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The human cysteine protease cathepsin V can compensate for murine cathepsin L in mouse epidermis and hair follicles

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Mice lacking the ubiquitously expressed lysosomal cysteine protease cathepsin L, show a complex skin phenotype consisting of periodic hair loss and epidermal hyperplasia with hyperproliferation of basal epidermal keratinocytes, acanthosis and hyperkeratosis. The recently identified human cathepsin L-like enzyme cathepsin V, which is also termed cathepsin L2, is specifically expressed in cornea, testis, thymus, and epidermis. To date, in mice no cathepsin V orthologue with this typical expression pattern has been identified. Since cathepsin V has about 75% protein sequence identity to murine cathepsin L, we hypothesized that transgenic, keratinocyte-specific expression of cathepsin V in cathepsin L knockout mice might rescue the skin and hair phenotype. Thus, we generated a transgenic mouse line expressing cathepsin V under the control of the human keratin 14 promoter, which mimics the genuine cathepsin V expression pattern in human skin, by directing it to basal epidermal keratinocytes and the outer root sheath of hair follicles. Subsequently, transgenic mice were crossed with congenic cathepsin L knockout animals. The resulting mice show normalization of epidermal proliferation and normal epidermal thickness as well as rescue of the hair phenotype. These findings provide evidence for keratinocyte-specific pivotal functions of cathepsin L-like proteolytic activities in maintenance of epidermis and hair follicles and suggest, that cathepsin V may perform similar functions in human skin.

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Introduction

Proliferation and differentiation of epidermal keratinocytes as well as morphogenesis and cycling of hair follicles are highly dynamic with regard to proliferation, differentiation and migration of cells. It has been shown that multiple, tightly regulated and specific proteolytic activities are essential for correct execution of these processes (Ekholm et al., 2000; Liu et al., 2000; Zhou et al., 2000; Franzke et al., 2002; Egberts et al., 2004; Zeeuwen et al., 2004). Cathepsins are proteolytic enzymes with a principal subcellular localization in the endosomal/lysosomal compartment. The largest family of cathepsins comprises the papain-like cysteine proteases with 11 human enzymes (Turk et al., 2001). In this protease family the expression of cathepsins B, C, F, H, L, O, and X/Z is ubiquitous in mammalian tissues although the level of an individual cathepsin shows considerable variation in different cell types. Other papain-like cysteine proteases, i.e. cathepsins S, W, K, and notably cathepsin V (CTSV), exhibit a more restricted expression pattern. Cathepsin S is selectively expressed in peripheral antigen-presenting cells such as dendritic cells and macrophages as well as in smooth muscle cells and plays a critical role in antigen presentation, while cathepsin W is exclusively expressed in natural killer cells (Honey and Rudensky, 2003). Cathepsin K is highly and specifically expressed in osteoclasts, which are essential for bone resorption. Inactivating cathepsin K mutations cause the inherited bone dysplasia known as pycnodysostosis (Gelb et al., 1996). CTSV, which is alternatively termed cathepsin L2, was first identified by molecular cloning in 1998 and since then expression has been reported in only a few cell lines or tissues such as colon tumour cells, thymic epithelial cells, testis, and corneal epithelium (Adachi et al., 1998; Santamaria et al., 1998; Brömme et al., 1999; Tolosa et al., 2003). Recently, CTSV was shown to contribute largely to the „stratum corneum thiol

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protease" activity of human epidermis (Watkinson, 1999; Bernard et al., 2003).

