et al.*, 2004; Kobiak *et al.*, 2003; Ming Kwan *et al.*, 2004; Yuhki *et al.*, 2004), a phenotype more severe than but analogous to the failure of the hair medulla to differentiate in the *Bmpr2/Acvr2a* double mutants. Thus, the phenotypes of the *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice appear more closely associated with the impairment of Bmp function than activin function. If the phenotypes of the *Tyr-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice have a similar basis, then the defects in melanosome biogenesis also result primarily from a loss of Bmp signaling, though other explanations cannot be ruled out, such as synergistic effects of losses in activin and Bmp signaling.

Previous studies have suggested a key role for the Bmp pathway in the initiation of hair growth, as Bmp signaling was found to prevent the activation of stem cells in quiescent follicles (Kobiak *et al.*, 2007; Plikus *et al.*, 2008; Zhang *et al.*, 2006) and hence to prevent the start of new anagens (Plikus *et al.*, 2008). Our results support these findings. The *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice display shortened telogens and premature re-entry into anagen, showing that *Bmpr2* and *Acvr2a* are necessary for the proper timing of telogen-to-anagen transitions. As the effects of *Bmpr2/Acvr2a* deficiency on anagen entry closely resemble the effects of the *Krt14-Nog* transgene (Plikus *et al.*, 2004; Plikus *et al.*, 2008), the defects of the type II receptor mutant appear to result from a lack of Bmp signaling. Likewise, Bmp signaling has been implicated in other aspects of hair-cycle regulation. In particular, a deficiency of *Bmpr1a* in cutaneous epithelial cells abolished the hair cycle, as follicular progenitor cells exhibited sustained proliferation, ultimately forming cysts and tumors (Andl *et al.*, 2004; Kobiak *et al.*, 2003; Ming Kwan *et al.*, 2004; Yuhki *et al.*, 2004; Zhang *et al.*, 2006). Additionally, mice carrying an *NSE-Noggin* transgene, which produces noggin in hair matrix cells, exhibited accelerated follicular cycling, due primarily to premature exit from anagen (Guha *et al.*, 2004). As shown in the present study, the *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice exhibit a shortened anagen, premature entry into catagen, and slowed progression through catagen, resulting in delayed entry into telogen. As such, *Bmpr2* and *Acvr2a* function are necessary for the correct duration of anagen and catagen as well as telogen. In particular, as *Bmpr2/Acvr2a* deficiency accelerates entry into anagen and catagen, Bmp signaling seemingly antagonizes or inhibits inducers of both of these hair-cycle phases. In all, taking our study together with others, Bmp signaling appears to be a key determinant of the timing and time-span of every phase of the hair cycle and to regulate the transition from each phase to the next.

The type II receptor mutants of this study display defects during anagen that are similar in kind, but different in extent, to the anagen defects of other Bmp pathway mutants. As shown here, the ablation of *Bmpr2* alone in epithelial cells (*K14-Cre;Bmpr2^{flox/flox}* mice) affects

the differentiation of one follicular cell lineage, namely, the medullary cell lineage, and this lineage exhibits more severe defects when the *Bmpr2* ablation is coupled to a reduction in *Acvr2a* (*K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice). In comparison, *Msx2-Noggin* transgenic mice, which produce the Bmp inhibitor noggin from a promoter active in hair-shaft precursor cells, lack all three differentiated lineages of the hair (the medullary, cortical, and cuticular lineages; Kulesa *et al.*, 2000). The epithelial ablation of *Bmpr1a* blocks the differentiation of six follicular lineages, namely, the three lineages of the hair shaft and three lineages of the inner root sheath (Henle's layer, Huxley's layer, and cuticle) (Andl *et al.*, 2004; Kobiela *et al.*, 2003; Ming Kwan *et al.*, 2004; Yuhki *et al.*, 2004).

