The role of cathepsin E in terminal differentiation of keratinocytes

Kawakubo, Tomoyo
Proteolysis Research Laboratory, Department of Pharmacology, Kyushu University

Yasukochi, Atsushi
Proteolysis Research Laboratory, Department of Pharmacology, Kyushu University

Okamoto, Kuniaki
Department of Dental Pharmacology, Graduate School of Biomedical Sciences, Nagasaki University

Okamoto, Yoshiko
Department of Biochemistry, Daiichi University College of Pharmaceutical Sciences

他

出版情報：Biological Chemistry. 392 (6), pp.571-585, 2011-06. De Gruyter
バージョン：

Biol. Chem., Vol. 392, pp. 571–585, June 2011 • Copyright © by Walter de Gruyter • Berlin • New York. DOI 10.1515/BC.2011.060

The role of cathepsin E in terminal differentiation of keratinocytes

Tomoyo Kawakubo[1,a], Atsushi Yasukochi[1,2,a], Kuniaki Okamoto[3], Yoshiko Okamoto[4], Seiji Nakamura[2] and Kenji Yamamoto[1,*]

[1] Proteolysis Research Laboratory, Department of Pharmacology, Kyushu University, Higashi-ku, Fukuoka 812-8582, Japan
[2] Department of Oral and Maxillofacial Surgery, Graduate School of Dental Science, Kyushu University, Fukuoka, Japan
[3] Department of Dental Pharmacology, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki 852-8588, Japan
[4] Department of Biochemistry, Daiichi University College of Pharmaceutical Sciences, Fukuoka 815-8511, Japan

* Corresponding author
e-mail: kyama@dent.kyushu-u.ac.jp

Abstract

Cathepsin E (CatE) is predominantly expressed in the rapidly regenerating gastric mucosal cells and epidermal keratinocytes, in addition to the immune system cells. However, the role of CatE in these cells remains unclear. Here we report a crucial role of CatE in keratinocyte terminal differentiation. CatE deficiency in mice induces abnormal keratinocyte differentiation in the epidermis and hair follicle, characterized by the significant expansion of corium and the reduction of subcutaneous tissue and hair follicle. In a model of skin papillomas formed in three different genotypes of syngeneic mice, CatE deficiency results in significantly reduced expression and altered localization of the keratinocyte differentiation induced proteins, keratin 1 and loricrin. Involvement of CatE in the regulation of the expression of epidermal differentiation specific proteins was corroborated by *in vitro* studies with primary cultures of keratinocytes from the three different genotypes of mice. In wild-type keratinocytes after differentiation inducing stimuli, the CatE expression profile was compatible to those of the terminal differentiation marker genes tested. Overexpression of CatE in mice enhances the keratinocyte terminal differentiation process, whereas CatE deficiency results in delayed differentiation accompanying the reduced expression or the ectopic localization of the differentiation markers. Our findings suggest that in keratinocytes CatE is functionally linked to the expression of terminal differentiation markers, thereby regulating epidermis formation and homeostasis.

[a] These authors contributed equally to this work.

Keywords: aspartic proteinase; cathepsin E; keratinocyte; skin papilloma; terminal differentiation.

Introduction

The epidermis is a stratified and keratinized squamous epithelium mainly composed of keratinocytes. Epidermal differentiation results in the formation of several distinct cell layers characterized by their ultrastructure, mitotic state and expression of specific markers. The basal keratinocytes have a high proliferating potential and are characterized by specific expression of keratin 5 (K5) and K14, in addition to their cuboidal structure and attachment to the underlying basement membrane. It has long been considered that the basal keratinocytes migrate to the suprabasal layer and directly become spinous cells expressing K1, K10 and involucrin when terminal differentiation is initiated. However, recent evidence has shown that most of the basal keratinocytes do not really migrate, but arise from dividing basal layer cells in which the mitotic spindle is perpendicular to the basement membrane, thus resulting in the development of suprabasal cells that commit to enter a program of terminal differentiation (reviewed in Blanpain and Fuchs, 2009). The spinous cells concomitantly switch from the expression of such differentiation markers as K1, K10 and involcrin to the expression of the late differentiation markers, loricrin and filaggrin, according to the levels of differentiation, thereby forming the granular layer characterized by the existence of keratohyalin granules. The granular keratinocytes terminally differentiate to form the cornified layer at the outmost layer, which consists of extremely flat, keratin filled and anucleated keratinocytes and is believed to be primarily responsible for the barrier function of the skin. Dermatological disorders ranging from minor cosmetic problems to life threatening conditions are commonly due to abnormal differentiation of the keratinocytes. Elucidation of the intracellular molecules involved in the cellular differentiation processes and the regulation of epidermal homeostasis is thus of particular importance for the understanding and therapy of these disorders.