Most of the human cysteine cathepsins possess a highly homologous murine counterpart, which allows the generation and analysis of cathepsin knockout mice in order to identify critical in vivo functions of these proteases (Reinheckel et al., 2001). The prominent skin phenotype of mice lacking cathepsin L (CTSL) is characterized by epidermal hyperproliferation, acanthosis and hyperkeratosis as well as by abnormalities in development and cycling of hair follicles that result in periodic hair loss (Roth et al., 2000; Tobin et al., 2002). In addition, CTSL knockout mice show reduced numbers of $CD4^+$ T cells and develop a dilative cardiomyopathy (Nakagawa et al., 1998; Stypmann et al., 2002). Mice deficient for CTSL exhibit reduced levels of the neuropeptide [Met]enkephalin in the brain with increased levels of the pro-hormone (Yasothornsrikul et al., 2003). Furthermore, it has been shown that CTSL is involved in the solubilization and degradation of thyroglobulin in the thyroid gland (Friedrichs et al., 2003).

CTSV represents a notable exception with regard to the feasibility of its functional analysis by means of knockout mice because it is not present in the mouse genome (Puente et al., 2003). As the result of a gene duplication, the human genome encodes for two cathepsin L-like proteases, namely the "classic" human CTSL, which is produced by a wide range of tissue and cell types, as well as CTSV/cathepsin L2 with a more restricted expression pattern (Fig. 1A). In contrast, the mouse genome contains only one CTSL gene, which is ubiquitously expressed. All three enzymes, murine CTSL, human CTSL and human CTSV, are highly homologous with about 75% amino acid identities (Fig. 1A). In order to identify potential in vivo functions of CTSV in humans, we generated transgenic mice expressing CTSV under the control of the human cytokeratin 14 promoter (K14) which mimics the genuine human expression of CTSV in some epithelial cell types. Subsequently, the Tg(K14-CTSV) mice were bred into the CTSL knockout ($ctsl^{-/-}$) mouse strain. We show that the resulting Tg(K14-CTSV);$ctsl^{-/-}$ mice express CTSV in the skin and are able to compensate for the lack of murine CTSL in epidermis and hair follicles, suggesting an important role of CTSV in human skin homeostasis.

Materials and methods

Generation of K14-CTSV transgenic mice and Tg(K14-CTSV);$ctsl^{-/-}$ mice

The full-length human CTSV cDNA (1.1 kb) including the stop codon but lacking the polyadenylation signal was inserted into an expression cassette that includes the cloning vector pBluescript (Stratagene, La Jolla, California), a 2.1-kb human K14 promoter followed by a 0.65-kb rabbit β-globin intron and a transcription termination/polyadenylation fragment [poly(A), 0.63 kb] of the human growth hormone gene (Brömme et al., 1999; Munz et al., 1999) (Fig. 1B). For generation of transgenic mice, standard procedures were followed. The purified expression cassette was microinjected into the pronuclei of one-cell-stage embryos (FVB/n background) that were subsequently retransferred into the oviducts of pseudo-pregnant recipient females. Mouse tail DNA was analyzed for integration of the transgene (founder analysis and routine genotyping) by PCR with primers for the CTSV cDNA that were designed with maximal differences to the murine CTSL sequence. About half of the offspring of founders carried the transgene. Subsequently transgenic mice (FVB/n) were crossed with the congenic CTSL knockout mouse strain (Roth et al., 2000).

RNA isolation and RT-PCR

Total RNA from murine back skin was prepared using the "RNeasy Mini kit" (Qiagen, Hilden, Germany). Briefly, 30 mg of murine back skin were triturated using a mixer mill. The homogenate was suspended in 350 µl lysis buffer (included in the kit) and passed 5 times through a 26-gauge needle. Reverse transcription for the generation of cDNA from total RNA was performed by using a first strand cDNA synthesis kit (Invitrogen, Karlsruhe, Germany). For expression analysis of CTSV and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), PCR amplification of the reverse transcribed cDNA was performed using equivalent amounts of cDNA/RNA, Taq polymerase, and specific primers (CTSV: 5′-caatggtggcttcatggctagg-3′ and 5′-ccttctcctttccaggtgcga-3′; GAPDH: 5′-tgcaccaccaactgcttag-3′ and 5′'-gatgcagggatgatgttc-3′') under the following conditions: 1 cycle for 1 min at 72 °C, 50 cycles (94 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s) and 1 cycle at 72 °C for 7 min in parallel. The resulting PCR products were visualized by ethidiumbromide staining after separation on 2% (w/v) agarose gels.

