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## Transgenic mice display hair loss and regrowth overexpressing mutant *Hr* gene

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**Abstract:** Mutations in the hairless (*Hr*) gene in both mice and humans have been implicated in the development of congenital atrichia, but the role of *Hr* in skin and hair follicle (HF) biology remains unknown. Here, we established transgenic mice (TG) overexpressing mutant *Hr* to investigate its specific role in the development of HF. Three transgenic lines were successfully constructed, and two of them (TG3 and TG8) displayed a pattern of hair loss and regrowth with alternation in the expression of HR protein. The mutant *Hr* gene inhibited the expression of the endogenous gene in transgenic individuals, which led to the development of alopecia. Interestingly, the hair regrew with the increase in the endogenous expression levels resulting from decreased mutant *Hr* expression. The findings of our study indicate that the changes in the expression of *Hr* result in hair loss or regrowth.

**Key words:** hair follicle, hairless, skin, transgenic mice

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### Introduction

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The hairless gene (*Hr*; previously known as *hr*) that encodes a 130-kDa protein (HR), a transcriptional co-repression, is involved in the growth cycle of the mature hair follicle (HF), which consists of growth, regression, and rest stages [3]. Various mutations in *Hr* have been studied to elucidate the development of the hairless phenotype, in which the skin and HF are normal until the first hair cycle is nearly complete and the hair does not grow back even after shedding, and the phenotype exhibits a striking similarity to the change caused by the *Hr* mutation associated with HF dysfunction in mice and humans [1, 4, 6]. The phenotype of the *Hr* mutation likely results from a perturbation of gene expression, as

HR is a transcriptional regulator, and the coding sequence of human *Hr* has been revealed to share 84% identity with that of the mouse, which confirms that *Hr* is highly conserved between them [10].

We have previously reported the existence of a novel nonsense mutation in the coding region of the *Hr* gene designated rhinocerotoc and short-lived (symbol *hr<sup>rhtl</sup>*) (MGI Accession No. 2678250) and successfully isolated and established a mutant mouse strain termed “Yuyi hairless mice.” Our studies identified a homozygous transition, G→A, at nucleotide position 3110 (numbered according to Genbank No. Z32675) leading to the substitution of tryptophan by a nonsense codon. The mutant mice grew a first coat of hair and appeared normal until approximately 12 days after birth. Subsequently,

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hair loss began around the eyelid and progressed caudally, resulting in a completely hairless condition within two weeks with the exception of the vibrissae. As the affected mice aged, the skin became progressively thickened, with a loose and redundant rhinocerotoid appearance, and the mice had a shorter life span [12]. However, the conclusions of the above studies were heavily based on investigations of *Hr* spontaneous mutations. We are particularly interested in the effects of HR regulation of HF growth. Mouse transgenesis affords an excellent approach to investigate the contribution of a particular gene defect to the observed phenotype by targeting the expression of the respective mutant gene in transgenic mice. Here, we produced transgenic mice overexpressing a mutant *Hr* gene to study the effect of its expression on hair structure and development. Surprisingly, the transgenic lines exhibited an interesting phenotype with a pattern of hair loss and regrowth in different domains of the body. We analyzed its pathogenesis and revealed that the altered protein levels were associated with the change in phenotype in the transgenic mice.

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## Materials and Methods

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### *Plasmid construction and preparation of transgenic mice*

Transgenic founder mice were produced on a C57BL/6 background. Full-length cDNAs of the hairless gene (*Hr*) were cloned, and a point mutation (G3427A, RefSeq NM\_021877) was introduced as in our previous study [12]. The entry clones (pDown-mHairless) were obtained by BP reaction cloning (Invitrogen) between attB1-mHairless/IRES/eGFP-attB2 and the entry vector of pDonr221 according to the instructions for Gateway® BP Clonase™ II Enzyme Mix (Invitrogen). The expression vector pRP (Exp)-EF1A>mHairless mutant>IRES/EGFP was successfully constructed by Gateway LR reactions according to manufacturer's recommendation for Gateway® LR Clonase™ II Plus Enzyme Mix (Invitrogen) between the entry clones and pRP.Des3d vector (TGBS140526Q1, Cyagen Biosciences Inc., Guangzhou, China). The recombinant vector synthesized by linearization with *NotI* was purified from agarose gel using a QIAquick Gel Extraction Kit (Qiagen, Chatsworth, CA, USA) and adjusted to a final concentration of 3 ng/μl in 10 mM Tris/0.1 mM EDTA (pH 7.4) buffer for microinjection. Male C57BL/6 mice were mated with female C57BL/6 mice that were superovu-

