# Targeted Expression of Human Vitamin D Receptor in the Skin Promotes the Initiation of the Postnatal Hair Follicle Cycle and Rescues the Alopecia in Vitamin D Receptor Null Mice

Juan Kong, Xiao Jian Li,† Donna Gavin,† Yulei Jiang,* and Yan Chun Li

Departments of Medicine and *Department of Radiology, The University of Chicago, Chicago, Illinois, U.S.A.; †Histopathology Laboratory, Genetics Institute, Andover, Massachusetts, U.S.A.

**Alopecia is a predominant feature of vitamin D receptor inactivation in mice and humans. To determine the role of vitamin D receptor in the regulation of hair growth directly, we used the human keratin 14 promoter to target human vitamin D receptor expression to the skin of transgenic mice, and generated vitamin D receptor null mice that express the human vitamin D receptor transgene. Parallel studies were carried out in littermates of wild-type, vitamin D receptor null, transgenic, and human vitamin D receptor-expressing null mice in two transgenic lines. The transgenic mice were grossly normal. The vitamin D receptor null and vitamin D receptor null/human vitamin D receptor mice were growth retarded and developed hypocalcemia, secondary hyperparathyroidism, and rickets. In contrast to the vitamin D receptor null mice that developed alopecia, however, the vitamin D receptor null/human vitamin D receptor mice displayed a normal hair coat, and their hair shaft and skin histology were indistinguishable from those of the wild-type mice. Immunohistochemical analyses revealed that the human vitamin D receptor was highly expressed in the basal layer of the epidermis and outer root sheath of the hair follicle. During follicular morphogenesis, no major histologic differences were seen in the skin of wild-type, vitamin D receptor null, transgenic, and vitamin D receptor null/human vitamin D receptor littermates. When anagen was induced by hair depilation at day 20 after birth, the vitamin D receptor null mice failed to initiate the hair cycle, whereas the vitamin D receptor null/human vitamin D receptor mice displayed the same pattern of anagen follicle formation as the wild-type mice. Interestingly, the transgenic mice initiated the follicular cycle earlier than the wild-type and vitamin D receptor null/human vitamin D receptor mice in a gene concentration-dependent manner. Taken together, these data provide direct evidence that vitamin D receptor is required for the initiation of the postnatal hair follicular cycle in mice.** ***Key words: alopecia/hair cycle/hair follicles/keratin 14 promoter/keratinocytes/vitamin D receptor. J Invest Dermatol 118:631–638, 2002***

Hair growth consists of two phases: the follicular morphogenesis and the postnatal hair cycle. Follicles form from the embryonic epidermis as a result of signals arising in both the primitive epithelium and the underlying mesoderm. In the process, the epithelium grows down into the dermis and joins at its proximal end a mesenchymal condensation called dermal papilla, which is thought to play an important part in follicular morphogenesis and cycling (Stenn and Paus, 2001). In mice, follicular morphogenesis extends into the third week after birth, which is followed by the first genuine follicular cycle (Muller-Rover *et al*, 2001). The follicular cycle is a regeneration process in the adult tissue, through which new hair shafts are generated to replace the old ones. Each cycle consists of anagen (the growth stage), catagen (the regression stage), and telogen (the quiescence stage) (Hardy, 1992; Stenn and Paus, 2001), and the distinct stages of the murine hair cycle have been well characterized histologically (Muller-Rover *et al*, 2001). Although follicular morphogenesis and cycling are different processes, both are controlled by the interaction between the epidermis and the underlying mesenchyme that involves multiple gene families (Stenn and Paus, 2001).

The vitamin D receptor (VDR) is a member of the nuclear receptor superfamily that mediates the action of 1,25-dihydroxyvitamin $D_3$ [$1,25(OH)_2D_3$], the most active metabolite of vitamin D (Haussler *et al*, 1998). The primary function of the vitamin D endocrine system is to regulate calcium homeostasis in the body (DeLuca, 1988). The notion of VDR playing multiple parts is implicated by its wide expression in various tissues involved in calcium metabolism as well as other functions. Genetic inactivation of the VDR gene in mice resulted in hypocalcemia, secondary hyperparathyroidism, osteomalacia, rickets, and alopecia (Li *et al*, 1997; Yoshizawa *et al*, 1997), phenotypes closely resembling the symptoms of patients with hereditary hypocalcemic vitamin D-resistant rickets (HVDRR) who bear mutations in the VDR gene

Manuscript received September 22, 2001; revised December 10, 2001; accepted for publication December 21, 2001.

Reprint requests to: Dr. Yan Chun Li, Department of Medicine, MC 4080, The University of Chicago, 5841 S. Maryland Avenue, Chicago, IL 60637. Email: cyan@medicine.bsd.uchicago.edu

0022-202X/02/$15.00 · Copyright © 2002 by The Society for Investigative Dermatology, Inc.

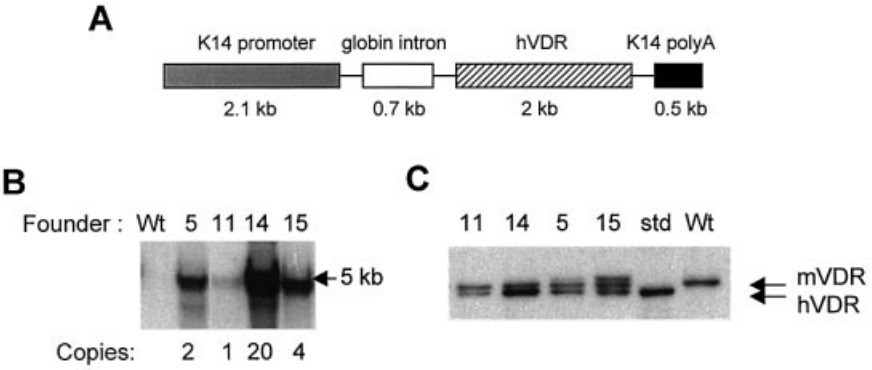



**Figure 1. Generation of Tg mice expressing hVDR.** (*A*) Schematic map of the DNA construct used for microinjection. The 2 kb full-length coding sequence of hVDR cDNA was driven by the human K14 promoter. (*B*) Southern blot analysis of tail genomic DNA from Wt mice and different Tg founders. The DNA (10 μg per lane) was digested with *Bgl*II and hybridized with $^{32}$P-labeled hVDR cDNA probe. The transgene copy number in each founder was shown below the blot. (*C*) Western blot analysis of protein extract (20 μg protein per lane) isolated from the tail skin of Wt mice and different Tg founder mice. The blot was analyzed with anti-VDR antibody. Note hVDR and mVDR display different electrophoretic mobility and are well separated. std, hVDR standard.