Histology and immunohistochemistry

For histological assessment, back skin sections of 5 µm thickness were deparaffinized in xylene, hydrated in graded ethanol solutions and stained with hematoxylin/eosin. Goat anti-mouse CTSL antibody (R&D Systems, Wiesbaden, Germany; at 0.2 µg/ml), anti-Ki67 (Dako-Cytomation, Hamburg, Germany; at 1:200 dilution) and rabbit anti-filaggrin (Covance Inc., New Jersey, USA; at 1:500 dilution) were used for the detection of CTSL, the proliferation marker Ki67 and the stratum corneum protein filaggrin, respectively. Peroxidase-based detection of the primary antibodies was performed according to the instructions of the "Vectastain Elite ABC Kit" (Vector Laboratories, Burlinghame, CA). Microscopy was performed with an Axioplan microscope (Zeiss, Stuttgart, Germany), and digital images were obtained with an Axiocam camera (Zeiss, Stuttgart, Germany).

Results and Discussion

CTSV expression in the skin of Tg(K14-CTSV);$ctsl^{-/-}$ mice

The human genome encodes, in addition to a homologue of the classical, ubiquitously expressed murine CTSL, the highly conserved lysosomal cysteine peptidase CTSV (Fig. 1A). For analysis of CTSV functions in epithelial mouse tissue we subcloned the human CTSV cDNA into an expression vector containing the human keratin 14 (K14) promoter (Fig. 1B), which directs expression to keratinocytes of the basal epidermal layer and the hair follicle outer root sheath (Munz et al., 1999). Subsequently, transgenic Tg(K14-CTSV) mice were generated. These mice are viable and fertile, and do not show any gross phenotype (data not shown). We chose one of the two K14-CTSV-positive founder mice for breeding with congenic FVB/n $ctsl^{-/-}$ mice. RT-PCR on total RNA from mouse back skin confirmed the specific expression of the CTSV mRNA in the skin of the resulting mouse line Tg(K14-CTSV);$ctsl^{-/-}$ as compared to wild-type or CTSL knockout mice (Fig. 2A). In human epidermis, CTSV has been shown to be present in all epidermal layers with a high abundance in basal keratinocytes (Bernard et al., 2003). Thus, the K14 promoter used in the present experiments should largely mimic the genuine expression pattern of CTSV in human epidermis and hair follicle epithelia. In contrast, murine CTSL was detected by immunohistochemistry in wild-type, but not in $ctsl^{-/-}$ or Tg(K14-CTSV);$ctsl^{-/-}$ mice (Fig. 2B – D). CTSL is present at high levels in the stratum corneum of wild-type epidermis (Fig. 2B). However, in situ hybridization for detection of murine CTSL mRNA has shown that CTSL transcription occurs in the

