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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 Tgf_{α}^{wa1} and $Egfr^{wa2}$, and Eda^{Ta} and $Edar^{de}$ (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 (Kulessa 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 precortex 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 mainly 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 cytomeglovirus (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*^{tm1Rilm}/*Msx2*^{tm1Rilm}) were generated by inserting a *neo* cassette into the *Nde*I 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*^{tm1Rilm} mice with ICR +/+ mice. Angora (*Fgf5*^{go}/*Fgf5*^{go}) 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-[^{35}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 (Biotecx 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′-TGTCAGGCTCTGGAGAACAGC-3′) and Tgfa-R (5′-CGGC-ACCACTCACAGTGTTTG-3′) on reverse transcribed skin total RNA. The resulting 350 bp fragment was cloned into the *Sma*I site of pGEM4Z (Promega, WI). An antisense probe for *Tgfa* was generated by digesting the above plasmid with *Eco*RI followed by in vitro transcription with T7 RNA polymerase. The *Foxn1* antisense RNA probe was generated by linearizing pBSK-*Foxn1* with *Xho*I 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 4×10^5 cpm of α- [^{32}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

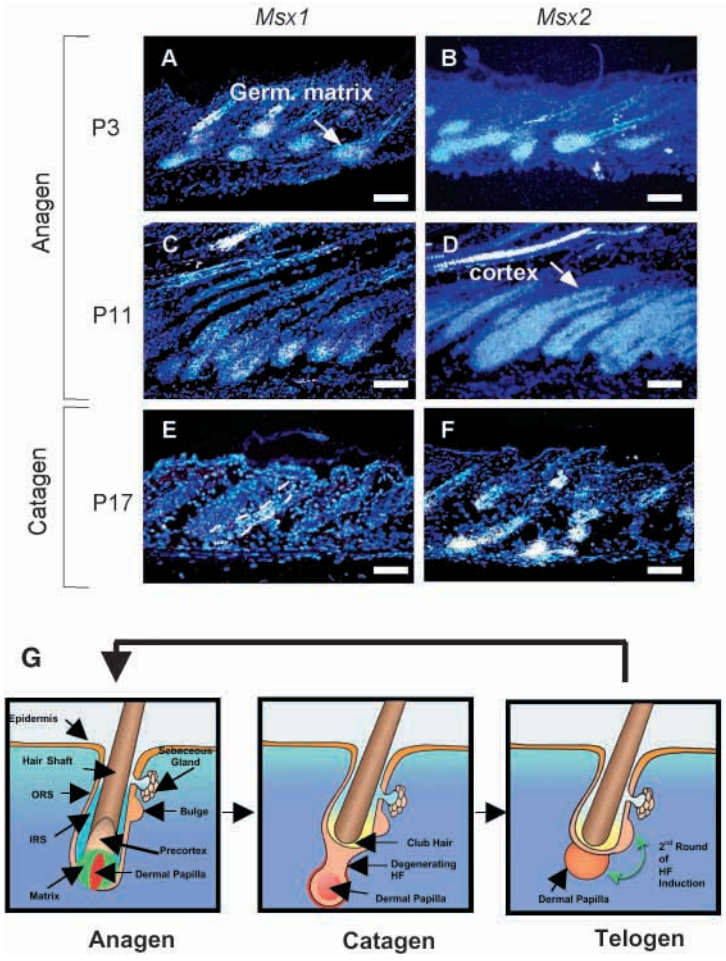


Fig. 1. Expression of *Msx1* and *Msx2*. Wild-type ICR pups were sacrificed at indicated time-points and their skin harvested, sectioned and hybridized to ^{35}S-labeled *Msx1* or *Msx2* cRNA probes. *Msx1* and *Msx2* are initially co-expressed in the developing hair follicle. Expression of *Msx1* is 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 precortical 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.