One explanation for these differing phenotypes involves threshold effects: that is, the various lineages require different threshold levels of Bmp signaling to differentiate and the differing phenotypes reflect the differing extents to which this signaling is inhibited. A second explanation involves the complexity and likely specialization of Bmp signal transducers. Mammals possess at least 10 different Bmps (reviewed in Schmierer and Hill, 2007), and their signals can be transduced by 3 different type II receptors (*Bmpr2*, *Acvr2a*, and *Acvr2b*) and 3 different type I receptors (*Bmpr1a*, *Bmpr1b*, and *Acvr1a*) (Ebisawa *et al.*, 1999; Fujii *et al.*, 1999; Koenig *et al.*, 1994; Lavery *et al.*, 2008; Liu *et al.*, 1995; Macias-Silva *et al.*, 1998; Nishitoh *et al.*, 1996; Nohno *et al.*, 1995; Rosenzweig *et al.*, 1995; ten Dijke *et al.*, 1994; Yamashita *et al.*, 1995; Yu *et al.*, 2005). These 6 receptors in turn can interact in a variety of combinations and form a variety of signaling complexes, as for example *Acvr2a* forms signaling complexes with all 3 Bmp-binding type I receptors (Lavery *et al.*, 2008; Liu *et al.*, 1995; Nishitoh *et al.*, 1996; Yamashita *et al.*, 1995; Yu *et al.*, 2005). Moreover, each combination of receptors appears to bind a particular subset of Bmps preferentially (Ebisawa *et al.*, 1999; Lavery *et al.*, 2008; Macias-Silva *et al.*, 1998; Nishitoh *et al.*, 1996; Nohno *et al.*, 1995; ten Dijke *et al.*, 1994; Yu *et al.*, 2005) and, upon binding these ligands, potentially activates a distinct set or subset of Bmp responses (Fujii *et al.*, 1999; Piscione *et al.*, 1997). As such, the differing phenotypes above may reflect differing downstream targets or outputs of the various receptors. In particular, a decrease in *Bmpr2*/*Acvr2a* function, rather than yield a general deficit in Bmp signaling, may instead produce a specific defect, such as an abnormality in the activity of a particular Smad or other (Smad-independent) factor. This specific defect may then specifically affect the differentiation of the hair medulla. Consistent with this idea, we note that, as judged by immunofluorescent staining, the *K14-Cre;Bmpr2^{flox/flox}* and *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice did not exhibit any obvious differences with wild-type mice in the total (combined) cutaneous levels of phosphorylated Bmp-effector Smads (Smad1/5/8; data not shown), which mediate Bmp effects in the canonical signaling mechanism. As no general phospho-Smad deficit was detected (despite the robust phenotypes of the *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice), *Bmpr2* and *Acvr2a* may preferentially promote a subset of Bmp responses that are especially critical for the differentiation of the hair medulla. As an additional explanation, consistent with the previous two, the differing phenotypes may reflect differing functional redundancies, as *Bmpr2* is clearly redundant with *Acvr2a* (and perhaps with other type II receptors), while *Bmpr1a* is plainly less redundant, at least in cutaneous epithelial cells.

While failing to generate proper hair medullas, the *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice also fail to produce hair clubs, and this absence presumably results from an impairment of club differentiation (similar to the impairment of medulla differentiation) and/or the abnormal activation of degradative processes, such as those thought to promote hair shedding (exogen; Milner *et al.*, 2002). In conjunction with their differentiation defects, the *K14-Cre;Bmpr2^{flox/flox}* and *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice display ectopic cell proliferation in the hair medulla, suggesting that the aberrant differentiation of medullary cells is caused by a cell cycle abnormality. Analogous ectopic cell proliferation is observed in *Msx2-Noggin* mice at the central follicular locations where hair shafts should form

(Kulesa *et al.*, 2000). Thus, as the lack of *Bmpr2*/*Acvr2a* and a gain of *noggin* yield similar effects on cell division, the *Bmp* pathway appears to play an essential role in the inhibition of cell proliferation during hair shaft differentiation.

