Development 127, 2421-2432 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV3126

Activation of the Notch pathway in the hair cortex leads to aberrant differentiation of the adjacent hair-shaft layers

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Accepted 15 March; published on WWW 10 May 2000

SUMMARY

Little is known about the mechanisms underlying the generation of various cell types in the hair follicle. To investigate the role of the Notch pathway in this process, transgenic mice were generated in which an active form of Notch1 (Notch$^{\Delta E}$) was overexpressed under the control of the *mouse hair keratin A1* (*MHKA1*) promoter. MHKA-Notch$^{\Delta E}$ is expressed only in one precursor cell type of the hair follicle, the cortex. Transgenic mice could be easily identified by the phenotypes of curly whiskers and wavy, sheen pelage hair. No effects of activated Notch on proliferation were detected in hair follicles of the transgenic mice. We find that activating Notch signaling in the cortex caused abnormal differentiation of the medulla and the cuticle, two neighboring cell types that did not express activated Notch. We demonstrate that these non-autonomous effects are likely caused by cell-cell interactions between keratinocytes within the hair follicle and that Notch may function in such interactions either by directing the differentiation of follicular cells or assisting cells in interpreting a gradient emanating from the dermal papilla.

Key words: Notch, Hair follicle, Mouse, *mouse hair keratin A1*, Cell-cell interaction, Transgenic mice

INTRODUCTION

The molecular mechanisms governing the emergence of specific cellular identities in the hair follicle remain a mystery. The epithelial cells of the hair follicle form a bulb surrounding specialized mesenchymal cells, dermal papilla (DP) cells, which are the source of inductive, short-range signals required for follicular growth and differentiation (Fig. 1A; Hardy, 1992; Sengel, 1976). The lower bulb region contains undifferentiated cells, called matrix cells, whose descendents differentiate into six distinct cell types arranged in concentric rings. The inner three rings, constituting the hair shaft, are the medulla, cortex and cuticle of the hair shaft. The next three rings form the inner root sheath (IRS) and are the IRS cuticle, Huxley's layer and Henle's layer. The outermost ring is outer root sheath (ORS) which is contiguous with the epidermis and may have a separate embryonic history (Coulombe et al., 1989; Kamimura et al., 1997; Kopan and Fuchs, 1989). Each hair follicle regenerates throughout the adult life in a process called the hair cycle, passing through three phases – anagen (growing), catagen (regression) and telogen (resting).

While the progenitor cells generating individual follicles during each growth phase are located in the matrix, the stem cells contributing to rebuilding cycling hair follicles are thought to be located in the bulge, a structure located at the upper third of the follicle (Costarelis et al., 1990). Hair follicles are derived from at least two, and possibly more, stem cells (Mintz and Silvers, 1970). Three models have been proposed for the mechanism by which cells acquire fates in the hair follicle matrix (see Fig. 7 in Kopan and Weintraub, 1993). The first model proposes that matrix cells are derived from lineage-restricted progenitors formed in response to different short-range, instructive DP signals, such that each progenitor generates only a limited subset of follicular cell identities. The existence of such heterotypic (mesenchymal-epithelial) and instructive interactions was demonstrated in avian skin development where dermal mesoderm control the differentiation of ectodermal cells (Sengel, 1976). The second model proposes that multipotent progenitors exist and an instructive, diffusible and long-range DP signal is differentially read at different zones by progeny in the matrix, their fate selected according to their position in the gradient. The third model assumes that the DP signal is permissive and cell-cell interactions among matrix cells ultimately determine cell fates. The formation of hair follicle by epidermal cells exposed to a dental papilla signal supports the permissive signal model (Reynolds and Jahoda, 1991). A combination of the latter two models is possible whereby cell-cell communication may be used to interpret a gradient, as is the case for the R3/R4 cell-fate determination in the fly eye (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999).

Notch is one of multiple signaling molecules, acting as

short- or long-range signals, involved in the formation of the hair follicle (for review, Millar, 1997; Stenn et al., 1994; van Steensel et al., 2000). However, the role for Notch signaling in mammalian hair organogenesis is unclear (Kopan and Weintraub, 1993; Powell et al., 1998). Experimental manipulation revealed that Notch signaling can play multiple roles in development, maintaining cells as undifferentiated progenitors in one tissue and promoting differentiation in another (Bray, 1998; Greenwald, 1998; Kimble and Simpson, 1997; Kopan and Turner, 1996; Panin and Irvine, 1998; Robey and Fowlkes, 1998). Notch is a large transmembrane receptor, which undergoes ligand-induced, Presenilin-dependent, proteolytic release of the Notch intracellular domain (NICD) from the membrane (Chan and Jan, 1998; De Strooper et al., 1999; Kidd et al., 1998; Kopan et al., 1996; Lecourtois and Schweisguth, 1998; Schroeter et al., 1998; Struhl and Adachi, 1998). NICD translocates to the nucleus where it acts as a component of a transcription complex with CBF1/Su(H)/Lag1 (CSL) proteins (Jarriault et al., 1995). Vertebrate targets of the Notch/CSL complex may include *Hairy/Enhancer of Split* genes (Jarriault et al., 1995), *Bcl2*, *Deltex* and *Notch* itself (Deftos et al., 1998). Four *Notch* genes have been identified in vertebrates (Greenwald, 1994; Gridley, 1997). Ligands for Notch are also membrane-bound and they include Delta, Jagged-1 and Jagged-2 in vertebrates (Fleming, 1998). By altering *Notch* gene dosage or Notch activity, it has been shown, in *Drosophila*, that levels of Notch signaling constitutes a feedback loop that can bias cell-fate selection (Heitzler and Simpson, 1991, 1993; Tomlinson and Struhl, 1999). For example, wild-type (2N) cells will adopt the neuronal fate if they are adjacent to cells containing *Notch* trisomy (3N) or activated Notch. In contrast, wild-type cells always adopt the epidermal fate if adjacent cells contain a single copy of the *Notch* gene (1N) or a Notch hypomorph. Mechanistically, Notch activity promotes the postmitotic epidermal fate by cell-autonomously blocking proneural gene activity. Adjacent cells respond by failing to activate Notch and remaining mitotically active as neuroblasts.

Notch1 RNA (Fig. 1C; Kopan and Weintraub, 1993; Powell et al., 1998) and protein (M.-H. L. and R. K., unpublished data) are detected in matrix cells that are not in contact with DP cells, the mesenchymal source of the induction required for follicular growth. Therefore, Notch1 is unlikely to function as a receptor for the short-range mesenchymal signal (see discussion in Kopan and Weintraub, 1993). The expression of *Notch1* overlaps that of the ligand *Jagged1* in precursor cells of the cortex, cuticle and IRS (Powell et al., 1998; M.-H. L. and R. K., unpublished data) and is therefore consistent with a possible role for Notch in proliferation, differentiation or homotypic cell-cell interactions within the follicular epithelium. Notch3 protein is expressed in the precursor and differentiating cortex and cuticle in the hair follicle, overlapping part of the expression domain of Notch1 (M.-H. L. and R. K., unpublished data). On the contrary, *Notch2* is not expressed in the whisker (Weinmaster et al., 1992) or hair follicle (M.-H. L. and R. K., unpublished data). Global activation of Notch signaling via overexpression of Delta in chick embryos results in blocking the initiation of feather buds (Chen et al., 1997; Crowe et al., 1998; Crowe and Niswander, 1998; Viallet et al., 1998), suggesting a role for Notch signaling in patterning the feather follicles. However, these results do not address the role of Notch in the differentiation of epithelial cells within individual follicles. *Notch* loss-of-function alleles cannot be analyzed in knockout mice in many tissues, including the skin, because null alleles of *Notch1* result in embryonic lethality (Conlon et al., 1995; Swiatek et al., 1994).

In an effort to elucidate the role of Notch in developing hair follicles, we examined the development of hair follicles in which Notch1 activity was elevated in one precursor cell type, the cortex of the hair follicle. Our results suggest that homotypic cell-cell interactions play an important role in the differentiation of epithelial cells within the follicle, either to directly regulate differentiation or to interpret a gradient emanating from the DP (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999).

