The disruption of Sox21-mediated hair shaft cuticle differentiation causes cyclic alopecia in mice

Makoto Kiso[a,1], Shigekazu Tanaka[b,1], Rie Saba[a], Satoru Matsuda[c], Atsushi Shimizu[d], Manabu Ohyama[d], Hirotaka James Okano[c], Toshihiko Shiroishi[b,e], Hideyuki Okano[c], and Yumiko Saga[a,e,2]

Divisions of [a]Mammalian Development and [b]Mammalian Genetics, National Institute of Genetics, Yata 1111, Mishima, Shizuoka 411-8540, Japan; Departments of [c]Physiology and [d]Dermatology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan; and [e]Department of Genetics, SOKENDAI, The Graduate University for Advanced Studies, 1111 Yata, Mishima, Shizuoka 411-8540, Japan

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Hair is maintained through a cyclic process that includes periodic regeneration of hair follicles in a stem cell-dependent manner. Little is known, however, about the cellular and molecular mechanisms that regulate the layered differentiation of the hair follicle. We have established a mutant mouse with a cyclic alopecia phenotype resulting from the targeted disruption of *Sox21*, a gene that encodes a HMG-box protein. These mice exhibit progressive hair loss after morphogenesis of the first hair follicle and become completely nude in appearance, but then show hair regrowth. Sox21 is expressed in the cuticle layer and the progenitor cells of the hair shaft in both mouse and human. The lack of this gene results in a loss of the interlocking structures required for anchoring the hair shaft in the hair follicle. Furthermore, the expression of genes encoding the keratins and keratin binding proteins in the hair shaft cuticle are also specifically down-regulated in the Sox21-null mouse. These results indicate that Sox21 is a master regulator of hair shaft cuticle differentiation and shed light on the possible causes of human hair disorders.

hair follicle | keratin | epidermal hyperplasia

Hair is maintained through a cyclic process that includes the periodic regeneration of hair follicles in a stem cell-dependent manner (1–3). Two types of hair loss mutations have been described to date, permanent and nonpermanent alopecia. The defects in the maintenance of specific stem cell populations, mainly caused by a defective β-catenin signaling pathway, may result in permanent hair loss (4, 5). Defects in the maintenance of hair shafts result in a nonpermanent and periodic alopecia, which is exemplified by the Msx2 KO mouse phenotype (6). The germinative layer of the hair follicle is suggested to harbor early progenitors that give rise to the diverse lineages that differentiate into the individual hair follicle compartments (7, 8). The distinctly-layered expression patterns of specific hair keratins in the hair follicle matrix suggest a high level of organization therein, indicating that compartment-specific lineages originate at distinct proximo-distal levels (9, 10). The regulatory mechanisms that govern the layered and restricted expression of these genes are only partly understood however, and no lineage-restricted transcription factor had been functionally identified before our current study to our knowledge.

Sox genes encode evolutionarily conserved DNA binding domains (HMG domains), and *Sox21* is known as a member of the B group of this gene family that also includes *Sox1–Sox3* and *Sox14* (11, 12). Sox21 has been shown to be a repressor of neuronal differentiation in PC12 cells (13). However, this protein has also been shown to promote neuronal differentiation in the chick neural tube (14). In either case, Sox21 functions as a transcriptional repressor, although the resulting effects of this activity in the cell appear to be context-dependent. In addition, although Sox21 is known to be expressed in other cell types, its function has only been reported in neuronal tissues thus far. In our present study, we report our finding of an unexpected function of Sox21 in hair follicle development.

Results

The Lack of Sox21 Results in Cyclic Alopecia. As a first step to elucidate in vivo function of Sox21, we have generated a Sox21 KO mouse (Fig. S1). The homozygous mice were born normally but showed an unexpected and striking phenotype, cyclic alopecia. The mice started to lose their fur from postnatal day 11 (P11), beginning at the head and progressing toward the tail region of the back (Fig. 1*A*). Between ≈P20 and P25, these Sox21-null mice eventually lost all of their body hair, including the whiskers, thus manifesting a completely hairless phenotype. Intriguingly, new hair regrowth was initiated a few days later but was followed by renewed hair loss. Although it became gradually disrupted with age, such synchrony was clearly observable for at least 2 more cycles with an ≈20-day interval (Fig. S2*A*), and the asynchronous cyclic alopecia continued for >2 years with no obvious sex influence (Fig. S2 *C* and *D*).