Recent analyses of patients with defective protease genes (Toomes et al., 1999; Chavanas et al., 2000) and mouse mutants with defects in proteases (Roth et al., 2000; List et al., 2002; Egberts et al., 2004, Demerjian et al., 2008) have highlighted their crucial roles in the regulation of dermal differentiation and the maintenance of epidermal homeostasis (reviewed in Ovaere et al., 2009). Several studies have also indicated the importance of protease inhibitors in dermal differentiation using mouse mutants with their defects (Sundberg et al., 1997; Zeeuwen et al., 2002). Accordingly, it is

likely that the protease inhibitor balance is an important factor for normal progression of dermal differentiation processes (reviewed in Zeeuwen, 2004; Candi et al., 2005).

We have previously identified two analogous aspartic proteinases of the pepsin superfamily, CatE and cathepsin D (CatD), in human and rat epidermis (Sakai et al., 1989; Hara et al., 1993; Igarashi et al., 2004). Both enzymes have similar enzymatic properties but their tissue distribution and cellular localization were apparently different (reviewed in Yamamoto, 1999). In rat and human skin, CatD was concentrated in granular cells, whereas CatE was diffusely detected in the cytoplasm of almost all epidermal cells (Hara et al., 1993). Additionally, immunoelectron microscopic studies demonstrated that CatD was localized mainly on desmosomes of human stratum corneum, whereas CatE was present intracellularly within the squames (Igarashi et al., 2004). These observations strongly suggest that these enzymes have different functions in the dermal differentiation processes. Egberts et al. (2004) demonstrated that CatD deficiency in mice resulted in reduced transglutaminase 1 activity essential for the correct cornified envelope formation and the reduced expression of the cornified envelope proteins involucrin and loricrin, thereby suggesting a functional link of CatD to the regulation of epidermal differentiation. By contrast, recent studies have demonstrated that $CatE^{-/-}$ mice spontaneously develop atopic dermatitis-like skin lesions when reared under conventional conditions, but not under specific pathogen free conditions (Tsukuba et al., 2003), and an increase in susceptibility to hapten-induced experimental dermatitis (Tsukuba et al., 2003) and to bacterial infection (Tsukuba et al., 2006), suggesting that CatE plays a crucial role in protection against various environmental stresses, such as water loss and microorganism infection. These observations also suggest that CatD and CatE are differentially involved in keratinocyte differentiation and maintenance of dermal homeostasis. Therefore, to assess the role of CatE in the multistage processes important for dermal differentiation, we manipulated this protein in both *in vivo* and *in vitro* experiments using a model of skin papilloma induced in three different genotypes of syngeneic mice; CatE-deficient ($CatE^{-/-}$), wild-type ($CatE^{+/+}$), and CatE-overexpressing transgenic ($CatE^{Tg}$) mice and in the primary cultures of keratinocytes from each genotype of mice, respectively.

Results

CatE deficiency induces abnormal differentiation processes in epidermis and hair follicle *in vivo*

