DEVELOPMENT AND DISEASE

‘Cyclic alopecia’ in Msx2 mutants: defects in hair cycling and hair shaft differentiation

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SUMMARY

Msx2-deficient mice exhibit progressive hair loss, starting at P14 and followed by successive cycles of wavelike regrowth and loss. During the hair cycle, Msx2 deficiency shortens anagen phase, but prolongs catagen and telogen. Msx2-deficient hair shafts are structurally abnormal. Molecular analyses suggest a Bmp4/Bmp2/Msx2/Foxn1 acidic hair keratin pathway is involved. These structurally abnormal hairs are easily dislodged in catagen implying a precocious exogen. Deficiency in Msx2 helps to reveal the distinctive skin domains on the same mouse. Each domain cycles asynchronously – although hairs within each skin domain cycle in synchronized waves. Thus, the combinatorial defects in hair cycling and differentiation, together with concealed skin domains, account for the cyclic alopecia phenotype.

Key words: Alopecia, Hair cycle, Hair differentiation, Homeobox genes, Msx2, Foxn1, Ha3, Fgf5, Mouse

INTRODUCTION

The mammalian hair follicle is a representative but highly complex epithelial organ that develops during late embryogenesis from reciprocal interactions between the epidermis and the underlying dermis (for reviews, see Hardy, 1992; Chuong, 1998; Fuchs et al., 2001). Similar to other skin appendages, hair follicle induction requires complex signaling between the two apposing tissue layers, which involves Fgf, Bmp, Msx and Hox genes, and Shh (Chuong et al., 1996; Oro and Scott, 1998; Paus and Cotsarelis, 1999; Widelitz and Chuong, 1999). Initially, β-catenin expression becomes highly localized to appendage placode regions, probably through interactions among signaling molecules such as Eda (Tabby) (Kere et al., 1996; Laurikkala et al., 2002), and Fgf and Bmp genes (Jones et al., 1991). As a result of diffusion and lateral inhibition, periodically arranged skin appendage domains emerge (Turing, 1952; Jiang et al., 1999b). After initial follicle induction, molecules such as Shh appear and promote cell proliferation (Oro et al., 1997; St. Jacques et al., 1998; Chiang et al., 1999). Epithelial invagination leads to the formation of the hair follicle, which contain the dermal papilla, proliferating matrix cells and slowly cycling stem cells. Imbalances in signaling pathways, exemplified by the mouse mutants Tgfa<sup>wa1</sup> and Egfr<sup>wa2</sup>, and Eda<sup>Ba</sup> and Eda<sup>de</sup> (downless), which correspond to defects in ligand-receptor signaling, cause derangements in hair follicle morphology that produce abnormal hair.

Less is known about the cellular and molecular mechanisms that regulate the differentiation and cycling of the hair follicle. During differentiation, germinative matrix cells in the hair bulb (TA or transient amplifying cells) actively divide to produce progenitor cells that differentiate and give rise to the hair shaft and to the inner root sheath or IRS (Rogers et al., 1998) (see Fig. 1G). These cell types are arranged in concentric layers from outside to inside, respectively. Several pathways are involved in the specification and differentiation of matrix cells into hair shaft and sheath cells. BMP signaling is required during hair differentiation, as attenuation of BMP signaling by ectopic Noggin abolishes hair filament differentiation but not that of the IRS (Kulksa et al., 2000). The Wnt signaling pathway is also implicated in hair follicle morphogenesis, with Lef1 constituting a key nuclear effector in this pathway (Zhou et al., 1995; DasGupta and Fuchs, 1999; Millar et al., 1999).
Wnt signals act on the dermal papilla and probably prompt the epidermis to induce hair development (Kishimoto et al., 2000). Although LeF1 RNA is expressed in matrix cells during anagen, LeF1 protein accumulates in the nucleus of postmitotic precortical cells undergoing terminal differentiation (DasGupta and Fuchs, 1999). Cell-cell interactions involving the Notch signaling pathway are also crucial for proper hair differentiation (Powell et al., 1998; Lin et al., 2000). In addition, the homeobox gene Hoxc13 has been implicated in the regulation of hair differentiation, as both overexpression (Tkatchenko et al., 2001) and genetic ablation (Godwin and Capecchi, 1998) lead to defective hairs and alopecia and HOXC13 can regulate hair keratin promoters directly (Jave-Suarez et al., 2002). Last, the nude gene product Foxn1 (also known as Whn) is implicated in hair progenitor cell differentiation as matrix cells exit the cell cycle and migrate up along the hair shaft en route to terminal differentiation (Lee et al., 1999). However, the relationships between these different genes in hair differentiation are unresolved.