To investigate the process of periodic hair loss in Sox21-null mice in more detail, hair follicle morphogenesis and the subsequent hair cycle were analyzed. We examined the mid-dorsal skin histology between P1 and P50. First, we did not observe any differences in hair cycle progression among genotypes. Catagen commenced at P14, shifted to telogen by P20, and reentered the anagen stage at P26 (Fig. 1*B*). To confirm that normal hair cycle regulation occurred in the Sox21-null mice, we performed a hair stripping test. Five days after stripping, anagen hair follicles reappeared in both WT and Sox21-null mice and displayed a normal progression of the hair cycle in both cases (Fig. S2*B*). In addition to hair loss, histological abnormalities in the homozygous skin were observed. Epidermal hyperplasia was detectable at P6 and a thickened epidermis was very prominent at P15–P16 (Fig. 1*B*). Another visible abnormality was an enlargement of the sebaceous glands (Fig. 1*B*) that was already evident by Oil-red O staining at P12 (Fig. 1*C*) and became a major component of the dermal layer at P14–P15. These abnormalities were also found to be cyclic and linked to the hair cycle (Fig. S2*E*).

The Expression of Sox21 Is Well Associated with Phenotype Observed in Sox21-Null Mouse. To investigate the cause of phenotype observed in Sox21-null mouse, we examined the time and place of expression of this gene. The strong and specific expression of *Sox21* was observed within a layer of the hair follicle bulb and suprabulbar area, but was completely absent in the mutant (Fig. 2 *A* and *B*). We next examined protein expression by using an anti-Sox21 antibody. Although the strongest expression levels of

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[1]M.K. and S.T. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: ysaga@lab.nig.ac.jp.

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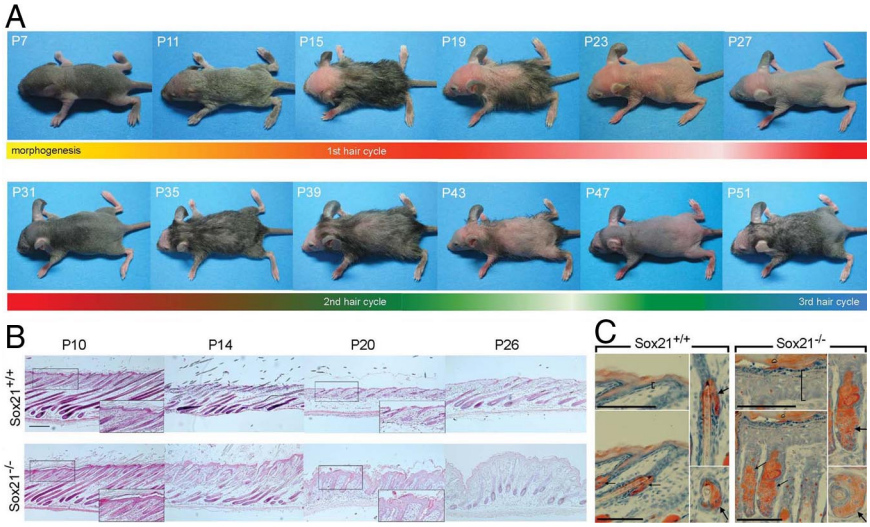


Fig. 1. Cyclic alopecia phenotype and histological features of Sox21 KO mice. (*A*) Hair loss in the same mouse from P7 to P55; images were taken every 4 days. (*B*) Histological changes in the Sox21 mutant mice during different hair cycle stages. (*C*) Oil-red O staining of sections at P12 in Sox21-null mice. Arrows indicate sebaceous glands. Brackets indicate epidermal layers. Magnified images are provided to show epidermal layers and sebaceous glands. Cross-sections for sebaceous gland are also shown. (Scale bars: *B*, 200 μm; *C*, 50 μm.)

this protein were restricted to the hair follicle (Fig. 2), we could also detect weaker expression in the infundibulum and interfollicular epidermis, in which the Sox21 signals did not overlap with those for Filaggrin or K10 but in many cases merged with K14 and proliferating cell nuclear antigen (PCNA)-positive cells (Fig. S3*A*). The expression of Sox21 appears to be regulated in a context-dependent manner; the strong expression in anagen-stage hair follicle became undetectable in telogen stage as hair follicle regressed, whereas the epidermal expression remained relatively unchanged (Fig. S3*C*). However, the epidermal hyperplasia was prominent at catagen stage, and it was accompanied with up-regulation of cell proliferation of Sox21-GFP positive cell lineage from anagen to catagen stage (Fig. S3 *B* and *C*), indicating that Sox21 has repressive effect on cell proliferation.