We used three different genotypes of the syngeneic C57BL/6 mice at three weeks of age, $CatE^{-/-}$ (n=8), $CatE^{+/+}$ (n=5), and $CatE^{Tg}$ (n=5). No significant differences in gross appearance and body size were detected between $CatE^{+/+}$ and $CatE^{Tg}$, but $CatE^{-/-}$ mice were smaller than other genotypes (data not shown). Also, the epidermis and hair follicles of $CatE^{-/-}$ mice showed abnormalities in epidermal differentiation processes, which were characterized by a more expanded corium and significantly reduced subcutaneous tissue and hair follicles compared with those of wild-type littermates (Figure 1A). It is known that hair follicles undergo repeated cycles of proliferation and differentiation (anagen stage), apoptosis (catagen stage) and resting (telogen stage) of epithelial cells (Stenn and Paus, 2001). According to well defined morphological criteria (Muller-Rover et al., 2001), it seems likely that the majority of hair follicles in each genotype of mice at three weeks of age are in the telogen stage and that a significant number of hair follicles in $CatE^{+/+}$ mice at this age are in the transitional process of preparing for the anagen stage (Figure 1A). To investigate the specific functions of CatE in epidermal differentiation, we first determined whether and which cell types in mouse skin express CatE. For this purpose, we prepared the primary culture of skin keratinocytes from $CatE^{+/+}$ mice and analyzed the cell extract by Western blotting under reducing conditions. CatE was expressed mainly as a 46 kDa proenzyme and slightly as a 42 kDa mature form (Figure 1B). By contrast, CatE expression in hair follicles was determined by *in situ* hybridization (ISH) and was found to be expressed and localized in close vicinity to the inner root sheath (Figure 1D), which was consistent with the previous observation that CatE was expressed in the cuticle of the keratogenous zone (Ishimatsu-Tsuji et al., 2005).

Characterization of skin papillomas generated in each genotype of mice by DMBA/TPA treatment

The first tumors generated by the two stage mouse skin tumorigenesis protocol using the initiator 7, 12-dimethylbenzanthracene (DMBA) and the promoter, 12-*O*-tetradecanoylphobol-13-acetate (TPA) have been demonstrated to be premalignant lesions, called papillomas (reviewed in Klein-Szanto, 1989), which further progress to squamous cell carcinoma at different rates by continuous TPA application (Burns et al., 1976; Aldaz et al., 1987). Histopathological analysis of surgical specimens of tumor lesions of each genotype revealed no distinct invasion or intravascular metastasis in tumors generated in each genotype of mice (Figure 2A, B, E, H), indicating that most of the tumors were premalignant under the conditions employed. All of the $CatE^{+/+}$ and $CatE^{Tg}$ mice induced multiple skin papillomas, whereas more than 20% of $CatE^{-/-}$ mice did not generate the papillomas under the conditions used (Figure 3A, B). Notably, $CatE^{-/-}$ mice developed only a small number of relatively large size papillomas, whereas $CatE^{Tg}$ mice had a large number and small size of papillomas compared with other genotypes (Figure 3A). These results suggest that papilloma size tended to decrease with the increase of CatE expression. The number of papillomas per mouse was positively associated with the expression levels of CatE, increasing according to the rank order $CatE^{-/-} < CatE^{+/+} < CatE^{Tg}$ mice, with the effect of CatE expression on the incidence of papillomas per $CatE^{Tg}$ mice being approximately 12 times more apparent for $CatE^{-/-}$ mice (Figure 3C).

Histological analysis also revealed that the extent of keratinocyte differentiation, called keratinization, was positively associated with the expression levels of CatE, increasing according to the rank order $CatE^{-/-} < CatE^{+/+} < CatE^{Tg}$ mice