(Malloy *et al*, 1999). The parathyroid and skeletal abnormalities in VDR null (Ko) mice were prevented by a diet with high calcium and phosphorus content, which normalized their blood ionized calcium levels, but alopecia persisted in the mutant mice with the normal calcium status (Li *et al*, 1998). Similar results, including alopecia, were recently observed in VDR/retinoid X receptor (RXR)γ double mull mutant mice (Yagishita *et al*, 2001); however, alopecia was not seen in mice ablated for the 25-hydroxyvitamin D 1α-hydroxylase gene or in patients with vitamin D-dependent rickets (VDDR) who carry a mutant 1α-hydroxylase gene (Fu *et al*, 1997; St-Arnaud *et al*, 1997; Takeyama *et al*, 1997; Panda *et al*, 2001). These results suggest that VDR may function in the regulation of hair growth by a ligand-independent mechanism. Most recent studies suggest that alopecia in Ko mice is due to a defect in epithelial–mesenchymal communication required for anagen initiation (Sakai and Demay, 2000; Sakai *et al*, 2001). In the hair follicle, VDR is highly expressed in the outer root sheath (ORS) and dermal papilla (Reichrath *et al*, 1994), but the exact role of VDR in the epithelial and mesenchymal follicular compartments remains unclear.

To assess the role of VDR in hair follicle homeostasis directly, we have used the human keratin 14 (K14) promoter to target human VDR (hVDR) expression to the skin of transgenic (Tg) mice and generated VDR knockout mice expressing hVDR (Ko/hVDR) through breeding. Through parallel analyses of wild-type (Wt), Tg, Ko, and Ko/hVDR mice, we provide direct evidence that VDR is required for the initiation of postnatal hair follicular cycling.

## MATERIALS AND METHODS

**Generation of Tg mice** The K14 promoter was used to drive hVDR expression in Tg mice. To this end, the full-length coding region of hVDR cDNA (ATTC, Manassas, VA) was inserted into the *Bam*HI site of the K14 β-globin cassette (Saitou *et al*, 1995) (kindly provided by Dr Elaine Fuchs, the University of Chicago) in the sense orientation. This cassette contains 2100 bp human K14 promoter sequence that has been shown to direct transgene expression specifically in the basal layer of the epidermis and the ORS of hair follicles (Vassar *et al*, 1989; Wang *et al*, 1997). The K14–hVDR construct (**Fig 1*A***) was released from the plasmid vector by *Eco*RI and *Hin*dIII digestion, purified, and microinjected into FVB mouse embryos at the single cell stage according to the standard procedure (Hogan *et al*, 1986). Founder Tg mice were identified by polymerase chain reaction analysis of tail genomic DNA with the hVDR primers 5′-CGTGTGAATGATGGTGGAGGGAGCC-3′, and 5′-GTCTTGGTTGCCACAGGTCCAGGAC-3′, and by western blot analyses of tail skin homogenates using an anti-VDR antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Two Tg lines derived from founders 11 and 14 (line 11 and line 14) were bred into homozygosity $Tg^{+/+}$, which was verified by southern and western blot analyses as well as by breeding the $Tg^{+/+}$ mice with Wt mice. All offspring from this breeding were heterozygotes ($Tg^{+/-}$).

**Generation of Ko mice expressing hVDR** The generation and characterization of $Ko^{-/-}$ mice have been described elsewhere (Li *et al*, 1997). Two steps of breeding were used to generate $VDR^{-/-}$ mice that bear the hVDR transgene: first, $Tg^{+/+}$ mice from both line 11 and line 14 were bred with $VDR^{+/-}$ mice to produce $Tg^{+/-}VDR^{+/-}$ mice. Then crossing of the $Tg^{+/-}VDR^{+/-}$ mice was used to generate the following littermates that were evaluated in parallel: $Tg^{+/+}VDR^{+/-}$ (12.5%), $Tg^{+/-}VDR^{+/-}$ (25%), $Tg^{+/+}VDR^{-/-}$ (6.25%), $Tg^{+/-}VDR^{-/-}$ (12.5%), $Tg^{+/+}$ (6.25%), $Tg^{+/-}$ (12.5%), $VDR^{+/-}$ (12.5%), $VDR^{-/-}$ (6.25%), and $VDR^{+/+}$ (6.25%). Genotypes obtained in the offspring of both lines approximately followed the expected ratio. All mice were fed an autoclaved rodent chow containing 1% calcium, 0.85% phosphorus, and 4 IU per g vitamin $D_3$.

**Southern blot** Mouse tail genomic DNA was prepared as described (Hogan *et al*, 1986). Genomic DNA was digested with *Bgl*II at 37°C overnight, separated on a 0.7% agarose gel, and transferred on to a nylon membrane. Hybridization was performed using a $^{32}$P-labeled hVDR cDNA probe as described previously (YC Li *et al*, 2001).

**Protein extraction and western blot** Freshly dissected tissues were placed in ice-cold Laemmli sample buffer (Laemmli, 1970) and processed as described previously (YC Li *et al*, 2001). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis according to Laemmli (Laemmli, 1970), and western blot analyses were performed using an antibody against VDR (Santa Cruz Biotechnology) as described before (YC Li *et al*, 2001).

**Blood parameters** Blood ionized calcium levels were determined using a Ciba/Corning 634 calcium-pH analyzer (Chiron Diagnostics, East Walpole, MA). Serum intact parathyroid hormone levels were determined using an enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (Immutopics, San Clemente, CA).

**Bone analysis** The X-ray radiographic analysis of the long bone was conducted using a MX20 Specimen Radiograph System (Faxitron X-ray Corporation, Wheeling, IL) at 18 kVp. For histologic evaluation, the femur and tibia were fixed in 4% formaldehyde in phosphate-buffered saline (pH 7.2) overnight. Specimens were dehydrated in graded concentrations of alcohol, and embedded in methyl methacrylate. Ten microgram consecutive sagittal longitudinal sections were cut from the mid-plane of each bone with a Polycut Microtome (Leica Inc., Deerfield, IL), and stained with either Goldner's trichrome or Von Kossa/toluidine blue for histologic evaluations.

