The lysosomal cysteine protease cathepsin L regulates keratinocyte proliferation by control of growth factor recycling

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Summary

Mice deficient for cathepsin L (CTSL) show epidermal hyperplasia due to a hyperproliferation of basal keratinocytes. Here we show that the critical function of CTSL in the skin is keratinocyte specific. This is revealed by transgenic re-expression of CTSL in the keratinocytes of *ctsl*$^{-/-}$ mice, resulting in a rescue of the *ctsl*$^{-/-}$ skin phenotype. Cultivation of primary mouse keratinocytes with fibroblast- and keratinocyte-conditioned media, as well as heterologous organotypic co-cultures of mouse fibroblasts and human keratinocytes, showed that the altered keratinocyte proliferation is caused primarily by CTSL-deficiency in keratinocytes. In the absence of EGF, wild type and CTSL-knockout keratinocytes proliferate with the same rates, while in presence of EGF, *ctsl*$^{-/-}$ keratinocytes showed enhanced proliferation compared with controls. Internalization and degradation of radioactively labeled EGF was identical in both *ctsl*$^{-/-}$ and *ctsl*$^{+/+}$ keratinocytes. However, *ctsl*$^{-/-}$ keratinocytes recycled more EGF to the cell surface, where it is bound to the EGF-receptor, which is also more abundant in *ctsl*$^{-/-}$ cells. We conclude that the hyperproliferation of keratinocytes in CTSL-knockout mice is caused by an enhanced recycling of growth factors and growth factor receptors from the endosomes to the keratinocyte plasma membrane, which result in sustained growth stimulation.

Key words: Cathepsins, Epidermis, Hair follicle, Lysosomes, Mice, Knockout

Introduction

The molecular processes controlling proliferation and differentiation of epidermal keratinocytes are not yet fully understood (Fuchs and Raghavan, 2002). Since epidermal homeostasis is highly dynamic with regard to proliferation, differentiation and migration of cells, it is likely that proteolytic activities are essential for the execution of these processes. Consequently, the expression patterns and functions of multiple proteases and their inhibitors have been established to understand better the physiology and pathology of the epidermis. These proteases belong to a variety of distinct protease families, i.e. matrix metalloproteases (MMPs) (Liu et al., 2000), adamalysin-related disintegrin- and metalloproteases (ADAMS) (Franzke et al., 2002), proteases and inhibitors of the plasminogen activator/plasmin cascade (Zhou et al., 2000), trypsin- and chymotrypsin-like serine proteases of the stratum corneum (Ekholm et al., 2000), as well as cysteine- and aspartyl-type lysosomal proteases (Egberts et al., 2004; Horikoshi et al., 1999; Watkinson, 1999).

Lysosomal cysteine proteases belong to the family of papain-like proteolytic enzymes (clan CA, family C1) with their principal subcellular localisation in the endosomal/lysosomal compartment (Turk et al., 2001). Seven of these peptidases, the cathepsins B, C, F, H, L, O and X/Z, are ubiquitously expressed in mammalian tissues, while the expression of other papain-like cysteine peptidases, i.e. cathepsins K, S, V and W, is restricted to specific cell types.

Cathepsin L (CTSL) is a highly potent endoprotease with maximal proteolytic capacity at an acidic pH of about 5.5 and primary endosomal/lysosomal localisation suggestive of an involvment of CTSL in lysosomal bulk proteolysis. However, there is growing evidence for specific intra- and extracellular functions for CTSL in MHC-II antigen presentation (Honey and Rudensky, 2003), prohormone processing (Friedrichs et al., 2003; Yasothornsrikul et al., 2003) and other processes involving limited proteolysis (Turk et al., 2001). Major insights into the function of cathepsin L in the skin result from analysis of mice with targeted inactivation of the CTSL gene (*ctsl*$^{-/-}$ mice) and from the spontaneous mouse mutations *nackt* (*ctsl*^{nkt}/*ctsl*^{nkt}) and *furless* (*fs*$^{-}$/*fs*$^{-}$) for which the cathepsin L gene has been identified as the target (Benavides et al., 2002; Roth et al., 2000). Cathepsin L-deficient (*ctsl*$^{-/-}$) mice develop periodic hair loss, gingival acanthosis and epidermal hyperplasia with hyperkeratosis (Nishimura et al., 2002; Tobin et al., 2002). Impaired differentiation of hair follicle epithelial cells and hyperproliferation of basal epidermal keratinocytes are the primary characteristics of the *ctsl*$^{-/-}$ phenotype. Organ cultures of neonatal *ctsl*$^{-/-}$ mouse skin (Roth et al., 2000) and

crossing of *Rag2*$^{-/-}$ mice with *ctsl*^{mkt}/*ctsl*^{mkt} mice (Benavides et al., 2002) revealed that the skin phenotype is independent of systemic, i.e. immunological, effects.

The present study was initiated to investigate the relationship of genotype to phenotype in the epidermis of *ctsl*$^{-/-}$ mice. Specifically, we aimed to identify the cell type and the cell-biological processes in which CTSL exerts essential functions in the skin. Transgenic epithelial-specific re-expression of CTSL in *ctsl*$^{-/-}$ mice, together with organotypic skin cultures and conditioned cell culture media revealed that CTSL activity is critically important in keratinocytes. Furthermore, we provide evidence for an increased proliferative response of CTSL-deficient keratinocytes to EGF, which is due to an increased level of EGF-receptor and increased recycling of internalized ligand in the absence of CTSL.

Materials and Methods

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

The generation, maintenance and breeding of the animals used in this study were performed in accordance with our institutional regulations. The full-length murine *CTSL* cDNA (1.2 kb), including stop codon but without polyadenylation signal, was inserted into an expression cassette that includes the cloning vector pBluescript (Stratagene), a 2.1 kb human keratin 14 promoter (K14) 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 (Munz et al., 1999). The CTSL cDNA was inserted between the intron and the poly(A) fragment. Standard procedures were followed to generate transgenic mice by microinjection of the purified expression cassette into the pronuclei of one-cell-stage embryos and their subsequent re-transfer into the oviducts of pseudopregnant recipient females. Mouse tail DNA was analyzed for integration of the transgene (founder analysis and routine genotyping) by a PCR spanning from the 5′ untranslated region to exon 5 of the *CTSL* gene allowing unambiguous identification of the integrated *CTSL* cDNA. About half of the offspring mice carried the transgene. Subsequently, transgenic mice were bred with the *CTSL*-knockout mouse strain establishing the new mouse line Tg(K14-CTSL);*ctsl*$^{-/-}$.