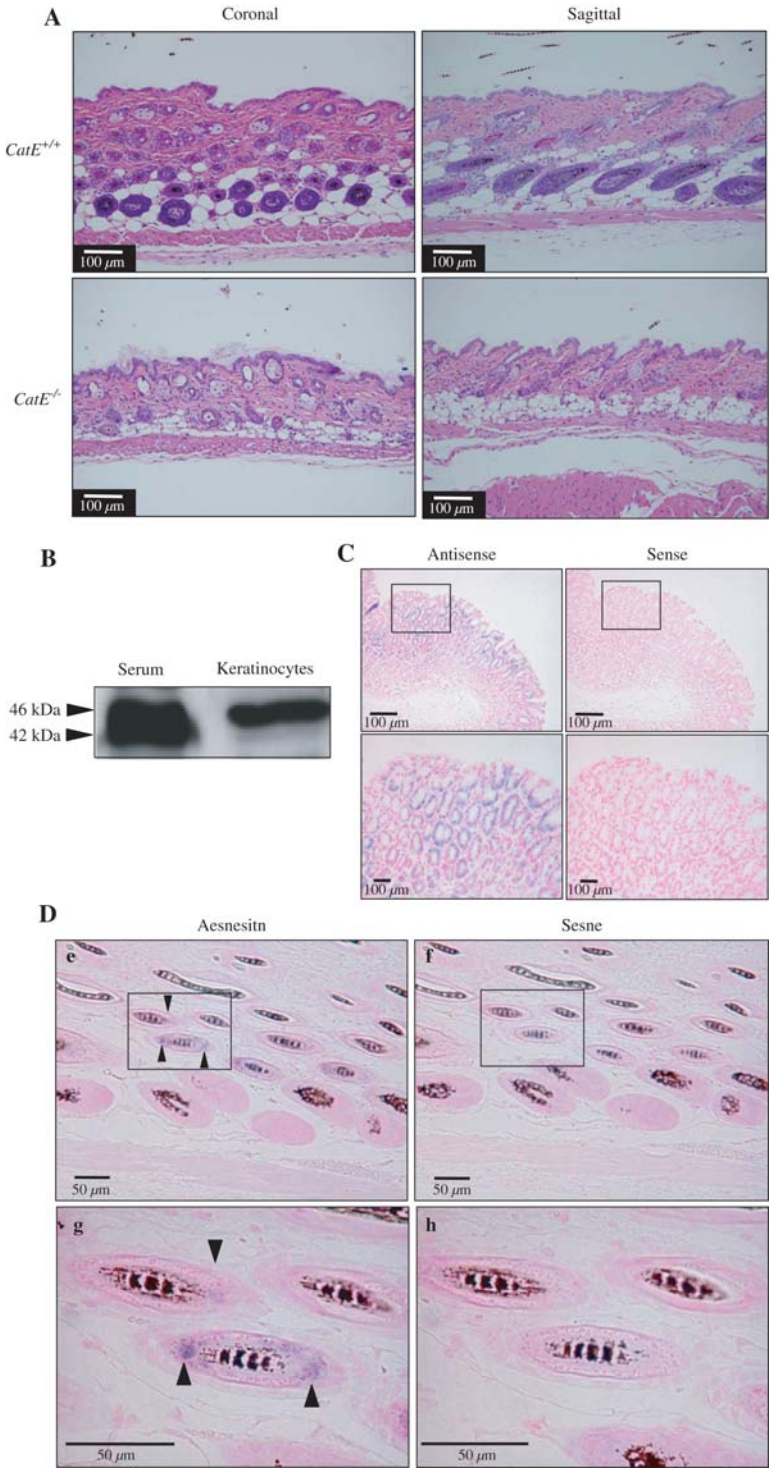

Figure 1 Impairment of differentiation in the epidermis and hair follicle in *CatE*$^{-/-}$ mice and CatE expression in these tissues in wild-type littermates.

(A) Histological analysis of surgical skin specimens from *CatE*$^{-/-}$ and *CatE*$^{+/+}$ mice at three weeks of age. The paraffin sections were processed for hematoxylin and eosin staining. The skin of *CatE*$^{-/-}$ mice was characterized by the significantly expanded corium and the significantly reduced subcutaneous tissue and the hair follicles in the anagen stage. Bars, 100 μm. (B) Western blot analysis of the cell extract of primary cultured keratinocytes, in addition to the serum, from *CatE*$^{+/+}$ mice with antibodies against rat CatE (1/500). Immunocomplexes on the nitrocellulose membranes were detected using chemiluminescence reagents. (C) ISH analysis of mouse gastric mucosa with DIG-labeled antisense and sense RNA probes for mouse CatE. ISH with mouse gastric mucosa was performed for validation of the probes used. Prominent expression of Cat E in stomach, particularly in epithelial cells, was observed as would be expected. (D) ISH analysis of hair follicles of *CatE*$^{+/+}$ mice. CatE was expressed around inner root sheath of hair follicles.