lated with exogenous gonadotrophins, and 196 fertilized eggs were collected from the oviducts the next morning. Transgenic mice were obtained by microinjection of the linearized DNA solution into fertilized oocytes and were identified by PCR analysis with *Hr*-specific primers (mHairless-F, 5'-CTTGGCACTTGATGTAAT TCTCC-3', and mHairless-R, 5'-CCCACAATGCC-GTTCACAGG-3'; PCR fragment=220bp) under the following conditions: 30 cycles of 94°C for 30 s, 58°C for 35 s and 72°C for 35 s, and then a final extension step of 72°C for 5 min.

Using PCR analysis, seven founders were found to contain the mutant *Hr* gene in their chromosomal DNA. Then, transgenic lines were established by mating with the C57BL/6 strain. Next, 5 of F1 animals shown to be transgenic for mutant *Hr* were intercrossed to produce F2. Five-eight F3 mice were then generated by the same method. Theoretically, a transgenic mouse homozygous for mutant *Hr* can be confirmed if all the offspring are found to be mutant *Hr* positive through mating of the mouse to wild-type partners according to Mendelian principles.

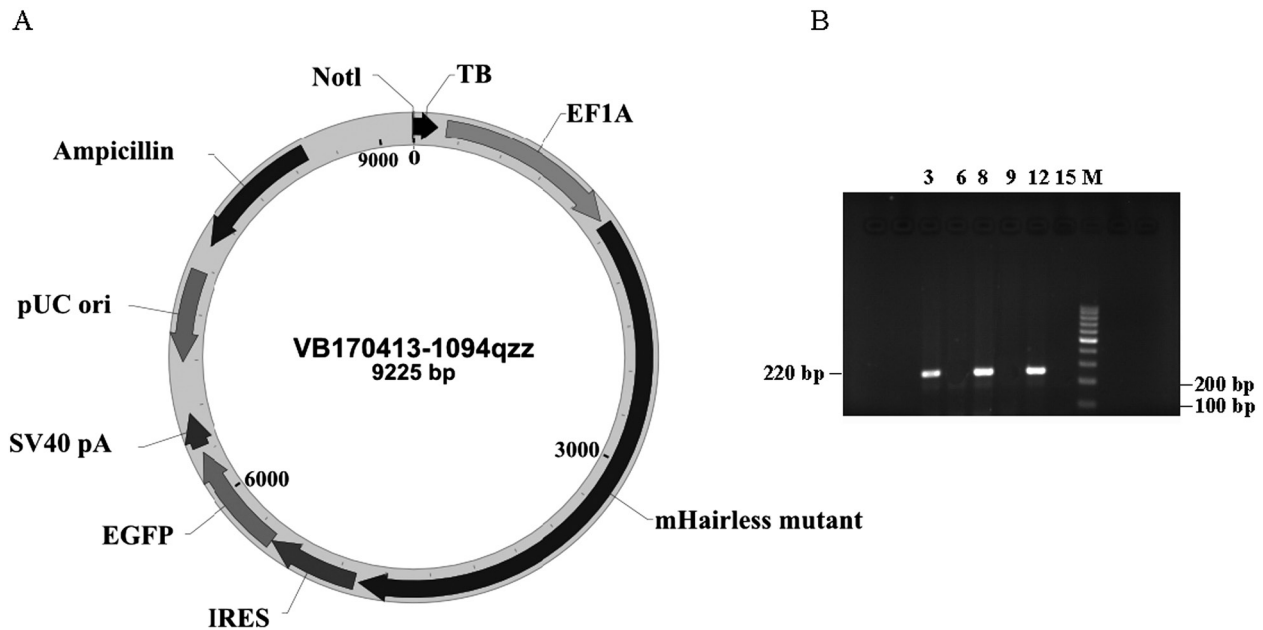
All procedures performed in studies involving animals were in accordance with the ethical standards approved by the Animal Ethics Committee of Zhengzhou University. Animals were maintained on a 12-h light: 12-h dark circadian schedule in a 20–24°C housing room and were provided water and mouse chow *ad libitum*.