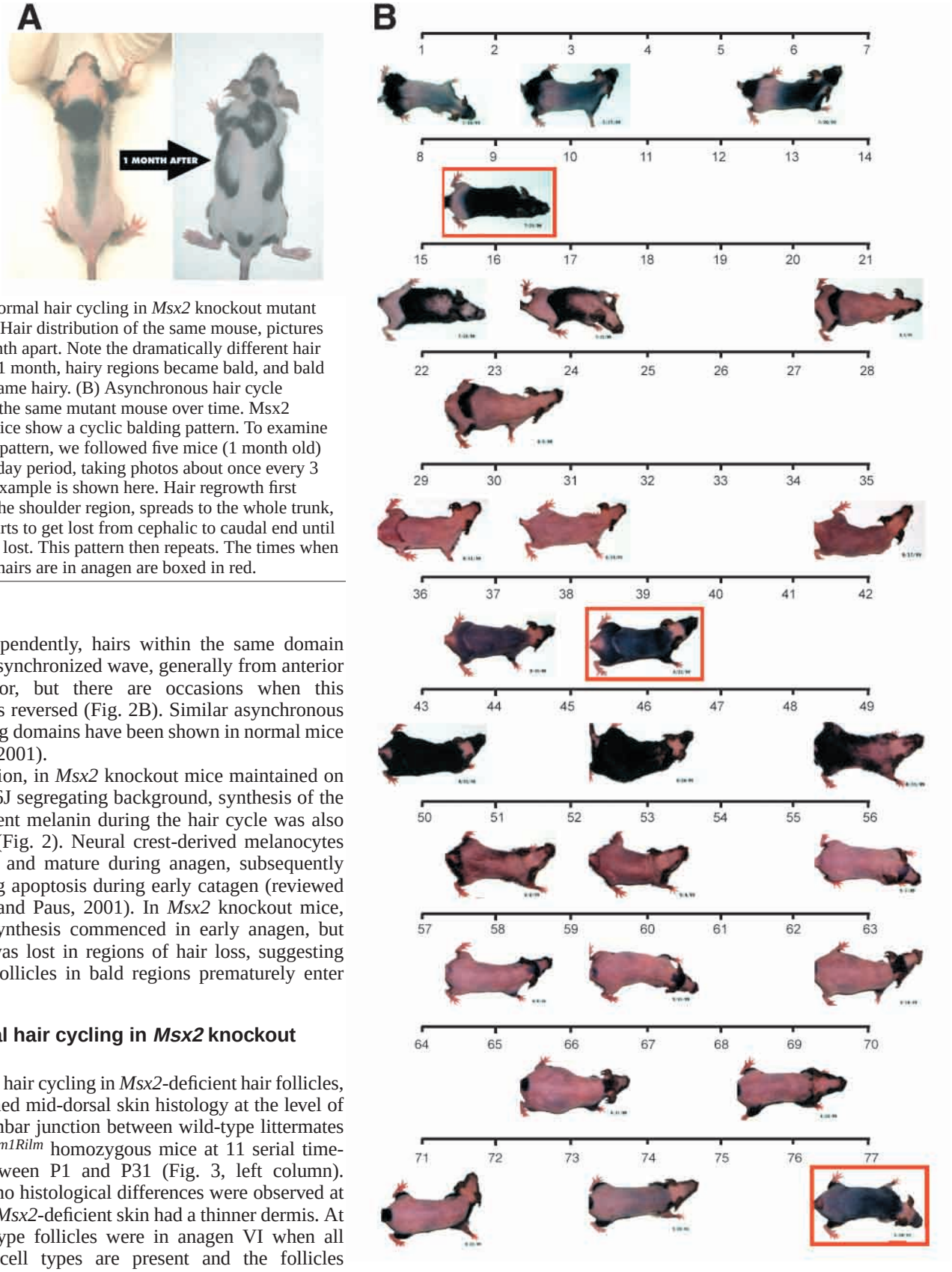


Fig. 2. Abnormal hair cycling in *Msx2* knockout mutant mouse. (A) Hair distribution of the same mouse, pictures taken 1 month apart. Note the dramatically different hair patches. In 1 month, hairy regions became bald, and bald regions became hairy. (B) Asynchronous hair cycle domains in the same mutant 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.

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.

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*^{tm1Rilm} 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