**Anagen induction** At day 20 after birth, mouse littermates were anesthetized and subject to depilation of the dorsal hair using wax strips

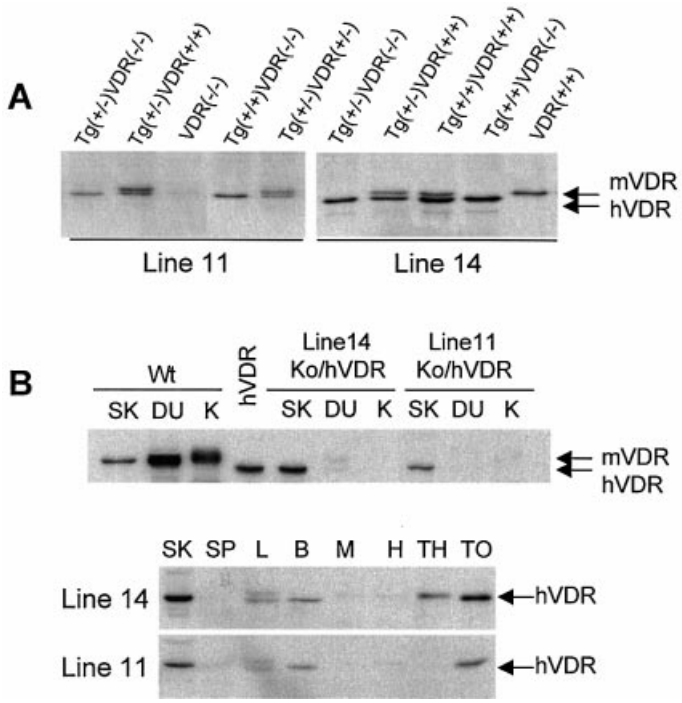



**Figure 2. Generation of Ko mice expressing the hVDR transgene.** (*A*) Western blot analysis of tail skin protein extract (20 μg protein per lane) isolated from line 11 and line 14 littermates generated by the breeding strategy described in *Materials and Methods*. The genotype of each mouse is shown on the top of each lane. (*B*) Tissue-specific expression of the hVDR transgene. Protein extracts were isolated from different tissues of $Tg^{+/-}VDR^{-/-}$ mice in both Tg lines and the expression of hVDR was determined by western blot with anti-VDR antibody (20 μg protein per lane). Ko/hVDR, $Tg^{+/-}VDR^{-/-}$; SK, skin; DU, duodenum; K, kidney; SP, spleen; L, liver; B, brain; M, skeletal muscle; H, heart; TH, thymus; TO, tongue.

according to the method described previously (Paus *et al*, 1990). Skin biopsies were taken from the mid-dorsum at days 4, 7, and 18 after depilation for histologic evaluations.

**Histology and immunohistochemistry** Skin specimen were fixed in 4% formaldehyde in phosphate-buffered saline (pH 7.2) overnight, processed, embedded in paraffin wax, and cut into 6 μm sections with a Leica Microtome 2030. Hematoxylin and eosin staining was performed according to standard procedures. Skin sections were immunostained with the anti-VDR antibody and visualized with a peroxidase substrate DAB kit (Vector Laboratories, Burlingame, CA) according to manufacturer's instructions.

**Bromodeoxyuridine (BrdU) labeling** Mice were injected i.p. with 50 mg per kg body weight of BrdU 2 h before skin samples were taken. The skin samples were fixed, embedded in paraffin, and cut into 5 μm sections. The sections were stained with a peroxidase-conjugated anti-BrdU monoclonal antibody (Roche Molecular Biochemicals, Indianapolis, IN) and visualized with the Vector DAB substrate kit.

**Scanning electron microscopy** Hair fibers were collected from 2 mo old mice, and placed directly on to aluminum stubs using double-stick tape. They were then sputter-coated with gold (approximately 15–20 nm thick) and viewed under a JEOL 840a scanning electron microscope (Peabody, MA) operated at 10 kV. Images were collected with a digital scan generator, DSG-1 (JEOL), at a working distance of 15 mm.

## RESULTS

**Generation of Tg and Ko/hVDR mice** We chose the human K14 promoter to drive hVDR expression in Tg mice because this promoter has been shown to target transgene expression specifically in the basal keratinocytes and the ORS of the hair follicles (Vassar *et al*, 1989; Wang *et al*, 1997), where a high level of VDR expression was seen previously (Stumpf *et al*, 1984; Reichrath *et al*, 1994). Embryonic injection of the K14–hVDR construct (**Fig 1*A***) resulted in eight pups positive for the transgene as identified by genomic polymerase chain reaction and confirmed by southern blot analyses (**Fig 1*B***), among which four (founders 5, 11, 14, and 15) expressed the hVDR protein as identified by western blot analyses of tail skin homogenates with an anti-VDR antibody (**Fig 1*C***). Although hVDR and the endogenous mouse VDR (mVDR) are highly homologous, they can be clearly separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (**Fig 1*C***). The transgene copy number for founders 5, 11, 14, and 15 was estimated to be 2, 1, 20, and 4, respectively (**Fig 1*B***), and the ratio of hVDR to mVDR content in these $Tg^{+/-}$ mice was about 1, 0.5, 2, and 1, respectively (**Fig 1*C***). Therefore, we chose founder 11 and 14 (their offspring were hereby designated as line 11 and line 14), which expressed hVDR at half and twice the amount of endogenous mVDR, for further investigations.

Line 11 and line 14 $Tg^{+/+}$ mice were used to breed with the $VDR^{+/-}$ mice ultimately to generate $Tg^{+/-}VDR^{-/-}$ and $Tg^{+/+}VDR^{-/-}$ mice, which were the Ko mice that expressed the hVDR transgene (hereby collectively designated as Ko/hVDR mice). As shown in **Fig 2(*A*)**, in both lines, as expected, $Tg^{+/-}VDR^{+/+}$, $Tg^{+/+}VDR^{+/+}$, $Tg^{+/-}VDR^{+/-}$, and $Tg^{+/+}VDR^{+/-}$ mice expressed both the hVDR and mVDR in the skin, $Tg^{+/-}VDR^{-/-}$ and $Tg^{+/+}VDR^{-/-}$ mice expressed only the hVDR, $VDR^{+/+}$ (Wt) mice expressed only the mVDR, and $VDR^{-/-}$ (Ko) mice did not express VDR at all. The tissue distribution of hVDR expression was examined using $Tg^{+/-}VDR^{-/-}$ mice to avoid the interference of the endogenous mVDR. As shown in **Fig 2*B***, no hVDR was detected in the duodenum and kidney, two organs crucial for calcium metabolism, as well as in the spleen, muscle, and heart; however, as reported previously (Wang *et al*, 1997), low expression of the transgene was seen in the tongue, brain, and esophagus, and in line 14, in the lung and thymus as well (**Fig 2*B*** and data not shown). In addition, no hVDR expression was seen in the testis, ovary, and uterus in both Tg lines (not shown). Thus, a high level of hVDR expression was specifically targeted to the skin in Tg and Ko/hVDR mice.