Quantitative real-time PCR

Total RNA from murine tissues was prepared using the 'RNeasy Mini kit' (Qiagen, Hilden, Germany). 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 cathepsin L and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), PCR amplification of the reverse-transcribed cDNA was performed using equivalent amounts of the intercalating SYBR-green dye, cDNA/RNA, Taq-polymerase and specific primers (CTSL: 5′-GCACGGCTTTTCCATGGA-3′ and 5′-CCACCTGCCTGAATTCCTCA-3′; GAPDH: 5′-TGCACCACCA-ACTGCTTAG-3′ and 5′-GATGCAGGGATGATGTTC-3′) under the following conditions: 1 cycle for 1 minute at 72°C, 50 cycles (94°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds) and 1 cycle at 72°C for 7 minutes in the MyiQ^{TM} single-color real-time PCR detection system (BioRad, München, Germany). The resulting PCR products were visualized by ethidium bromide staining after separation on 2% (w/v) agarose gels.

Histology and immunohistochemistry

For histological assessment, back skin sections of 5 μm were deparaffinized in xylene, hydrated in graded ethanol solutions and stained with hematoxylin/eosin. Goat anti-mouse CTSL antibody (R&D Systems, Wiesbaden, Germany; 0.2 μg ml^{-1}) and rat anti-mouse Ki67 antibody (DakoCytomation, Hamburg, Germany; dilution 1:200) were used for the detection of CTSL and of the proliferation marker Ki67, respectively. In sections from organotypic co-cultures, antibodies directed against Ki67 (ab833; Abcam, Cambridge, UK; dilution 1:100), transglutaminase (ab421; Abcam; dilution 1:100) and cytokeratin 10 (ab9026; Abcam; dilution 1:100) were used. 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).

Detection of CTSL enzyme activity

CTSL proteolytic activity was determined in epidermal lysates (100 μg protein) by degradation of the fluoropeptide Z-Phe-Arg-4-methyl-coumarin-7-amide (20 μM; Bachem) in the presence of the CTSB-specific inhibitor CA074 (1.5 μM; Bachem) at pH 5.5. The release of 7-amino-4-methyl-coumarin was continuously monitored for 1 hour by spectrofluorometry at excitation and emission wavelengths of 360 nm and 460 nm, respectively.

Preparation of primary dermal fibroblasts and keratinocytes from mouse skin

For isolation of dermis and epidermis the skin of 3-day-old wild-type or *ctsl*$^{-/-}$ mice was incubated in 0.25% trypsin solution at 4°C for 24 hours. Subsequently, dermis and epidermis were carefully separated. For preparation of primary dermal fibroblasts, the dermis was cut into small pieces and digested in 5 ml M-199 medium containing 0.35% (w/v) collagenase at 4°C for 45 minutes. The cell suspension was cleared through a 100 μm cell strainer, cells were collected by centrifugation and resuspended in DMEM containing 5% FCS. The fibroblast culture was incubated at 37°C with 5% CO_2 and the medium was changed every 48 hours. For preparation of primary keratinocytes epidermal pieces of four mice were pooled, cut into small pieces and a single cell suspension was prepared by stirring in 6 ml 'self made' low calcium keratinocyte-growth medium (Calautti et al., 1995; Hennings et al., 1980) at 4°C for 1 hour. The cell suspension was cleared through a 100 μm cell strainer and plated on a 150 cm^2 cell culture dish. The keratinocyte culture was incubated at 37°C with 7% CO_2 and the medium was changed every day.

Organotypic co-cultures (OTC)

Heterologous OTC were performed as previously described (Maas-Szabowski et al., 2001). In brief, normal epidermal keratinocytes (NEK) derived from adult human skin were seeded (1×10^6 per insert) onto collagen type I gels (rat tail tendon, 3.2 mg ml^{-1}) cast in cell culture filter inserts (pore size 3.0 μm, Falcon, Becton Dickinson, Heidelberg, Germany) containing 1.5×10^5 ml^{-1} fibroblasts (two different isolations of mouse *ctsl*$^{+/+}$ and *ctsl*$^{-/-}$ skin fibroblasts as well as human fibroblasts, respectively). After 24 hours, medium was replaced by DME-medium with 10% FCS and 50 μg ml^{-1} L-ascorbic acid (Sigma, Deisenhofen, Germany) and cultures were raised to the air-liquid interface. Medium was replaced every second day for 7 days. Cultures were fixed in 3.7% phosphate-buffered formaldehyde and embedded in paraffin according to a standardized protocol for routine histology. Paraffin sections were stained in haematoxylin and eosin and by immunohistochemistry.

Measurement of keratinocyte proliferation by [^{3}H]thymidine incorporation

Three hours after the last medium change, [^{3}H]thymidine (3μCi ml^{-1} final) was added and cells were further incubated for 90

minutes at 37°C with 7% CO_2. Cells were washed twice with ice-cold PBS followed by addition of 2 ml of ice-cold 10% trichloroacetic acid (TCA) and an overnight incubation at 4°C. Precipitates were washed twice with cold 10% TCA and dissolved with 0.2 M NaOH for 5 minutes at room temperature. The sample was neutralized with an equal volume of 0.4 M HEPES buffer and [^{3}H]thymidine incorporation was determined by scintillation counting.

Conditioned cell culture media

Fibroblast- and keratinocyte-conditioned media, respectively, were produced by incubation of subconfluent cells with low calcium keratinocyte growth medium. After 24 hours the medium was harvested, contaminating cells were removed by centrifugation and the conditioned medium was supplemented with 50% fresh keratinocyte medium to provide sufficient nutrition for the cells. To measure the effects of conditioned media toward keratinocyte proliferation, keratinocytes were incubated with 50% conditioned medium for 3 hours followed by [^{3}H]thymidine incorporation for 90 minutes.