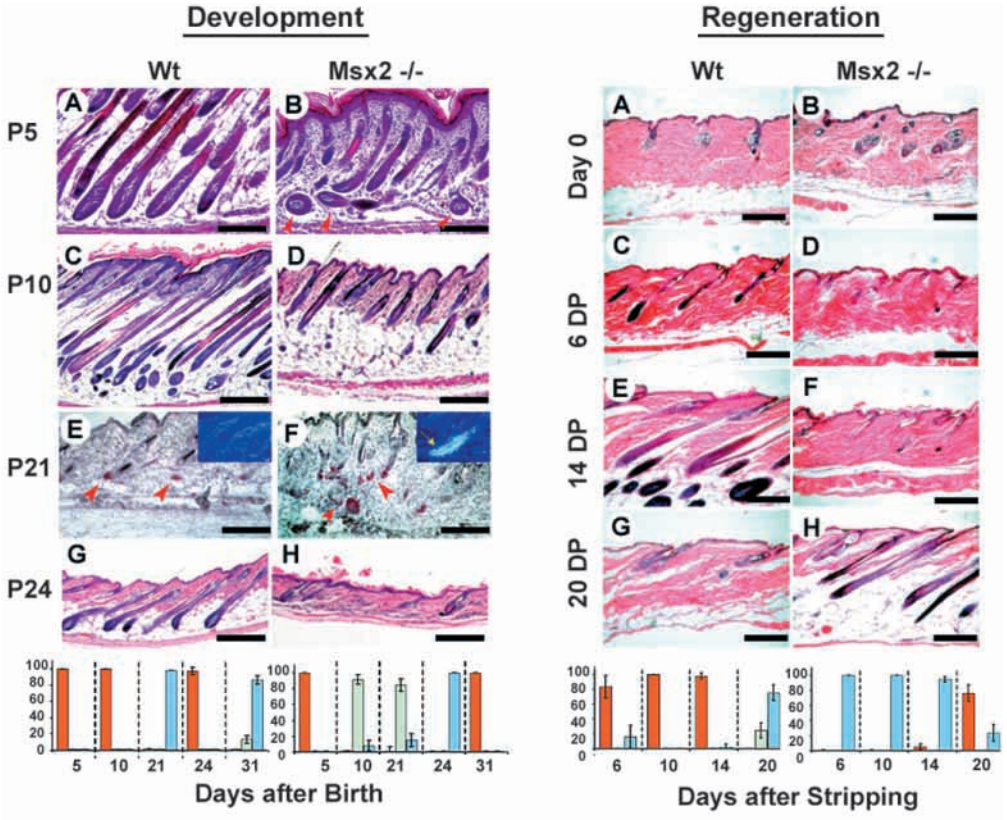


Fig. 3. Histopathological changes of *Msx2* knockout mice in different hair cycle stages. Left column, normal cycling showed shortened anagen. Differences in skin and hair follicle morphology between wild-type and *Msx2* knockout mice start to show at postnatal day 3 (P3). There is a lack of hair cortex differentiation at P5 (A,B). Anagen in *Msx2* knockout mutant is shorter and hair follicles at P10 have already entered catagen (C,D). Catagen progression is also delayed compared with wild type. At P21, wild-type follicles have entered telogen, whereas *Msx2* knockout mutant follicles are still in catagen (E,F). At P24, control skin has entered anagen, while *Msx2* knockout mutants are still in telogen (G,H). M*sx2* knockout mutant skin eventually re-entered anagen at P31. Examination of *Tgfa* expression supported the histological observation (E,F, insets). No *Tgfa* expression was detected in wild type hair follicles at P21 whereas strong *Tgfa* expression was still present in *Msx2* knockout mutant hair follicles (arrow, in inset of F). Scale bars: 500 μm. Quantification (below) was carried out by counting about 50 hair follicles (at designated days after birth) and converting them into percentage of hair follicles in different hair cycle stages. Orange is anagen, green is catagen and blue is telogen. Error bar represents one standard deviation. (Right column) Regeneration after plucking showed defect in re-entry into anagen. One-month-old mice were stripped with hot wax and followed at 6, 10, 14 and 20 day post-stripping. Note that normal hair follicles re-enter anagen at day 6 (C,E), while those of *Msx2* knockout mutants remain in telogen (D,F), and do not enter anagen until 20 days after stripping (H). At this time, control skin has re-entered telogen (G). Quantification is achieved as described in the development column. Scale bar: 500 μm. Arrowheads in E,F indicate dermal papilla stained with alkaline phosphatase.

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.