**Impaired calcium homeostasis in Ko/hVDR mice** The Ko/hVDR mice from both line 11 and line 14 were evaluated in parallel with Wt, Ko, and Tg littermates. Similar to the Ko mice (Li *et al*, 1997), the Ko/hVDR mice displayed growth retardation, and developed hypocalcemia after weaning (**Fig 3*A***), with the blood ionized calcium level decreasing by about 30% at 35 d of age compared with the Wt littermates. Secondary hyperparathyroidism was evident, as the serum parathyroid hormone level was continually elevated with age, reaching a concentration 200 times higher than the Wt level by 90 d (**Fig 3*A***). Contact X-ray radiographic analyses of the long bone revealed a clear decrease in bone calcification in Ko and Ko/hVDR mice, with an expansion in the epiphyseal growth plate and a decrease in cortical width (**Fig 3*B***). Histologic evaluation confirmed a dramatic increase in the width of the hypertropic zone of the growth plate, poor mineralization of the cartilage and cortical bone, and an increase in osteoid in trabecular and cortical bone (**Fig 3*C***), which are typical features of advanced rickets. The calcemic and skeletal phenotypes of the Tg mice in both lines were indistinguishable from those of the Wt mice (**Fig 3**). These results demonstrated that targeted expression of hVDR in Ko mice had no effect on their calcium metabolism.

**Rescue of alopecia and skin abnormalities by the hVDR transgene** One dramatic difference between the Ko and Ko/hVDR littermates was that, in both Tg lines, the Ko/hVDR mice displayed a normal hair coat like the Wt mice. No alopecia was seen in the Ko/hVDR mice 8 mo after birth, whereas alopecia was already prominent in the Ko mice at 4 mo of age (**Fig 4*A***). The hair loss in the Ko mice was evident on both dorsal and ventral skins, with dermal cysts clearly seen in the hairless regions at 5 mo of age. The morphology or fiber pattern of the hair shaft of both

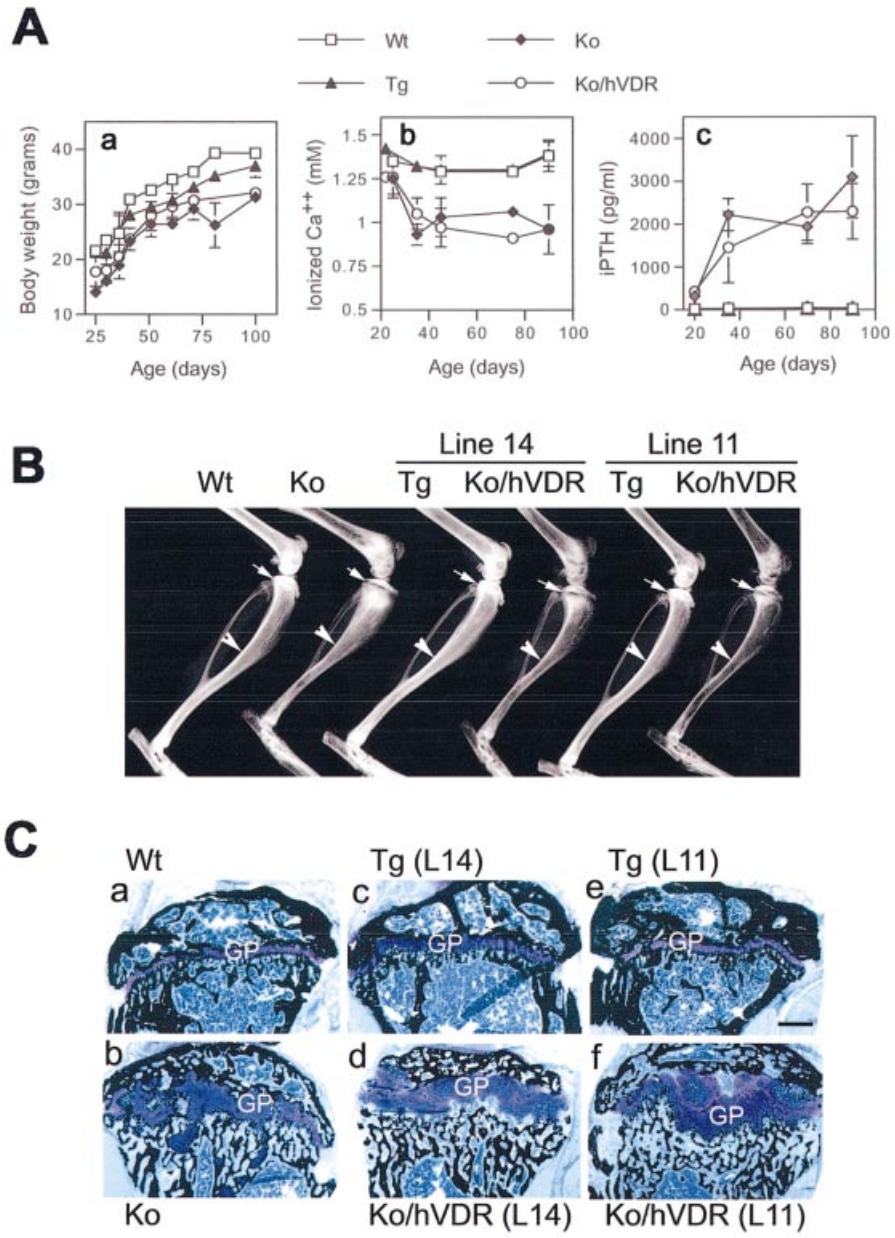



**Figure 3. Impaired calcium metabolism in Ko/hVDR mice.** (*A*) The Ko/hVDR mice are growth retarded and develop hypocalcemia and secondary hyperparathyroidism. (*a*) Growth curves of Wt, Ko, Tg, and Ko/hVDR mice. The data were generated from male mice. n = 7 in each group. (*b*) Blood ionized calcium levels of Wt, Ko, Tg, and Ko/hVDR mice determined at different times after birth. n = 7 in each group. (*c*) Serum intact parathyroid hormone (iPTH) concentrations of Wt, Ko, Tg, and Ko/hVDR mice determined at different ages. n = 5 in each group. Curve legend: Wt, □; Ko, ◆ Tg, ▲; Ko/hVDR, ○. (*B*) The Ko/hVDR mice develop rickets. Contact X-ray radiograph of the long bones from 75 d old Wt, Ko, Tg, and Ko/hVDR mice in both lines. *Arrowheads* indicate the cortical bone, and *arrows* indicate the epiphyseal growth plates. (*C*) Von Kossa/toluidine blue staining of their undecalcified bones. *Panel a*, Wt; *panel b*, Ko; *panel c*, Tg line 14; *panel d*, Ko/hVDR line 14; *panel e*, Tg line 11; and *panel f*, Ko/hVDR line 11. GP, growth plate. *Scale bar*: 100 μm.

Ko/hVDR lines, as revealed by scanning electron microscopic analysis, was the same as that of Wt and Tg littermates (**Fig 4*B***), indicating that the rescued hair fiber structure was normal. Histologic analyses demonstrated that the skin microscopic structure of the Ko/hVDR mice in both lines was indistinguishable from that of the Wt mice, whereas the Ko mice skin already displayed dilated piliary canals and large dermal cysts by 75 d (**Fig 4*C***). Of particular note is a complete rescue of the alopecia in line 11 $Tg^{+/-}VDR^{-/-}$ mice that only expressed hVDR at about half the endogenous mVDR level (**Fig 1**). The interfollicular epidermis of the Ko mice is generally one to two cell layers thicker than that of the Wt mice, which was not seen in the Ko/hVDR mice either (**Fig 4*D***). Normal skin histology was also seen in the two Tg lines (**Figs 4*C*, *D***). Similar histologic results were observed in mice at 90 d of age (data not shown).