^{125}I-EGF internalisation, degradation and recycling

Primary mouse keratinocytes were cultured for 5 days without EGF. ^{125}I-EGF (10 nM, ICN Biomedicals GmbH, Eschwege, Germany) was added to cells at 4°C for 30 minutes. Subsequently, cells were washed twice with PBS and chased with keratinocyte medium. At appropriate chase time points the cells were washed in PBS, followed by the removal of surface-localized radioactivity by an isotonic lysine-buffered solution, pH 3.5. Internalized ^{125}I-EGF was measured by counting radioactivity in cell lysates after an acidic glycine (pH 3.5) wash. Recycling and degradation of EGF was analyzed after loading cells with 10 nM ^{125}I-EGF for 30 minutes. Upon loading, the surface-localized ^{125}I-EGF was removed by a glycine-buffered solution (pH 3.5) and the fate of internalized ^{125}I-EGF was followed during chase at 37°C. The medium was analyzed for degraded EGF (soluble in 10% TCA with 0.5% BSA as tracer) and intact recycled EGF (precipitable by TCA) and the cells were analyzed for recycled EGF (released by the pH 3.0 buffer). In addition, degraded and intact ^{125}I-EGF was determined in cell lysates. The sum of radioactivity in all measured fractions obtained from a cell culture dish represents the total, i.e. 100%, radioactivity.

Detection of the EGF-receptor by western blotting

Subconfluent keratinocytes were lysed in TBS/0.1% SDS and 10 µg protein sample was applied to SDS-PAGE (8-16% gradient) and subsequently blotted onto a PVDF membrane. Monoclonal anti-mouse EGFR (non-phospho-Y1173) antibody (Upstate, Lake Placid, NY; at 1:1000 dilution) and mouse monoclonal anti-mouse actin antibody (ICN Biochemicals, Aurora, OH; at 1:2500 dilution) were used. The binding of secondary antibody (anti-mouse IgG-POD) was detected by the SuperSignal™ Chemiluminescent Substrate (Pierce, Rockford, IL).

Data presentation and statistical analysis

Data in graphs are expressed as means±s.e.m. Statistical comparison between the *ctsl*$^{+/+}$ and the *ctsl*$^{-/-}$ group at various time intervals was done by one-way ANOVA and by the Student's *t*-test for independent samples. Differences were considered significant at a level of $P<0.05$. Data presentation was performed with ORIGIN for Windows (Microcal Software, Northampton, MA).

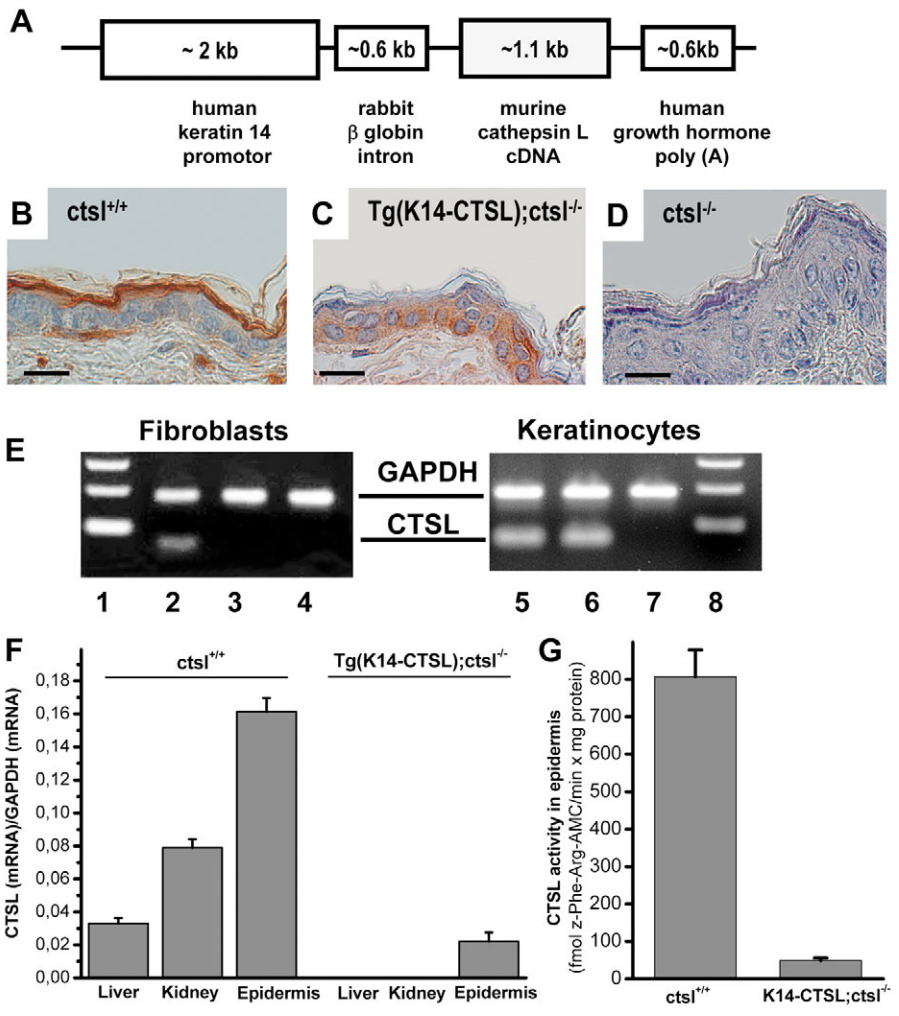

Fig. 1. Expression of murine cathepsin L (CTSL) in skin from the backs of *ctsl*$^{+/+}$, *ctsl*$^{-/-}$ and Tg(K14-CTSL);*ctsl*$^{-/-}$ mice. (A) Transgene construct. Functional elements are the human keratin 14 promoter, the rabbit β-globin intron, the full-length mouse CTSL cDNA, and the human growth hormone poly(A)signal. (B-D) Immunohistochemistry for detection of CTSL (brown staining; bars 20 µm). (B) Wild type; (C) Tg(K14-CTSL);*ctsl*$^{-/-}$; and (D) *ctsl*$^{-/-}$. (E) RT-PCR (endpoint amplification; 50 cycles) for detection of CTSL and GAPDH mRNA in primary dermal fibroblasts and epidermal keratinocytes. Lanes 1 and 8: 100 bp ladder; lanes 2 and 5: *ctsl*$^{+/+}$; lanes 3 and 6: Tg(K14-CTSL);*ctsl*$^{-/-}$; lanes 4 and 7: *ctsl*$^{-/-}$ (representative of three independent experiments). (F) Quantitative RT-PCR (real-time) for quantification of CTSL and GAPDH mRNA in liver, kidney and epidermis of wild type (*ctsl*$^{+/+}$) and Tg(K14-CTSL);*ctsl*$^{-/-}$ mice (n=3). (F) CTSL proteolytic activity in epidermal lysates of wild type (*ctsl*$^{+/+}$) and Tg(K14-CTSL);*ctsl*$^{-/-}$ mice (n=2).