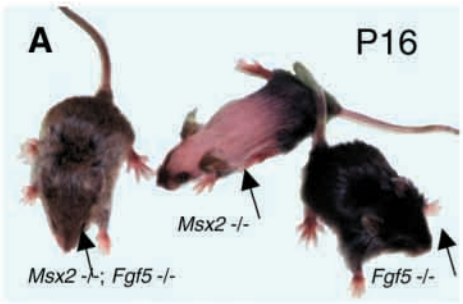
To confirm this result, we examined the expression of *Tgfa*, a marker for the inner root sheath (IRS) and keratogenous zone (Luetteke et al., 1993). By P17, the number of *Tgfa*-expressing follicles was much reduced, and no *Tgfa* expression could be detected in wild-type P21 telogen hair follicles. 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*^{go}/*Fgf5*^{go}) 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



B

Genotype	Time of hair loss P14	P18	P24	P30
Msx2 -/-	48/48	N/A	N/A	N/A
Msx2 -/-; Fgf5 -/-	0/41	9/41	17/41	15/41

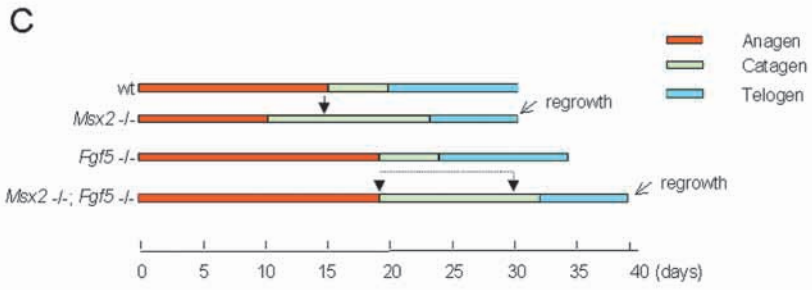


Fig. 4. Cross between Msx2 and Fgf 5^{go}/Fgf5^{go} (Angora) mice. To address the genetic relationship between *Msx2* and *Fgf5* in hair cycle regulation, double mutant mice for *Msx2* and *Fgf5* were generated by crossing the two mutants. Hair loss in *Msx2* knockout mice occurs invariably at P14 (A,B). By contrast, *Fgf5^{go}/Fgf5^{go}* mice grow long pelage hairs as a result of prolonged anagen (A). Mice doubly homozygous for both mutations exhibited long pelage hairs and no longer lose their pelage hairs at P14. Instead, hair loss in these mice eventually occurs between P18 and P30, depending on the genetic background of the mouse (B). (C) The hair cycle length in each genetic mutant. Anagen in double mutant is prolonged, similar to *Fgf 5* mutants. Hair loss still occurs, but is also delayed accordingly. Catagen and telogen in double mutants are approximately similar to that of *Msx2* mutants. The result suggests that hair loss in *Msx2* knockout mutants is associated with a specific time-point in catagen and is delayed by mutation in *Fgf5*, which prolongs anagen length.

crossed to generate *Fgf5^{go}*, *Msx2^{tm1Rilm}* double homozygotes. Examination of 48 *Msx2* knockout mice revealed that hair loss always occurred at P14, regardless of genetic background (Fig. 4A,B). *Msx2^{tm1Rilm}* homozygotes carrying one *Fgf5^{go}* allele exhibited the same cyclic alopecia phenotype as *Msx2^{tm1Rilm}* 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^{tm1Rilm}* homozygous mice, *Fgf5^{go}*, *Msx2^{tm1Rilm}* double homozygotes grew long pelage hairs characteristic of the angora (*Fgf5^{go}*) 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*, *Fgf5* 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^{go}*, *Msx2^{tm1Rilm}* 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 mice 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 mice 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 mice anagen hair follicles showed 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