The *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice exhibit cyclic alopecia (Ma *et al.*, 2003), as each anagen produces dense coat hair, and each catagen brings the total loss of this hair. This type of alopecia has been observed in two other murine mutants: *Msx2* null mutants (*Msx2^{-/-}*), which like the *Bmpr2*/*Acvr2a* double mutants exhibit hair loss combined with premature entry into and slow progression through catagen (Ma *et al.*, 2003), and repeated epilation mutants (*Sfn^{Er/+}*), which are heterozygous for a frameshift mutation in stratifin (14-3-3 σ ; Hunsicker, 1960; Herron *et al.*, 2005; Li *et al.*, 2005) and like the *Bmpr2*/*Acvr2a* double mutants lose hair in part due to defects in hair club formation (Xin *et al.*, 2010). Additionally, loss-of-function mutations in *Hr* (hairless) lead to the total loss of a dense coat as well as excessive nail growth (similar to the nail phenotype observed in the *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice), and while the lost hair does not re-grow, the alopecia results from multiple defects in catagen (reviewed in Panteleyev *et al.*, 1999). Based on immunofluorescent staining, the *Msx2*, *Sfn*, and *Hr* proteins do not exhibit substantial changes in level or localization in the *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice (data not shown), suggesting that other changes (in these or other factors) are responsible for the type II receptor phenotypes. Nonetheless, the similar phenotypes of the *Sfn*, *Msx2*, *Hr*, and *Bmpr2*/*Acvr2a* mutants suggest that these proteins are linked or synergistic regulators of the various events of catagen. One major event of catagen — the shortening of the hair follicle — is slowed in *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* mice, yet the pattern of apoptosis, the process that produces the shortening, appears grossly normal and appropriately matched to follicular morphology, as judged by TUNEL staining. Potentially, the slowed shortening is explained by the increased cell proliferation observed in the follicle's permanent segment during catagen (Fig. 6e): that is, excessive growth in the permanent segment drives cells 'downward' into the transient segment, thus replenishing the transient segment and slowing its regression. An alternative possibility is that the length of the cell death program itself increased: i.e., individual follicular cells take longer to die, or to be eliminated after they die, and the end result is slower regression. In all, the results suggest that *Bmpr2*/*Acvr2a* function is not essential for the induction of apoptosis but is important for other processes that affect the rate of follicular regression.

While necessary for the retention of hair, *Bmpr2* and *Acvr2a* are also essential for the pigmentation of hair, as a gray coat color results from a deficiency of these receptors in melanocytes. This loss of pigmentation stems primarily from a miniaturization of the melanosomes, as the melanosomes fill with pigment but are substantially smaller than normal, leading to the accumulation of less melanin per organelle. This result suggests that melanosome growth is a process separable from melanosome differentiation (the acquisition of pigment-making capacity) and that signaling by *Bmps*, and perhaps *activins*, promotes melanosome growth. Overall, as miniaturized melanosomes result from *Bmpr2*/*Acvr2a* deficiency, the *Bmpr2* and *Acvr2a* receptors are key determinants of melanosome size, providing insight into how cells regulate the development and dimensions of their organelles. As such, the epithelial and melanocytic phenotypes reported here highlight the different uses to which different cells apply the *Bmp* pathway.

METHODS

Mice

All alleles and transgenes used in this study — *Bmpr2^{flox}* (Beppu *et al.*, 2005), *Bmpr2⁻* (Beppu *et al.*, 2000), *Acvr2a⁻* (Song *et al.*, 1999), *K14-Cre* (Li *et al.*, 2001), and *Tyr::Cre*

(Delmas *et al.*, 2003) — were described previously. All animal studies were conducted under IACUC-approved protocols.