One of the more remarkable characteristics of the hair follicle biology is its growth cycle, which includes anagen, catagen and telogen (reviewed by Fuchs et al., 2001; Stenn and Paus, 2001) (see Fig. 1G). Anagen is the stage of active cell proliferation and differentiation. During anagen, the follicle lengthens and penetrates into the dermis. Meanwhile, descendants of the hair matrix cells at the base of the follicle bulb are gradually pushed upwards, differentiating into the hair shaft that emerges from the follicle. In catagen, hair production ceases and the hair bulb region is converted into an anchoring club with a thin epithelial strand connecting the inner root sheath (Hardy, 1992; Koch et al., 1998). Cellular apoptosis in follicular keratinocytes is associated with catagen progression (Lindner et al., 1997). By the end of catagen, the dermal papilla relocates to the vicinity of the hair follicle bulge. Telogen is the resting stage, but an exogen stage also has been defined that represents the stage of controlled hair shaft-extrusion (Paus et al., 1999). After telogen, the next round of hair growth begins with interaction between the dermal papilla and the multipotent epithelial stem cells that reside in the outer root sheath (ORS) and in the bulge region (Cotsarelis et al., 1990; Oshima et al., 2001). Through these interactions, a group of TA cells are induced and anagen restarts when new follicle pushes old one to the side. At some point in telogen the old fiber (club hair) is lost. Several molecules and diffusible growth factors exhibit oscillatory expression during different phases of the hair cycle, and transgenic overexpression of some of these leads to acceleration or arrest of the hair cycle (reviewed by Paus et al., 1999; Stenn and Paus, 2001). However, only a few genes have been shown to directly influence hair cycling.

One family of genes involved in signal transduction between interacting tissue layers during organogenesis is the mammalian Msx homeobox family, homologs of the Drosophila msh (muscle segment homeobox) gene (for a review, see Davidson, 1995). During mouse embryogenesis, Msx1 and Msx2 are expressed in hair follicle placode ectoderm, and subsequently in epithelial matrix cells. Both genes are expressed during anagen in the matrix cells of the hair bulb (Reginelli et al., 1995). Mice doubly homozygous for Msx1 and Msx2 have ~1/3 the wild-type number of induced follicles, and these incipient follicles are associated with reduced Patched expression, suggesting a threshold requirement for Msx protein in hair follicle induction (Satokata et al., 2000). Whereas transgenic mice overexpressing Msx2 under the cytomegalovirus (CMV) promoter exhibit retarded hair growth and a reduced hair matrix (Jiang et al., 1999a), preliminary analysis of Msx2-deficient hair follicles suggests that catagen onset occurs prematurely (Satokata et al., 2000). However, the mechanisms by which Msx2 acts during the mouse hair growth cycle and by which its deficiency leads to hair loss are unclear. Msx2 knockout mice exhibit a curious balding phenotype, characterized by repeated cycles of hair loss and regrowth in different domains of the body, which we named cyclic alopecia. We analyzed its pathogenesis and revealed defects in all hair cycle stages hair differentiation.

**MATERIALS AND METHODS**

**Mice and genotyping**

Msx2 knockout mice (Msx2<sup>−/−</sup>/<sup>−/−</sup>) were generated by inserting a neo cassette into the NdeI site, 5’ of the Msx2 homeobox in exon 2 as previously described (Satokata et al., 2000). Skin samples used in this study came from crossing C57BL/6J to ICR congenic genetic background. ICR congenic strain was generated by breeding B6;129 Msx2<sup>−/−</sup>/<sup>−/−</sup> mice with ICR +/+ mice. Angora (Fg550/Fg550) mutant mice on an agouti colored outbred stock were provided by Dr Gail Martin (UCSF) as described previously (Hébert et al., 1994). Some variations were observed because angora was used on a segregating background (Sundberg et al., 1997).

**In situ hybridization**

Radioactive in situ hybridization was performed by hybridizing α-[<sup>35</sup>S]-labeled cRNA probes to skin sections as described (Wawersik and Epstein, 2000; Maas et al., 1996; Zhang et al., 1999).

**RNase protection assays**

Whole-cell RNA was prepared from dorsal skin of wild type and Msx2 knockout mice at different stages during the first anagen using the Ultraspec RNA isolation system (Biotec Laboratory, TX). Antisense probes for Bmp4 and LeF1 were generated as described (Chen et al., 1996). A Tgfa probe was generated by PCR using primers Tgfa-F (5’-TGTCAAGCTCTGGAGAACAGC-3’) and Tgfa-R (5’-CGGC-ACCACCCATGGTGGT-3’) on reverse transcribed skin total RNA. The resulting 350 bp fragment was cloned into the Smal site of pGEM4Z (Promega, WI). An antisense probe for Tgfa was generated by digesting the above plasmid with EcoRI followed by in vitro transcription with T7 RNA polymerase. The Foxn1 antisense RNA probe was generated by linearizing pBSK-Foxn1 with XhoI followed by transcription with T3 polymerase (Nehls et al., 1994). RNA probes for β-actin and Rpl19 were respectively prepared according to manufacturer’s instructions (Ambion, TX) or as described (Ma et al., 1998). Total skin RNA (20 μg) was hybridized overnight at 45°C with 4×10<sup>5</sup> cpm of α-[<sup>32</sup>P]-UTP labeled antisense RNA probes for Bmp4, LeF1, Ha3, Tgfa or Foxn1, with β-actin or Rpl19 as loading controls. After digestion with 20 μg/ml RNase A and 1.5 μg/ml RNase T1, protected fragments were precipitated and separated on a 6% denaturing polyacrylamide gel and band intensities were quantified by phosphorimager (Molecular Dynamics).