### *Histology*

The dorsal skins of the wild-type (WT) and transgenic mice with hair loss were removed immediately, and biopsies were taken from both for histological studies. The skin tissues were cut into pieces fixed in 10% neutral buffered formalin overnight, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E).

### *Expression of transgene*

Total RNA was isolated from skin tissues of wild-type (WT) and transgenic mice with hair loss using TRIzol® reagent (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), according to the manufacturer's instructions. Then, reverse transcription of total RNA into cDNA was carried out using an AMV First Strand cDNA Synthesis Kit (Takara Bio Inc., Kusatsu, Japan). Next, qRT-PCR was performed to detect the mRNA expression of the mutant and endogenous *Hr* genes to compare the differ-



**Fig. 1.** *Hr* expression vector and genotyping of the transgenic mice. (A) The scheme for construction of the expression vector that was used to obtain transgenic mice expressing *Hr*. The ORF of *Hr* was linked into vector pRP.Des3d by Gateway® cloning technology. (B) The transgenic mice were genotyped by PCR analysis as described in detail in the Materials and Methods. Mice #3, #8, and #12, mutant *Hr* transgenic pups recovered; #6 and #9, non-transgenic mice; #15, a wild-type mouse as the negative control. M, 1 kb DNA marker.

ences in the mRNA expression of the *Hr* gene between transgenic and non-transgenic animals. The sequences of the primers for detecting the endogenous *Hr* were as follows: forward, 5'-CAGCGGAAAGTGGAGTTC-TAA-3', and reverse, 5'-TCAGAGCACACAG-GATAGG-3'. QRT-PCR was performed with SYBR Premix Ex Taq (Takara) using a LightCycler 480 Software setup (Roche, Indianapolis, IN, USA). The cycling conditions were initial denaturation for 3 min at 95°C followed by 45 cycles of denaturation at 95°C for 7 s, annealing at 57°C for 10 s, and extension at 72°C for 15 s. The sequences of the primers for detecting the mutant *Hr* were as follows: forward, 5'-AGTGGGTGGAGACTGAAGTT-3', and reverse, 5'-GAGTGGTGTCTACCGAA-3'. The PCR conditions were 3 min at 95°C followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s, and the final extension was for 15 s at 72°C. Data analysis was performed using the  $2^{-(\Delta\Delta C_t)}$  method, in which beta-actin was used as a reference gene [5, 7].

For Western blot, protein samples from skin tissues of wild-type and transgenic mice were prepared according to the protocols of the manufacturer (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Protein concentra-

tion was determined by Bio-Rad protein assay using BSA as a standard. Separated proteins in the polyacrylamide gel were transferred onto a PVDF membrane. The membrane was blocked with 5% nonfat dry milk for 90 min and incubated with the following primary antibodies: HR (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The membrane was then incubated with an HRP-conjugated secondary antibody at room temperature for 1 h. The relative protein expression was normalized against the internal control,  $\beta$ -tubulin.