To localize the expression of the hVDR transgene in the skin specifically, immunohistochemical staining with an anti-VDR antibody was used to examine the Ko/hVDR mouse skin, in which no interference of the endogenous mVDR was expected. As shown in **Fig 5**, a high level of hVDR expression was mainly detected in the nuclei of the basal cells of the epidermis (**Fig 5*a***) and the ORS cells of the anagen hair follicle (**Fig 5*b–d***), but no expression was seen in the dermal papilla (**Fig 5*c***), or in the ORS of Ko mice (**Fig 5*e***). These results demonstrated that targeted expression of hVDR in the ORS and/or in the basal keratinocytes effectively rescued the alopecia.

**Stimulation of hair cycle initiation by the hVDR transgene** To define further the role of VDR in the regulation of hair cycle, we performed detailed histologic analyses of the skins from Wt, Ko, Tg, and Ko/hVDR littermates in both lines at different stages of the follicular cycle. At 3 and 8 d after birth, when the hair follicles were still in the morphogenesis phase (Stenn and Paus, 2001), anagen follicles with the same morphologic pattern were seen in the skins of Wt, Ko, Tg, and Ko/hVDR pups (data not shown), suggesting that VDR is dispensable during follicular morphogenesis. This observation reflected the fact that Ko mice developed a normal hair coat after birth; however, after hair depilation at 20 d of age, Ko mice failed to initiate the follicular cycle, whereas the Ko/hVDR mice displayed approximately the same pattern of anagen follicle induction as the Wt mice, and most

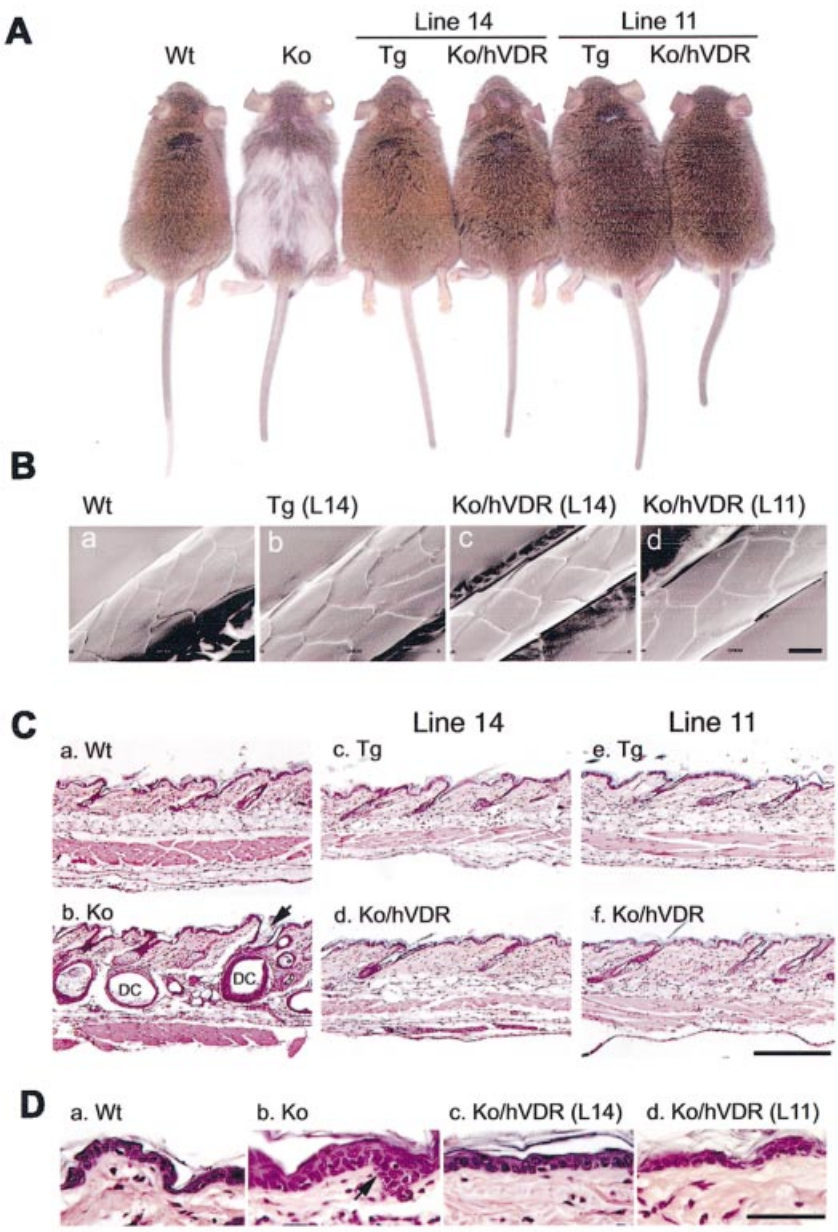



**Figure 4. The hVDR transgene rescues the alopecia of VDR knockout mice.** (*A*) Four month old Wt, Ko, Tg, and Ko/hVDR littermates in line 11 and line 14. (*B*) Scanning electron micrographs of the back hair shaft of these mice. L14, line 14; L11, line 11. *Scale bar*: 10 μm. (*C*) Hematoxylin and eosin staining of back skin obtained from 75 d old Wt, Ko, Tg, and Ko/hVDR littermates in both Tg lines. DC, dermal cyst. *Arrow* indicates the dilated piliary canal. *Scale bar*: 100 μm. (*D*) High magnification of the epidermal region of the skin. *Arrow* points to the epidermis of Ko mice. *Scale bar*: 25 μm.

strikingly, the Tg mice initiated the follicular cycle earlier than the Wt and Ko/hVDR littermates. In our examination of multiple litters in both Tg lines, a clear gene concentration-dependent effect of VDR was observed on the induction of the follicular cycle, so that in each litter the $Tg^{+/+}VDR^{+/+}$ littermate always displayed the most advanced anagen follicles at the initiation phase. As shown in **Fig 6**(***A***, ***B***), 4 d after depilation, no anagen follicle formation was seen in the skin of $VDR^{-/-}$ mice, whereas anagen II, IIIa, or IIIb follicles, as defined recently by Muller-Rover *et al* (2001), were seen in $Tg^{+/-}VDR^{-/-}$, $VDR^{+/-}$, or $Tg^{+/-}VDR^{+/-}$ littermates, and $Tg^{+/+}VDR^{+/-}$ and $Tg^{+/+}VDR^{+/+}$ littermates already formed anagen IIIc or IV follicles, characterized by longer follicles extending deep into the subcutis, mass accumulation of melanin in the follicle bulbs, and thicker skin (Muller-Rover *et al*, 2001). Similar results were observed at day 7 after depilation (data not shown). Consistent with these observations, the $Tg^{+/+}VDR^{+/+}$ mice had the longest hair shaft as determined at day 17 after depilation (data not shown).