MATERIALS AND METHODS

Production of transgenic mice

The active form of Notch1 (Notch$^{\Delta E}$) was described previously (Jarriault et al., 1995; Kopan et al., 1996). It includes a Myc tag inserted into the *Hin*dIII site at amino acid 2293 in the Notch1 sequence. The *MHKA1* promoter and *Notch*$^{\Delta E}$ in the *pMHKA-Notch*$^{\Delta E}$ construct was derived from *pCS2-Notch*$^{\Delta E}$ (Jarriault et al., 1995) and pMHK113-3.6R (Kaytes et al., 1991), respectively. To generate transgenic mice, the *MHKA-Notch*$^{\Delta E}$ DNA insert was purified and microinjected into the male pronucleus of fertilized egg of FVB/N mice followed by reimplantation of injected eggs into pseudopregnant Swiss Webster females. The purification and microinjection of DNA were performed as described (Hogan, 1994). The transgenic mice were screened by polymerase chain reaction using a *MHKA1* promoter sequence (5′-GCAATGGCTGAAGAAGAGTCCTGA-3′) and a *Notch1* sequence (5′-CTCACTCTTCACGGCCTCAATCTT-3′) as primers. The identities of candidate mice were confirmed by Southern blot analyses. Tail genomic DNA was extracted as described (Hogan, 1994), digested with *Eco*RV and hybridized with a probe from the Exon J of *Notch1* gene (Huppert et al., 2000). The signals were then detected by autoradiography and the number of integrated transgenes were estimated from the ratio of the intensities of the transgenic band to the genomic band using a phosphoimager (Molecular Dynamics).

Hair-pulling test

Hair-pulling tests were performed on the anterior back of mice every 3-7 days starting from P12 (postnatal day 12). Adhesive tapes were applied to the back and pulled gently in the direction of hair growth (Koch et al., 1998). Two of each gender from transgenic mice and one of each gender from wild-type littermates were tested during the 6 month duration.

Monitoring of hair cycling

Hairs of the right back of mice were clipped using a beard clipper every 3-7 days starting from P12. The hair growth was recorded during the period of 4 months to monitor the hair cycling. Several of each gender from transgenic mice and wild-type littermates were tested.

Scanning electron microscopy (SEM)

Hairs plucked from the anterior back of mice were attached to carbon adhesive tabs on aluminum mounts and coated with 350 angstrom gold using Polaron E-500 sputter coater. Samples were observed under Hitachi S-450 scanning electron microscope operated at 20 kV accelerating voltage. Photographs were taken using Polaroid 55P/N film.

Immunohistological analysis

Anterior dorsal skins were collected from mice on P10, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), embedded in paraffin and sectioned at 5 μm. Sections were subjected to deparaffinization (15 minute immersion in XS-3 xylene substitute; StatLab Medical product, Inc., Lewisville, TX) and rehydration. Rehydrated sections were incubated at 37°C for 10 minutes in 0.1% trypsin in PBS (for S100A3 antibody) or boiled for 10 minutes in 10 mM sodium citrate (pH 6.0) in a rice cooker (for other antibodies) to retrieve the antigen and then incubated in 0.3% H_2O_2 in PBS for 30 minutes to block endogenous peroxidase activity. The immunohistological analyses were performed using the peroxidase Vectastain ABC kit (Vector Laboratories, Burlingame, CA) as described in the manufacture's instructions. Sections were incubated in primary antibodies at 4°C overnight. The dilutions of antibodies are 1:10 for AE13 antibody (Lynch et al., 1986), 1:25 for Notch1 antibody (Huppert et al., unpublished data), 1:500 for Myc antibody (c-Myc, A-14; Santa Cruz Biotechnology, Santa Cruz, CA), 1:1000 for S100A3 antibody (Kizawa et al., 1996, 1998) and plasminogen activator 2 (PAI-2) antibody (Lavker et al., 1998), and 1:2000 for GP19 antibody, GP16 antibody (Heid et al., 1988), AE1 antibody (Boehringer Mannheim, Germany) and PCNA antibody (PCNA, PC10; Santa Cruz). The TSA-Indirect kit (NEN Life Science Products, Boston, MA) was used to further amplify signals detected by Notch1 antibody. The signals given by S100A3 antibody were then visualized by the AEC substrate kit (red stain; Vector Laboratories, Burlingame, CA) and those given by other antibodies were visualized by the metal-enhanced DAB substrate kit (brown stain; Pierce, Rockford, IL) as described in the manufacture's instructions. The sections were finally mounted in Crystal/Mount (Biomeda Corp., Foster City, CA) and postmounted in Permount (Fisher Scientific, Fair Lawn, NJ). Some slides were counterstained by hematoxylin before mounting.

Double immunohistological analyses to detect expression of both Myc and S100A3 were performed by adding simultaneously 9E10 antibody (1:1000; Sigma, St Louis, MO) and S100A3 antibody (1:1000) onto trypsin-treated sections. The Myc signal was detected using the peroxidase ABC method with DAB as a substrate as described above. The S100A3 signal was then detected using the peroxidase ABC method with AEC as a substrate.

For detecting trichohyalin granules in the medulla and inner root sheath, rehydrated sections were subjected to Dane's staining as described (Luna, 1968).

In situ hybridization

Anterior dorsal skins were collected from mice on P10 or P33. Skin sections were prepared as described above. For detecting the RNA expression of *MHKA1*, skin sections were hybridized to a digoxigenin-labeled *MHKA1* probe and signals were detected using anti-digoxigenin antibody coupled to alkaline phosphatase as described previously (Kopan and Weintraub, 1993). To detect both *Notch1* and *MHKA1* expression, skin sections were hybridized simultaneously with a digoxigenin-labeled *Notch1* probe and a biotin-labeled *MHKA1* probe. The signals were visualized using anti-digoxigenin antibody coupled to alkaline phosphatase and streptavidin-β-galactoside conjugate, respectively, as described (Kopan and Weintraub, 1993). For detecting the RNA expression of *HeyL*, skin sections were hybridized to a digoxigenin-labeled *HeyL* probe and signals were detected as described (Leimeister et al., 1998).

Keratin extraction and two-dimensional gel electrophoresis

Hairs were plucked from the anterior back skin of mice and the lower 1 mm was cut off from the hair. Hair keratins were extracted as described (Kopan and Fuchs, 1989). The hair (1.4 mg) was sonicated (4× 15 second) at 4°C in 3 ml solution of 0.6 M KCl, 1% Triton X-100, 0.1 M Tris-HCl (pH 7.4), and 0.3 mg/ml phenylmethyl sulfonylfluoride and then centrifuged for 10 minutes at 4°C. The pellet was resuspended and sonicated in the same solution and centrifuged as above. These steps were repeated twice more. The pellet was then resuspended at room temperature in 0.5 ml of 8.5 M urea and 1% dithiothreitol (pH9.5) followed by sonication (4× 5 second) at 4°C to solubilize the hair keratins. The lysate was finally centrifuged for 20 minutes at 4°C and the supernatant was aliquoted and stored at –80°C. For two-dimensional gel electrophoresis, extracted proteins (10 μl) were resolved by isoelectric focusing in the first dimension using the immobilized pH gradient of 4-7 (Amersham Pharmacia Biotech, Piscataway, NJ) as described in the manufacture's instructions. The second dimension was performed by SDS-PAGE with 10% acrylamide mix. The gels were then stained by silver nitrate as described (Oakley et al., 1980).

RESULTS

Elevated Notch activity in the cortex of the hair follicle leads to gross hair and whisker phenotypes in transgenic mice

Although *Notch* RNA and protein are detected in the hair follicle, it is unknown in which cell types of the hair follicle Notch activity is required and whether Notch activity promotes proliferation or differentiation of specific cell types. To investigate the role of the Notch pathway in hair follicle organogenesis, we generated transgenic mice in which a hair-specific promoter from the *mouse hair keratin A1* (*MHKA1*) (Bertolino et al., 1988, 1990; Kaytes et al., 1991) drives ectopic expression of activated Notch1. The activated *Notch* construct used lacks most of the extracellular domain but retains the transmembrane domain as well as the complete intracellular domain. A single Myc epitope tag was inserted to distinguish this molecule from endogenous *Notch1* (see Materials and methods). This *Notch* allele (*Notch*$^{\Delta E}$) is active in the absence of ligands (Kopan et al., 1996; Nye et al., 1994). The *MHKA1* promoter drives expression in precursor and differentiating cells of the hair cortex (Fig. 1B; Kaytes et al., 1991), in the nails and in the tongue. The expression domain of *MHKA1*, and thus *MHKA-Notch*$^{\Delta E}$, overlaps a subset of the expression domain of endogenous *Notch1* in the hair follicle (Fig. 1C; Kopan and Weintraub, 1993). The *MHKA1* promoter is expected to maintain the pathway activated ectopically only in cortical cells that would otherwise turn *Notch* off as they begin to differentiate (Kopan and Weintraub, 1993). If Notch was involved in fate selection, no changes are expected if Notch is normally active in the cortex or if elevated Notch activity does not antagonize cortical fate. In contrast, we expect to see a fate change in Notch-expressing cells if molecules involved in cortex fate are substrates to Notch-mediated repression. Independent of its effect on the cortex, if homotypic cell-cell interactions within the follicular epithelium exist, elevated Notch activity in the cortex could affect its two neighboring cell types, the medulla and cuticle. If Notch plays no role in selecting cell fate within the follicle, elevated Notch activity in the cortex could affect proliferation or onset of cortex differentiation. If Notch were to maintain matrix cells as uncommitted progenitors, we would expect activated Notch in the cortex to result in uncontrolled growth, similar to the effect of activated Notch on mammary epithelium (Dievart et al., 1999; Smith et al., 1995).