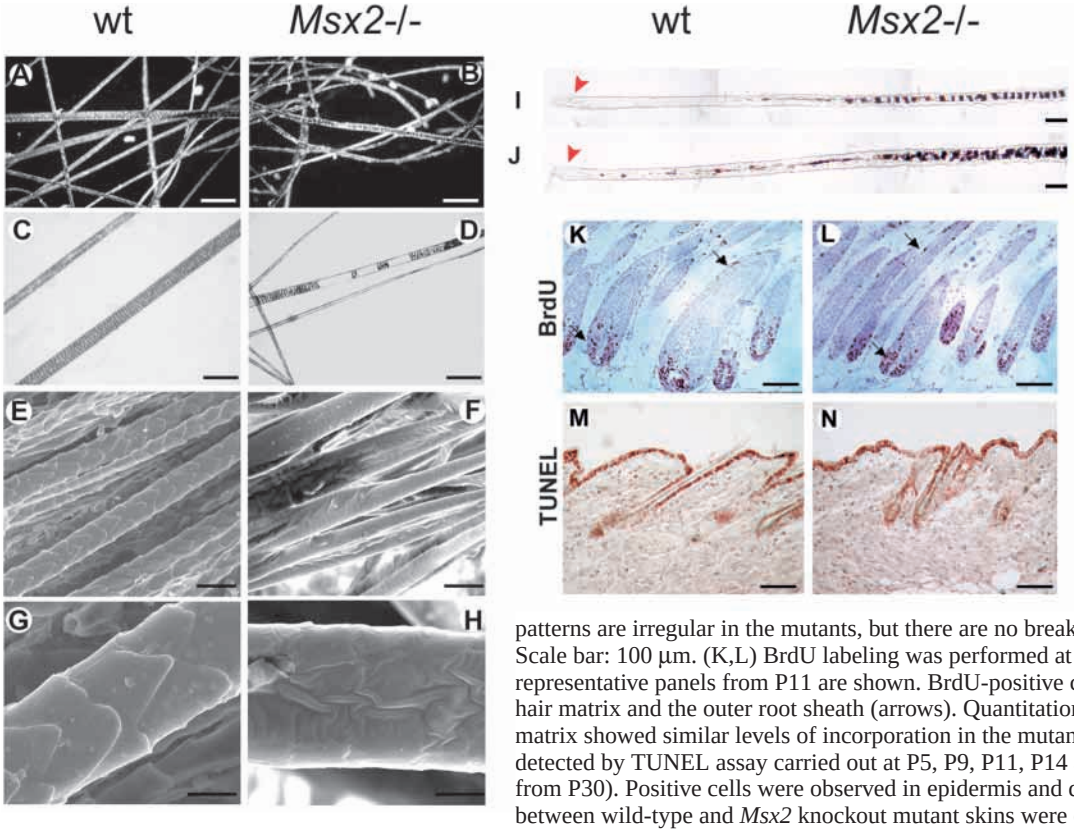


Fig. 5. Changes of hair filament differentiation in *Msx2* knockout mice. (A,B) Plucked hairs from *Msx2* knockout mice are short and curly, reflecting defects in hair structures. Scale bar: 100 μm. (C,D) Medulla patterning is affected as suggested by the irregular septations in the hair. Scale bar: 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.