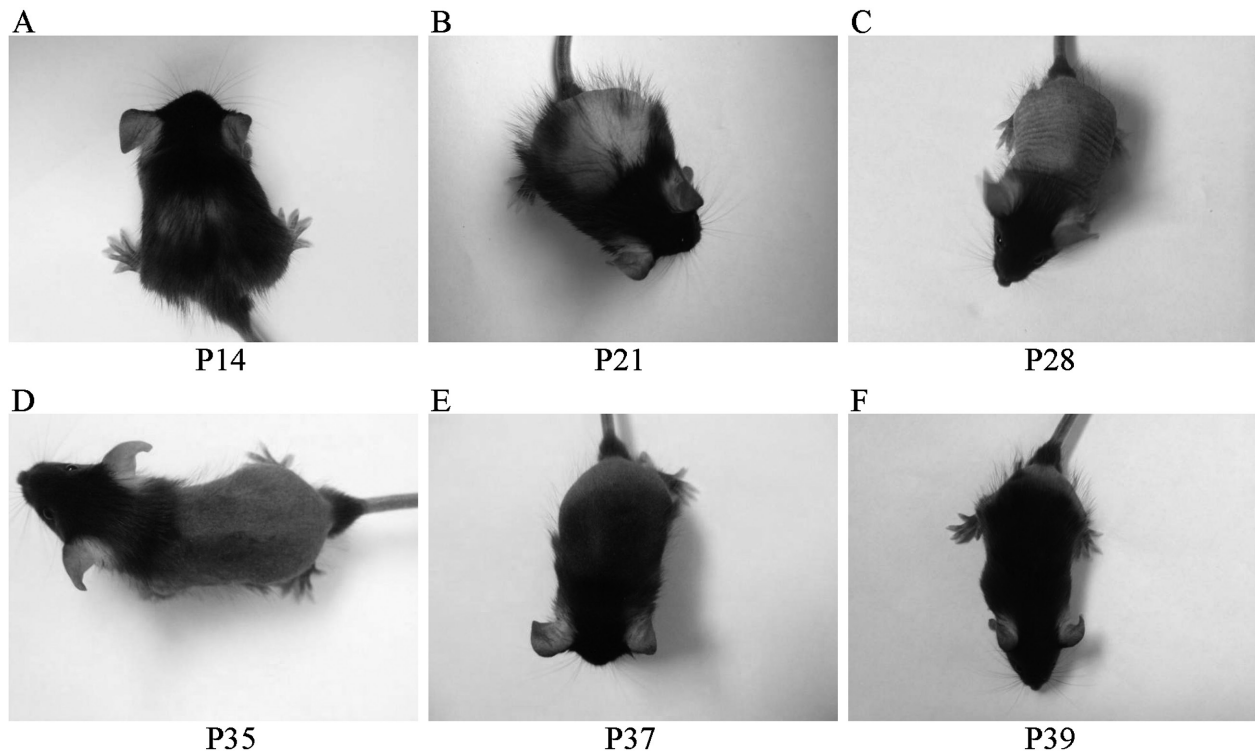
#### Statistical analysis

The data obtained from each mouse were averaged per group, and the standard deviation of the mean values was calculated. Statistical analysis was conducted using Student's *t*-test. The results were expressed as the mean value  $\pm$  SD, and differences at  $P < 0.05$  were considered statistically significant.

## Results

#### Generation of transgenic mice

Transgenic mice were obtained by microinjection of the following constructs into fertilized oocytes (Fig. 1A).



**Fig. 2.** The patterns of hair loss and regrowth in the TG3 mice. (A) Note the initiation of hair loss in the transgenic mouse at P14. (B, C) The hair loss in the mouse proceeds from dorsal regions towards the tail, and no hair loss occurs in the skin of the head from 14 to 28 days of age. (D–F) The regrowth of hair occurs rapidly over the body from P35.

Of 26 pups recovered, seven (referred to as mice #3, #5, #7, #8, #12, #16, and #21) were found to contain the mutant *Hr* gene in their chromosomal DNA using PCR analysis (Fig. 1B). Seven independent lines were subsequently established by mating with C57BL/6 strains, but only three lines (TG3, TG8, and TG12) were successfully constructed. The other lines could not be established due to death or infertility. Approximately 79% F3 mice (46/58) had an abnormal phenotype with hair loss in the T3 and T8 lines, while the hair developed normally in all the F1 and F2 pups. By mating the mice with C57BL/6 strains, all of their offspring were shown to be transgenic for mutant *Hr*. Forty-six F3 mice with hair loss were confirmed to be homozygous for mutant *Hr* (see the Materials and Methods section for the judgment method) and used in the study.