Next, we concentrated our analyses in the hair follicle because the Sox21 expression in this place was likely to be responsible for the hair loss phenotype. To identify the specific cell types within the layered hair follicle, we used several known keratin markers (listed in Fig. 2*C*). Stronger nuclear Sox21 signals were consistently observed along the hair shaft and were found not to merge either with cells expressing K14 in the outer root sheath (ORS) or K71 in the inner root sheath (IRS) of the hair follicle (Fig. 2 *D* and *E*). Sox21 signals were observed inside the IRS, indicating that the expression occurs in the outer layer of the hair shaft. As expected, the Sox21 signals overlapped with cells expressing K32, a marker for the hair shaft cuticle (Fig. 2 *F*, *F′*, and *F″*) and partially merged with cells expressing K31 (Fig. 2*G*), a known cortex marker (9). However, in WT follicles, K31 appears to be expressed in 2 cell layers, the cuticle of the hair shaft and the most external layer of the cortex. In this regard, Sox21 nuclear signals were detectable only in cells located outside of the cortex (Fig. 2 *G′* and *G″*), indicating that the expression of this gene is restricted to the cuticle of the hair shaft (Cuh).

We next used an anti-GFP antibody to delineate Sox21-expressing cells. No structural differences were detected in the ORS (Fig. 2 *I* and *J*) or IRS (Fig. 2 *K* and *L*) between *Sox21*$^{+/-}$ and *Sox21*$^{-/-}$ mice. However, it was noted that the *Sox21-GFP* signals in the *Sox21*$^{-/-}$ follicle had become thicker and more diffuse (Fig. 2 *J′*, *J″*, *L′*, and *L″*). These signals were observed even in the cortex layer, as shown by double staining with an anti-K31 antibody (Fig. 2 *M* and *N*). *Sox21*-GFP signals were restricted to the cuticle of the hair shaft in the *Sox21*$^{+/-}$ mice, whereas these signals could be detected also in the cortex cell layer in the *Sox21*$^{-/-}$ follicle (Fig. 2 *N′* and *N″*). To elucidate whether the cuticle layer of the hair shaft exists normally in the *Sox21*$^{-/-}$ hair follicle, we examined the expression of K32 and K82 markers, which are cuticle-specific type I (acidic) and type II (basic/neutral) hair keratins, respectively (9, 15). A stronger K82 expression signal was observed in the *Sox21*$^{+/-}$ Cuh (Fig. 2*O* and Fig. S4*A*) but this was markedly decreased in the *Sox21*$^{-/-}$ Cuh (Fig. 2*P* and Fig. S4*A*). K32 exhibited a speckled expression pattern in the *Sox21*$^{+/-}$ mice (Fig. 2*Q*) but showed a uniform pattern in their homozygous counterparts (Fig. 2*R*). These results indicate that the expression of these keratins is attenuated in the *Sox21*$^{-/-}$ hair shaft cuticle. We next examined Sox21 expression in the human hair follicle because this is a candidate as a causative gene for human alopecia. As expected in human, Sox21 expression in the nucleus was restricted to cells in the Cuh monolayer (Fig. 2 *S* and *S′*).

We further examined whether Sox21 is expressed in the progenitor cells or postmitotic differentiated cells by double staining with anti-PCNA antibodies. PCNA signals were indeed detected in the progenitor cells (matrix) that constitute the germinative layers lining the dermal papilla. Moreover, although Sox21 expression was detected mainly in postmitotic cells (Fig. 2*H*), this overlapped with PCNA in the proximal cells (arrowheads in Fig. 2 *H′* and *H″*), indicating that Sox21 expression initiates in the progenitor cells before their entry into the cuticle cell lineage. Hence, Sox21 might be involved in Cuh cell differentiation.