Detection of *Bmpr2* ablation and *Acvr2a* alleles

To detect the *K14-Cre*-driven conversion of the *Bmpr2^{fllox}* allele to a null allele, tail skins of one-month-old mice were heated for 1 min. in phosphate-buffered saline (PBS) at 60°C and transferred immediately to cold PBS (4°C). The epidermis was then scraped off the dermis with a scalpel. DNA was prepared separately from the epidermal and dermal compartments by heating samples in 50 mM NaOH (600 µl) at 95°C for 30 min., neutralizing with 1 M Tris-HCl, pH 8.0 (50 µl), and centrifuging for 5 min. at room temperature. The extracted DNA was then used for PCR analysis. Primers A and 6R were used to amplify a fragment of *Bmpr2* floxed allele before Cre-mediated deletion; primers A and C were used to amplify a fragment of *Bmpr2* sequence only after Cre-mediated deletion. The sequences of primers were: primer A, 5'-CAC ACC AGC CTT ATA CTC TAG ATA C-3'; primer C, 5'-TTA TTG TAA GTA CAC TGT TGC TGT C-3'; primer 6R, 5'-CAC ATA TCT GTT ATG AAA CTT GAG-3' (Beppu *et al.*, 2005).

To perform routine genotyping and to detect the *Tyr::Cre*-driven conversion of the *Bmpr2^{fllox}* allele to a null allele, tail DNA was probed by PCR; the conversion to the null allele was assayed using primers A and C above. To detect the *Acvr2a⁻* allele, the following primers were used: forward, 5'-TCA GGA CAT AGC GTT GGC TAC C-3'; reverse, 5'-GCT GAA GTA AGA GGA ACC TGC TC-3'. The forward primer recognizes a sequence within the *neo^R* insertion of *Acvr2a⁻*, while the reverse primer recognizes a sequence downstream from the insertion; the resulting product thus spans the downstream insertion site. To detect the *Acvr2a⁺* allele by PCR, the following primers were used: forward, 5'-GAA TTG GCT TCT CGT TGC ACT G-3'; reverse, 5'-CTC CAC CTA CAC CCT ACT TGA G-3'. These primers amplify a region that is deleted in the *Acvr2a⁻* allele (i.e., parts of exon 9 and intron 9). To detect the Cre transgenes, the following primers were used: forward, 5'-GGT CGA TGC AAC GAG TGA TGA GGT-3'; reverse, 5'-CAG CAT TGC TGT CAC TTG GTC GTG-3'.

Histological Analyses, Immunofluorescence, Tyrosinase Assays, Hair Fiber Analyses, Anagen Measurement, and Electron Microscopy

Back skin was removed from mice and either embedded in OCT and flash-frozen or fixed in 10% buffered formalin (Fisher Scientific) and embedded in paraffin. Frozen skin sections were fixed in 1:1 methanol:acetone at -20°C immediately after adherence to glass slides. For histological analyses, sections were stained with hematoxylin and eosin.

Immunofluorescent analyses were carried out as described (Weiner and Green, 1998) with little modification. Frozen sections were blocked in SuperBlock T20 PBS blocking buffer (Thermo Scientific), incubated sequentially with rabbit polyclonal anti-trichohyalin antibody (1:3000; the gift of Dr. George E. Rogers, University of Adelaide), goat-anti-rabbit IgG (1:2000; Santa Cruz Biotechnology), and streptavidin-CY3 conjugates (1:700, Sigma-Aldrich, Inc.), and counterstained with Hoechst dye 33258 (Fluka).

Tyramide-based tyrosinase assay (TTA) was performed as described (Han *et al.*, 2002) on frozen sections.

Hair fibers were collected from back of mice with a forceps or comb and then mounted on glass slides with Cytoseal XYL mounting medium (Richard-Allan Scientific).

The length of anagen was evaluated by monitoring skin pigmentation according to the method of Plikus *et al.*, 2008. To ensure the comparison of similar follicle types and body

sites, measurements were made exclusively on skin located in the anterior dorsal region of the body, between the front shoulders and on or near the body's midline (i.e., the 'upper back').

For electron microscopy, back skin was removed from mice, fixed in 4% methanol-free formaldehyde, and embedded in epon epoxy. Ultrathin sections were viewed in a transmission electron microscope.