Results

Rescue of the *ctsl*$^{-/-}$ skin phenotype by transgenic expression of CTSL under the control of the keratin 14 promoter

To analyze CTSL functions in epithelial tissue we expressed the mouse CTSL cDNA under the control of the human keratin 14 (K14) promoter in transgenic mice (Fig. 1A). The Tg(K14-CTSL) mice are viable, fertile and did not show a skin phenotype. One of the two transgenic founder mice with single locus integration was chosen for breeding with *ctsl*$^{-/-}$ mice. The resulting mouse line, Tg(K14-CTSL);*ctsl*$^{-/-}$, exhibits an expression pattern for CTSL in the skin which differs from that of wild-type mice (Fig. 1). By immunohistochemistry CTSL expression in wild-type skin is pronounced in the corneal layer of interfollicular epidermis (Fig. 1B), whereas Tg(K14-CTSL);*ctsl*$^{-/-}$ mice exhibit CTSL expression primarily in basal keratinocytes without accumulation in the upper epidermal layers (Fig. 1C). As expected, CTSL was not detectable in the skin of CTSL-knockout mice (Fig. 1D). RT-PCR revealed that CTSL is specifically expressed in primary keratinocytes of Tg(K14-CTSL);*ctsl*$^{-/-}$ mice, but CTSL mRNA is not present in the corresponding dermal fibroblast culture (Fig. 1E). Quantitative RT-PCR revealed that Tg(K14-CTSL);*ctsl*$^{-/-}$ epidermis contained 10-15% CTSL-mRNA when compared with wild-type epidermis (Fig. 1F). In agreement with this finding, epidermal lysates of Tg(K14-CTSL);*ctsl*$^{-/-}$ mice exhibit about 5% CTSL activity compared with the wild type (Fig. 1G). Despite these differences in CTSL expression between wild type and Tg(K14-CTSL);*ctsl*$^{-/-}$ mice, the epidermal thickening and hyperproliferation of basal keratinocytes observed in *ctsl*$^{-/-}$ mice are rescued in Tg(K14-CTSL);*ctsl*$^{-/-}$ mice (Fig. 2A-E). In addition, the characteristic transient hair loss occurring around day 30 in CTSL-knockout mice is almost completely reversed by CTSL expression controlled by the keratin 14 promoter (Fig. 2F,G). Since the K14-promoter directs expression to keratinocytes of the basal epidermal layer as well as to the outer root sheath of hair follicles (Munz et al., 1999), these data provide strong evidence that the keratinocyte represents the cell type in which CTSL is critically important for homeostasis of mouse epidermis and hair cycling.

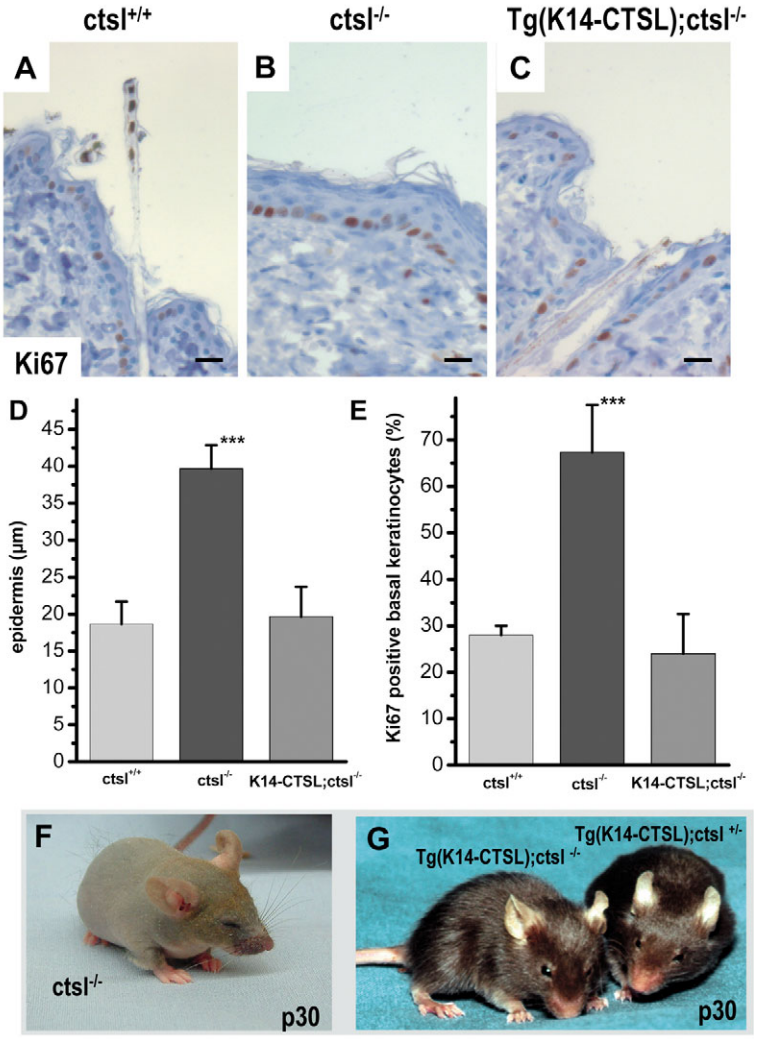

Fig. 2. Rescue of the *ctsl*$^{-/-}$ skin phenotype by keratinocyte-specific re-expression of CTSL. (A-C) Immunohistochemical detection of the proliferation marker Ki67 in basal keratinocytes (brown staining; bars 20 µm). (D) Quantitative analysis of epidermal thickness of mouse back skin (Axiovision; Zeiss). (E) Ki67 proliferation index (%) calculated as Ki67-positive basal layer cells divided by total basal layer cells×100. ***P<0.001 compared with all other groups (n=3, four paraffin sections per mouse). (F,G) Rescue of the periodic hair loss of CTSL-deficient mice in Tg(K14-CTSL);*ctsl*$^{-/-}$ mice photographed 30 days after birth.