Four heterozygous transgenic lines carrying the *MHKA-Notch*$^{\Delta E}$ construct were identified by polymerase chain reaction (PCR) analyses. Both PCR-positive and -negative pups were further analyzed by Southern blotting. PCR-positive pups showed both endogenous and transgenic *Notch* fragments

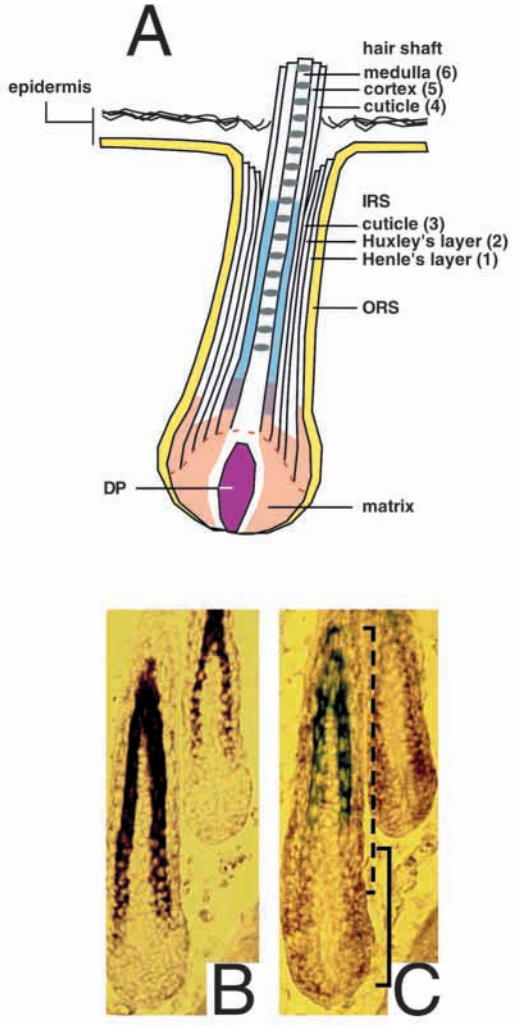

Fig. 1. Expression of *Notch1* and *MHKA1* in the hair follicle. (A) The hair follicle is composed of seven concentric rings of differentiated cell types. The inner six cell types (numbered 1 through 6) are derived from matrix cells (confined by the dashed red line). These epithelial cells form a bulb surrounding the dermal papilla (DP, purple), the source of inductive signals required for follicular growth and differentiation. The mRNA expression domains of *Notch1* (peach) and *mouse hair keratin A1* (*MHKA1*, blue) are indicated, with the overlapped expression domain marked in gray. The keratin K14 expression in the outer root sheath (ORS) layer and epidermal basal layer is marked in yellow. IRS, inner root sheath. (B) Mouse skin sections on P33, the anagen of the second hair cycle, were hybridized with a digoxigenin-labeled *MHKA1* RNA probe. The *MHKA1* expression (brown) was detected in precursor and differentiating cortical cells. The hybridization pattern is similar to that of first cycle follicles (Kopan and Weintraub, 1993). (C) An adjacent section was hybridized simultaneously with a digoxigenin-labeled *Notch1* RNA probe (brown) and a biotin-labeled *MHKA1* RNA probe (blue). The full expression domain of *MHKA1* (dashed bracket) was not detected since the biotin-labeled *MHKA1* probe is less sensitive than the digoxigenin probe. *Notch1* RNA expression (black bracket) was observed in matrix cells not adjacent to DP and in precursor cells of the cortex, cuticle and inner root sheath. *Notch1* expression in the cortex overlaps with part of the *MHKA1* expression domain.

on the blot while PCR-negative pups showed only endogenous *Notch* fragments (Fig. 2A). Measured by the ratio of the intensities between these two bands, we estimated that pups from lines A7 and A5 carry about 6 copies of the transgene, pups from line A3 inherit 4 copies of the transgene, and pups from line A4 carry about 40 copies of the transgene.

The abnormal morphology of whiskers, pelage (coat) hair (Fig. 2B-D) and nails (the nail phenotype will be reported elsewhere) could easily identify animals from lines A7 and A5. Animals from lines A4 and A3 did not show gross hair or whisker phenotypes. Pelage hair from lines A7 and A5 had a sheen appearance from P9 (postnatal day 9) onwards (Fig. 2B). Histological analyses of skin from pups on P9 and P11 failed to detect a difference in the number or distribution pattern of pilosebaceous units between transgenic and wild-type pups (not shown). Skin thickness, DP size and hair follicle orientation were also indistinguishable (not shown). However, the transgenic pups appeared pinkish for a period of time after hair shafts had erupted from the skin. We did not determine if this is due to hair breaking after maternal grooming, shorter hair or delayed eruption of transgenic hair. Transgenic pups appeared covered with wavy hair on P12 and the waviness was more pronounced by P16 (Fig. 2C). Except for a brief period in the second anagen (from P28 to P35) during which the wavy appearance was diminished, wavy, sheen pelage hair persisted throughout the 20 month observation period. Individuals from lines A5 and A7 also displayed curved or curly whiskers upon birth compared to the straight whisker in their wild-type littermates. The whisker phenotypes remained unchanged throughout the 20 month observation period (Fig. 2D).

Elevated Notch activity in the cortex of the hair follicle leads to hair-loss phenotypes and changes in the hair cycle

A hair-loss phenotype, much more prominent in females (Fig. 3A), was observed in line A7. The gross hair-loss phenotype was first detected on about P40 when the hair is in the second catagen or at the start of the second telogen. Hair loss was prominent on the head, anterior back and abdomen with alternating periods of severe and mild hair loss during 6 months of observation. Line A5 displayed similar but milder hair loss than line A7 that was also more prominent in females. Lines A4 and A3 did not show gross hair-loss phenotypes during 6 months of observation.

Hair loss may result from loose attachment of the hair shaft to the cuticle of the IRS (loose anagen), fragile hair shaft, reduced number of hair follicles or premature transition into telogen. No differences in follicle numbers were observed in the transgenic lines in comparison to the wild-type littermates (data not shown). In order to assay if hair loss results from hair fragility or/and loose attachment, coat hair was pulled from the back of these mice along the direction of hair growth (head to tail) using adhesive tape (Koch et al., 1998). These tests were performed on mice every 3-7 days starting from P12. Coat hair from females of line A7 could be pulled out very easily from P18 onwards (the first catagen or at the start of the first telogen) whereas no or only few hairs could be pulled out from wild-type animals (compare Fig. 3B and C). Coat hair from males of line A7 could also be pulled out but to a lesser degree than females (Fig. 3D). Hair removal with adhesive tape can result from either hair fragility or loose attachment. To distinguish between these possibilities, coat hairs were plucked from the back skin of A7 mice and examined under the microscope.