Cell Proliferation Assays

5-bromo-2'-deoxyuridine (BrdU; Invitrogen) was diluted in PBS to 10 mg/ml and sterile filtered. BrdU solution was then injected intraperitoneally into mice at 100 µg/g body weight. After two hours, mice were sacrificed, and skin was removed from the back. Skin samples were then embedded in OCT compound, flash-frozen, sectioned, and fixed in methanol immediately after adherence to glass slides. To detect incorporated BrdU, sections were re-hydrated with PBS, denatured by treatment with freshly made 2N HCl for 30 min at room temperature, and then neutralized by three washes (5 min. each) with 0.1M sodium borate, pH 8.5. Sections were next washed three times with PBS, incubated with Alexa Fluor 488-conjugated anti-BrdU (murine monoclonal PRB-1, diluted 1:20–1:50; Invitrogen) for 2 hours at room temperature, washed three times with PBS, and counterstained with Hoechst 33258 dye.

Melanocyte Cultures

Primary melanocytes were cultured from the epidermis of newborn wild-type mice as described (Halaban *et al.*, 1998). Melanocytes were grown in Ham's F10 medium (Invitrogen) supplemented with 7% horse serum (Hyclone), 7% fetal bovine serum (Hyclone), 50 mM dbcAMP (N6, 2'-dibutyryl adenosine 3':5'-cyclic monophosphate; Sigma), 1 mM Na₃VO₄, 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen).

mRNA and Protein Analyses

Bmpr2 and *Acvr2a* mRNA were detected by RT-PCR in primary melanocytes. Total RNA was isolated using the RNeasy Mini Kit (Qiagen), and reverse transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). cDNA and primers were added to GoTaq Green master Mix (Promega) for a 40-cycle PCR reaction, and the products were run on 2% agarose gels. For *Bmpr2*, the primers were 5'-CAT CAA AGC CCA GAA GAG CAC AG-3' and 5'-CAC CTG ATC CTG ATT TGC CAT C-3', generating an expected product 120 bp. For *Acvr2a*, the primers were 5'-TCC AAA GGG ACG CAT TTC TGA G-3' and 5'-GAT GCT GGC CAA TTT CTT CCT C-3', generating an expected product 138 bp. Both primer sets spanned introns and thus specifically amplified cDNA.

Bmpr2 and *Acvr2a* proteins were analyzed in primary melanocytes by immunoblotting. Cell lysates were generated using 2% SDS, 50 mM Tris-HCl, pH 6.8, separated on 7.5% SDS-polyacrylamide gels, and transferred to Immobilon membranes (Millipore). Blots were blocked with 5% milk/TBST (Tris-buffered saline, 0.1% Tween-20) and probed under the same conditions with murine monoclonal antibodies to *Bmpr2* (1:500; BD Biosciences) or goat polyclonal antibodies to *Acvr2a* (N-17, 1:250; Santa Cruz Biotechnology). Primary antibody reactions were detected using horseradish peroxidase (HRP)-conjugated anti-mouse antibodies (for the antibodies to *Bmpr2*; GE Healthcare) and HRP-conjugated donkey anti-goat IgG (for the antibodies to *Acvr2a*; Santa Cruz Biotechnology). Antibody reactions were visualized using the SuperSignal West Femto Maximum Sensitivity Substrate (for *Bmpr2* immunoblots; Thermo Scientific) or the Western Lightning Chemiluminescence Reagent Plus (for *Acvr2a* immunoblots; PerkinElmer).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank George E. Rogers for antibodies to trichohyalin, Yimin Ruan for technical assistance, Pierre Chambon for *K14-Cre* mice, and Mary McKee and Wei Quan for assistance with electron microscopy. This work was supported by grants from NIH/NIAMS to J.L.B. (AR045284 and AR055218), NIH/NICHD to J.L.B. and E.L. (HD035286), and the Cutaneous Biology Research Center via the Massachusetts General Hospital/Shiseido Co. Ltd. Agreement to J.L.B. L.L. was supported by the Ligue Nationale Contre le Cancer (Equipe labellisée).