**Cell proliferation and apoptosis assay**

Mice were injected subcutaneously with 50 μg/g body weight of BrdU at a concentration of 5 mg/ml in PBS. Anti-BrdU-peroxidase antibody staining was processed according to the manufacturer’s instructions (Boehringer Mannheim, #1585860). TUNEL staining of dorsal skin of P45 mice was performed using the In Situ Cell Death Detection Kit (Boehringer Mannheim, #1684809).
Immunohistochemistry
Primary antibodies used were rabbit/mouse anti-Foxn1 (Lee et al., 1999), rabbit anti-K14 (1:400, Berkeley Antibody Company); anti-K10 (1:200, Sigma), monoclonal AE 13, 14, 15 (gift of Dr T. T. Sun) (Lynch et al., 1986). Immunohistochemistry was performed using the peroxidase substrate kit (Vector Laboratories, Inc.) or immunofluorescence as described in the manufacturer’s instructions.

Hair stripping
Hair stripping experiments were performed by coating the dorsal skin of P45 mice with wax at 60°C. Wax was pulled off along with hairs from the dorsal skin after the wax had cooled to room temperature.

Scanning electron microscopy (SEM)
Hairs from the dorsal skin of mice were attached to carbon adhesive tabs on aluminum mounts and coated with a Polaron sputter coater. Samples were analyzed by scanning electron microscope at 20 kV (Bechtold, 2000).

RESULTS
Dynamic expression of Msx genes in the postnatal hair follicle
Although several studies have documented Msx expression in skin (Noveen et al., 1995; Jiang et al., 1999a; Reginelli et al., 1995; Stelnicki et al., 1997), Msx1 and Msx2 expression during the hair cycle has not been systematically examined. We therefore examined Msx1 and Msx2 expression in wild-type skin during different phases of the highly synchronized first hair cycle. At P3, Msx1 and Msx2 are both expressed in the hair bulb, including the germinative matrix (Fig. 1A,B). At P11, the Msx1 expression pattern remained unchanged, although the level was downregulated. By contrast, Msx2 expression is upregulated and expands into the upper region of hair follicle, including the hair cortex (Fig. 1C,D). By P17 catagen, extending to P21 telogen, Msx2 expression in the hair follicle persists while that of Msx1 becomes undetectable (Fig. 1E,F and data not shown). Neither Msx1 nor Msx2 is expressed in the dermal papilla at any time point. Thus, Msx1 and Msx2 are coordinately expressed during early phases of the hair cycle, but thereafter Msx2 is the sole Msx family member expressed.

Cyclic alopecia in Msx2 knockout mice
Msx2-deficient mice were analyzed on both the segregating C57BL/6J and congenic ICR Backgrounds, with only slight differences in phenotype. Newborn Msx2 knockout mice could be distinguished from wild-type littermates by their short, curly vibrissae at P5. At P3, homozygous mutants began to exhibit retarded pelage hair growth. Although control hairs were straight, mutant hairs were wavy and exhibited irregular diameters along the hair shaft (Fig. 2; see Fig. 5). Measurements showed that mutant hairs were 25-30% shorter, which was not related to the slight overall growth retardation of these mutant mice. All four hair types (awl, auchene, guard and zigzag) are present in Msx2 knockout mice.

In contrast to heterozygous and wild-type littermates, at P14, Msx2 knockout mice began to lose hair. This is followed by progressive hair loss and regrowth. The mouse showed dynamic patches of hairiness and baldness. The distinct appearance is illustrated by the two pictures of the same mouse, taken one month apart (Fig. 2A). To analyze this phenomenon carefully, we observed the pattern of alopecia of five homozygote mice twice a week over 3 months (Fig. 2B). The hair cycle-dependent hair loss helped to bring out the concealed mouse skin domains that cycle asynchronously. Although hairs in different domains