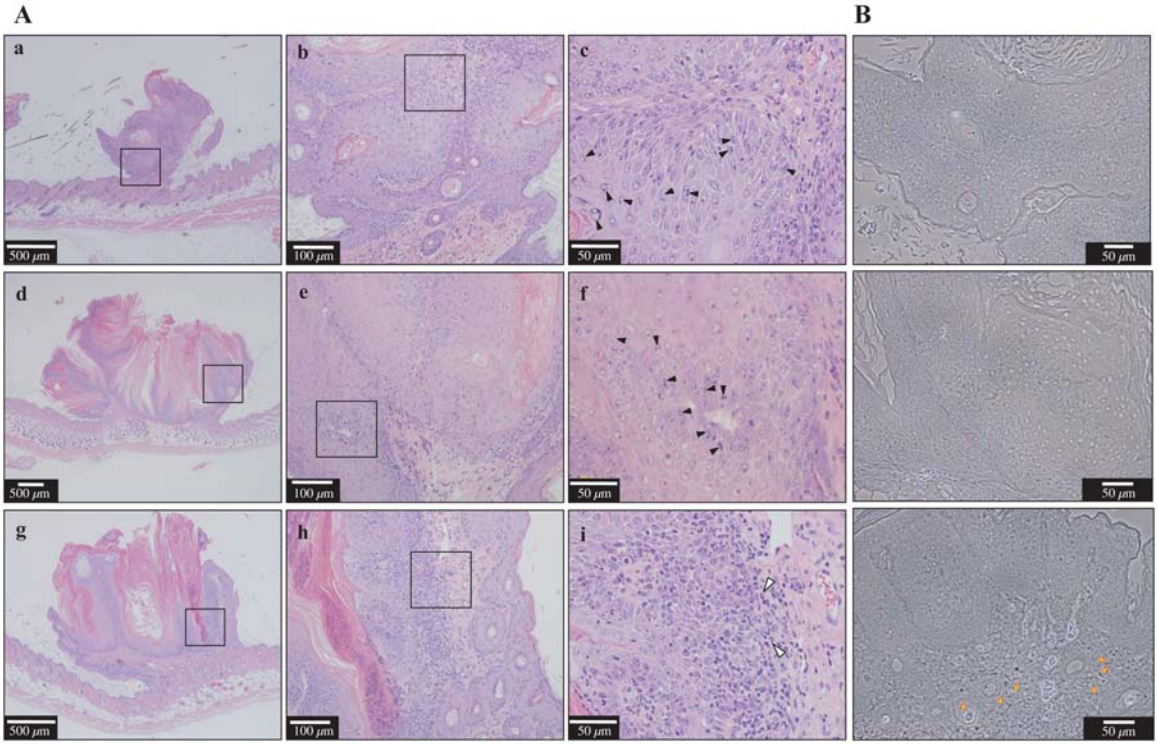

Figure 2 Histopathology of skin from each genotype of mice initiated with DMBA and promoted with twice weekly treatment of TPA for 14 weeks.
(A) The photographs from top to bottom in each column represent the representative histological sections stained with hematoxylin and eosin of papillomas in *CatE*$^{-/-}$, *CatE*$^{+/+}$ and *CatE*^{Tg} mice, respectively. The photographs from left to right in each column show low and higher magnification of the respective histological sections of papillomas in each genotype. The specimens in each row are the same magnification. Histological analyses revealed that all tumors from each genotype were squamous cell papillomas, but not squamous cell carcinomas. Black and white arrowheads indicate mitosis and tumor infiltrated neutrophils, respectively. Data are representative of results obtained with eight animals for each group. (B) Immunohistochemical detection of Gr-1 in sections of papillomas generated in *CatE*$^{-/-}$, *CatE*$^{+/+}$ and *CatE*^{Tg} mice (from top to bottom). Yellow arrowheads indicate tumor infiltrated neutrophils.