Sox21 Is Required for the Normal Cuticle Formation. The expression of Sox21 in the Cuh strongly suggested that defects in this structure were the most likely cause of the hair loss in the *Sox21*$^{-/-}$ mutant. To address this possibility, we further examined the hair shaft by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Unlike the scaly tile pattern of the cuticle that covers the surface of WT hairs (Fig. 3 *A* and *B*), *Sox21*$^{-/-}$ mouse hairs exhibited a flattened and smooth cuticle by SEM analysis (Fig. 3 *C* and *D*). Furthermore, TEM analysis revealed distinct differences in the cuticle structures between the WT and null animals. It is known that the interface between the Cuh and the inner root sheath cuticle (Cui) form an interlocking structure to anchor the developing

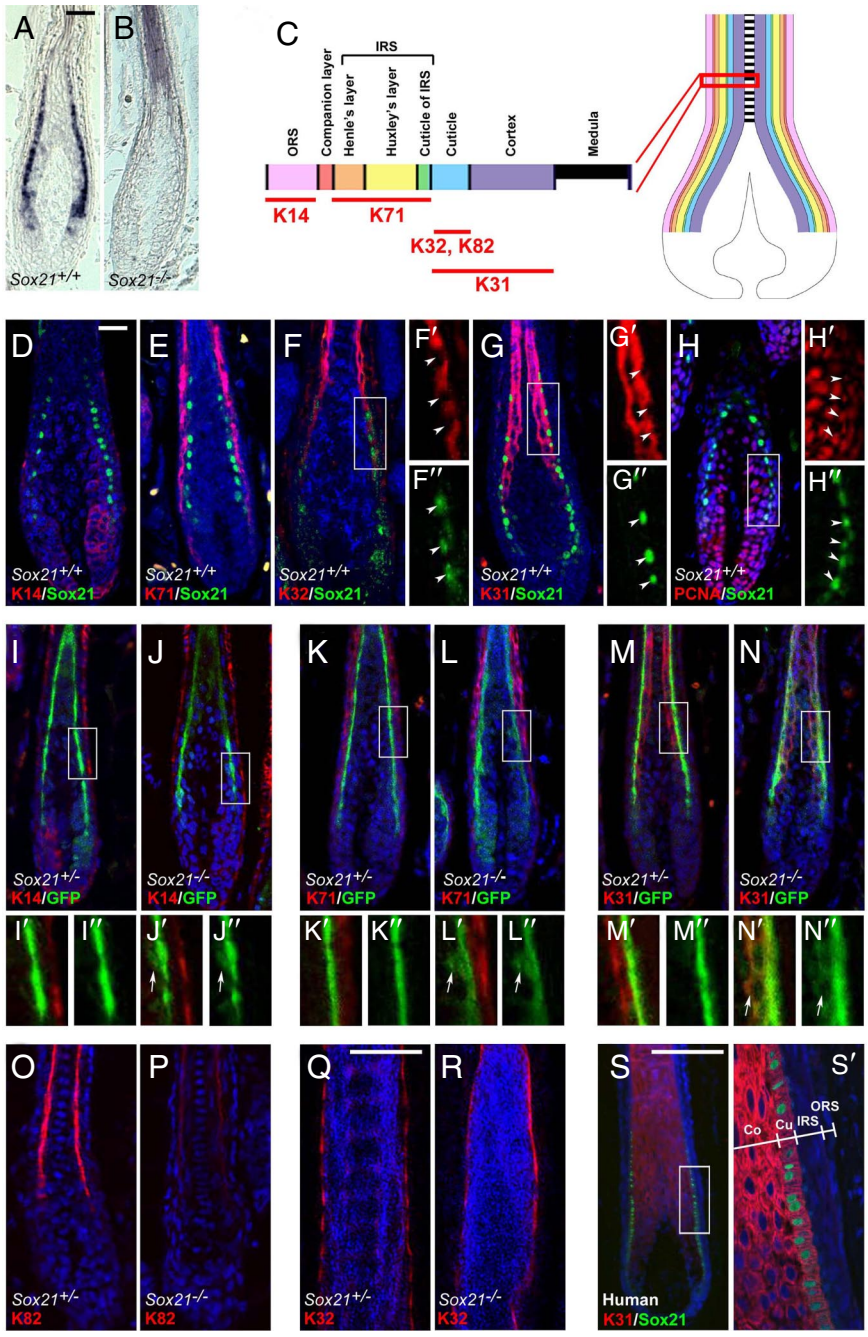
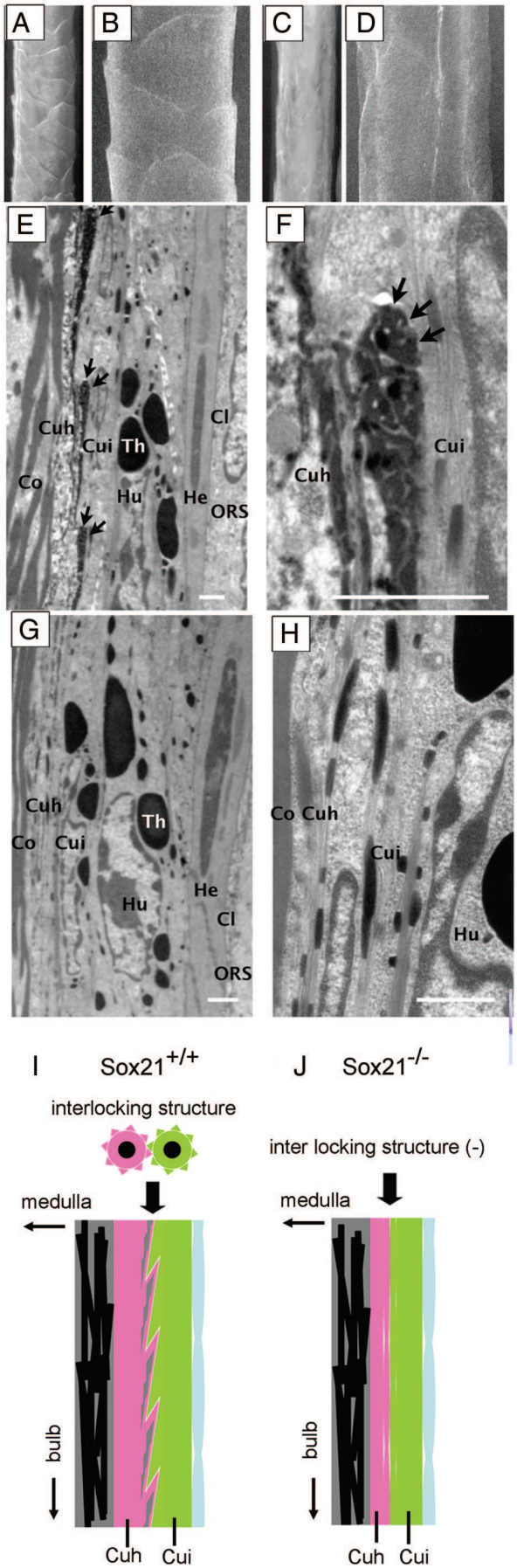