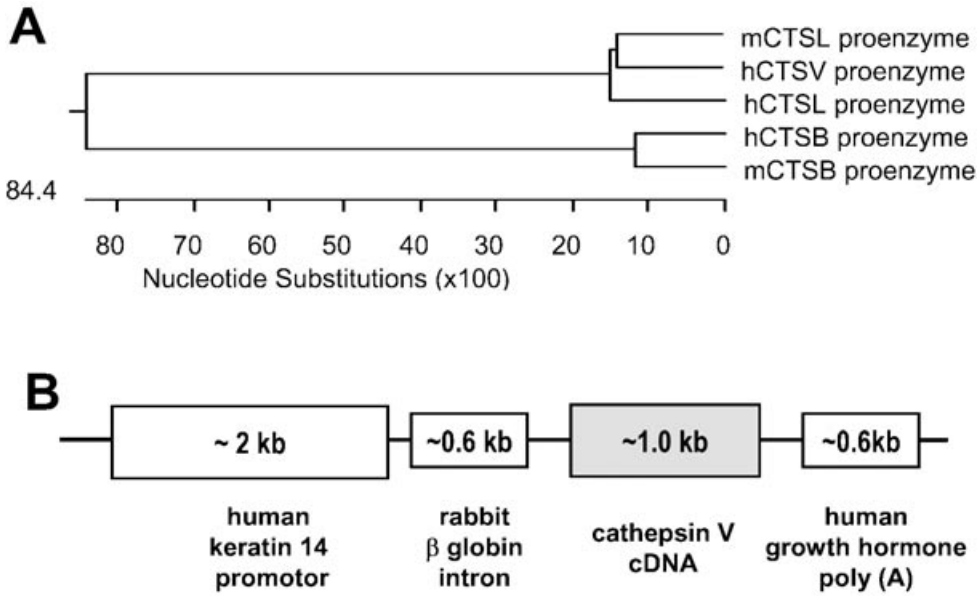

Fig. 1. Phylogenetic tree of murine and human cathepsin proenzymes (A). Murine cathepsin L (mCTSL), human cathepsin V (hCTSV), and human cathepsin L (hCTSL) are compared to human and murine cathepsin B (hCTSB, mCTSB) as prototypic members of lysosomal papain-proteases. Scheme of the transgene construct (B). Functional elements are the human keratin 14 promoter, the rabbit β-globin intron, the full-length human CTSV cDNA, and the human growth hormone poly(A)signal.