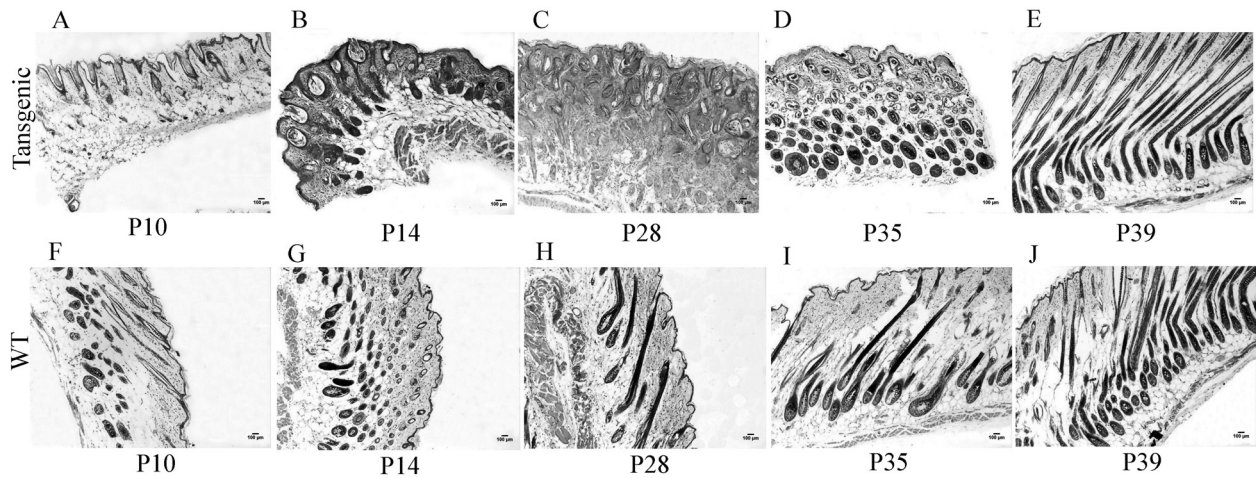
#### *Abnormal phenotype and pathological features in Hr transgenic mice*

Surprisingly, two lines of transgenic mice (TG3 and TG8 but not TG12) exhibited a pattern of hair loss and regrowth. Fifty-eight F3 mice from the TG3 and TG8

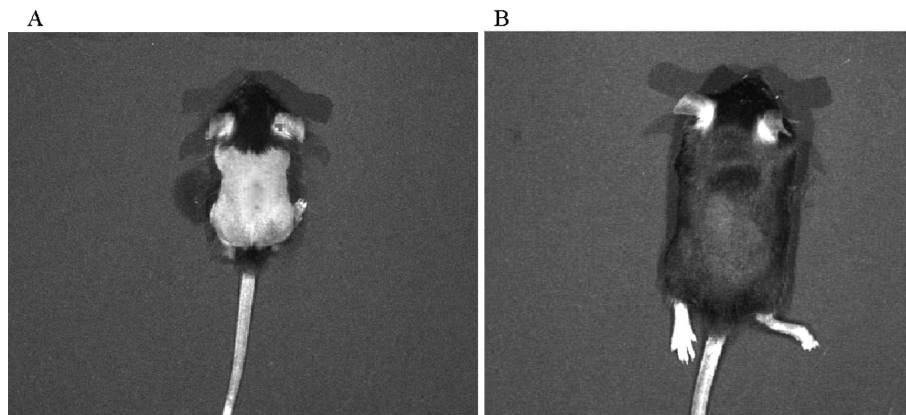
lines were analyzed, and 46 of them transgenic mice showed initial and normal hair development in terms of appearance, but the signs of hair loss were visible at postnatal day 14 (P14) and continued after that (Figs. 2A and B). By postnatal day 28 (P28), the mice developed alopecia, and only a band of hair remained in the skin of the scalp (Fig. 2C). This bald condition lasted for seven days, but afterwards, new hair growth was initiated in the patches of baldness, and the hair coat developed normally until the end of their lives (Figs. 2D–F). There were no differences in the changes in phenotype between TG3 and TG8 mice.

Histological analysis revealed that the first sign of pathological effects was a visible change in the skin of the transgenic mice, namely, obvious dilatation near the top of the pilary canal (infundibulum) at postnatal day 10 (P10). The transgenic hair follicles entered catagen (Fig. 3A), while the wild-type follicles were still in anagen (Fig. 3F). Numerous intradermal follicular cysts formed in follicles of the naked skin of the transgenic mice at P14 (Fig. 3B) and P28 (Fig. 3C) in contrast to the age-matched wild-type mice (Figs. 3G and H). Histo-





**Fig. 3.** Histological photographs of back skin from TG3 and wild-type mice. Note the dilatation of the pilary canal at P10 (A) and utricule formation of the transgenic HF at P14 (B) compared with wild-type follicles (F, G). Anagen is shorter in the transgenic hair follicle, and by P10, the hair follicles have already entered catagen, while wild-type follicles are still in anagen. Note the multiple dermal cysts at P28 (C) and hair regrowth beginning at P35 in the transgenic mouse with hair loss (D, E) when compared with the aged-match controls (H–J).