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Bmpr2/Acrv2a double mutants exhibit defects in hair development and retention. **(a)** DNA was isolated from the epidermal (ep) or dermal (de) compartments of *K14-Cre;Bmpr2^{fllox/flox};Acrv2a^{+/-}* skin and assayed by PCR for the *Bmpr2* floxed allele (no Cre-mediated recombination) and *Bmpr2* deletion allele (generated by Cre-mediated recombination). Representative PCR products are shown on an agarose gel. The *K14-Cre* transgene yields the efficient ablation of *Bmpr2*, as the floxed alleles are replaced by deletion (null) alleles in cutaneous epithelial cells. **(b–e)** Skin sections of newborn control **(b, d)** and *K14-Cre;Bmpr2^{fllox/flox};Acrv2a^{-/-}* mice **(c, e)** are shown after staining with

hematoxylin and eosin (H & E). Development of coat (c) and vibrissa (e) follicles is inhibited in the double mutant. HF, hair follicle; HP, hair peg; VF, vibrissa follicle. Scale bar for panels b–c, 50 μ m; d–e, 100 μ m. (f) Hind feet of 6-month-old normal (left) and *Bmpr2*/*Acvr2a*-deficient (right) mice are shown. Genotypes are indicated, with *Bmpr2* *f/f* representing *Bmpr2*^{*flox/flox*} and *Bmpr2* *cko* (*Bmpr2* conditional knockout) representing *K14-Cre*;*Bmpr2*^{*flox/flox*}. Nails grow abnormally large in *Bmpr2*/*Acvr2a* deficient mice. (g, h) *K14-Cre*;*Bmpr2*^{*flox/flox*};*Acvr2a*^{*+/-*} mice are shown at 23 days (g) and 7.5 months (h) of age. The hair coats of these double mutants undergo cycles of loss and re-growth throughout life.

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FIG. 2.

Bmpr2/Acvr2a double mutants display aberrant catagens. Skin sections of control (**a, c, e**) and *K14-Cre;Bmpr2^{flx/flx};Acvr2a^{+/-}* (**b, d, f**) mice of different ages are shown after staining with H & E. (**a, b**) P11. (**c, d**) P15. (**e, f**) P20. *Bmpr2/Acvr2a* double mutants experience premature yet prolonged catagens. HS, hair shaft; HB, hair bulb; CH, club hair; ES, epithelial strand. Scale bars, 50 μm.

FIG. 3.

Bmpr2/Acvr2a double mutants display aberrant anagens and telogens. Skin sections of control (**a, c, e**) and *K14-Cre;Bmpr2^{flx/flx};Acvr2a^{+/-}* (**b, d, f**) mice of different ages are shown after staining with H & E. (**a, b**) P24. Insets show higher magnification images of the epidermis. (**c, d**) P35. (**e, f**) P50. *Bmpr2/Acvr2a* double mutants experience shortened anagens and telogens. HB, hair bulb; ES, epithelial strand; CH, club hair. Scale bars, 50 μ m.

FIG. 4.

Mutations in *Bmpr2* and *Acvr2a* cause differentiation defects in hair medulla. Skin sections of P11 control (a, d), *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* (b, e) and *K14-Cre;Bmpr2^{flox/flox}* (c, f) mice are shown after staining by H & E (a–c) or immunofluorescence (d–f) using antibodies to trichohyalin (red; DNA is stained in blue). A lack of type II receptor function leads to structural disorganization and trichohyalin deficiency in the hair medulla (M). C, cortex; I, IRS; O, ORS. Scale bar, 20 μ m.

FIG. 5.

Bmpr2/Acvr2a double mutants exhibit macrostructural defects in hair fibers. **(a, b)** Full-length views of intact coat hair from control **(a)** and *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* **(b)** mice are shown. Examples of the four hair types — guard (gu), awl (aw), auchene (au), and zigzag (z) — are indicated. The double mutants display a shortening of the awl, auchene, and zigzag fibers and a curvature of the guard and awl fibers. **(c–e)** High magnification views of intact hair fibers from control **(c)**, *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* **(d)**, and *K14-Cre;Bmpr2^{flox/flox}* **(e)** mice are shown. Double-mutant awl and auchene hair follicles **(d)** fail to produce multiple columns of medullary cells, generating a single column instead. The hair

medulla is structurally abnormal in all hair types of the double mutant (**d**) and in occasional hairs of the single mutant (**e**). (**f–g**) High magnification views of intact hair-shaft bases from control (**f**) and *K14-Cre;Bmpr2^{flox/flox};Acvr2a^{+/-}* (**g**) mice are shown. The hair club (HC) develops on control hairs but not on double-mutant hairs, which exhibit tapered, wavy ends instead. Scale bar for panels **c–g**, 25 μm .