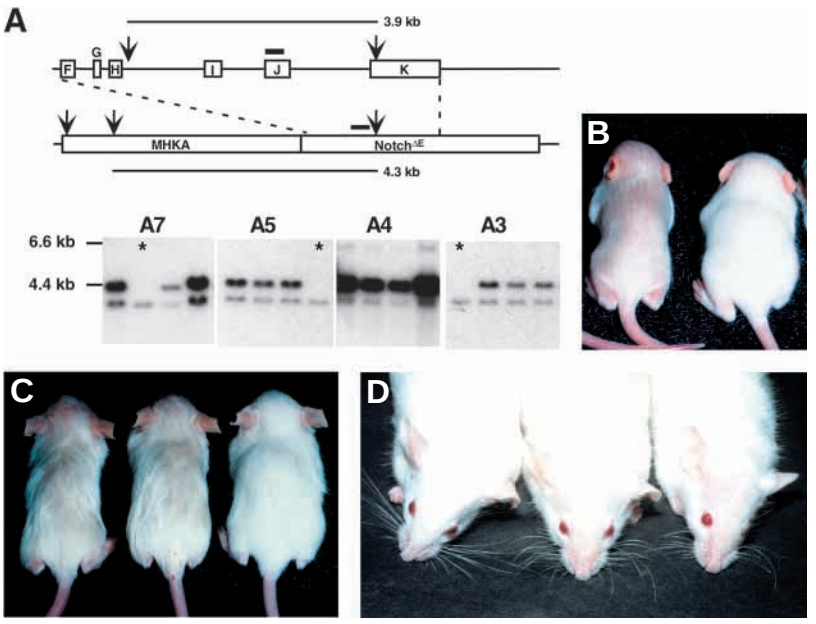

Fig. 2. Identification of heterozygous transgenic lines carrying the *MHKA-Notch*$^{\Delta E}$ construct by Southern blot analyses and gross hair and whisker phenotypes. (A) The upper panel shows the partial genomic structure of *Notch1* (exon F to K) and the *MHKA-Notch*$^{\Delta E}$ fragment used for microinjection, with the corresponding boundaries indicated by dashed lines. The probe used in Southern blot analyses is contained in exon J and marked with a black bar. The size of the hybridized *Eco*RV (arrow) fragments from endogenous and transgenic *Notch1* are indicated. The lower panel shows the Southern blot analyses of *Eco*RV-digested tail genomic DNA of four different lines carrying the *MHKA-Notch*$^{\Delta E}$ construct. Asterisks identify lanes containing DNA from wild-type littermates, exhibiting only bands derived from the endogenous *Notch1* locus. In contrast, heterozygous transgenic mice were identified by the appearance of a second band derived from the integrated transgenic *Notch*$^{\Delta E}$. The ratio of the intensities of these two bands indicates that line A7 and A5, line A4 and line A3 inherited about 6, 40 and 4 copies of the transgene, respectively. Southern blot analyses of tail genomic DNA digested with other restriction enzymes confirm that these lines contain independent integration sites (not shown). (B-D) Hair and whisker phenotypes exhibited by line A7. (B) The transgenic animal (left) appeared pinkish and displayed a sheen appearance of pelage hair on P10 compared to its wild-type littermate (right). (C) The transgenic animals (left and middle) exhibited a wavy appearance of pelage hair on P16 compared to their wild-type littermate (right). (D) The transgenic male (middle) and female (right) showed curved or bent whiskers compared to their wild-type littermate (left). All three animals are 2-months old.

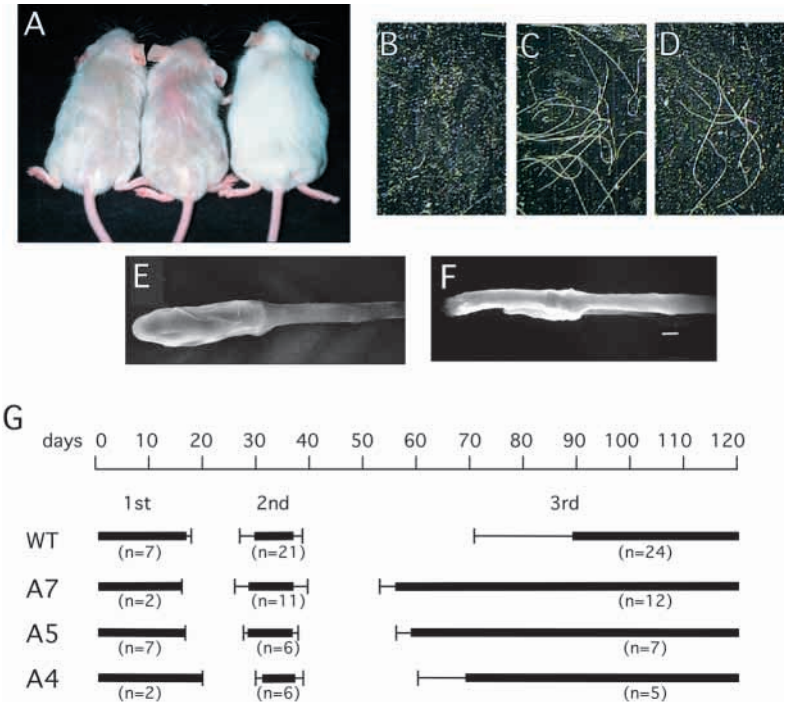

Fig. 3. Elevated Notch activity in the cortex of the hair follicle leads to hair-loss phenotypes and changes in the hair cycle. (A) The transgenic male (left) and female (middle) from line A7 exhibited a hair-loss phenotype compared to their female wild-type littermate (right). All three animals are 6 months old. The hair-loss phenotype was observed from about P40 onwards and was more pronounced in the female. (B-D) Representative pictures of adhesive tape used in the hair-pulling assays on 4-month-old mice. No hair came out from the back of wild-type females (B). More hairs could be pulled out onto the adhesive tape from line A7 females (C) in comparison with A7 males (D), consistent with the more severe hair-loss phenotype in females. (E,F) Analyses by SEM showed that plucked hairs from 19-day-old wild-type animals end in a bulb (E) while those from age-matched line A7 animals have broken ends (F). Bar, 5 μm. (G) Transgenic lines carrying *MHKA-Notch*$^{\Delta E}$ showed changes in the hair cycle. Coat hairs of transgenic lines and their wild-type littermates were clipped on the right back every 3-7 days starting from P12 to monitor the hair growth. Results from three to four independent experiments of each line were combined. Days after birth are indicated on the top. The duration of the first, second and third (and possibly later cycles) anagen is indicated in black bars with standard deviation marked. The numbers (*n*) of animals monitored are also indicated. In all cases, equal numbers of males and females were assayed but no gender differences were observed. In each line, the first two anagen were not recorded in every experiment, so the '*n*' numbers are different from those displayed in the third anagen. The first two anagens of line A7, A5 and A4 appeared to be normal. However, the third anagen in both line A7 and A5 initiated earlier than in wild-type animals (P56±3 in line A7, P59±2 in line A5, and P89±18 in wild-type animals). Line A4 also showed an early onset of the third anagen in five of the seven animals tested (P69±9). Hairs grew in patched patterns in the third and later cycles, in contrast to the synchronous growth pattern in the first two cycles, making it difficult to define later cycles using the assay performed here.