(Figure 2A, D, G). The papillomas of *CatE*$^{-/-}$ and *CatE*$^{+/+}$ mice contained a significant number of cells, indicating mitotic divisions, which might be progressing to squamous cell carcinoma (Figure 2A, B, C, F, I). In contrast, the cells showing mitotic divisions were scarcely found in the papillomas of *CatE*^{Tg} mice. It is also noted that papillomas of *CatE*^{Tg} mice, but not *CatE*$^{-/-}$and *CatE*$^{+/+}$ mice, contained a large number of tumor-infiltrating effector cells such as neutrophils (Figure 2A–I). This was further confirmed by immunohistochemical staining of papillomas from the different genotypes with anti-Gr1 antibody showing the presence of neutrophils (Figure 2B). Anti-Gr1-positive cells were the most abundant in papillomas of *CatE*^{Tg} mice, whereas papillomas of *CatE*$^{-/-}$ mice had only a small amount of anti-Gr1-positive cells. These results were consistent with our previous data indicating that the expression of CatE is positively associated with the number and the extent of activation of macrophages infiltrated both within and nearby the tumors formed 23 days after the subcutaneous injection of mouse B16 melanoma cells in the mice of each genotype (Kawakubo et al., 2007). Thus the present results strongly suggest that a significant decrease in the number of papillomas in *CatE*$^{-/-}$ mice is due to an altered epidermal differentiation processes, whereas the appearance of multiple papillomas in *CatE*^{Tg} mice is due to stimulation of the keratinocyte differentiation rate by the increased expression of CatE. Taken together, it is assumed that CatE is associated with differentiation leading keratinization and enhanced infiltration of inflammatory cells into tumorous parenchyma to remove malignant squamous cells, probably developed from occasional papillomas.

Expression of differentiation specific markers in normal skin and papillomas

Epidermal differentiation is known to begin with proliferation of basal keratinocytes and to end with the formation of the cornified layer. The process of keratinocyte proliferation and differentiation is also characterized by the expression of specific marker proteins. To determine whether epidermal differentiation was altered by the CatE expression level, we thus analyzed the protein expression and localization of differentiation specific proteins in the epidermis of each genotype of mice by immunohistochemical studies. K1 is one of the proteins to be expressed during keratinocyte differentiation. Control *CatE*$^{+/+}$ mice treated with acetone and without

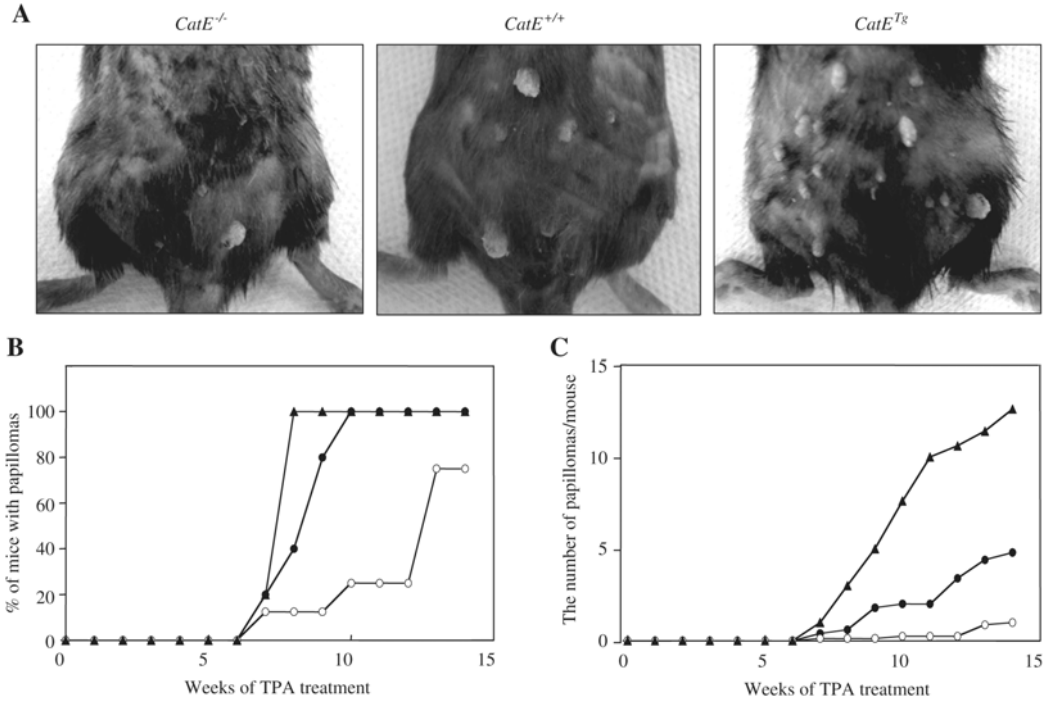

Figure 3 Papillomas occurring in the three different genotypes of mice treated with DMBA and TPA.
Each genotype of skin was initiated with 400 nmol DMBA and promoted with twice weekly treatments of 20 nmol TPA. (A) Gross appearance of lesions in each genotype. Data are representative of photographs obtained using eight animals in each group. (B) Papilloma incidence per mouse was plotted against time. The data are means values from eight animals in each group (○, $CatE^{-/-}$; ●, $CatE^{+/+}$; ▲, $CatE^{Tg}$). (C) Number of papillomas per mouse was plotted against time (○, $CatE^{-/-}$; ●, $CatE^{+/+}$; ▲, $CatE^{Tg}$). The data are means values using eight animals in each group.