Hyperproliferation of *ctsl*$^{-/-}$ keratinocytes is an autocrine process

Epidermal homeostasis involving proliferation and terminal differentiation of keratinocytes is largely regulated by mesenchymal growth factors (Fusenig, 1994). The consequence of CTSL deficiency in dermal fibroblasts for the formation of stratified keratinizing epithelia was addressed by heterologous organotypic co-cultures in which human keratinocytes grow air-exposed on a matrix of collagen I containing viable mouse fibroblasts (Maas-Szabowski et al., 2001). In skin equivalents containing either human fibroblasts, ctsl$^{+/+}$ or ctsl$^{-/-}$ mouse fibroblasts, human keratinocytes form well-stratified and differentiated epithelia (Fig. 3). No differences were detected for the proliferation marker Ki67, nor for the epidermal differentiation markers cytokeratin 10 and transglutaminase. To further assess the para- and autocrine signaling on CTSL-deficient keratinocytes, we measured the proliferative effects of fibroblast- and keratinocyte-conditioned media on cultured *ctsl*$^{-/-}$ and *ctsl*$^{+/+}$ primary mouse keratinocytes (Fig. 4). The source of conditioned medium (CM) had no relative effect on proliferation rates of *ctsl*$^{+/+}$ keratinocytes. However, ctsl$^{-/-}$ keratinocytes were significantly affected by the source of CM, with reduced proliferation in CM from *ctsl*$^{-/-}$ fibroblasts compared with CM from *ctsl*$^{+/+}$ fibroblasts and enhanced proliferation of *ctsl*$^{-/-}$ keratinocytes compared with *ctsl*$^{+/+}$ keratinocytes in medium conditioned by *ctsl*$^{+/+}$ keratinocytes. Most importantly, *ctsl*$^{-/-}$ keratinocyte CM

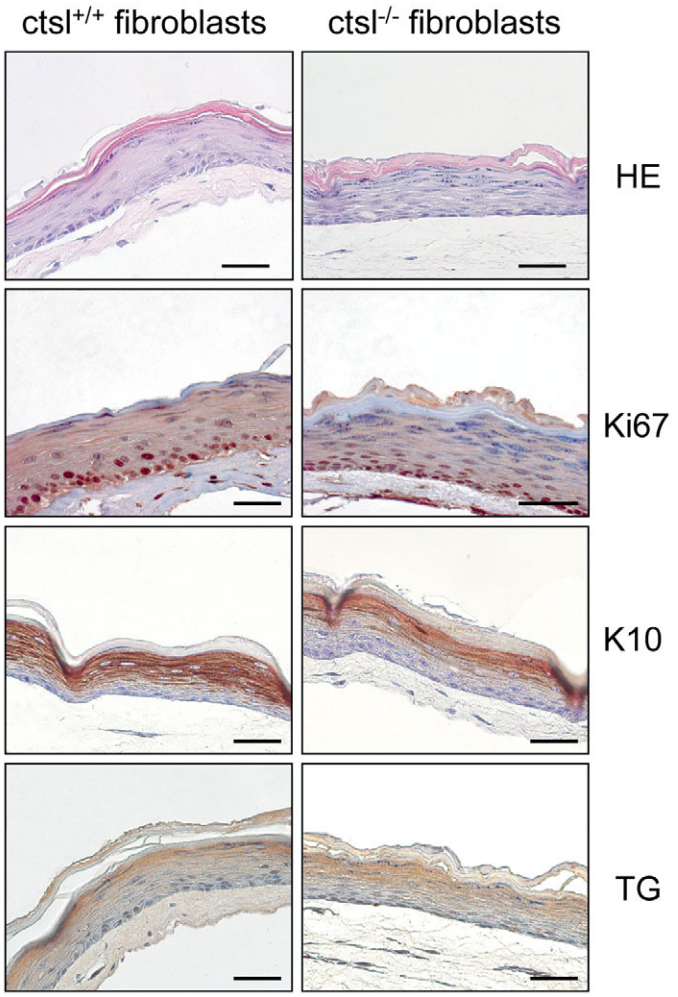

Fig. 3. Mesenchymal-epidermal interactions assessed by heterologous organotypic co-cultures (OTC). Heterologous OTC consisting of *ctsl*$^{+/+}$ fibroblasts or *ctsl*$^{-/-}$ fibroblasts in collagen type I gels topped by normal human primary keratinocytes were grown air-exposed for 7 days. Paraffin sections were stained in haematoxylin and eosin (HE) or by immunohistochemistry for the proliferation marker Ki67 (Ki67, brown nuclear staining) and the differentiation markers cytokeratin 10 (K10, brown staining) and transglutaminase (TG, brown staining). The experiment is representative for a total of 16 OTC grown with primary dermal fibroblasts from *ctsl*$^{+/+}$ or *ctsl*$^{-/-}$ skin (two independent fibroblast preparations per genotype). Bars, 100 μm.

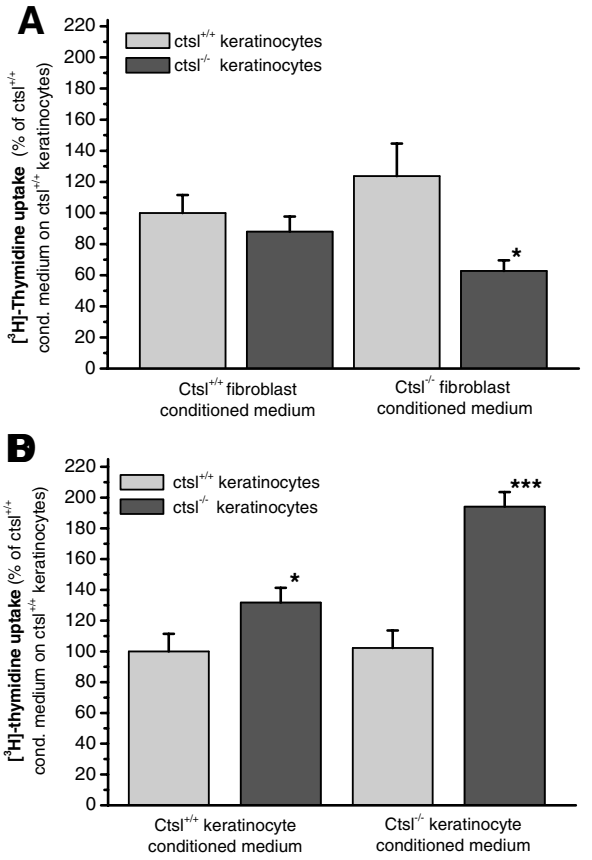

Fig. 4. Paracrine and autocrine effects on keratinocyte proliferation in absence of CTSL. (A) Effects of fibroblast conditioned media on proliferation of *ctsl*$^{+/+}$ (pale gray columns) and *ctsl*$^{-/-}$ keratinocytes (dark gray columns). Proliferation was measured by [^{3}H]thymidine incorporation into DNA. **P*<0.05 compared with all other groups. (B) Effects of keratinocyte conditioned media on proliferation of *ctsl*$^{+/+}$ (pale gray columns) and *ctsl*$^{-/-}$ keratinocytes (dark gray columns). **P*<0.05 compared with *ctsl*$^{+/+}$; ****P*<0.001 compared with all other groups.

significantly stimulated proliferation of *ctsl*$^{-/-}$ keratinocytes more than twofold compared with CM from *ctsl*$^{+/+}$ keratinocytes, suggesting an altered status of growth factor receptors on CTSL-deficient keratinocytes and enhancement of *ctsl*$^{-/-}$ keratinocyte proliferation by autocrine mechanisms.