**Fig. 4.** Detection of green fluorescent protein (EGFP) expression in postnatal day 28 (A) and postnatal day 37 (B) TG3 mice.

logical examination showed that by approximately postnatal day 35 (P35), a contiguous region of transgenic HF regrew, and normal follicles developed with age (Figs. 3D and E), which was similar to the status observed in age-matched wild-type mice (Figs. 3I and J).

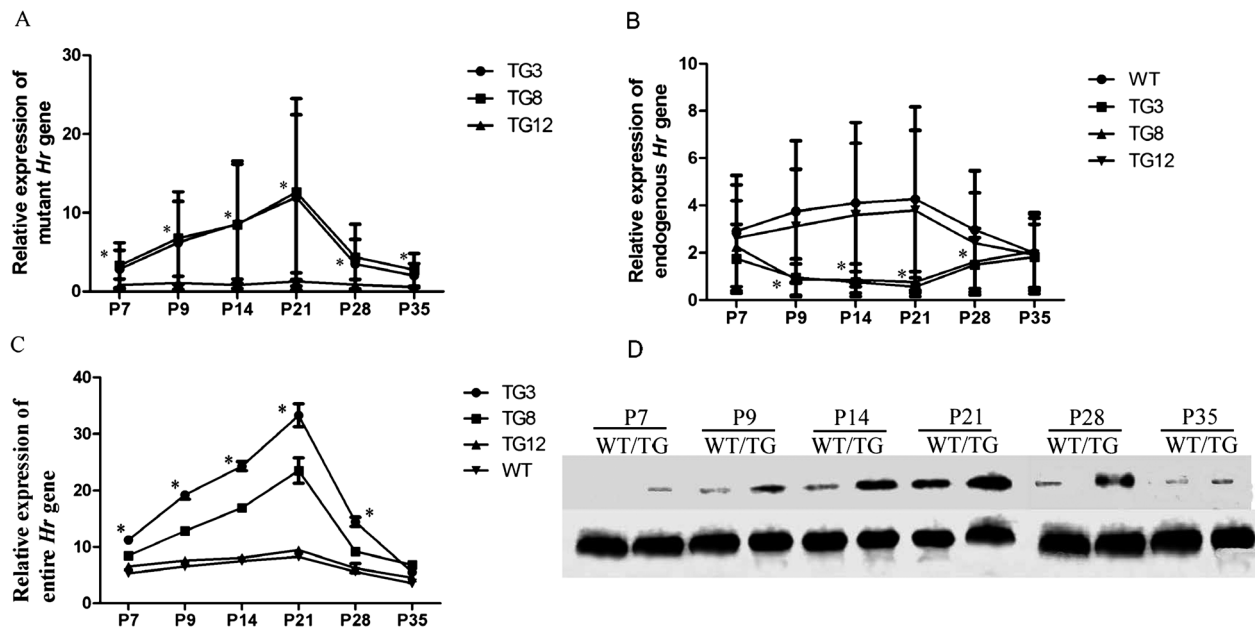
#### *Observation of fluorescence in the transgenic mice*

Green fluorescent protein expression in live mice was evaluated using an *in vivo* imaging system (PerkinElmer, Waltham, MA, USA). The TG3 transgenic strain expressed strong EGFP fluorescence in the naked dorsal skin, which was entirely absent in the haired scalp in a 28-day-old mouse (Fig. 4A). Green fluorescence was not

detected in any of the surface features of the entire body in a 37-day-old TG mouse when its hair regrew (Fig. 4B).