DMBA and TPA did not result in papilloma formation (Figure 4 and Table 1). K1 staining in the skin of the control mice was exclusively confined to the outer cell layer, hair follicles and sebaceous glands (Figure 4A), which was comparable with that reported previously (Kartasova et al., 1993). Both the horny layer and the epidermis of $CatE^{+/+}$ mice treated with DMBA and TPA showed similar K1 staining patterns to those obtained with the control mice. K1 staining was also observed in all the papillimas of each genotype of mice (Figure 4A). No significant differences in the extent of K1 expression in normal skin regions and papillomas of both the horny layer and the epidermis were detected among genotypes. Notably, $CatE^{-/-}$ mice treated with DMBA and TPA displayed the increased ectopic expression and localization of K1 in the muscle layer, implying abnormal keratinocyte differentiation by CatE deficiency. Loricrin is a major component of the epidermal cornified envelop proteins and is localized in the granular layer during cornification of the human epidermis (Sundberg et al., 1994; Tiano et al., 2002). Loricrin staining was found in the terminal differentiated horny layer of $CatE^{+/+}$skin treated with acetone alone (Figure 4B). The expression and localization of loricrin was significantly reduced in normal skin regions in the horny layer of $CatE^{+/+}$mice treated with DMBA and TPA (Table 1) and inversely increased in papillomas formed in these mice (Figure 4B and Table 1). CatE deficiency induced the significantly decreased expression of loricrin in both normal and papillomous regions in the horny layer by DMBA/TPA treatment and the markedly increased ectopic localization in the muscle layer, also suggesting abnormal keratinocyte differentiation by CatE deficiency. These data thus indicate a close link of CatE expression to keratinocyte differentiation. K13 expression is known to not occur simultaneously with keratinocyte differentiation but instead follows squamous cell differentiation (Sutter et al., 1991). Therefore, K13 is likely to be expressed and localized mainly in differentiating and/or differentiated keratinocytes of non-cornifying squamous epithelia. K13 expression was found in cells mainly in the granular layer and epidermis of $CatE^{+/+}$ and $CatE^{Tg}$ papillomas, and partly in the hair follicle of the control mice treated with acetone alone (Figure 4C). Notably, the extent of K13 expression in papillomas in the horny layer and epidermis of $CatE^{-/-}$ mice was very low compared with that of other genotypes. The expression and localization of K13 in the papillomas generated in the horny layer of DMBA/TPA treated $CatE^{+/+}$ and $CatE^{Tg}$ mice was significantly reduced by CatE deficiency. Given that K13 is an early marker of DMBA/TPA induced papilloma progression (Gimenez-Conti et al., 1990; Robles et al., 1993), CatE appeared to be essential for the papilloma promotion step in the process of keratinocyte terminal differentiation. The *in vivo* experiments therefore suggest that a significant decrease in the number of papillomas in $CatE^{-/-}$ mice is due to the altered expression of the epidermal differentiation proteins,