EGF-induced proliferation of *ctsl*$^{-/-}$ keratinocytes

The EGF-receptor is a major regulator of keratinocyte proliferation (Hashimoto, 2000). Thus, we tested the proliferative response of *ctsl*$^{-/-}$ and *ctsl*$^{+/+}$ keratinocytes to exogenously added murine EGF (Fig. 5). Without addition of EGF, [^{3}H]thymidine incorporation as a measure of cell proliferation is not significantly different for either CTSL genotype (Fig. 5A). However, culturing of keratinocytes in the presence of murine EGF resulted in significantly increased proliferation of CTSL-knockout keratinocytes as compared with wild-type cells (Fig. 5B). This finding could be explained by a higher abundance of EGF-receptor in *ctsl*$^{-/-}$ keratinocytes, which has also been confirmed by western blotting (Fig. 5C). This higher EGF-receptor abundance in *ctsl*$^{-/-}$ keratinocytes is not caused by an increase in EGF-receptor gene transcription as revealed by quantitative RT-PCR (Fig. 5D).

EGF internalization, degradation and recycling by *ctsl*$^{-/-}$ keratinocytes

Receptor-mediated endocytosis was assessed by internalization of ^{125}I-EGF into keratinocytes (Fig. 6). In this experiment *ctsl*$^{+/+}$ and *ctsl*$^{-/-}$ keratinocytes showed identical rates of growth factor internalisation. To follow the fate of the endocytosed ^{125}I-EGF, keratinocytes were loaded with radioactive growth factor at 37°C. ^{125}I-EGF that was not internalized was removed from the cell surface by an acidic-glycine wash and the degradation as well as the re-appearance (i.e. recycling) of ^{125}I-

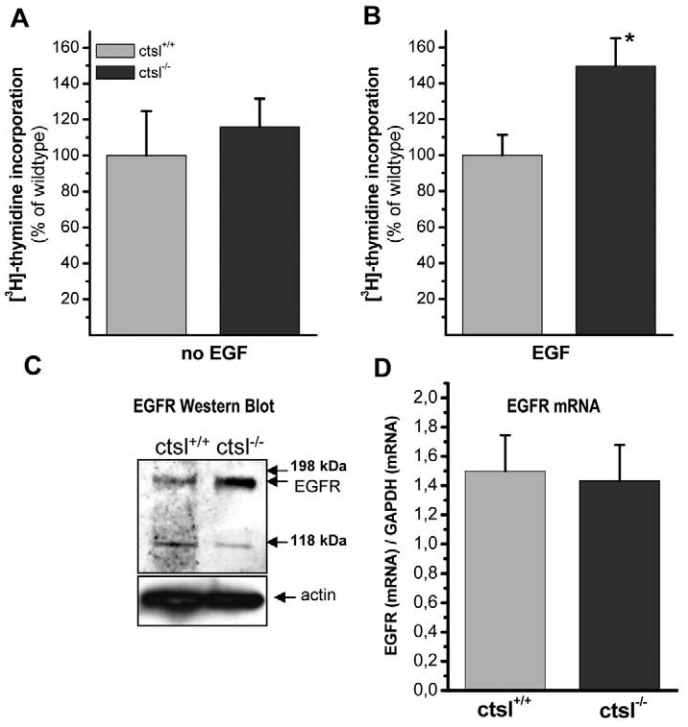

Fig. 5. CTSL-knockout keratinocytes are more responsive to EGF stimulation than wild-type cells. Proliferation of *ctsl*$^{+/+}$ (pale gray columns) and *ctsl*$^{-/-}$ keratinocytes (dark gray columns) grown (A) in absence (no EGF) or (B) in presence (EGF) of exogenously added murine EGF (10 ng ml^{-1}). The last supplementation with EGF was performed simultaneously with the addition of [^{3}H]thymidine. *P<0.05 compared with *ctsl*$^{+/+}$ keratinocytes. (C) Western blot for detection of EGF-receptor (EGFR) on keratinocytes and (D) quantitative RT-PCR (real-time) for quantification of EGF-receptor and GAPDH mRNA in absence of exogenous EGF.

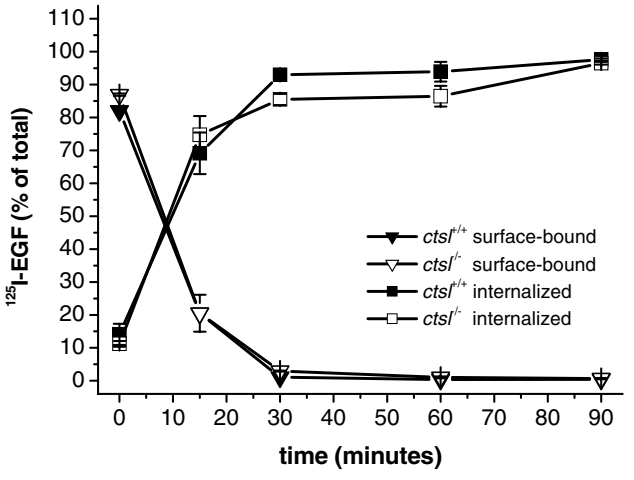

Fig. 6. Internalization of ^{125}I-EGF by wild type (closed symbols) or CTSL-knockout (open symbols) primary keratinocytes. Cells were incubated with murine ^{125}I-EGF for 30 minutes at 4°C. During the chase at 37°C, cell-surface-bound ^{125}I-EGF (triangles) and intracellular ^{125}I-EGF (squares) were quantified.