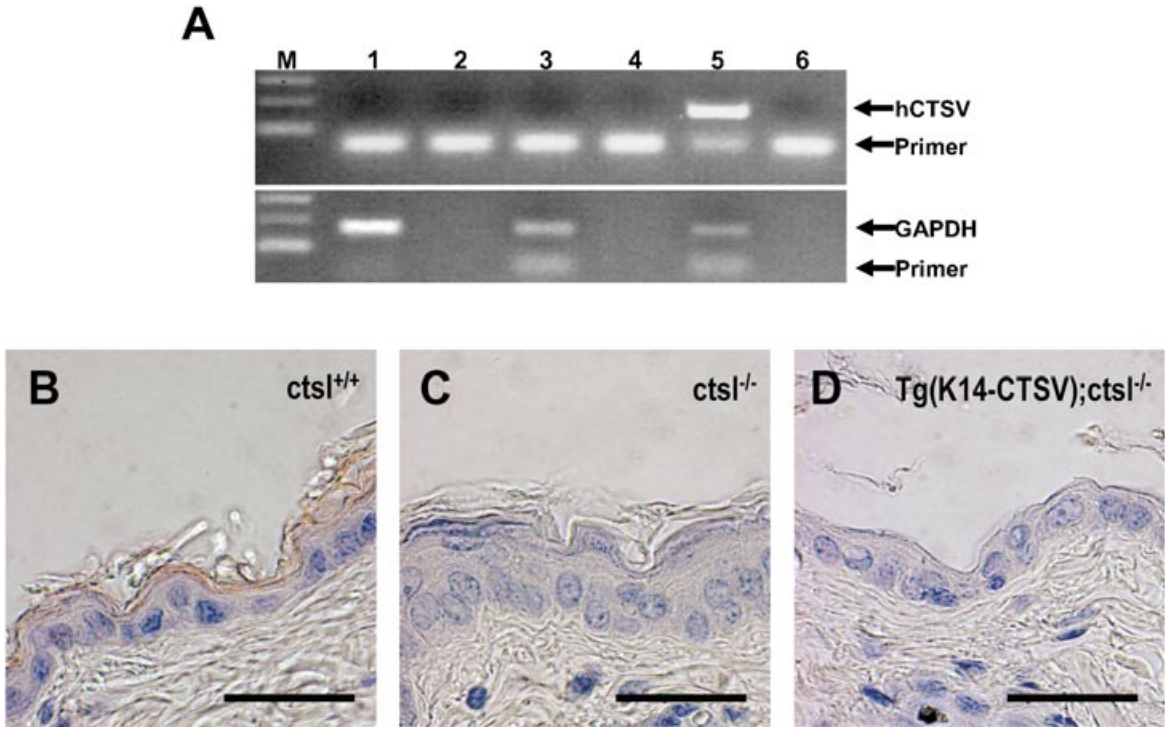

Fig. 2. Expression of human CTSV and murine CTSL in mouse back skin. RT-PCR detection of cathepsin V (hCTSV) expression (A). Lanes 1,2: wild-type mouse; 3,4: CTSL-deficient mouse (ctsl$^{-/-}$); 5,6: CTSL-deficient mouse expressing CTSV under control of the human cytokeratin 14 promoter Tg(K14-CTSV);ctsl$^{-/-}$; 1, 3 and 5: cDNA samples with reverse transcription; 2, 3 and 6: total RNA without reverse transcription; M: 100 bp ladder. Immunohistochemical detection of mCTSL in murine skin (B–D). Wild type (B); ctsl$^{-/-}$ (C); Tg(K14-CTSV);ctsl$^{-/-}$ (D). Bars indicate 50 μm.

keratinocytes of the basal epidermal layer (Roth et al., 2000). This suggests a high stability and relative accumulation of CTSL during shrinkage of differentiating epidermal keratinocytes. However, the absence of murine CTSL from the epidermis of ctsl$^{-/-}$ and Tg(K14-CTSV);ctsl$^{-/-}$ mice indicates that phenotypic differences between the two genotypes must be caused by the K14-controlled transgenic expression of CTSV in the skin of Tg(K14-CTSV);ctsl$^{-/-}$ mice.