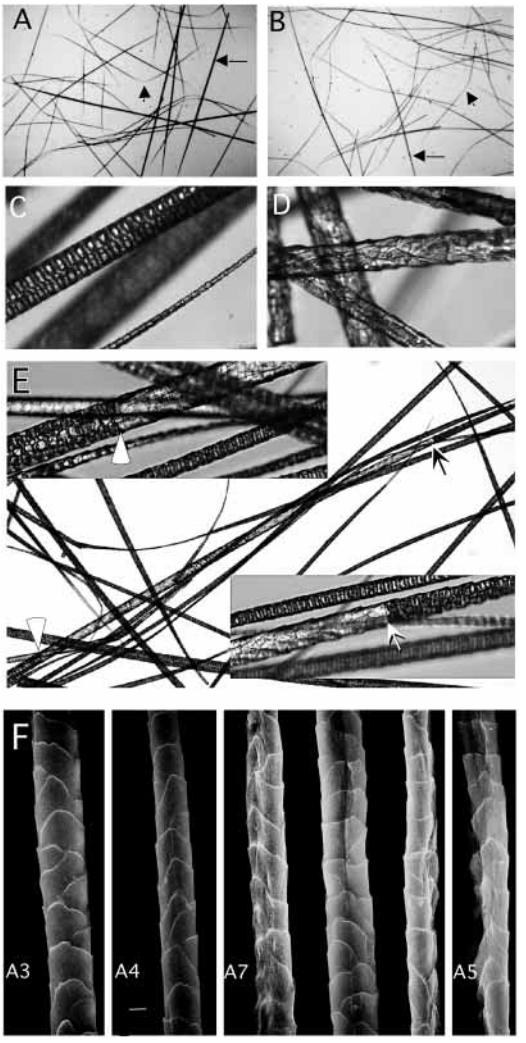

Fig. 4. Elevated Notch activity in the cortex of the hair follicle results in microscopic abnormalities in the coat hair. (A-E) Abnormal morphologies of hair shaft from transgenic mice carrying the *MHKA-Notch*$^{\Delta E}$ construct observed under the light microscope. All hairs were obtained from 16-day-old mice. Comparison of zigzag hair from wild-type (arrowhead in A) and A7 transgenic (arrowhead in B) animals revealed differences in the curvature of this hair type. In addition, non-zigzag hairs (arrow in B) were curved in the A7 transgenic animals and were straight in the wild-type animals (arrow in A). Strikingly, absence of medullar air spaces was detected in all hairs from transgenic line A7 (D) compared to wild-type animals (C). About 40% of hairs from line A4 displayed the same medullar phenotype as in line A7. One hair showing an intermittent phenotype in the medulla is shown in E. This hair showed a transition from wild-type to mutant phenotype and back to wild type. The boundary of wild-type to mutant phenotype is magnified in the upper inset and indicated by arrowheads. The transition of mutant phenotype back to wild type is magnified in the lower inset and indicated by arrows. (F) Morphologies of hair shaft from different lines under the SEM. All hairs were obtained from 16-day-old mice. Coat hair from line A3, like its wild-type littermate, displayed a smooth, organized appearance of the cuticle. In contrast, hairs from line A7 and A5 exhibited a rough, disorganized appearance of the cuticle. Hair from line A4 exhibited a mild cuticle phenotype. Bar, 5 μm.

Most of the hairs plucked from line A7 on P19 (first catagen/telogen) and P26 (first telogen) showed tapered ends, compared to bulb ends of hairs plucked from the wild-type littermate (not shown). Scanning electron microscopy (SEM) confirmed that A7 hairs had broken ends (compare Fig. 3E and F), suggesting that hair fragility was a contributing factor to hair loss. The hair-loss phenotype assayed by hair-pulling tests was also evident in A5 animals, with females showing a more severe hair loss than males. In addition, hair loss in A5 males was initiated during the third anagen (P66, see Fig. 3G) while, in A5 females, hair loss was initiated during the second telogen (P40). To test for changes in the hair cycle, animals from wild type, line A7, line A5 and line A4 were hair-clipped on the right dorsal side every 3-7 days starting from P12 to monitor the hair cycle (Dry, 1926). In all animals, the first anagen ended between P17 and P20, and the second anagen initiated between P29 and P32 and ended between P37 and P38 (Fig. 3G). However, the third anagen in lines A7 and A5 initiated on P57±3 in all animals examined (n=19; A7 at P56±3, n=12 and A5 at P59±2, n=7), compared to wild-type littermates which initiated the third cycle after P89 (P89±18, n=24; Fig. 3G). Finally, five out of seven animals from the A4 line demonstrated an early onset of the third cycle on P69 (P69±9), while the two remaining had an onset of anagen after P100, similar to wild-type animals. Collectively, these results indicate that the hair-loss phenotype and the changes in the third hair cycle observed in our transgenic lines are independent events.

Expression of activated Notch in the cortex results in abnormal differentiation of the neighboring medulla and cuticle

Coat hairs from line A7 and A5 were examined microscopically and showed normal proportion of zigzag hairs to the non-zigzag hair types (guard, awl and auchene). However, zigzag hair from line A7 and A5 displayed reduced curvature while non-zigzag hairs were curved in these lines (Fig. 4A,B). Most strikingly, the air spaces seen normally in the medulla were not detected in the hair collected from these two lines (Fig. 4C,D). Their absence indicates a change in the differentiation program of the medulla, a cell type not expected to be expressing the *MHKA-Notch*$^{\Delta E}$ transgene. To determine if the hair and whisker phenotypes observed correlate with the expression of the transgene, all four lines were analyzed for the expression of the Myc tag used to identify the MHKA-Notch$^{\Delta E}$ protein. Immunohistological analyses of dorsal skin follicles of P10 mice showed that the expression of Notch$^{\Delta E}$ was detected in precursor and differentiating cortical cells of line A7 (Fig. 5B) and A5 (not shown). Notch$^{\Delta E}$ accumulated in nuclei as expected from the expression of this activated form of Notch (Kopan et al., 1996). The expression of transgenic Notch was not detected in the cuticle as the expression of Myc tag (brown in Fig. 5D,F) did not overlap with that of the cuticular marker, S100A3 (red in Fig. 5E,F), a cysteine-rich calcium-binding protein (Kizawa et al., 1996, 1998). The expression of transgenic Notch was not detected in the medulla, either. Line A3 did not show any detectable Notch$^{\Delta E}$ staining (not shown). Surprisingly, line A4 showed a heritable, mosaic expression of the transgene. About half the follicles show transgene expression, but activated Notch was detected in only a subset of cortical cells within these follicles (Fig. 5C). Closer examination of hairs from line A4 uncovered loss of air cells in at least part of the medulla in a