FIG. 6.

Mutations in *Bmpr2* and *Acvr2a* cause increases in cell proliferation. Skin sections of control (**a, d, f**), *K14-Cre;Bmpr2^{fllox/fllox};Acvr2a^{+/-}* (**b, e, g**) and *K14-Cre;Bmpr2^{fllox/fllox}* (**c**) mice were stained by immunofluorescence using antibodies to BrdU (green; DNA is stained in blue). (**a–c**) P11. Whereas BrdU-labeled (proliferating) cells are normally absent from the differentiating hair shaft (**a**), the hair medulla (M) possesses many BrdU-labeled cells in double-mutant hair follicles (**b**). To a lesser extent, single-mutant hair follicles (**c**) also exhibit medullary BrdU-labeling (marked by arrowheads). (**d, e**) P18. During catagen, BrdU-labeled cells are elevated in the superficial segments of double-mutant hair follicles. Asterisks denote background staining in the stratum corneum of the epidermis. (**f, g**) P21. At the start of a new anagen, BrdU-labeled cells are more prominent in the epidermis of the double mutant. Dotted lines in panels **d–g** indicate the epithelial-dermal boundary. HM, hair matrix. Scale bar, 50 μm.

FIG. 7.

Loss of *Bmpr2* and *Acvr2a* in melanocytes inhibits melanosome maturation. (a) *Bmpr2* and *Acvr2a* expression are detected in primary murine melanocytes by RT-PCR (upper panel) and immunoblotting (middle and lower panels). M, markers. (b) Tail DNA was probed by PCR for the *Tyr::Cre*-driven conversion of the *Bmpr2^{flox}* allele to the deletion (null) allele. Representative PCR products from *Bmpr2^{flox/flox};Acvr2a^{+/-}* (ctl, control), *Tyr::Cre;Bmpr2^{flox/flox}* (cko, conditional knockout), and *Tyr::Cre;Bmpr2^{flox/flox};Acvr2a^{-/-}* (cko -/-) mice are shown on an agarose gel. The *Tyr::Cre* transgene generates the *Bmpr2* deletion allele. (c) *Tyr::Cre;Bmpr2^{flox/flox}* (left; *Bmpr2* cko) and

Tyr::Cre;Bmpr2^{flox/flox};Acvr2a^{-/-} (right) mice are shown at P10. The *Bmpr2/Acvr2a* double knockout develops gray hair during coat development. (**d, e**) Skin sections of P10 *Tyr-Cre;Bmpr2^{flox/flox}* (**d**) and *Tyr-Cre;Bmpr2^{flox/flox};Acvr2a^{-/-}* (**e**) mice are stained for melanocytes by the TTA assay (red; DNA is stained in blue). Double-mutant hair follicles develop a melanocyte cluster (MC) of normal size and location. (**f, g**) Bright-field views are shown of the sections in panels **d** and **e** above. Despite their robust melanocyte clusters (**e**), the double-mutant hair follicles generate low levels of melanin (**g**). (**h-k**) Skin sections from P10 *Tyr-Cre;Bmpr2^{flox/flox}* (**h, j**) and *Tyr-Cre;Bmpr2^{flox/flox};Acvr2a^{-/-}* (**i, k**) mice are shown in transmission electron micrographs. Melanosomes are visible in melanocytes (M; panels **h, i**) and medullary cells (ME; panels **j, k**) as black, spherical or ovoid bodies. The melanosomes of the double mutant are darkly pigmented but substantially reduced in size. HS, hair shaft. Scale bar for panels **d-g**, 50 μm ; **h-k**, 0.1 μm .