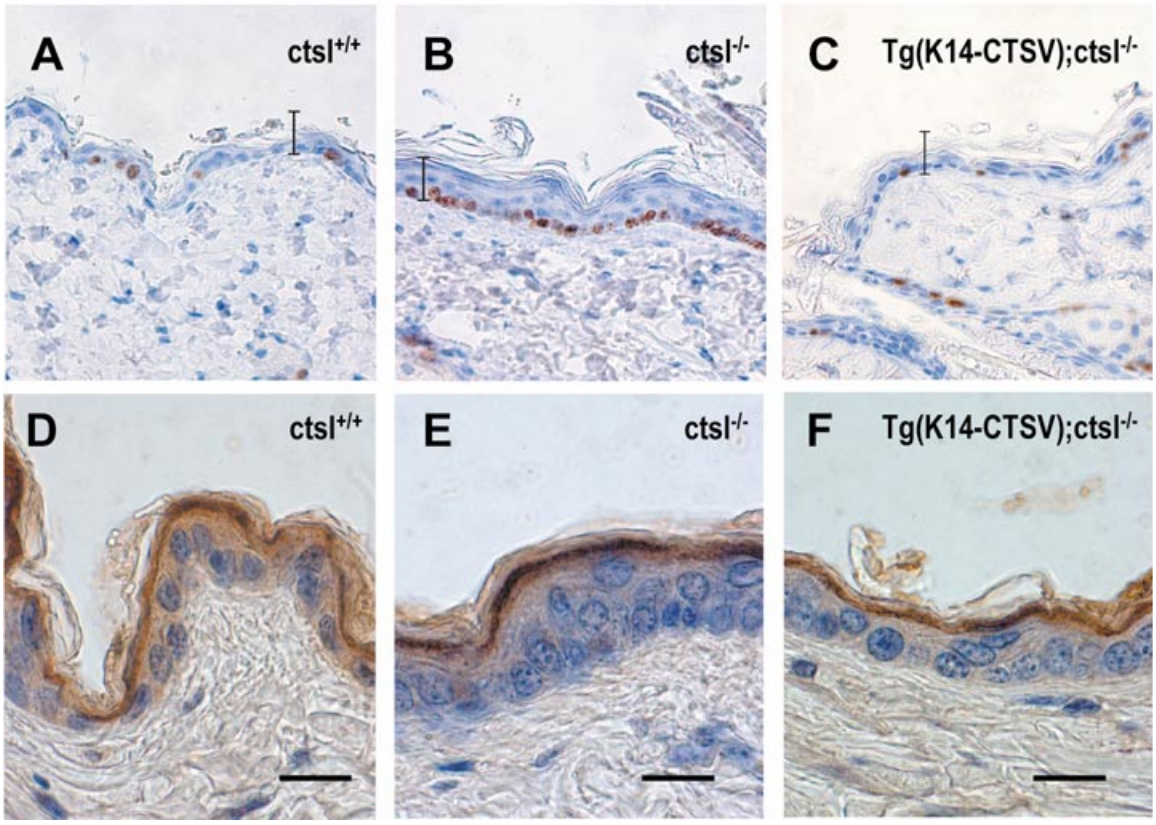

Fig. 3. Proliferation and differentiation in mouse back skin. Immunohistochemical staining of Ki67 in basal keratinocytes (A – C, bars indicate 50 μm) and stratum corneum filaggrin (D – F, bars indicate 20 μm). Wild type (A, D); ctsl$^{-/-}$ (B, E); Tg(K14-CTSV);ctsl$^{-/-}$ (C, F).

Phenotype of interfollicular epidermis in Tg(K14-CTSV);ctsl$^{-/-}$ mice

Ctsl$^{-/-}$ mice develop epidermal hyperplasia with acanthosis and hyperkeratosis as a result of a hyperproliferation of basal keratinocytes (Roth et al., 2000). In order to assess the proliferation state of keratinocytes in epidermis of ctsl$^{+/+}$, ctsl$^{-/-}$ and Tg(K14-CTSV);ctsl$^{-/-}$ mice, skin sections were stained for the proliferation marker Ki67 (Fig. 3A – C). In agreement with previous reports epidermal sections of ctsl$^{-/-}$ mice showed a clear increase in the number of Ki67-positive basal keratinocytes compared to the wild type (Roth et al., 2000; Tobin et al., 2002) (Fig. 3A, B). Most interestingly, the number of proliferating keratinocytes in Tg(K14-CTSV);ctsl$^{-/-}$ epidermis was reduced to the wild-type level (Fig. 3A – C). In addition to normalized proliferation, the epidermis of Tg(K14-CTSV);ctsl$^{-/-}$ mice shows a clear amelioration of acanthosis and hyperkeratosis as compared to the CTSL knockout (Fig. 3A – F). Interestingly, a CTSL-like protease has been shown to be able to cleave the stratum corneum protein filaggrin in vitro (Kawada et al., 1995). This finding would suggest an accumulation of filaggrin in the cathepsin L-knockout. However, ctsl$^{-/-}$ mice do not show a clear increase of filaggrin staining and Tg(K14-CTSV);ctsl$^{-/-}$ transgenic mice do not exhibit altered filaggrin immunoreactivity (Fig. 3D – F). Thus, a cathepsin L-like activity is not critical for filaggrin turnover in mouse epidermis. Other proteases of the stratum corneum like the "stratum corneum chymotryptic enzyme", the "stratum corneum tryptic enzyme", the aspartic protease cathepsin D or the cysteine protease legumain are likely to compensate for the alterations in cathepsin L activity with regard to the degradation of stratum corneum proteins (Egberts et al., 2004; Elias, 2004; Zeeuwen et al., 2004). We assume that the amelioration of hyperkeratosis in Tg(K14-CTSV);ctsl$^{-/-}$ mice is more likely to be caused by the reversal of keratinocyte hyperproliferation resulting in lower cell numbers that undergo the epidermal differentiation process.