Fig. 2. The restricted expression of Sox21 within the hair follicle. (*A* and *B*) *Sox21* expressions detected by in situ hybridization using a *Sox21* cRNA probe in $Sox21^{+/+}$ (*A*) and $Sox21^{-/-}$ (*B*) at P28. (*C*) A schematic illustration of the layered structure and specific keratins expressed in each follicular layer. (*D–H*) Immunohistological detection of Sox21 in P5 $Sox21^{+/-}$ hair follicles using K14 for the ORS (*D*), K71 for the IRS (*E*), K32 for the Cuh (*F*), K31 for the cortex (*G*), and the cell cycle marker PCNA (*H*). Enlarged images are provided with single confocal channels: *F′* and *F″* for *F*, *G′* and *G″* for *G*, and *H′* and *H″* for *H*. Arrowheads indicate Sox21 signals. (*I–N*) Immunohistological detection of *Sox21*-GFP in $Sox21^{+/-}$ (*I*, *K*, and *M*) and $Sox21^{-/-}$ (*J*, *L*, and *N*) mice using K14 (*I* and *J*), K71 (*K* and *L*), and K31 (*M* and *N*). Enlarged images are shown for double and single (GFP) channels in *I′–N″*. Arrows indicate ectopic *Sox21*-GFP expression in $Sox21^{-/-}$ samples. (*O–R*) Immunohistological detection of the cuticle specific proteins K82 (*O* and *P*) and K32 (*Q* and *R*) in $Sox21^{+/-}$ (*O* and *Q*) and $Sox21^{-/-}$ hair follicles at P5. (*S*) Immunohistological detection of human *Sox21* and K31 in the hair follicle of the calvarial skin. An enlarged image is shown in *S′*. (Scale bars: 25 μm in *A–R*, 250 μm in *S.)*