EGF in cell culture medium and at the cell surface receptor was then analyzed (Fig. 7). The intra- and extracellular amount of trichloroacetic acid-soluble EGF-degradation products was not significantly different in *ctsl*$^{+/+}$ and *ctsl*$^{-/-}$ keratinocytes (Fig. 7A,B). However, intact EGF was significantly elevated in the cell culture medium from *ctsl*$^{-/-}$ keratinocytes after 90 minutes (Fig. 7C). Most importantly, in comparison with wild-type cells, *ctsl*$^{-/-}$ keratinocytes showed significantly increased pH-sensitive binding of intact ^{125}I-EGF to its receptor at the cell surface during the entire time course of the experiment (Fig. 7D). Thus, *ctsl*$^{-/-}$ keratinocytes recycle more intact growth factor to the cell surface, where it is specifically bound to its receptor, which is itself more abundant on *ctsl*$^{-/-}$ cells (Fig. 5C).

Together, these data strongly suggest sustained proliferation stimuli from growth factors/growth factor receptors that are, in the absence of CTSL, recycled at higher rates from the endosomal compartment of keratinocytes resulting in the observed hyperproliferation of basal keratinocytes of *ctsl*$^{-/-}$ mice.

Discussion

Regulated proliferation is a key aspect of keratinocyte biology. CTSL-knockout mice exhibit an increased number of cells staining positive for the proliferation marker Ki67 in the epidermal basal layer and throughout the entire hair follicle epithelium (Roth et al., 2000; Tobin et al., 2002). Notably, the aberrant occurrence of proliferating cells during hair follicle regression largely contributes to the disturbed hair cycle of ctsl$^{-/-}$ mice (Tobin et al., 2002). Organ cultures of neonatal mouse skin suggested an autonomous deregulation of keratinocyte proliferation in the skin of *ctsl*$^{-/-}$ mice (Roth et al., 2000). However, whole skin is composed of the epidermal and dermal layers each containing a variety of specialized cell types. Keratinocytes and fibroblasts are the prominent epidermal and dermal cell types, respectively. These epithelial and mesenchymal cells secrete multiple para- and autocrine growth factors that form a complex signaling network for the regulation of epidermal proliferation and differentiation (Fusenig, 1994). By three complementary experimental approaches we provide evidence for a critical role of CTSL in the proliferation of basal epidermal keratinocytes and exclude a contribution of mesenchymal cells to the epidermal thickening of *ctsl*$^{-/-}$ mice. We first performed a genetic rescue experiment by expressing mouse CTSL specifically in the basal epidermal layer. Breeding of these transgenic mice with *ctsl*$^{-/-}$ mice resulted in a normal proliferation index for basal keratinocytes, physiological epidermal thickness and normal hair coat in the otherwise CTSL-deficient mice. Interestingly, an mRNA expression level of 10-15% and an enzyme activity of about 5% as compared with the wild-type was sufficient to rescue the epidermal and hair cycle phenotype in the Tg(K14-CTSL);*ctsl*$^{-/-}$ mice. Secondly, we employed heterologous organotypic co-cultures that have been used previously to investigate the effects of growth factors released from genetically modified mouse fibroblasts on the formation of a stratified epithelium composed of human keratinocytes (Szabowski et al., 2000). Our results obtained with this system show that CTSL-deficiency in dermal fibroblasts does neither affect the proliferation nor the differentiation of keratinocytes,