#### *Expression pattern of Hr during HF morphogenesis*

To determine the *in vivo effects* of the Hr-TG G3427A mutation at the molecular level, we compared the expression levels of *Hr* in transgenic and wild-type mice by qRT-PCR and Western blotting. The striking difference in the expression of *Hr* in the skins of the wild-type and transgenic littermates clearly showed that the pattern of hair loss might be deeply affected by the expression level. First, we investigated the mRNA expression of the mutant and endogenous *Hr* gene. The mutant *Hr* expres-



**Fig. 5.** Analysis of *Hr* gene expression in back skin of wild-type (WT) and transgenic (TG) mice. (A–C) The relative mRNA expression levels were measured by qRT-PCR. (A) The mRNA expression levels of the mutant *Hr* gene in wild-type and transgenic mice at P14. The mutant (B) and endogenous (C) *Hr* genes displayed a complex relative mRNA expression pattern during the course of the hair cycle (P7–P35). (D) The total amounts of mRNA expression of the *Hr* gene in the WT and TG mice. (E) Western blot analysis showing HR protein expression in WT and TG mouse skin at the indicated postnatal ages. Tubulin (lower) was used as a protein loading control. All experiments were performed independently in triplicate, and the results were expressed as the mean  $\pm$  SD of three replicates, \* $P < 0.05$  versus the control in each experiment.

sion was significantly increased from  $5.70 \pm 0.36$  to  $24.50 \pm 0.70$  in TG3 mice and from  $5.20 \pm 0.45$  to  $22.43 \pm 1.05$  in TG8 mice from postnatal day 7 (P7) to postnatal day 21 (P21), which corresponds to the time of the hair cycle from the late anagen to telogen stages in the wild-type mice, and then it decreased to  $4.83 \pm 0.65$  in TG3 mice and  $3.50 \pm 0.47$  in TG8 mice at P35 (Figs. 5A and B). The relative mRNA level of endogenous *Hr* in the hair-loss littermates of the TG3 and TG8 lines ( $3.20 \pm 0.30$  to  $0.93 \pm 0.15$  and  $4.20 \pm 0.30$  to  $1.20 \pm 0.30$ , respectively) seemed to be lower than that in the TG12 line and the wild-type mice that did not suffer from hair loss between P7 to P21, with the relative mRNA level ranging from  $4.86 \pm 0.37$  to  $7.16 \pm 0.41$  in TG12 mice and from  $5.26 \pm 0.55$  to  $8.16 \pm 0.35$  in the wild-type mice, respectively, indicating that the mutant *Hr* gene might have an inhibitory effect on expression of the endogenous *Hr* gene (Fig. 5C). The superposition expressions of the mutant and endogenous *Hr* gene demonstrated that the total amount of *Hr* expression of the transgenic individual was significantly higher than that of the non-transgenic one during hair loss (Fig. 5D).

The effect of the transgene on HR protein expression

was assessed by Western blot analysis. HR is a mouse monoclonal antibody raised against amino acids 1–300 mapping at the N-terminus of HR according to the manufacturer's instructions (<https://datasheets.scbt.com/sc-514686.pdf>), which allows for detecting both endogenous and exogenous HR with a mutation at amino acid position 911 (RefSeq NP\_068677). At P9, the anagen phase, the TG3 mice had HR protein expression, which was not detected in age-matched wild-type control mice. HR expression was detected in the wild-type mice at P14, and it gradually increased, with the highest level of expression at P21, but the amount of HR protein in the transgenic mice was greater than that in the age-matched wild-type mice. HR expression was decreased in the transgenic mice from P21 to P28, the period of initiation of a new anagen stage. The expression of HR was low in both TG and wild-type mice at P35, which corresponded to the mid-anagen phase of the secondary hair cycle (Fig. 5E).

## Discussion

We performed gene identification and functional analysis in transgenic animal models to reconstruct the