hair shaft in the base of the hair follicle, which can be seen from an electron-dense aggregate structures in the outermost layer of the WT Cuh (Fig. 3 *E* and *F*) (16). This electron-dense structure is likely to be aggregated keratin filaments as it was found to show positive immunoreactivity to K82 and K32 (Fig. S4*A*). However, in the $Sox21^{-/-}$ mouse, the Cuh layer was ill-formed and the interlocking structure appeared to be missing because the characteristic electron-dense aggregate structure was almost absent in the Cuh, suggesting keratinization failure (Fig. 3 *G*, *H*, and *J*). In contrast, the Cui remained intact in the $Sox21^{-/-}$ mouse (Fig. 3*H*). Although the cuticle of the hair shaft is severely affected in the $Sox21^{-/-}$ mice, the club ends of plucked hairs were intact in these null animals (Fig. S4*B*). However, we frequently observed abnormal detachment of the hair shaft in



tissue sections of *Sox21*$^{-/-}$ skin samples. This frequency was equivalent to that in WT during the anagen stage but increased significantly in the catagen stage in the *Sox21*$^{-/-}$ skin, indicating that the hair shaft can be held in the hair follicle even in the absence of an interlocking structure during anagen. However, the hair shaft cannot be maintained without this structure upon progression to the catagen stage (Fig. S4*C*).

Sox21 Is a Master Regulator of Keratin Gene Network in the Cuh. In an effort to define the molecular mechanisms underlying the distinct structural defects in the *Sox21*$^{-/-}$ mice, we performed microarray analysis of total skin from Sox21 KO versus WT mice at P5. Differential expression was scored by the following criteria: (*i*) genes had to be identified as "present" by Genespring GX software in at least 1 of the 3 genotypes analyzed, 26,223 probe sets; (*ii*) $P \leq 0.05$ in heterozygous versus homozygous, 1,444 probe sets; (*iii*) a >2-fold change in heterozygous versus homozygous, 162 probe sets; and (*iv*) a >2-fold change in WT versus homozygous, 119 probe sets (Fig. 4*A*). Among the 119 probe sets identified as differentially expressed in the mutant skin (5 up-regulated and 114 down-regulated) 18 probe sets showed a >10-fold reduction in the Sox21 null mutant.

The gene ontology software we used revealed that many structural genes involved in the cytoskeleton and cellular adhesiveness were also markedly down-regulated in the Sox21-null mice (Fig. 4*A*). Among these, we identified keratin and its related genes, *Krt32*, *Krt82*, *Krtap5–1*, *Krtap5–4*, and *Krtap12–1*, which are known to be expressed only in the Cuh (9, 10, 17, 18) and validated their down-regulation in *Sox21*$^{-/-}$ skin by quantitative PCR (Q-PCR) (Fig. 4*B*). The results are consistent with the decreased protein expression found for K82 (Fig. 2*P*) but not K32 (Fig. 2*R*). Nevertheless, it is conceivable that the unbalanced expression of structural proteins may lead to a disruption of normal keratin fiber assembly. The down-regulation of *Krt82* and *Krt32* was already evident at P0, which is the early stage of follicle differentiation (Fig. S5). We also examined other genes, which were known to be expressed in the cortex (*Foxn1*, *Krt35*, *Krt85*) (9, 10, 19), and matrix (*Hoxc13*, *Msx2*) (20, 21). None, however, showed a significant reduction in *Sox21*$^{-/-}$ mice (Fig. 4*B*). Because a similar cyclic alopecia is observed in the Msx2-null mutant mouse (6), we speculated that *Sox21* might be one of the downstream target genes of Msx2. However, *Sox21* expression was found to be unaltered in the Msx2-null mouse (Fig. S5), indicating that Sox21 functions in a genetic pathway that is independent of Msx2 and/or Foxn1. The restricted expression of Sox21 in the Cuh cells and the down-regulation of genes exclusively expressed in the Cuh in the absence of Sox21 thus strongly indicate that Sox21 is a master regulator of normal cuticle formation.

Discussion

In this study, we found that the lack of Sox21 resulted in the manifestation of striking phenotypes associated with the specific expressions in the hair follicle and epidermis. The lack in the epidermis resulted in the epidermal hyperplasia, which was

Fig. 3. Defective Cuh in the Sox21-null mouse. (*A–D*) Comparison of hair samples during the second hair cycles from *Sox21*$^{+/-}$ (*A* and *B*) and *Sox21*$^{-/-}$ (*C* and *D*) mice by SEM analyses. (*E–H*) Transelectron micrographs of longitudinally-sectioned anagen hair follicles of P14 WT (*E* and *F*) and *Sox21*$^{-/-}$ (*G* and *H*) mice. Micrographs were obtained from the same anatomical level in the suprabulbar area, where trichohyaline granules (Th) emerge in Huxley's layer. Note that the interface between Cuh and the Cui form an interlocking structure (arrows) to anchor the developing hair shaft at the base of hair follicle (*E* and *F*). Co: cortex; Hu: Huxley's layer, He: Henle's layer, Cl: companion layer, Th: trichohyaline granule. (Bars: 1 μm.) (*I* and *J*) Schema illustrating the ultrastructural anomalies observed in the Sox21 KO mouse.