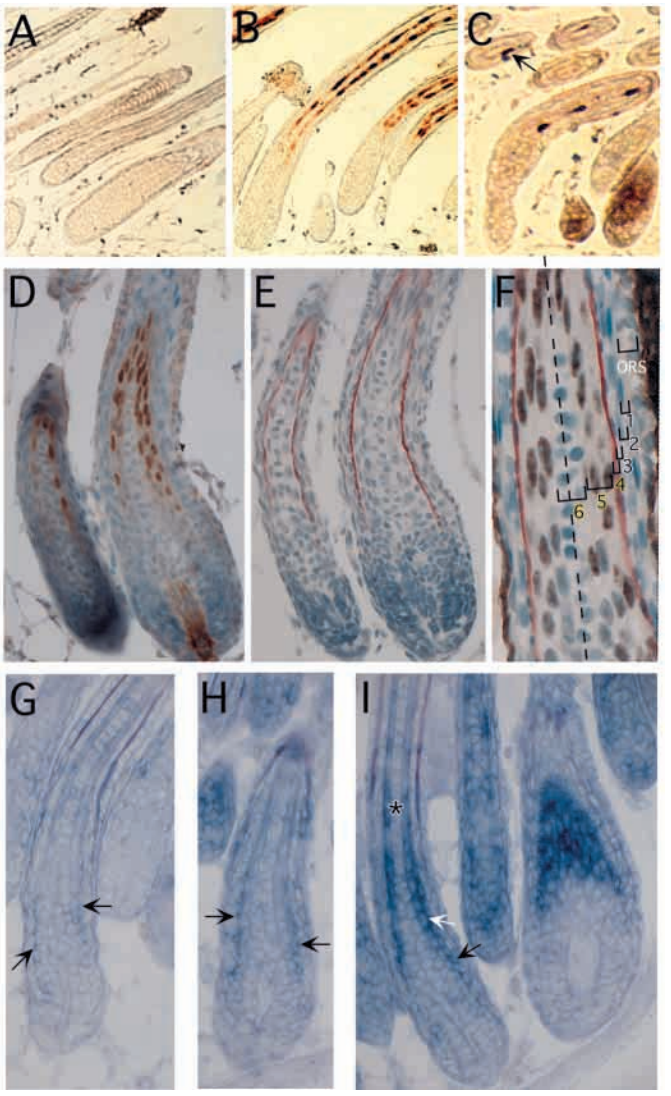

Fig. 5. Immunohistological and in situ analyses of animals carrying the *MHKA-Notch*$^{\Delta E}$ construct. (A-F) Immunohistological analyses on longitudinal sections of dorsal skins from mice on P10. While wild-type littermates showed no expression of Myc-tagged Notch$^{\Delta E}$ (A), skin sections from line A7 showed expression of Myc-tagged Notch$^{\Delta E}$ in the nuclei of precursor and differentiating cortical cells (B). The cortical expression of Myc-tagged Notch$^{\Delta E}$ in transgenic animals persisted within the lower half of the follicle. Notch$^{\Delta E}$ expression in line A4 was mosaic, detected in 40% of hair follicles and in only a subset of cortical cells within those follicles (C). Multiple follicles contain only one cortical cell (arrow in C) expressing the transgene along the circumference of the hair follicle. This expression pattern was heritable, most likely due to position effect. (D,E) Adjacent sections of line A7, immunostained with antibody against the Myc tag and the cuticular marker S100A3, respectively. Despite staining until non-specific Myc-staining is detected in these sections (brown in D; the signal in the DP and ORS is non-specific), Notch$^{\Delta E}$ expression in the transgenic line is restricted to the cortex. Tagged Myc is expressed in cells neighboring to but exclusive from the medulla and the S100A3-expressing cuticular cells (red in E). (D-F) Counterstained with hematoxylin (blue nuclear stain). (F) Higher magnification of the area of an A7 hair follicle above the DP, double immunostained with Myc (brown) and S100A3 (red) antibodies, showing that Myc was only detected in the nuclei of cortical cells. The dashed line marks the axis of symmetry of the hair follicle. Refer to Fig. 1 for the numbering. ORS, outer root sheath. (G-I) In situ hybridization on longitudinal sections of dorsal skins from mice on P10. Wild-type samples showed weak expression of *HeyL* in the precursor and differentiating cuticle (arrows in G and H). In contrast, line A5 showed induced *HeyL* expression in the precursor and differentiating cortex (white arrow in I) in addition to the expression in the cuticle (black arrow in I). *HeyL* expression was never observed in the medulla (asterisk in I).

fraction (up to 40%) of hairs examined. In addition, some hairs show an intermittent phenotype in the medulla (Fig. 4E), consistent with the discontinuous expression of the transgene in this line. Thus, the hair and whisker phenotypes correlate perfectly with the expression of the transgene in the hair follicle. Under SEM, a second phenotypic change was observed. Cuticular cells of lines A7 and A5 displayed rough, disorganized appearances (Fig. 4F). As shown in Fig. 4E, the A4 medullar phenotype, loss of air spaces, detected in Notch$^{\Delta E}$-expressing hairs is indistinguishable from those seen in A7 and A5 hairs. However, only a mild cuticle phenotype was detected in some hairs of line A4 (Fig. 4F). Hairs from the non-expressing line A3 are indistinguishable from wild-type hairs (Fig. 4F).

Several markers were used to perform immunohistological analyses of the skin to investigate if the elevated Notch activity affects the differentiation of various cell types within the hair follicle. Immunoreactivity to AE1 antibody, which detects keratins in ORS (Tseng et al., 1982; Woodcock-Mitchell et al., 1982), appeared to be unaffected in line A7 and A5 (not shown). Distribution of trichohyalin in the medulla and IRS in the lower part of the hair follicle was normal as judged by Dane's staining (Fig. 6A,E). Expression patterns of hair-specific keratins in the cortex and cuticle detected by AE13 (Lynch et al., 1986), GP19 and GP16 (Heid et al., 1988) antibodies were not affected (not shown). Immunostaining to S100A3, a cysteine-rich calcium-binding protein (Kizawa et al., 1996, 1998), was also normal in the cuticle (Fig. 5E) and the cortex (not shown). The morphology of the lower part of the hair follicle also appeared to be normal (Fig. 6A,E). Thus, no overt changes in the differentiation program could be detected in the lower half of hair follicles of line A7 and A5. In contrast, the upper hair follicle in line A7 and A5 was different from that in wild-type animals. Air spaces normally form in the medulla when fully keratinized cells shrink (Fig. 6B-D). In the upper part of follicles in line A7 and A5, cells in the medulla did not shrink and the air spaces did not form (Fig. 6F-H). At the level of the skin surface, the A7 and A5 hairs lacked air spaces, altering the refractive properties of the coat (Figs 5D, 7H). In conclusion, the altered morphology of the hair shaft in line A7 and A5 did not result from an abnormal morphology of the IRS/ORS or subsequent abnormal hair outgrowth, nor did it result from global changes in keratin or trichohyalin expression.

These results suggest that an elevated Notch activity in the cortex results in abnormal differentiation of the two neighboring cell types, the medulla and cuticle. A major concern in interpreting these results stems from the demonstration that Notch is active at nuclear protein concentration below the detection sensitivity of the Myc antibody (Schroeter et al., 1998). Therefore, Notch may be also expressed in the medulla and the cuticle at low levels. To control for this possibility, RNA

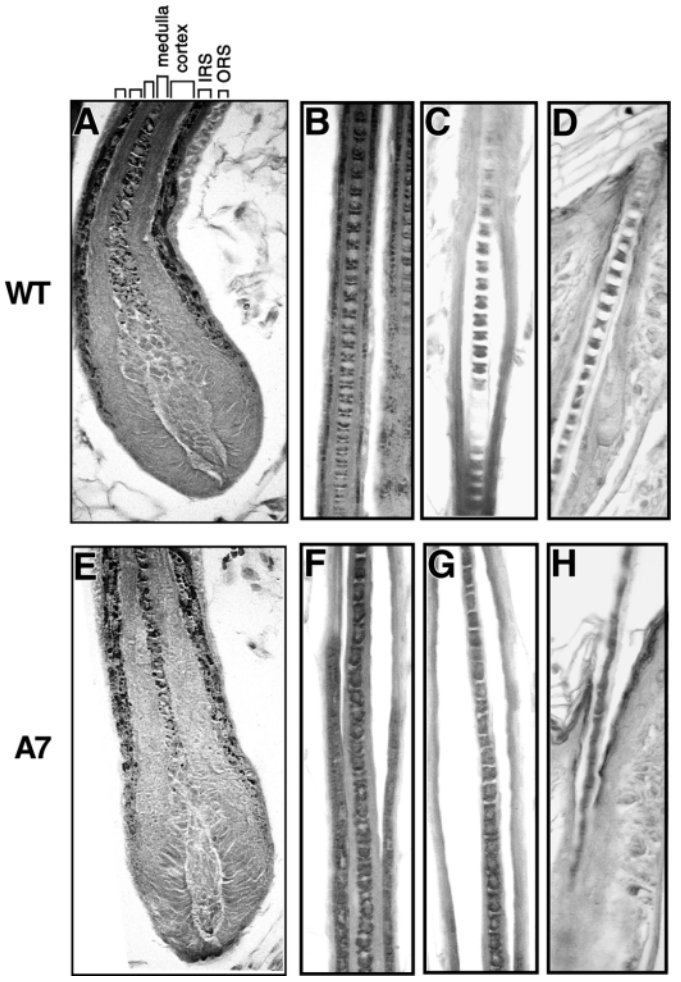

Fig. 6. Elevated Notch activity in the cortex of the hair follicle results in the abnormal differentiation of the medulla. Longitudinal sections of the dorsal skins from mice on P10 were detected for the expression of trichohyalin by Dane's staining. (A-D and E-H) Discontinuous parts of two individual hair follicles from wild-type and line A7 mice, respectively, with D and H the most upper part of the hair follicle. Accumulation of trichohyalin granules in the medulla and IRS in line A7 was normal in the lower part of the hair follicle (A,E). The morphology of the lower part of the hair follicle also appeared to be normal. In contrast, the upper part of the hair follicle in line A7 was abnormal. The medullar cells did not shrink, forming medulla lacking air spaces (F-H). Compare to the organized appearance of air spaces in the medulla from wild-type littermate (B-D).

in situ hybridization analyses were conducted to detect the expression of a novel Notch target in the skin, *HeyL*, a basic helix-loop-helix protein gene (C. L. and M. G., unpublished data). Wild-type samples showed weak expression of *HeyL* in the precursor and differentiating cuticle (Fig. 5G,H). In addition to the expression in the cuticle, both line A7 and A5 showed induced *HeyL* expression in the precursor and differentiating cortex (line A5 shown in Fig. 5I). While we cannot rule out the possibility that ectopic Notch activity is present in the cuticle, the expression of the Notch target was never detected in the medulla in these transgenic lines. Thus, the abnormal differentiation of the medulla (and possibly the cuticle, see below) is triggered by ectopic Notch activity in the cortex.