We then used BrdU labeling to confirm cell proliferation during anagen induction. As shown in **Fig 6**(***C***, ***D***), 4 or 7 d after depilation massive BrdU incorporation was seen in the hair matrix cells of Ko/hVDR, Wt, and Tg mice, which are the hair-forming progenitor cells (Oshima *et al*, 2001). In contrast, little BrdU labeling was detected in the hair follicles of Ko mice; instead, most of the BrdU was incorporated into interfollicular keratinocytes and dermal fibroblasts. These data indicated that the hVDR transgene was required for the restoration of matrix cell proliferation in the anagen follicles.

## DISCUSSION

The data presented in this study provide direct evidence that VDR is required for the progression of the postnatal follicular cycle in mice. This conclusion is based on several lines of evidence: (i) genetic ablation of the VDR gene does not affect prenatal and postnatal hair morphogenesis, but abrogates the initiation of the following hair cycle; (ii) targeted expression of hVDR in the skin of Ko mice specifically rescues the alopecia and other skin abnormalities, but does not prevent the development of other nonskin phenotypes; (iii) the hVDR transgene is targeted to the keratinocytes of the basal layer and the ORS, with little or no

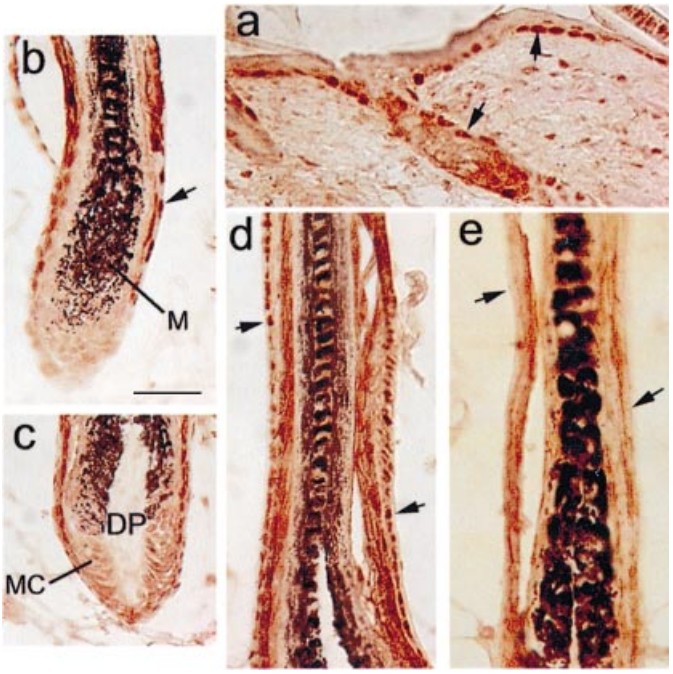



**Figure 5. The hVDR transgene is expressed in keratinocytes in the basal layer of the epidermis and the ORS.** Dorsal skins of 7 d old $Tg^{+/-}VDR^{-/-}$ (*a–d*) and $VDR^{-/-}$ (*e*) mice were analyzed by immunohistochemical staining with an anti-VDR antibody. (*a*) Interfollicular epidermal region. *Arrows* indicate positive keratinocytes in the basal layer and distal ORS. (*b*, *c*) Proximal hair follicle. *Arrows* indicate the positive cells in the ORS. DP, dermal papilla; M, melanin; MC, matrix cells. (*d*) Middle and distal hair follicle. *Arrows* point to the positive cells in the ORS. (*e*) Ko mouse hair follicle. *Arrows* indicate the negative ORS. *Scale bar*: 25 μm.

expression in other tissues; and (iv) the hVDR transgene restores the initiation of the hair cycle and anagen hair matrix cell proliferation in Ko mice, and overexpression of the transgene in Tg mice accelerates the formation of anagen follicles. These results suggest that VDR is required either for the initiation of the postnatal follicular cycle or for the orderly progress of catagen/telogen in the follicular morphogenesis phase so that the next anagen can be initiated properly. In either situation, the genetic inactivation of VDR should lead to a blockade of the onset of the postnatal anagen.

That VDR is dispensable during the morphogenesis of hair follicles but is crucial for its postnatal initiation is consistent with the fact that Ko mice develop a normal hair coat after birth. Their hair loss is, therefore, due to the failure of postnatal follicular cycle initiation. Follicular morphogenesis arises from a primitive epidermis, and follicular cycle is generated from adult tissues. Although these two processes share many features, they are regulated by different factors (Stenn and Paus, 2001). The exact expression pattern of VDR in follicular morphogenesis is not known, but the lack of VDR function may well be compensated by the redundant pathways known to exist in follicular morphogenesis. Indeed, so far no gene has been found absolutely crucial to hair follicle morphogenesis (Stenn and Paus, 2001).

Hair follicle formation is a result of cross-talks between the epithelial and mesenchymal compartments (Hardy, 1992; Stenn and Paus, 2001), and molecules expressed in both compartments have been shown to be important for follicular morphogenesis and cycling (Stenn and Paus, 2001). It is known that VDR is expressed in both ORS keratinocytes and dermal papillae, the key mesenchymal structure of the hair follicle (Reichrath *et al*, 1994); however, it is unclear whether the presence of VDR in the epithelial or in the mesenchymal component is important to follicle formation. We show here that hVDR expression in the ORS can fully restore hair growth in Ko mice, indicating that the presence of VDR in the epithelial, rather than the