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

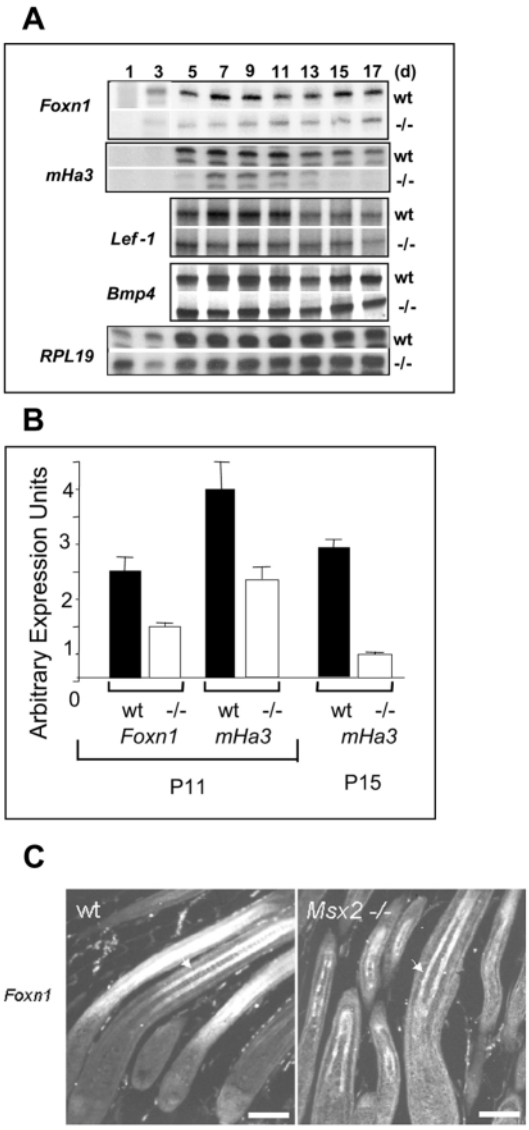


Fig. 6. Changes of other molecular pathways in Msx2 knockout mutant hairs. (A) Timecourse RNase protection assays. Expression analyses of molecules implicated in hair differentiation. A 1 cm^2 dorsal skin sample from indicated time points extracted for RNA. Examination of hair cortex differentiation markers by RNase protection assay revealed significantly lower levels of *Foxn1* and its target gene *Ha3* in *Msx2* knockout mutants. At P13 (postnatal day; d on figure) *Ha3* expression sharply decreases in the mutant skin and is barely detectable at P15 and P17 (A,C). This loss of Ha3 expression correlates with hair loss in *Msx2* knockout mutants. *Lef1* expression in the hair matrix cells and in the wild-type skin, increases from P7 to P11, which was not seen in *Msx2* knockout mutants. Although the difference is not striking, the trend is consistent in different experiments. Expression of two other genes, *Bmp4* and *Tgfa* (not shown) is not affected by the *Msx2* mutation. Scale bar: 200 μm. (B) Quantitation of *Foxn1* and *Ha3* message levels at P11 revealed that *Foxn1* and *Ha3* mRNA is downregulated 50% compared with that in wild-type littermates. A much more dramatic 72% reduction in *Ha3* expression was observed at P15. (C) Indirect immunohistochemistry with an affinity-purified Foxn1 antibody showed reduced Foxn1 protein in Msx2 knockout mutant cortex.

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

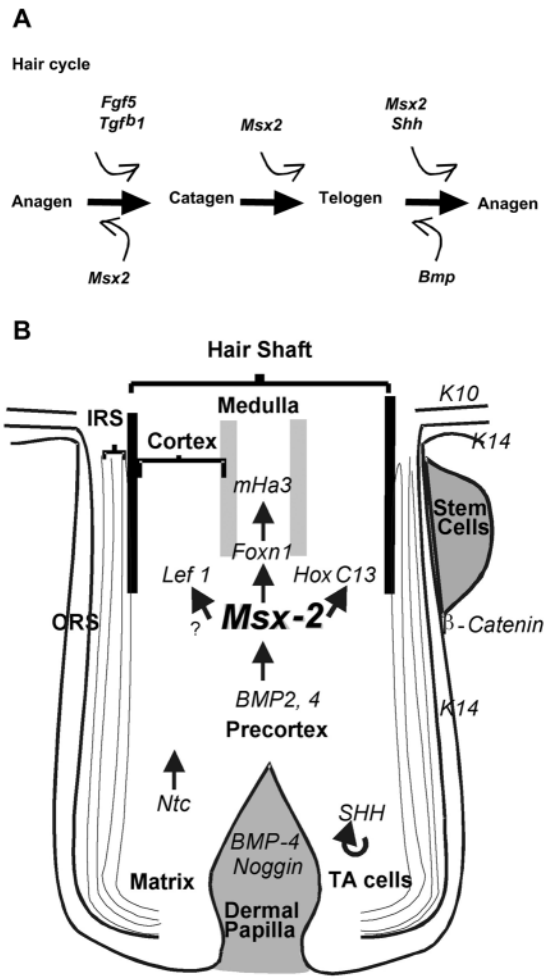


Fig. 7. Summary diagram for hair shaft differentiation and hair cycle regulators. (A) Regulators and check points at each hair cycle phase transition. In essence, the hair cycle is orchestrated by molecules that regulate the transition between anagen-catagen, catagen-telogen and telogen-anagen. Each checkpoint is likely to be regulated by a group of factors. Some promote and some suppress the transition. The observed length of each hair cycle phase reflects the summation of the activities that promote or suppress the entry to the next phase. In the *Msx2* knockout mutants, catagen starts earlier and lasts longer, and telogen hair has difficulty re-entering anagen. Therefore, it is most likely that the normal role of *Msx2* in hair cycling is to maintain hairs in anagen phase. (B) Role of *Msx2* in hair shaft differentiation. Upon induction of dermal papilla, stem cells in outer root sheath (ORS) generate TA cells that migrate to the matrix region. TA cells proliferate to generate cellular masses for making differentiated hair structures, and the regulation of this cellular flow can determine the size of hairs (Wang et al., 1999). The specified cell types are arranged in concentric layers from outside to inside. Several major molecular pathways are known to be involved in this specification and differentiation process (see text for detail). *Msx2* is one of the central integrators that transmits growth factor signals to regulate hair differentiation.

enhance the positive influences converging at anagen-catagen transition, thereby facilitating catagen entry. At the same time, the prolongation of catagen suggests that *Msx2* also plays a positive role in the transition to telogen. Similarly, *Msx2* is also required for anagen re-entry, as *Msx2* knockout mice exhibit a 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, $Fgf5^{go}/Fgf5^{go}$, 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 $Fgf5^{go}$ and $Msx2^{tm1Rilm}$. 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) and Wnt (Millar 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 provides 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 *Msx1* 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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