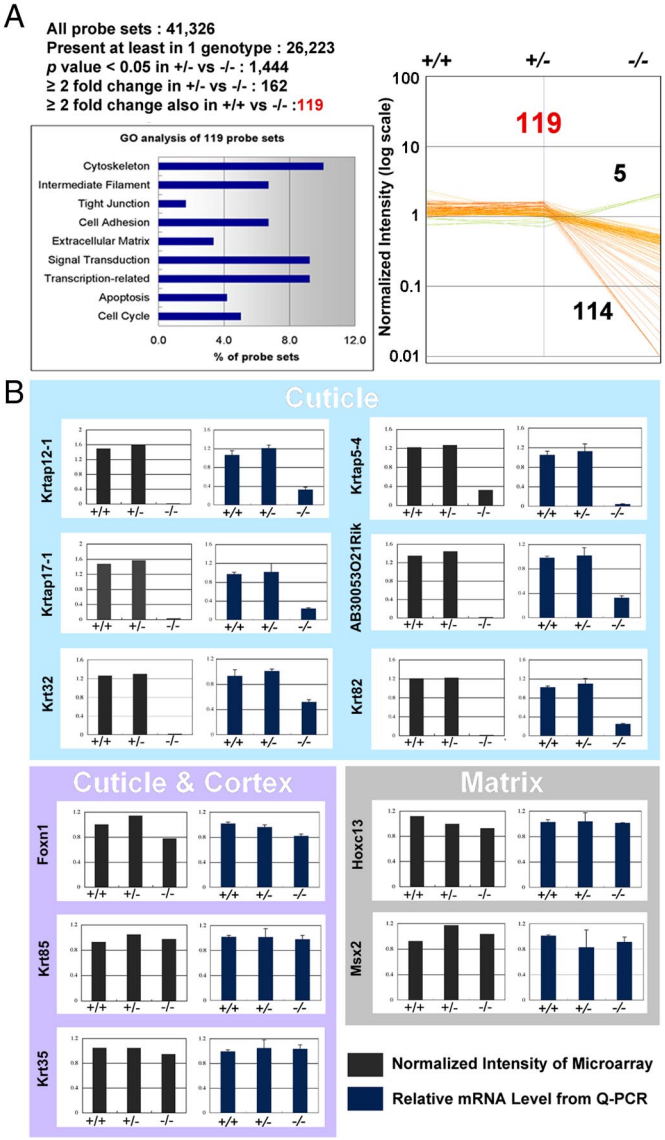


Fig. 4. Sox21 is a master regulator of normal keratinization in the cuticle of the hair shaft. (*A*) Summary of comparative microarray analyses of P5 back skin samples from WT, heterozygous, and homozygous mice. Gene ontology and expression profiling of the 119 probe sets are shown in Table S1 and a graph (*Right*). (*B*) Real-time Q-PCR analysis (blue) confirmed the results of the microarray analysis (black). All of the expression data were calculated relative to the WT levels.

associated with up-regulation of cell proliferation, indicating that Sox21 may play a role in the regulation of differentiation and proliferation of epidermal kerationocytes. However, because the epidermal expression was observed throughout the hair cycle, other factors may also be associated in the hair cycle-dependent hyperplasia. In the hair follicle, Sox21 was found to be a master regulator of hair keratin and keratin-associated protein (KRTAP) genes that are required for proper terminal differentiation of the Cuh (17, 22). The molecular mechanisms by which Sox21 regulates these keratin-related genes are currently unknown. However, the direct activation of these factors by Sox21 is unlikely because Sox21 belongs to the group B2 Sox family and has repressive functions that contrast with those of the group B1 proteins such as Sox1, Sox2, and Sox3, which act as transcriptional activators (11, 12). Indeed, studies in chick, mouse, and zebrafish neuronal cell lineages strongly indicate that Sox21 works as a transcriptional repressor via its C-terminal domain (13, 14, 23). This being the case, the direct target of Sox21 would almost certainly be another transcriptional repressor but that was not indicated by our array analysis. Alternatively, Sox21 might have a partnering factor that masks its repressor domain and functions as a transcriptional activator. Further studies are required to clarify this critical issue.