![Fig. 1. Expression of Msx1 and Msx2. Wild-type ICR pups were sacrificed at indicated time-points and their skin harvested, sectioned and hybridized to 35S-labeled Msx1 or Msx2 cRNA probes. Msx1 and Msx2 are initially co-expressed in the developing hair follicle. Expression of Msx1 is limited to the matrix cells (A,C) and its expression disappears during catagen (E). Msx2 expression in the hair follicle is more dynamic: first it is expressed only in the matrix and pre cortical cells, then it is expanded into the hair cortex and medulla (B,D). Msx2 continues to be expressed during catagen, when Msx1 expression is no longer expressed (E,F). Scale bar: 50 μm. (G) A schematic diagram of hair follicle structures and the hair cycle. During anagen, germinative matrix cells proliferate to generate progenitor cells, which receive signals from the dermal papilla to differentiate into either hair shaft cells or inner root sheath cells. During catagen, hair production ceases and hair follicle degenerates to form a club hair. During telogen, hair follicle rests and at the end of telogen, dermal papilla at the base of the hair follicle interact with the adjacent bulge region to initiate the second round of hair follicle morphogenesis. IRS, inner root sheath; ORS, outer root sheath; HF, hair follicle.]

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cycle independently, hairs within the same domain cycle in a synchronized wave, generally from anterior to posterior, but there are occasions when this direction is reversed (Fig. 2B). Similar asynchronous hair cycling domains have been shown in normal mice (Militzer, 2001).

In addition, in Msx2 knockout mice maintained on a C57BL/6J segregating background, synthesis of the skin pigment melanin during the hair cycle was also deranged (Fig. 2). Neural crest-derived melanocytes proliferate and mature during anagen, subsequently undergoing apoptosis during early catagen (reviewed by Tobin and Paus, 2001). In Msx2 knockout mice, melanin synthesis commenced in early anagen, but pigment was lost in regions of hair loss, suggesting that hair follicles in bald regions prematurely enter catagen.

Fig. 2. Abnormal hair cycling in Msx2 knockout mutant mouse. (A) Hair distribution of the same mouse, pictures taken 1 month apart. Note the dramatically different hair patches. In 1 month, hairy regions became bald, and bald regions became hairy. (B) Asynchronous hair cycle domains in the same mutant mouse over time. Msx2 knockout mice show a cyclic balding pattern. To examine the balding pattern, we followed five mice (1 month old) over an 80-day period, taking photos about once every 3 days. One example is shown here. Hair regrowth first appears in the shoulder region, spreads to the whole trunk, and then starts to get lost from cephalic to caudal end until all hairs are lost. This pattern then repeats. The times when most trunk hairs are in anagen are boxed in red.

Abnormal hair cycling in Msx2 knockout mice

To analyze hair cycling in Msx2-deficient hair follicles, we examined mid-dorsal skin histology at the level of thoracolumbar junction between wild-type littermates and Msx2<sup>2imRilm</sup> homozygous mice at 11 serial time-points between P1 and P31 (Fig. 3, left column). Although no histological differences were observed at P1, by P3 Msx2-deficient skin had a thinner dermis. At P5, wild-type follicles were in anagen VI when all follicular cell types are present and the follicles penetrate into the adipose layer. By contrast, Msx2-deficient skin had a thinner dermis and a significant region of the hair follicles were horizontal, parallel to the muscle layer (Fig. 3B, arrowheads). This may reflect asynchrony between the down growth of hair follicles and the ability of the dermis to accommodate them. At P10, when wild-type mice still
possessed anagen hair, hair in Msx2 knockout mice had already entered catagen. However, by P17, when wild-type mouse skin displayed catagen V-VII follicles, Msx2-deficient skin displayed early abnormal catagen II follicles characterized by small and long hair bulbs (data not shown). Moreover, by P21, wild-type mice displayed only telogen follicles, whereas Msx2 knockout mice displayed abnormal catagen VI-VII follicles, further indicating a significant delay in catagen progression.

To confirm this result, we examined the expression of Tgfa, a marker for the inner root sheath (IRS) and keratogenous zone (Luetteke et al., 1993). By P17, the number of Tgfa-expressing follicles was much reduced, and no Tgfa expression could be detected in wild-type P21 telogen hair follicles. By contrast, in Msx2-deficient hair follicles Tgfa continues to be strongly expressed at P21, consistent with a prolonged catagen (Fig. 3E,F, inset). At P24, control skin has re-entered anagen; however, hairs in Msx2-deficient skin remain in telogen until about P31, when they are seen to re-enter anagen.

We also quantify the results by histograms that show the percentage of hair follicles in anagen, catagen, and telogen phases in different ages of the mice. These results suggest that in Msx2-deficient skin, there is a shortened anagen phase, followed by an abnormal and prolonged catagen phase. The shortened hair in Msx2 knockout mice is also consistent with the conclusion that anagen is shortened.