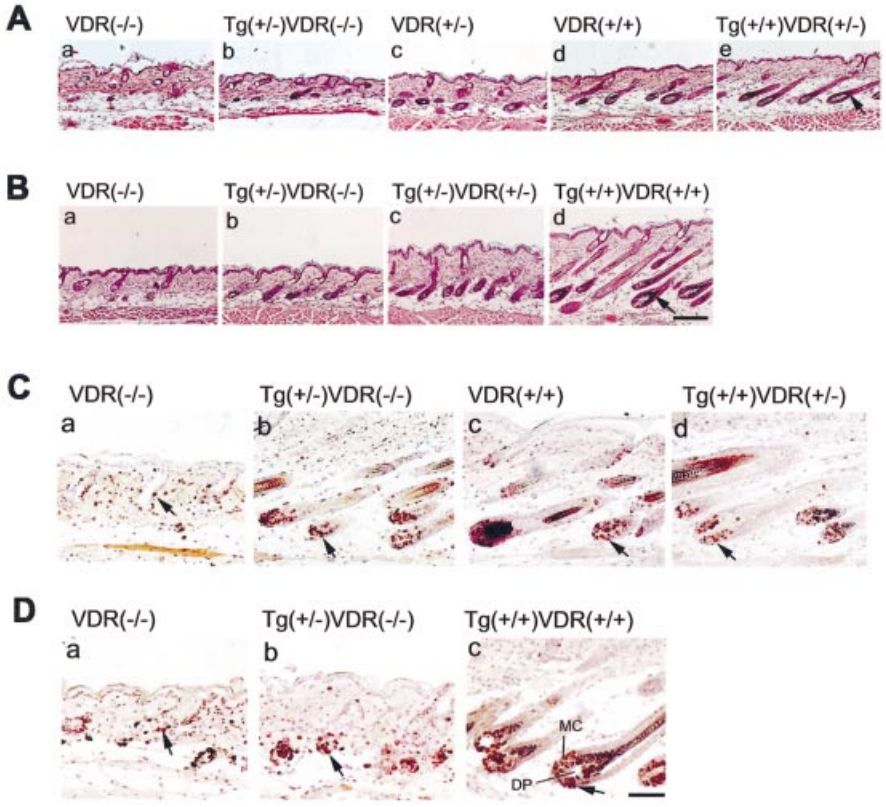



**Figure 6. The hVDR transgene stimulates the initiation of the follicular cycle by a gene concentration-dependent fashion.** (*A*, *B*) Dorsal skin biopsies were taken from each litter 4 d after depilation at day 20 after birth and stained with hematoxylin and eosin. The genotype of each mouse is shown on the top of each panel. *Scale bar*: 50 μm. (*A*) Female littermates from line 11. (*B*) Female littermates from line 14. Note the gene dose or the VDR content in the skin increases from panel *a* to *e* in line 11 or from *a* to *d* in line 14, which is correlated with the advance of the anagen stages. *Arrows* point to melanin in anagen IIIc and IV follicles. (*C*, *D*) Skin biopsies were taken from each litter after depilation at day 20 after birth and stained for BrdU incorporation. The genotype of each mouse is shown on the top of each panel. *Scale bar*: 25 μm. (*C*) Line 11 littermates at day 7 after depilation. (*D*) Line 14 littermates at day 4 after depilation. *Arrows* point to some BrdU-positive cells. MC, matrix cells; DP, dermal papilla.

mesenchymal compartment, is sufficient to initiate hair follicle cycle. This conclusion is consistent with the recent finding that alopecia in Ko mice is due to defects in keratinocytes rather than in dermal papilla cells (Sakai *et al*, 2001). Indeed, ORS, the keratinocyte layer lining the hair follicle, has been shown to be crucial for hair growth. ORS contains the multipotent stem cells required for the formation of hair follicles (Oshima *et al*, 2001). Increasing the survival rate of the ORS cells significantly shortens the anagen phase and prolongs the telogen phase, leading to shorter hairs (Pena *et al*, 1999), and inactivation of fibroblast growth factor-5 in ORS cells results in long hairs (Hebert *et al*, 1994). At present, the coupling between VDR expression in the ORS and the initiation of follicle formation is not known. One possibility can be that VDR, as a transcriptional factor, may be required for the production of some soluble paracrine molecules by the ORS that diffuse to the hair matrix or dermal papilla to facilitate the cross-talks between the epithelial and mesenchymal compartments. It is also plausible that VDR is required for the stem cells to respond to morphogenetic signals to generate hair follicles (Oshima *et al*, 2001).

Although many genes are known to be involved in hair morphogenesis (Stenn and Paus, 2001), factors proved to be required for postnatal hair follicle initiation are few. Localized transient expression of Sonic hedgehog has been shown to induce postnatal anagen and hair growth in mice (Sato *et al*, 1999). Skin specific inactivation of RXR$\alpha$, the heterodimeric partner of VDR, results in impaired anagen initiation after hair depilation (M Li *et al*, 2001). Here we demonstrate that targeted expression of hVDR in the skin not only restores the follicular cycle without altering other abnormalities in Ko mice, but also accelerates the induction of anagen follicle formation when it is overexpressed. These data directly prove that VDR plays an indispensable and stimulatory role in postnatal hair cycle initiation.

Alopecia is a predominant feature of the HVDRR patients and VDR knockout mice (Li *et al*, 1997; Malloy *et al*, 1999), but is not seen in mice genetically ablated for the 1$\alpha$-hydroxylase gene or in VDDR patients (Fu *et al*, 1997; St-Arnaud *et al*, 1997; Takeyama *et al*, 1997; Panda *et al*, 2001), suggesting that VDR regulates hair growth by a $1,25(OH)_2D_3$-independent mechanism. Previous studies have shown that alopecia is independent of hypocalcemia, secondary hyperparathyroidism or the high level of vitamin D metabolites associated with VDR inactivation (Li *et al*, 1998; Sakai *et al*, 2001), but whether VDR inactivation indeed directly causes the alopecia still needs to be further proved. Our data presented in this study unequivocally demonstrate that it is VDR inactivation *per se* that directly causes the hair loss. Therefore, VDR is among the growing list of transcriptional factors (Stenn *et al*, 1996; Stenn and Paus, 2001) that are indispensable for the maintenance of normal hair follicle cycle. VDR heterodimerizes with RXR, but only RXR$\alpha$ inactivation leads to alopecia, whereas RXR$\gamma$ knockout mice show no overt abnormalities, and the skin phenotype of VDR/RXR$\gamma$ double knockout mice is the same as VDR knockout mice (Krezel *et al*, 1996; M Li *et al*, 2001; Yagishita *et al*, 2001). Furthermore, it is of interest to note that hair loss resulted from the inactivation of VDR, RXR$\alpha$ and hairless genes in mice shares some common features, including the apparent normal hair morphogenesis and similar skin histologic abnormalities such as dilation of piliary canals and formation of dermal cysts (Sundberg, 1994; YC Li *et al*, 1998; M Li *et al*, 2001). These observations strongly suggest that the formation of VDR-RXR$\alpha$ heterodimer is required for hair follicle cycling (Li *et al*, 2001); however, the relationship between VDR and hairless gene remains to be elucidated.

In summary, by targeting hVDR expression to the skin, we have demonstrated that the hVDR transgene rescues the alopecia of Ko mice, and accelerates anagen follicle formation when overexpressed in Tg mice. Our data indicate that VDR is required for the initiation of the postnatal hair follicle cycle.