Expression of structural proteins is unaffected in transgenic mice

Since the antibodies used in this study, AE13 (Bertolino et al., 1988; Lynch et al., 1986), GP19 and GP16 (Heid et al., 1988), crossreact with several hair type I or type II keratins, it is possible that changes in the expression pattern of only a subset of keratins were not detected. Therefore, the phenotypic abnormalities of the coat hair seen in Notch$^{\Delta E}$-expressing animals may still be the result of abnormal keratinization of the hair shaft. To test this possibility, the hard keratin content of the hair shaft from transgenic mice was compared to that of their wild-type littermates by two-dimensional gel electrophoresis. Overall, the repertoire of the type I and type II keratins expressed in animals from line A7 was indistinguishable from those found in wild-type extracts (not shown; Bertolino et al., 1988; Langbein et al., 1999).

Expression of activated Notch in the cortex does not alter proliferation in the hair follicle

Activated Notch is thought to be involved in maintaining epithelial cells as undifferentiated progenitors, since its ectopic activation leads to the oncogenic phenotype in some epithelial cells (Capobianco et al., 1997; Girard and Jolicoeur, 1998; Gray et al., 1999; Smith et al., 1995; Zagouras et al., 1995). The expression of MHKA-Notch$^{\Delta E}$ in line A7 and A5 overlapped with that of proliferating cell nuclear antigen (PCNA) (Szepesi et al., 1994; A7 follicle shown in Fig. 7), indicating that the transgene was expressed in cells prior to their withdrawal from the cell cycle. However, no alterations in PCNA expression were observed in follicles overexpressing MHKA-Notch$^{\Delta E}$, compared to wild-type littermates (not shown). No tumors have arisen in our colony over 20 months. These data indicate that, in the upper bulb region of the hair follicle, cortex cells withdrew from cell cycle at the correct timing in the presence of activated Notch 1. Furthermore, it appeared that the phenotypic abnormalities reported here did not result from changes in cell death in the hair follicle. Elevated Notch activity in line A7 and A5 did not lead to abnormal numbers of picnotic nuclei, a sign of necrotic or apoptotic cell death, nor was the expression of PAI-2, a molecule suggested to protect epithelial cells from apoptosis, changed (not shown; Lavker et al., 1998). Therefore, the activity of Notch reported here is most consistent with a role for Notch in promoting hair differentiation.

DISCUSSION

Notch affects differentiation rather than proliferation in the hair follicle

Notch1 expression is first detected in proliferating matrix cells and is lost as cells begin terminal differentiation. The expression of *Notch1* in hair follicles thus coincides with the transition of matrix cells out from the cell cycle and into six differentiated fates. One possible role for Notch signaling is to maintain matrix cells in an uncommitted and proliferating state. A similar role for Notch was observed in the chick retina where nascent neurons maintain their sibs as dividing precursors via Notch signaling (Henrique et al., 1997). It has also been shown that Notch expression is detected in various epithelial tumors (Gray et al., 1999; Zagouras et al., 1995) and ectopic activation of Notch by viral integration (Int3) can cause

Fig. 7. Elevated Notch activity in the cortex of the hair follicle does not affect proliferation within the hair follicle. Two consecutive, longitudinal sections of the dorsal skins of line A7 on P10 were subjected to immunohistological analyses for the detection of Myc-tagged Notch$^{\Delta E}$ (left) and PCNA (right), a marker associated with proliferation. The transgene expression shown in the left panel is traced in orange and overlaid on the PCNA expression shown in the right panel. The MHKA-Notch$^{\Delta E}$ expression in line A7 was detected in cells prior to their withdrawal from the cell cycle as indicated by the overlapping expression of these two antigens. Proliferation within the A7 hair follicles was identical to that of the wild-type littermate (not shown).

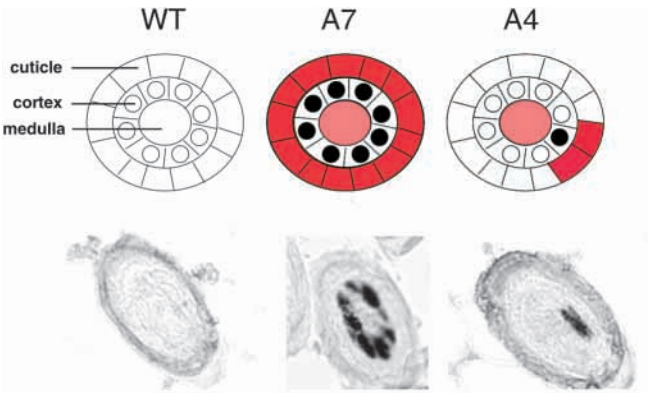

Fig. 8. Proposed mechanism by which cell-cell interactions exert in cell-fate selection within the hair follicle. Upper panels are cartoons depicting the transverse sections of hair shafts from different lines carrying activated Notch in the cortex, showing the correlation of transgene expression and severity of phenotypes. The transgene-expressing cortical cells are marked in black and the affected medullar and cuticular cells are marked in red. Elevated Notch activity in the cortex affects the differentiation of its two neighboring cell types, the cuticle and the medulla, suggesting that there are cell-cell interactions during cell-fate selection within the hair follicle. We hypothesize that activated Notch in the cortex induces the expression of a factor capable of altering the differentiation of adjacent cells. This model is supported by the phenotype of line A4 in which a single cortical cell expressing the transgene along the circumference of the hair follicle is capable of causing a complete medullar phenotype but only a mild cuticular phenotype. Lower panels are photographs of transverse sections of hair follicles corresponding to each line, exhibiting the different number of transgene-expressing cortical cells detected by the anti-Myc antibody

mammary epithelial tumors (Gallahan and Callahan, 1997; Gallahan et al., 1996; Smith et al., 1995). Another possibility is that Notch activity is involved in the differentiation choices of the postmitotic cells, similar to its role in *Drosophila* ectoderm where Notch activity is required in the differentiating epidermal cells but not in the proliferating sensory organ precursors (Campos-Ortega, 1996; Kopan and Turner, 1996).

To test the role of Notch in the development of the hair follicle, we generated transgenic mice in which Notch1 activity is elevated in the cortex at the onset of differentiation, driven by the promoter for the differentiation marker MHKA1. The expression domain of *MHKA1* overlaps that of *Notch1* in the cortex (Fig. 1; Kopan and Weintraub, 1993). In wild-type animals, cortical cells withdraw from the cell cycle earlier than their neighbors (Fig. 7). In the transgenic animals, cortex cells are supplemented with activated Notch as soon as they start to execute a differentiation program and, consequently, may stay longer in an uncommitted, proliferative state. Alternatively, activated Notch may have an effect on cellular differentiation program within the hair follicle.

The transgenic mice that we generated displayed four distinct, and possibly related, phenotypes in their pelage hair. These include sheen appearance of pelage hair, wavy hairs (both pelage and whiskers are affected), hair loss (more prominent in females) and early entry into the third anagen. A fifth phenotype (brittle nails) will be described elsewhere. Abnormal sheen, observed in transgenic lines A5 and A7, is most likely due to the change in refraction caused by the loss of air spaces in the medulla. Alteration in sheen due to changes in the air space within the medulla is reminiscent of the human condition pili annulati (Stroud, 1987). Pili annulati hair has a distinctive sheen, thought to be caused by formation of air spaces in the hair (human hairs do not have air spaces normally). The effects of ectopic Notch activity on hair differentiation may be mediated by maintaining cells in a precursor compartment or by affecting cellular differentiation programs. No change in cell death was observed in the hair follicle of these lines (not shown). We did not observe changes in the immunoreactivity to PCNA (Fig. 7), indicating that no change occurred in the proliferative potential of cortical cells or other cell types. In addition, over a period of 20 months, our mice show no sign of hyperplasia in the hair follicle or the skin. These observations rule out a role for Notch in cell-cycle regulation within the cortex of the hair follicle. Another possible role for ectopic Notch activity could be in regulating the differentiation program of the cortex and, through cell-cell interactions, the neighboring cell types. We show that elevated Notch activity in the cortex results in abnormal differentiation of neighboring cell types, the medulla and the cuticle.