To challenge the ability of Msx2-deficient hair follicles to re-enter anagen, we carried out wax stripping of hairs to synchronize hair cycling (Fig. 3, right column). Six days after stripping, anagen hair follicles first re-appeared in wild-type skin, and by 20 days after stripping, these hair follicles finished their cycling and entered telogen. By contrast, in Msx2-deficient skin, anagen follicles did not re-appear until 20 days after stripping, suggesting that hairs in Msx2 knockout mice have difficulty re-entering anagen phase. Measurement of percentages of hair follicles stages showed this clearly. Taken together, we conclude that while Msx2-deficient hair follicles can complete the hair cycle, their cycles are out of phase compared with normal hair follicles because of abnormalities in transitions between all three major phases of the hair cycle.

**Msx2 and Fgf5 in the genetic pathway controlling the hair cycle**

As Msx2 knockout mice have a shortened anagen, we used a well characterized spontaneous mutant mouse mutant, the angora (Fgf5<sup>50</sup>/Fgf5<sup>50</sup>) mouse, which is Fgf5-deficient and has an abnormal prolongation of anagen VI (Hébert et al., 1994; Sundberg et al., 1997). To probe the interactions between these genes, mice deficient for both Fgf5 and Msx2 were generated by crossing the mutant stocks. Compound heterozygotes were phenotypically indistinguishable from wild type and were
crossed to generate Fgf5<sup>500</sup>, Msx2<sup>2tm1Rilm</sup> double homozygotes. Examination of 48 Msx2 knockout mice revealed that hair loss always occurred at P14, regardless of genetic background (Fig. 4A,B). Msx2<sup>2tm1Rilm</sup> homozygotes carrying one Fgf5<sup>500</sup> allele exhibited the same cyclic alopecia phenotype as Msx2<sup>2tm1Rilm</sup> homozygous only. However, mice doubly homozygous for Msx2 and Fgf5 no longer lost their pelage hair at P14 (Fig. 4A,B). Despite the initial delay in hair growth in Msx2<sup>2tm1Rilm</sup> homozygous mice, Fgf5<sup>500</sup>, Msx2<sup>2tm1Rilm</sup> double homozygotes grew long pelage hairs characteristic of the angora (Fgf5<sup>500</sup>) mutation by P16. Between P18 and P30, all double homozygotes eventually lost their pelage hairs (Fig. 4B,C). The variation in the timing of hair loss in Msx2<sup>2tm1Rilm</sup>, Fgf5<sup>500</sup> double knockout mice could reflect variability in genetic background, as the hair cycle phases of different strains of mice have different lengths (Hébert et al., 1994). In angora mice, it has been shown that anagen is extended by 3 days (Pennycuik and Raphael, 1984; Sundberg et al., 1997). In Msx2 knockouts, hair loss is temporally linked to a specific point of time in catagen; the observation that both catagen onset and hair loss are postponed by 4 or more days in Fgf5<sup>500</sup>, Msx2<sup>2tm1Rilm</sup> double mutants (for Msx2 mutants, the C57BL/6J strain was used) further supports the idea that hair loss in Msx2 knockout mice is associated with catagen onset. It also suggests that the consequence of defective Fgf5 is dominant compared with those of mice with defective Msx2. However, in Msx2 knockout mice, Fgf5 in the ORS (Hébert et al., 1994; Rosenquist and Martin, 1996) was not significantly altered in anagen (data not shown). So the two pathways may not interact directly.

**Defective hair shaft differentiation in Msx2 knockout mice**

If a hair cycle defect was the only abnormality in Msx2 knockout mice, the hair length would vary but the hair should not be lost. Hair loss can be the result of breakage at the shaft region because of structural defect, or the loss of the club hair due to defect of the follicle to retain the club hair. To investigate the mechanism of hair loss further, we examined the structure of the hair shaft medulla, cortex and cuticle (Fig. 5A-J). Msx2 knockout mouse hairs were short, wavy and of uneven diameter, and their medullae contained disorganized septates, septulates and air bubbles (Fig. 5A-D,I,J). SEM analyses showed that, unlike the scaly tile pattern of the cuticle covering the surface of wild-type hairs, Msx2 knockout mouse hairs exhibited flattened cuticles with wrinkled surfaces (Fig. 5E-H) (Rogers et al., 1998). To differentiate whether hair loss is due to breakage of the shaft or dislodge of the club hair, we examined the plucked hairs. We observed that these hairs have their club ends, and none breaks in the middle. Application of the tape stripping test (Koch et al., 1998) showed that mutant hairs were much more easily removed than wild-type hairs. Although the club end appears to be morphologically similar between normal and mutant hairs (Fig. 5I,J), defects in the cuticle or the formation of the club are likely to contribute to the loose hair attachment between the club hair and the inner root sheath, resulting in the hair loss observed during catagen.