molecular alterations associated with alopecia. In the present study, the first hair coat of transgenic mice developed normally. However, beginning 14 days after birth, progressive hair loss was initiated that resembled that of the *Hr* mutant littermates sharing the same gene mutation [8, 9]. The most evident effect observed in the transgenic mice was hair regrowth in patches of baldness, which suggested a cycling defect in regulation of the hair cycle and/or a differentiated defect in the hair follicle cells. The histological characteristics of the transgenic mice during hair loss showed a significant widening of the follicular canal and the development of follicular keratosis, which resulted in disintegration of the hair follicle epithelium and the distal portion of outer root sheath (ORS; isthmus) as a result of the transgenic insertion. At P10, the transgenic HF exhibited histological signs of catagen, while those of the age-matched wild-type mice remained in anagen. Therefore, there was an imbalance in the complicated processes of cellular proliferation, differentiation, and apoptosis of pluripotent epidermal cells, which caused this loss of normal epithelial-mesenchymal interactions, which in turn might have led to morphological derangements in HF that produced hair loss in the transgenic mice. The hairs broke easily, and the development of cyst-like structures just below the skin surface and the hair bulb, which was a key structure for hair regeneration, was observed in the dermis of the transgenic mice during hair loss, whereas the skin in both the epidermis and the dermis in humans and mice with a mutation in the hairless gene show utricle formation and prominent deep cysts.

Why did the hairs not break until they reached their full lengths? Why did new hair growth initiate in patches of baldness? Use of the *Hr* transgenic lines generated unexpected results and raised these questions. The striking difference in the expression of *Hr* in the skin of wild-type and transgenic littermates from day 14 to day 35 clearly showed that the patterns of hair loss can be deeply affected by its expression level. First, we investigated the mRNA expressions of the mutant and endogenous *Hr* genes. The mutant *Hr* expression was significantly increased from P9 to P21, after which it began to decline. The endogenous *Hr* expression seemed to be lower in the hair-loss littermates when compared with the levels of TG12 and wild-type mice that did not suffer from hair loss from P7 to P21, which showed that the mutant *Hr* gene might have an inhibitory effect on the expression of the endogenous *Hr* gene, and during hair

loss, the total amount of *Hr* expression of the transgenic individual was considerably higher than that of the non-transgenic one.

Next, we decided to address the HR protein levels to assess whether the mRNA changes were associated with alterations at the protein level. Thus, using Western blot analysis, we found that the HR expression could be detected in the hair-loss mice at P9, the anagen phase of the first hair cycle. Interestingly, the mouse hair follicles in anagen had *Hr* mRNA but not protein expression, which indicated that the HR protein was not expressed concurrently with *Hr* mRNA during the anagen phase of the HF [11]. The expression of the HR protein was dramatically increased in TG mice during hair loss, but it began to decrease when the hair regrew, which suggested that HR played an important role in the development of the HF, most likely in the anagen initiation of HF cycling [2].

The mutant *Hr* transgene appeared to decrease the mRNA of endogenous *Hr*. This result might have been influenced by unknown factors and thus must be considered tentative. Since the exogenous cDNA was from mouse *Hr*, it shared almost the same sequence with the endogenous *Hr*. Given this, they might be under similar regulatory control, resulting in decreased endogenous RNA transcription in the presence of the mutant transgene. The current data support previous findings indicating that the hair follicles of the *Hr<sup>m1Enu</sup>/Hr<sup>m1Enu</sup>* mouse have higher expression of *Hr* mRNA during hair loss but lower expression during the growth of its first hair coat [8], which differ from the reported data indicating that little *Hr* mRNA remains in the skins of *Hr<sup>rh-R</sup>/Hr<sup>rh-R</sup>* and *Hr<sup>-/-</sup>* mice [4, 13]. This inconsistency may result from different mutations in the *Hr* gene. The gain or loss of function of HR leads to alopecia, suggesting that *Hr* mutations can contribute to critical imbalances in the regulation of unidentified pathways. Further research is underway in order to screen key pathways regulated by *Hr* to elucidate the mechanism of alopecia. In addition to elucidating the successive cycles of regrowth and loss, we were interested in examination of the distinctive skin domains of the region of the head in the same transgenic mouse without hair loss. The research on *Hr* expression could facilitate explanation of the abnormal phenotype, but key signal pathways regulated by *Hr* remain to be substantiated by further studies.

In conclusion, an elevated level of HR protein may lead to improper expression of genes that are normally

suppressed by HR protein, resulting in a defect in the development of the transgenic hair follicle. However, with the decrease in the expression of mutant *Hr*, the hair regrows through translational derepression of endogenous *Hr*. That is to say, the phenotype of transgenic mice does appear to be dependent on the expression level of *Hr*. Our findings demonstrate for the first time that the gain or loss of function of HR results in alopecia or regrowth. Transgenic animal models may be a useful tool for studying human hereditary alopecia.

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