The differential usage of keratins and KRTAP is a hallmark of the layered structure of the hair follicle (17, 24). The precursor cells are known to be supplied by secondary hair germ, originally derived from bulge stem cells, and finally line the dermal papilla to constitute the germinative layer (7, 25, 26). It has been proposed that upward and laterally moving cells derived from the upper germinative layer differentiate into precursors of the medulla and cortex, whereas cells derived from the lower germinative layer undergo amplification as transient-amplifying cells before differentiating into the cuticle and IRS unit (8). The sorting mechanism that regulates this lineage differentiation is of particular interest but still remains largely unknown. It is noteworthy in this regard that our current observations suggest that Sox21 progenitor cells might have contributed to the cortex layer in addition to cuticle cell layer in the absence of Sox21 (Fig. 2). This might indicate that the cuticle and cortex cells are derived from common precursors and further that Sox21 might be involved in a lineage restriction mechanism. Recently, it has been shown that *Hoxc13* is expressed in the precursors of all 3 hair shaft-forming compartments (cuticle, cortex, and medulla) and mainly regulates the expression of medulla-specific genes by directly regulating *Foxq1* expression (18). However, the expression of both *Hoxc13* and *Foxq1* was found unaltered in the Sox21-null mouse, indicating that Sox21 functions downstream or in parallel with the Hoxc13 cascade in the hair follicle.

Although a mutation in human Sox21 has not been reported to date, we confirmed that Sox21 is also expressed in the hair shaft cuticle in human. It should be noted that the impaired adhesion between the hair shaft and IRS cuticle layers could cause hair anchoring problems in individuals with loose anagen syndrome, a situation with some similarities to that in *Sox21*$^{-/-}$ mice (27). Our findings thus suggested that Sox21 mutations could be responsible for as yet poorly-defined hair loss conditions in humans.

Collectively, the maintenance of Sox21 expression in hair follicle progenitors and its early descendants of the cuticle of the hair shaft are likely to be indispensable for proper keratinization processes in the hair shaft cuticle, which anchors the hair shafts particularly in the catagen phase. The elucidation of the signaling cascades or cellular interactions leading to the induction and maintenance of Sox21 expression would enable new strategies to be developed to treat patients suffering from hair shaft anomalies and alopecia caused by impaired hair shaft anchoring.

Materials and Methods

Generation of Sox21 KO Mouse. Because the Sox21 gene is composed of a single exon, we replaced the coding sequence with EGFP cDNA in frame at the translational initiation site. The detailed targeting strategy is shown in Fig. S1.

Histological Methods. Skin tissue was always obtained from the midback region of age-matched mouse littermates. For HE staining, tissues were fixed in 4% paraformaldehyde before paraffin embedding. Details for immunohistochemistry and antibodies used are described in *SI Text*. For Oil-red O staining, frozen sections were treated in a solution of 60% isopropanol saturated with oil-red O and counter stained with hematoxylin. In situ hybridizations were carried out with frozen sections. The staging of the hair cycle was conducted according to the criteria defined by Muller-Rover et al. (28).

SEM. Hairs from the dorsal skin of mice were attached to carbon adhesive tabs on aluminum mounts and were analyzed by using a SEM (JEOL JSM-5800 LV).

TEM. Back skins of P14 WT and Sox21-KO mice were sampled and fixed in 2% glutaralaldehyde in 0.2 M cacodylate buffer (pH 7.4), followed by postfixation in 1% osmium tetroxide and samples were embedded in Epon 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate solution (29) and observed under a JEOL-1230 TEM (JEOL).

RNA Isolation and Microarray Analysis. Total RNAs were purified by using an RNeasy mini kit (Qiagen). For each hybridization assay, 500 ng of total RNA was labeled with Cy3 and then hybridized to a whole mouse genome oligo microarray (G4122F; Agilent) according to the manufacturer's protocols. Arrays were scanned with a microarray scanner system (G2565BA; Agilent) and the data were analyzed with Genespring GX software, version 7.3.1 (Agilent).

Real-Time Q-PCR. Real-time Q-PCR was performed on an ABI7700 light cycler (Applied Biosystems) using the Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) following the manufacturer's protocols. The results were normalized to β-actin levels by using the recommended DDCt method. The primer sequences are listed in Table S2.

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