The short abnormal hair shaft could result from either defective proliferation or differentiation of the hair matrix cells. We first examined whether matrix cell proliferation was affected in Msx2 knockout mice anagen hair follicles by injecting BrdU into P5, P9, P11 and P15 Msx2 knockout mice and wild-type littermates, collecting skin samples 2 hours after injection. At P11, germinative matrix cells in both wild-type and Msx2 knockout mouse hairs were shown similarly extensive cellular proliferation in the lower region of the hair bulb and outer root sheath (Fig. 5K,L). We quantified cellular proliferation by counting BrdU-positive cells in serial sections through the germinative matrix of 12 histologically...
similar follicles in Msx2 knockout mice and wild-type littermates. The average number of BrdU-positive cells per follicle section (±s.e.m.) was not significantly different between Msx2 knockout mice [34.6±7.6 (BrdU+ cells/follicle section)] and wild-type littermates [32.5 ± 6.3 (BrdU+ cells/follicle section)]. Similarly, no significant differences in matrix cell proliferation were observed between Msx2 knockout mice and wild-type littermates at P5, P9 and P15 (data not shown).

TUNEL-positive cells were found in hair follicles. They are associated with catagen and also other hair morphogenesis processes (Lindner et al., 1997). Examination of cellular apoptosis was also carried out on P5, 9, 11, 14 and 30 wild-type and Msx2 knockout mice skin sections. No apparent difference was detected and an example is shown (Fig. 5M,N).

To assay differentiation, we used immunohistochemistry and in situ hybridization to detect molecules that represent different layers of hair related epidermis. Keratin 10 (K10) is expressed in the suprabasal layer of the interfollicular epidermis and K14 in the basal layer and outer root sheath. There were no detectable changes relative to wild type (data not shown). Immunohistochemistry with AE13 (react with low sulfur hair keratin) and AE14 (react with high sulfur hair keratin) monoclonal antibodies were expressed in the hair cortex (Lynch et al., 1986), and reduced in the mutants (not shown). Levels of mouse acidic (type I) hair keratin (Ha3; Krt1-2 – Mouse Genome Informatics), another marker for hair cortex cells (Winter et al., 1994; Meier et al., 1999), were also reduced (Fig. 6A,B). A similar increase in the expression of Ha3 transcripts from P5 to P11 followed by a decrease was observed in both wild-type and in Msx2-deficient skin, but in mutants the levels were reduced to 31% from 72% (Fig. 6A,B). These results indicate a failure of shaft differentiation in Msx2-deficient hair follicles.

The Foxn1 regulatory pathway is altered in Msx2 deficient hair follicles

We next focused on signaling pathways that might be affected by Msx2 deficiency. Ha3 was shown to be a downstream target of Foxn1 (Meier et al., 1999; Schorpp et al., 2000). As Ha3 is much reduced in Msx2 knockout mice, we examined whether Foxn1 also resides downstream of Msx2. Like Msx2, Foxn1 is highly expressed in the hair cortex with lower levels in matrix and outer root sheath cells (Lee et al., 1999; Meier et al., 1999). Foxn1 expression was analyzed serially by RNase protection between P1 and P17 in wild-type and Msx2-deficient skin and was expressed at all days after P1. However, in Msx2-deficient skin, Foxn1 expression was markedly decreased at all time points (Fig. 6A,B). Quantification of 6 sets of RNase protection assays at P11 revealed a 50% reduction in Foxn1 expression in Msx2-deficient skin (Fig. 6B). Furthermore, immunostaining with an affinity purified polyclonal anti-Foxn1 antibody detected Foxn1 protein in the hair matrix and pre-cortex, with a relative enrichment in the cortex regions in wild-type follicles. This immunoreactivity was significantly reduced in Msx2 knockout mice (Fig. 6C), consistent with the conclusion that Foxn1 expression requires Msx2 function. To see whether Foxn1 is downstream to Fgf5, we examined the expression of Foxn1 in Fgf5-deficient mice. We did not observe difference in expression level or distribution pattern (not shown). We also examined the expression level of Lef1 and Bmp4. While no significant

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**Fig. 5.** Changes of hair filament differentiation in Msx2 knockout mice. (A,B) Plucked hairs from Msx2 knockout mice are short and curly, reflecting defects in hair structures. Scale bar: 100 μm. (C,D) Medulla patterning is affected as suggested by the irregular septations in the hair. Scale bar: 50 μm. (E-H) Scanning EM showing unevenness in diameter of Msx2 knockout mice hairs. Cuticles fail to form, resulting in a smooth, wrinkled surface. Scale bar: 30 μm in E,F; 10 μm in G,H. (I,J) Trunk hairs from wild-type (I) and Msx2 knockout (J) mice. Both club ends are morphologically similar (arrowheads). Septation patterns are irregular in the mutants, but there are no breakages in the middle of the shaft. Scale bar: 100 μm. (K,L) BrdU labeling was performed at P5, P9, P11 and P15, and representative panels from P11 are shown. BrdU-positive cells were detected in both the hair matrix and the outer root sheath (arrows). Quantitation of BrdU-positive cells in the matrix showed similar levels of incorporation in the mutants. (M,N) Apoptotic cells detected by TUNEL assay carried out at P5, P9, P11, P14 and P30 (an example is shown from P30). Positive cells were observed in epidermis and dermis. No apparent differences between wild-type and Msx2 knockout mutant skins were observed. Scale bars: 250 μm.
reduction of Bmp4 is detected, we observed a consistent low level reduction of Lef1 mRNA.