*We thank Merry Bolt and Michael Sitrin for technical assistance and helpful discussions. We are also grateful to Elaine Fuchs for providing the K14 promoter, to Linda Degenstein for microinjection of the DNA construct and to Edward Williamson for scanning electron microscopic analysis of the hair shaft. This work was supported by a start-up fund from the University of Chicago, a grant from the National Alopecia Areata Foundation and a NIH grant DK59327 (to Y.C.L.).*

## REFERENCES

DeLuca HF: The vitamin D story: a collaborative effort of basic science and clinical medicine. *FASEB J* 2:224–236, 1988

Fu GK, Lin D, Zhang MYH, Bikle DD, Shackleton CHL, Miller WL, Portale AA: Cloning of human 25-hydroxyvitamin D-1$\alpha$-hydroxylase and mutations causing vitamin D-dependent rickets type I. *Mol Endocrinol* 11:1961–1970, 1997

Hardy MH: The secret life of the hair follicle. *Trends Genet* 8:55–61, 1992

Haussler MR, Whitfield GK, Haussler CA, *et al:* The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. *J Bone Miner Res* 13:325–349, 1998

Hebert JM, Rosenquist T, Gotz J, Martin GR: FGF5 as a regulator of the hair growth cycle: evidence from targeted and spontaneous mutations. *Cell* 78:1017–1025, 1994

Hogan B, Costantini F, Lacy E: *Manipulating the Mouse Embryo. a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1986

Krezel W, Dupe V, Mark M, Dierich A, Kastner P, Chambon P: RXR gamma null mice are apparently normal and compound RXR alpha +/–/RXR beta –/–/RXR gamma –/– mutant mice are viable. *Proc Natl Acad Sci USA* 93:9010–9014, 1996

Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685, 1970

Li M, Chiba H, Warot X, Messaddeq N, Gerard C, Chambon P, Metzger D: RXR-alpha ablation in skin keratinocytes results in alopecia and epidermal alterations. *Development* 128:675–688, 2001

Li YC, Pirro AE, Amling M, Delling G, Baron R, Bronson R, Demay MB: Targeted ablation of the vitamin D receptor: an animal model of vitamin D-dependent rickets type II with alopecia. *Proc Natl Acad Sci U S A* 94:9831–9835, 1997

Li YC, Amling M, Pirro AE, *et al:* Normalization of mineral ion homeostasis by dietary means prevents hyperparathyroidism, rickets, and osteomalacia, but not alopecia in vitamin D receptor-ablated mice. *Endocrinology* 139:4391–4396, 1998

Li YC, Bolt MJG, Cao L-P, Sitrin MD: Effects of vitamin D receptor inactivation on the expression of calbindins and calcium metabolism. *Am J Physiol Endocrinol Metab* 281:E558–E564, 2001

Malloy PJ, Pike JW, Feldman D: The vitamin D receptor and the syndrome of hereditary 1,25-dihydroxyvitamin D-resistant rickets. *Endocr Rev* 20:156–188, 1999

Muller-Rover S, Handjiski B, van der Veen C, *et al:* A comprehensive guide for the accurate classification of murine hair follicles in distinct hair cycle stages. *J Invest Dermatol* 117:3–15, 2001

Oshima H, Rochat A, Kedzia C, Kobayashi K, Barrandon Y: Morphogenesis and renewal of hair follicles from adult multipotent stem cells. *Cell* 104:233–245, 2001

Panda DK, Miao D, Tremblay ML, Sirois J, Farookhi R, Hendy GN, Goltzman D: Targeted ablation of the 25-hydroxyvitamin D 1alpha-hydroxylase enzyme: evidence for skeletal, reproductive, and immune dysfunction. *Proc Natl Acad Sci USA* 98:7498–7503, 2001

Paus R, Stenn KS, Link RE: Telogen skin contains an inhibitor of hair growth. *Br J Dermatol* 122:777–784, 1990

Pena JC, Kelekar A, Fuchs EV, Thompson CB: Manipulation of outer root sheath cell survival perturbs the hair- growth cycle. *EMBO J* 18:3596–3603, 1999

Reichrath J, Schilli M, Kerber A, Bahmer FA, Czarnetzki BM, Paus R: Hair follicle expression of 1,25-dihydroxyvitamin D3 receptor during the murine hair cycle. *Br J Dermatol* 131:477–482, 1994

Saitou M, Sugai S, Tanaka T, Shimouchi K, Fuchs E, Narumiya S, Kakizuka A: Inhibition of skin development by targeted expression of a dominant- negative retinoic acid receptor [see comments]. *Nature* 374:159–162, 1995

Sakai Y, Demay MB: Evaluation of keratinocyte proliferation and differentiation in vitamin D receptor knockout mice. *Endocrinology* 141:2043–2049, 2000

Sakai Y, Kishimoto J, Demay MB: Metabolic and cellular analysis of alopecia in vitamin D receptor knockout mice. *J Clin Invest* 107:961–966, 2001

Sato N, Leopold PL, Crystal RG: Induction of the hair growth phase in postnatal mice by localized transient expression of Sonic hedgehog. *J Clin Invest* 104:855–864, 1999

St-Arnaud R, Messerlian S, Moir JM, Omdahl JL, Glorieux FH: The 25-hydroxyvitamin D 1-alpha-hydroxylase gene maps to the pseudovitamin D-deficiency rickets (PDDR) disease locus. *J Bone Miner Res* 12:1552–1559, 1997

Stenn KS, Combates NJ, Eilertsen KJ, Gordon JS, Pardinas JR, Parimoo S, Prouty SM: Hair follicle growth controls. *Dermatol Clin* 14:543–558, 1996

Stenn KS, Paus R: Controls of hair follicle cycling. *Physiol Rev* 81:449–494, 2001

Stumpf WE, Clark SA, Sar M, DeLuca HF: Topographical and developmental studies on target sites of 1,25(OH)2 vitamin D3 in skin. *Cell Tissue Res* 238:489–496, 1984

Sundberg JP: The hairless (hr) and rhino ($hr^{rh}$) mutations, chromosome 14. In: JP Sundberg (ed.). *Handbook of Mouse Mutations with Skin and Hair Abnormalities: Animal Models and Biomedical Tools*. New York, CRC Press, 1994: pp 291–312

Takeyama K, Kitanaka S, Sato T, Kobori M, Yanagisawa J, Kato S: 25-Hydroxyvitamin D3, 1alpha-hydroxylase and vitamin D synthesis. *Science* 277:1827–1830, 1997

Vassar R, Rosenberg M, Ross S, Tyner A, Fuchs E: Tissue-specific and differentiation-specific expression of a human K14 keratin gene in transgenic mice. *Proc Natl Acad Sci USA* 86:1563–1567, 1989

Wang X, Zinkel S, Polonsky K, Fuchs E: Transgenic studies with a keratin promoter-driven growth hormone transgene: prospects for gene therapy. *Proc Natl Acad Sci USA* 94:219–226, 1997

Yagishita N, Yamamoto Y, Yoshizawa T, *et al:* Aberrant growth plate development in VDR/RXRgamma double null mutant mice. *Endocrinology* 142:5332–5341, 2001

Yoshizawa T, Handa Y, Uematsu Y, *et al:* Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. *Nature Genet* 16:391–396, 1997