Hair phenotype of Tg(K14-CTSV);ctsl$^{-/-}$ mice

Hair follicles are complex and highly dynamic "mini-organs" that produce the hair coat of the body (Paus and Cotsarelis, 1999). Morphogenesis and cycling of hair follicles in mouse skin is a tightly controlled, synchronized process. CTSL knockout mice exhibit delayed hair follicle morphogenesis, a prolonged hair follicle regression (catagen), a truncated resting phase (telogen) and premature entry into the growth phase (anagen) of the hair cycle (Roth et al., 2000). A pathological hyperproliferation and disturbed cell death in ctsl$^{-/-}$ hair follicles together with the disturbance of inner root sheath differentiation result in periodic hair loss of ctsl$^{-/-}$ mice (Tobin et al., 2002). This hair loss is most prominent in the first hair cycle during which CTSL knockout mice completely lose their fur at around day 30 after birth (Fig. 4A). Remarkably, Tg(K14-CTSV);ctsl$^{-/-}$ mice do not develop hair loss (Fig. 4A). After the hair loss, CTSL knockout mice grow new hair and 40 days after birth ctsl$^{+/+}$, ctsl$^{-/-}$ and Tg(K14-CTSV);ctsl$^{-/-}$ mice (Fig. 4B) are nearly indistinguishable. However, closer inspection of Tg(K14-CTSV);ctsl$^{-/-}$ mice reveals that these mice still look "rough-haired" (Fig. 4A, B). Thus, expression of CTSV under

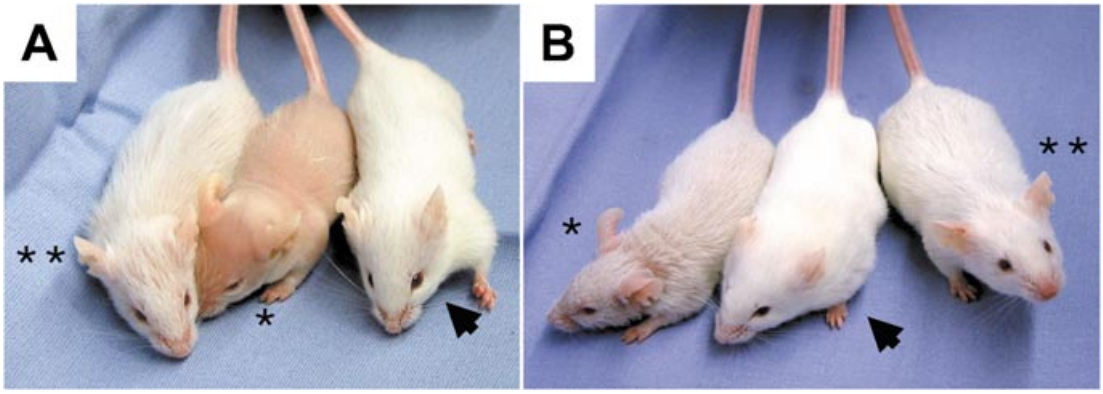

Fig. 4. Rescue of the periodic hair loss in K14-CTSV^{+}/ctsl$^{-/-}$ mice. Littermates were photographed 30 days (A) and 40 days (B) after birth. Wild-type mouse (arrow), ctsl$^{-/-}$ littermate (*) and Tg(K14-CTSV);ctsl$^{-/-}$ (**).

control of the K14 promoter might not be completely sufficient to mimic the murine CTSL expression pattern. Alternatively, and despite their high degree of homology (Fig. 1A), human CTSV and murine CTSL might have slightly different enzymatic properties in vivo (Brömme et al., 1999). These considerations highlight the requirement of regulated expression and function of proteases for the control of hair growth.

Conclusions

We report a compensation experiment in which the selectively expressed human CTSL-like cysteine protease CTSV can largely rescue the skin phenotype of CTSL knockout mice. This indicates that a keratinocyte-specific CTSL-like proteolytic activity has pivotal functions in the maintenance of epidermis and hair follicles. The results also imply that human CTSV and murine CTSL have similar substrate specificity in vivo. The ability of CTSV to normalize the proliferation rate of basal epidermal keratinocytes may direct further investigations on this "stratum corneum thiol protease" to epidermal layers and cell biological functions that are different from the desquamation processes in the stratum corneum.

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