**DISCUSSION**

We have analyzed the morphological and molecular defects in Msx2-deficient hair follicles. These investigations reveal two defects. A cycling defect occurs in the regulation of the hair cycle at the anagen-catagen, catagen-telogen and telogen-anagen transitions. The differentiation defect is manifested by short, curly pelage hairs and vibrissae that are subsequently lost because of structural abnormalities. The combination of these two defects results in a cyclic alopecia phenotype characterized by repetitive hair growth and loss. It also helps to reveal the asynchronous cycling in different body skin regions.

**The role of Msx2 in regulating the hair cycle**

Recently, both signaling molecules and transcription factors that exhibit altered expression during different cycle phases have been identified. Classical experiments suggest the existence of an intrinsic control of the hair cycle that resides in the hair follicle itself, and that can be modulated by outside factors (reviewed by Stenn and Paus, 2001). In mouse hair follicles, it has been proposed that such an intrinsic biological clock, termed the ‘hair cycle clock’, controls the length of each phase of the hair cycle (Paus et al., 1999; Stenn and Paus, 2001). Although the molecular nature of this clock is unknown, it may involve hormones or diffusible factors whose expressions oscillate in each hair cycle. This view implies that the hair cycle is controlled by sets of molecules that coordinately regulate the anagen-catagen, catagen-telogen and telogen-anagen transitions. According to this model (Fig. 7), sets of regulatory factors act at each checkpoint either to promote or suppress the transitions between the phases of the hair growth cycle. The observed length of each phase thus reflects the balanced strength of the promoting and suppressing factors impinging on each transition point. Analysis of the hair loss phenotype in Msx2 mutant mice permits the integration of Msx2 into this model.

In wild-type hair follicles, catagen is accompanied by changes in follicle morphology, including cessation in matrix cell proliferation and apoptosis in the hair bulb. However, in Msx2-deficient hair follicles, these events are uncoupled. As early as P10, Msx2-deficient hair follicles exhibit histological signs of catagen, while those of heterozygous littermates remain in anagen. However, matrix cells in both wild-type and Msx2-deficient follicles show comparable numbers of BrdU-positive cells. Therefore, there is an apparent uncoupling between proliferation and differentiation in the Msx2 deficient hair follicle. The precocious onset of catagen in Msx2 knockout mice suggests that Msx2 normally plays a role in maintaining hair follicles in anagen. Such a role is consistent with the role proposed for Msx genes in maintaining the proliferative potential of various cell populations (e.g. hair matrix cells) and in preventing their differentiation (Hu et al., 2001). Pursuant to this view, Msx2 would be predicted to suppress the anagen-catagen transition. In the model presented in Fig. 7, loss of Msx2 could thus either weaken the suppressive influences or
10-day delay in re-entering anagen after hair stripping. Premature hair loss in Msx2 knockout mice is not due to hair shaft breakage, but the result of the loss of whole club hairs. Therefore, there is a precocious entry of exogen.

Several growth factors have been implicated in the control of catagen onset, and we took advantage of one of these, Fgf5<sup>−/−</sup>/Fgf5<sup>−/−</sup>, to dissect further the genetic pathway controlling catagen entry (Hébert et al., 1994). Fgf5 is expressed in the ORS during late anagen and has been suggested to induce catagen by diffusion into the dermal papilla (Rosenquist and Martin, 1996). To test their relationship, we crossed Fgf5 knockout mice, which have long hairs as a result of a prolongation of anagen VI, into the Msx2-deficient background to generate mice doubly homozygous for Fgf5<sup>−/−</sup> and Msx2<sup>tm1Rilm</sup>. These mice also had long hairs, and while these hairs were eventually lost, the timing of hair loss was significantly delayed, suggesting an elongated anagen phase consistent with the angora phenotype. However, expression of Fgf5 is unaltered in Msx2 knockout mice. These results suggest that Fgf5 and Msx2 may act independently in their regulation of the length of anagen.