A possible role for Notch in cell-cell communication in cell-fate selection within the hair follicle

Ectopic expression of Notch in the cortex correlates with abnormal differentiation of the two adjacent layers, the central column of hair shaft, the medulla, and the outer layer of hair shaft, the cuticle (Fig. 4). Induction of the expression of Notch target, *HeyL*, was detected in the cortex but not in the medulla (Fig. 5I). Since *HeyL* is expressed in the hair cuticle in wild-type follicles, the possibility that ectopic expression of Notch$^{\Delta E}$

occurs in this cell type cannot be unequivocally ruled out. While it is possible that the addition of few active Notch molecules, undetectable by anti-Myc staining, to the nuclei of cuticle cells is responsible for the alterations detected in cuticular differentiation, we think this to be unlikely (see also Schroeter et al., 1998). Our results are most consistent with the idea that abnormal differentiation of the medulla and the cuticle results from non-autonomous effect of Notch expression in the cortex. One possibility is that the cortex is malformed, losing its ability to function as a scaffold for the differentiation of the adjacent layers. However, no overt change in cortex morphology were detected (Fig. 6). Alternatively, ectopic expression of Notch in the cortex affects the cell-cell interaction within the epithelial compartment of the hair follicle. Observations that support this interpretation come from the mosaic line A4: a single cortex cell expressing activated Notch along the follicle circumference (Fig. 5C) is sufficient to produce a fully penetrant phenotype in the medulla (no air spaces, Fig. 4E) but only a very mild cuticular phenotype (Fig. 4F). Such non-autonomous effects of Notch could be explained if activated Notch induced the expression of a surface or secreted protein in the cortex, capable of altering the differentiation program of neighboring cells. Since the cells in the medulla are encircled by the cortex, they make contact with every cortical cell. Such a hypothetical signal will be thus received by the medulla even if only one cell in the cortex is signaling, inducing the phenotype. On the contrary, the cuticle surrounds the cortex and, consequently, only one or two cells in the cuticle make direct contact with the Notch-expressing cortical cell and will be affected (Fig. 8).

It remains to be determined how the elevated Notch activity in the cortex affects the differentiation of neighboring cells within the hair follicle. No alterations were observed in the hair follicle of line A5 and A7 in the expression patterns of genes involved in the Notch pathway, including Notch1, Notch3 and Jagged1 (M.-H. L. and R. K., unpublished data). A complete study of the expression patterns of all known Notch pathway genes in wild-type and transgenic animals is underway. Several other signaling pathways have been shown to be involved in the development of hair follicles (for review, Millar, 1997; Stenn et al., 1994; van Steensel et al., 2000). The expression in the hair follicle of the transgenic mice appeared to be normal for phosphorylated ERK (a reporter for activated receptor tyrosine kinase; Marshall, 1995) and phosphorylated Smad2 (a reporter for activated TGF-β/activin receptor; Whitman, 1998), indicating that no gross changes occurred in these signaling inputs as a result of Notch activation (M.-H. L. and R. K., unpublished data). Wnt signaling is known to be involved in the inductive interactions leading to the formation of hair follicles (Gat et al., 1998; Kratochwil et al., 1996; van Genderen et al., 1994; Zhou et al., 1995) and in the differentiation of hair shaft (Millar et al., 1999). Notch interacts with components of the Wnt pathway genetically and biochemically (for example, see Axelrod et al., 1996). While we cannot rule out the possibility that Wnt signaling is perturbed in the transgenic mice, the distribution of β-catenin, an effector molecule in Wnt signaling, was unchanged (Willert and Nusse, 1998; M.-H. L. and R. K., unpublished data).

The results presented here demonstrate that cell-cell interactions do occur in the follicle and such interactions can affect follicular morphogenesis. The alteration in the morphology of the medulla and cuticle seen in A4 transgenic mice is reminiscent of the fly eye. Through the use of mosaic clones in *Drosophila* many molecular details involved in patterning and cell-fate selection were revealed. For example, Notch, in conjunction with a postulated gradient in Frizzled activity, acts to elaborate the R3 and the R4 fates in the eye (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999). In the hair follicle, Notch activity may either be directly involved in differentiation of follicular cells (model C of Fig. 7 in Kopan and Weintraub, 1993) or act to assist cells in interpreting their positions in a gradient emanating from the DP (combined model B and C in Kopan and Weintraub, 1993).

Hair loss and hair-cycle alteration in Notch-expressing transgenic mice

Elevated Notch activity in the cortex during anagen resulted in a hair-loss phenotype in line A7 and A5, which was more prominent in females. Our results show that this phenotype is very likely due to the brittleness of the hair. No morphological differences were detected by histology in the cortex, the cell types in which the majority of hair keratins are produced. Although analyses of hair shaft keratin revealed a normal repertoire of hair keratins in our transgenic mice, hair waviness and brittleness may result from changes in other proteins contributing to abnormal fiber formation. One possible explanation for the severity of hair loss in females is that it is enhanced by estrogen since subcutaneous implantation of estrogens has been shown to inhibit hair growth (Hale and Ebling, 1975). That possibility is under investigation. The hair-loss phenotype in line A5 initiated later (on P43 in females, P66 in males) than line A7 (on P18) and was less severe, even though both lines expressed equivalent amount of activated Notch in their cortex. It is possible that this phenotype is modified by the integration loci.

Finally, we observed a substantial shift in the onset of the third anagen in both males and females in our transgenic lines (Fig. 3G). One possibility is that hair loss, displayed by both males and females, contributes to the early onset of the third cycle, similar to the effects of hair depilation on hair cycle. However, we find that early onset of the third anagen is unlikely to be linked to the degree or initiation of hair loss since no correlation is detected between the premature entry into the third cycle (on P57±3) and the onset of the hair-loss phenotypes (P18 in A7, P43 in A5 females, P66 in A5 males).

Even when synchronicity is lost in the rodent hair cycle, hair appears to grow in a stereotypical pattern (Dry, 1926), indicating that regional ‘time keeping’ is in existence. In species that have asynchronous hair cycle, global signals can trigger hair cycle changes, for example during seasonal transitions between winter and summer coat (Houssay et al., 1966). Since MHKA1 is not expressed until after the initiation of anagen, it is possible that Notch$^{\Delta E}$ expression in the previous anagen results in resetting the cycle clock in our transgenic animals. Resetting the cycle clock locally, by delaying the start of anagen, can be accomplished by FGF injection (du Cros, 1993). Global delay in the onset of catagen is seen in *FGF5* null mice (Hébert et al., 1994). Combined, these observations suggest that FGF signaling stimulates transition from anagen to catagen and may be part of the clock mechanism. Agents promoting entry into the hair cycle have also been reported (Jiang et al., 1995; Paus et al., 1989; Takahashi et al., 1998).

The onset of third anagen in line A4 animals initiated at an intermediate time between line A7 and A5 and the wild-type

animals (Fig. 3G). In addition, while all A7 and A5 animals (n=19) displayed early onset of third anagen, in line A4, this was observed at a lower penetrance. Since MHKA-Notch$^{\Delta E}$ is expressed only after anagen initiation, the model most likely to account for the effects of ectopic Notch on the hair cycle is that, during late anagen in normal animals, a substance is produced that inhibits anagen reinitiation. The anagen-promoting effect of activated Notch is either due to reduction in this hypothetical substance or production of its antidote, and can be interpreted in one of two ways. If the cycle clock acts autonomously in each follicle, low numbers of Notch$^{\Delta E}$-expressing cells present in each A4 follicle are insufficient to produce a full effect. Alternatively, the cycle clock is affected by diffusible factors, and the length of telogen is controlled by average amounts of the inhibitor in the neighborhood. The different timing of anagen onset and the incomplete penetrance observed in line A4 could be explained in this case by the presence of wild-type hairs that dilute the anagen-promoting effect exerted by Notch$^{\Delta E}$-expressing hairs. The mechanism by which the cycle clock is affected by Notch$^{\Delta E}$ expression is under investigation.

We would like to thank Drs Paul Kaytes and Gabriel Vogeli, the Upjohn company for their providing with the pMHK113-3.6R plasmid, Dr Kenji Kizawa, Kanebo Ltd., Japan for the antibody against S100A3, Dr Tung-Tien Sun, New York University for AE13 antibody, Drs Hans Heid and Lutz Langbein, German Cancer Research Center, Germany for GP19 and GP16 antibodies, and Dr David Ginsburg, University of Michigan for PAI-2 antibody. We are also grateful to Drs Arthur Eisen and Donald Russ Johnson for the critical comments on the paper, Dr Ici Thalmann for the help in the analysis by two-dimensional gel electrophoresis, Dr Kevin Roth for the help in the immunohistological analysis, Xiang Hua for performing microinjection, Michael Veith for SEM analysis, and Darlene Stewart and Teresa Tolley for their technical assistance in making paraffin-embedded sections. MHL would like to thank the Dermatology Foundation and Pharmacia & Upjohn for supporting this research. RK is supported by PO1AR45254.

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