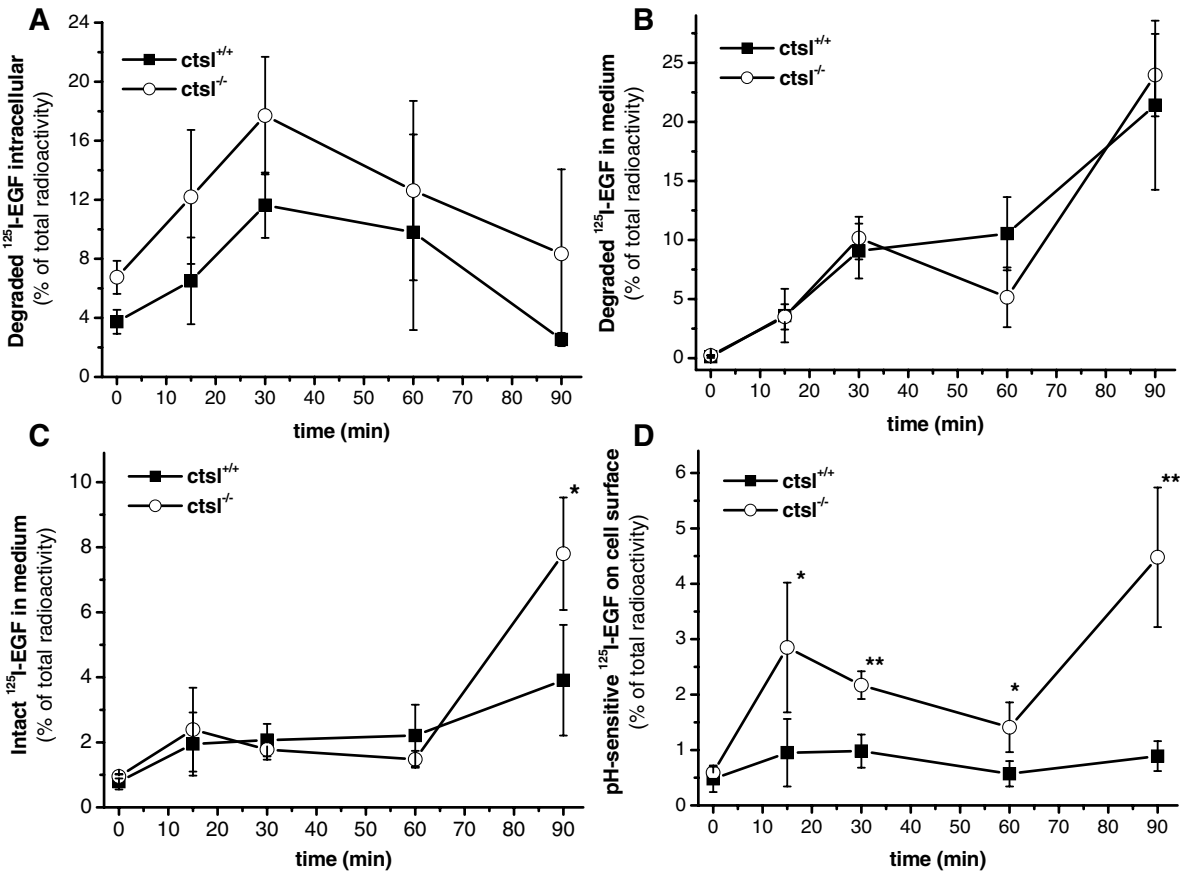

Fig. 7. Enhanced recycling of ^{125}I-EGF in *ctsl*$^{-/-}$ keratinocytes. After loading the cells for 30 minutes, cell-surface-bound ^{125}I-EGF was removed by an isotonic pH 3.5 wash and the fate of the internalized ^{125}I-EGF was followed during chase at 37°C. (A) Degradation of intracellular ^{125}I-EGF in *ctsl*$^{+/+}$ and *ctsl*$^{-/-}$ keratinocytes. (B) Degraded ^{125}I-EGF in the medium. (C) Intact ^{125}I-EGF in the medium. *P<0.05 between *ctsl*$^{+/+}$ and *ctsl*$^{-/-}$ of the corresponding time point. (D) Cell surface (receptor-bound) ^{125}I-EGF releasable by incubation at pH 3.5. *P<0.05, **P<0.01 between *ctsl*$^{+/+}$ and *ctsl*$^{-/-}$ of the corresponding time point.

which further excludes a CTSL-dependent role of dermal fibroblasts in epidermal proliferation. Thirdly, the results using conditioned media (CM) support a keratinocyte specific function of CTSL, since major effects on proliferation were only measured when CTSL-deficient keratinocytes were incubated with fibroblast CM or keratinocyte CM. The experiment with fibroblast CM suggests a paracrine effect of *ctsl*$^{-/-}$ fibroblasts inhibiting *ctsl*$^{-/-}$ keratinocyte proliferation, however, our transgenic rescue experiment and the work with organotypic co-cultures do not support this hypothesis. Most interestingly, the increased proliferation of *ctsl*$^{-/-}$ keratinocytes upon incubation with wild-type-keratinocyte CM was further enhanced when *ctsl*$^{-/-}$ keratinocyte CM was used. Two major hypotheses, each with very significant implications for regulation of epidermal cell homeostasis, can be drawn from these experiments. Firstly, *ctsl*$^{-/-}$ keratinocytes are more responsive to growth factors than wild-type cells. Secondly, in comparison with wild-type cells, *ctsl*$^{-/-}$ keratinocytes release larger amounts of active growth factors.

To test these hypotheses we investigated the EGF-receptor system, which is a prominent representative of endo-, para- and autocrine signaling regulating epidermal cell proliferation (Hashimoto, 2000). The mammalian ligands that bind to the EGF-receptor include EGF, betacellulin, epigen, amphiregulin, epiregulin, transforming growth factor-α and heparin-binding EGF-like growth factor (Harris et al., 2003). It has already been shown that the latter four ligands are produced by epithelial cells, are activated by the metalloprotease ADAM 17 and act in autocrine loops (Harris et al., 2003; Sahin et al., 2004). Transgenic overexpression of transforming growth factor-α in murine epidermis is linked to hyperplasia and hyperkeratosis, a phenotype of interfollicular epidermis similar to CTSL-deficient mice (Vassar and Fuchs, 1991). In the present study, we chose murine EGF as the model ligand to investigate the EGF-receptor system of CTSL-deficient keratinocytes. EGF is to a large extent synthesized by the submaxillary glands, but neither by fibroblasts nor by keratinocytes (Harris et al., 2003). Therefore, in keratinocyte cultures it is present only in minor amounts, these being derived from the fetal calf serum, which is essential for the cultivation of primary mouse keratinocytes. Thus, the measured EGF-induced proliferation can be contributed to the exogenously added growth factor. In these experiments CTSL-knockout keratinocytes were more responsive to EGF than wild-type keratinocytes. This is most likely due to the increased amount of EGF-receptor present in *ctsl*$^{-/-}$ keratinocytes. Since EGF-receptor gene transcription is not affected in *ctsl*$^{-/-}$ keratinocytes, increased EGF-receptor levels are due to post-transcriptional mechanisms. These could

be increased translation efficiency or, more likely, a prolonged half-life of the receptor. Plasma membrane signaling receptors, like the EGF-receptor and their ligands are internalized into endosomes and can be completely degraded after fusion of these endosome/multivesicular bodies with lysosomes (Sorkin and Von Zastrow, 2002). Inhibitor studies have suggested that the EGF-receptor can be degraded by a cathepsin L-like protease (Hiwasa et al., 1988) and that CTSL is involved in degradation of the insulin-like growth factor binding protein-3 (Zwad et al., 2002). It has also been shown that EGF is degraded in hepatocytes by cathepsin B, which is another representative of the papain-like cysteine peptidases (Authier et al., 1999). However, a part of the internalized receptors and/or ligands are recycled to the cell surface via recycling endosomes. Thus, balanced internalization, degradation and recycling of signaling receptors are each essential components for the regulation of cellular signal transduction, i.e. in signal termination and re-sensitization processes (Sorkin and Von Zastrow, 2002). Our results on the fate of ^{125}I-EGF in *ctsl*$^{-/-}$ keratinocytes indicate normal receptor mediated internalisation and no defect in EGF degradation. However, the recycling of intact EGF was significantly enhanced in *ctsl*$^{-/-}$ keratinocytes. We propose that increased recycling of active growth factors (e.g. EGF) in CTSL-knockout keratinocytes is the mechanism that results in the stimulation of keratinocyte proliferation by medium conditioned by *ctsl*$^{-/-}$ keratinocytes. In our investigation of ^{125}I-EGF recycling, most of the recycled EGF could immediately bind to its receptor (DeWitt et al., 2001), which is present at high levels on CTSL-deficient keratinocytes. Since EGF-receptor transcription does not vary between the CTSL genotypes, we assume that the recycling of not only the ligand but also the EGF-receptor is increased in *ctsl*$^{-/-}$ keratinocytes.

In conclusion, we have shown that critical CTSL functions in the skin are keratinocyte-specific and are most likely located in the endosomal/lysosomal compartment. We propose a model were enhanced recycling of plasma membrane receptors and their ligands, with the EGF-receptor system as a prominent example, results in increased proliferation of basal keratinocytes and, therefore, in epidermal thickening of CTSL-knockout mice. Since the epidermal phenotype of CTSL-deficient mice is unique among the existing knockout models for cysteine-cathepsins (Halangk et al., 2000; Ondr and Pham, 2004; Pham and Ley, 1999; Saftig et al., 1998; Shi et al., 1999), the present work provides clear evidence for a cell-type specific, non-redundant function of a ubiquitously expressed lysosomal cysteine-protease.

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