**Msx2 is required for hair shaft differentiation**

Both development of the hair follicle and subsequent hair growth involve signaling between the dermal papilla and matrix cells. During anagen, TA cells in the hair matrix proliferate in response to growth signals from the dermal papilla, differentiating into the several hair cell types of the hair shaft and IRS (Fig. 7). The fate of these epithelial cells may be determined as soon as they leave the cell cycle, or later during differentiation via cell-cell interactions (Kopan and Weintraub, 1993). Regulation of this process has been proposed to involve FGFs, BMPs (Kulessa et al., 2000), Notch (Lin et al., 2000), Foxn1 (Prowse et al., 1999) (Fig. 7). As Msx2 deficiency causes defects in all three layers of the hair shaft, we consider the role of Msx2 is to integrate the differentiation of TA cells, as Msx2 is expressed in regions where proliferating matrix cells migrate up to become precortical cells and subsequently differentiate into hair cortex cells.

Differentiation of hair cortex cells requires functional Foxn1 protein, a winged helix transcription factor. Loss of Foxn1 function gives rise to the nude mouse phenotype in which differentiation of hair progenitor cells is severely affected, resulting in short, bent hairs that rarely protrude beyond the skin surface where they break off (Nehls et al., 1994; Brissette et al., 1996). Recently, Foxn1 has been shown to be a transcriptional regulator of the mouse acidic hair keratin genes, providing one of the molecular mechanisms that may contribute to the nude phenotype (Meier et al., 1999; Schorpp et al., 2000; Baxter and Brissette, 2002). The acidic hair keratin Ha3 is specifically expressed in the hair cortex layer and thus serves as a terminal differentiation marker. Interestingly, downregulation of Ha3 and decreased immunoreactivity of low and high sulfur hair keratins are observed in Msx2-deficient hair, consistent with the observed structural defect in cortex cell differentiation. These results suggest that an Msx2/Foxn1/Ha3 pathway participates in the control of hair shaft growth and differentiation (Fig. 7). However, the lost hairs show a complete length, suggesting that defect is due to weakness in retaining club hairs, not due to breakage of the shaft.
What factors reside upstream of Msx2 in the matrix and precortex region? During hair differentiation, Bmp4 is expressed in hair matrix cells and in hair shaft cells in contact with the IRS. Bmp2 is specifically expressed in the precortex cells (Lyons et al., 1991; Kulessa et al., 2000), while noggin is expressed in the dermal papilla (Botchkarev et al., 1999). Ectopic expression of noggin in the hair matrix under a minimal Msx2 promoter disrupts hair differentiation, with the cells remaining in a highly proliferative state in the precortex and hair shaft regions; these results provide strong evidence that BMPs are required during hair differentiation (Kulessa et al., 2000). Msx2 expression is markedly reduced in these Msx2-noggin mice. Conversely, we find that Bmp4 expression is preserved in Msx2 mutant skin. In addition, as the defects in Msx2-deficient hair follicles are mainly restricted to the hair shaft and are less severe than those associated with abolition of Bmp signaling (which involves both IRS and hair shaft), Msx2 is likely to function downstream of Bmp genes during hair differentiation.

Another major pathway involved in hair morphogenesis is the Wnt/catenin pathway. β-Catenin participates in the maintenance of epidermal stem cells, and activation of β-catenin can induce new skin appendages in mouse and chicken (Chan et al., 1999; Gat et al., 1998; Widelitz et al., 2000; Kishimoto et al., 2000; Huelsken et al., 2001). Msx2 is co-expressed in hair matrix cells with Lef1, a key effector in the Wnt/catenin pathway. High levels of Lef1 expression are found in proliferating matrix cells, possibly transducing Wnt signals. Later, nuclear Lef1 protein accumulates in pre-cortex cells undergoing terminal differentiation (DasGupta and Fuchs, 1999), and several keratin promoters contain Lef1-binding sites (Zhou et al., 1995). These results suggest that Lef1 is required to prepare matrix cells for terminal differentiation. The reductions in Lef1 expression we observe in Msx2-deficient hair follicles are modest, but may indicate that Lef1 resides downstream of Msx2 in matrix and pre-cortical cell differentiation, similar to the genetic relationship proposed between Msxl and Lef1 in tooth development (Chen et al., 1996; Kratochwil et al., 1996).

An intricate signaling network in the hair matrix region regulates the specification and differentiation of the hair shaft and the IRS. From the analyses performed to date, Msx2 is unlikely to be the more upstream molecules in this network, but it is likely to constitute a key regulator in the differentiation of TA cells into hair shaft cells. For example, upon Msx2 deficiency, pre-cortical cells may fail to respond fully to signals such as BMPs, Wnts, Fgfs or Notch ligands. As a result, Foxn1 and Lef1 may fail to be expressed at the levels needed to ensure proper cortex differentiation and expression of hair keratins. The successful formation of a hair depends on the progression of hair progenitor cells through several major determination and morphogenetic events. Our results show that Msx2 is involved in regulating the switches between TA cells and pre-cortical cell and in the transitions between the different phases of the hair cycle.

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