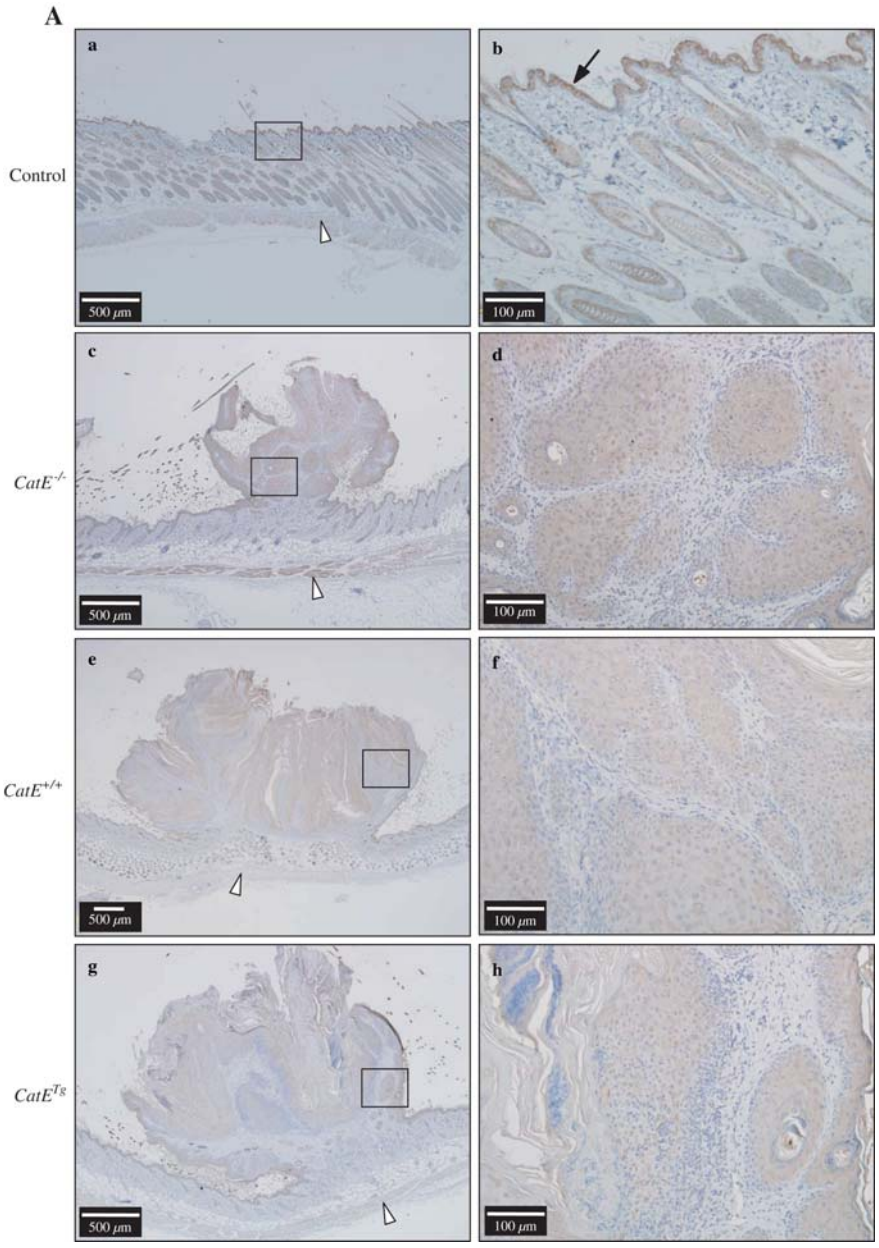

Figure 4 (Continued)

thereby leading to the impaired keratinocyte terminal differentiation.

Correlation of CatE expression and keratinocyte terminal differentiation in primary cultures of keratinocytes

To determine whether or not the pathohistological effects observed in skin papillomas of *CatE*$^{-/-}$ mice using DMBA and TPA were actually related to altered expression of CatE, we prepared primary cultures of epidermal keratinocytes from the three different genotypes of newborn syngeneic mice. We first examined the survival of these cells in response to DMBA treatment because this carcinogen is known to induce genetic defects and aberrant signaling pathways, and ultimately apoptosis in the damaged cells (Kim et al., 2009). When treated with acetone alone, primary keratinocyte cultures of each genotype had only a small amount of annexin V and/or propidium iodide positive cells and showed no significant differences in the extent of cell viability (Figure 5). In addition, DMBA treatment had little effect on the viability of keratinocytes from each genotype at any of the doses used. These data indicate that the DMBA induced initiation step in keratinocyte papillomagenesis is scarcely affected by CatE expression levels and that the increased number of papillomas in *CatE*^{Tg} mice induced by DMBA/TPA treatment is unlikely due to either enhancement in DMBA induced keratinocyte proliferation or reduction in

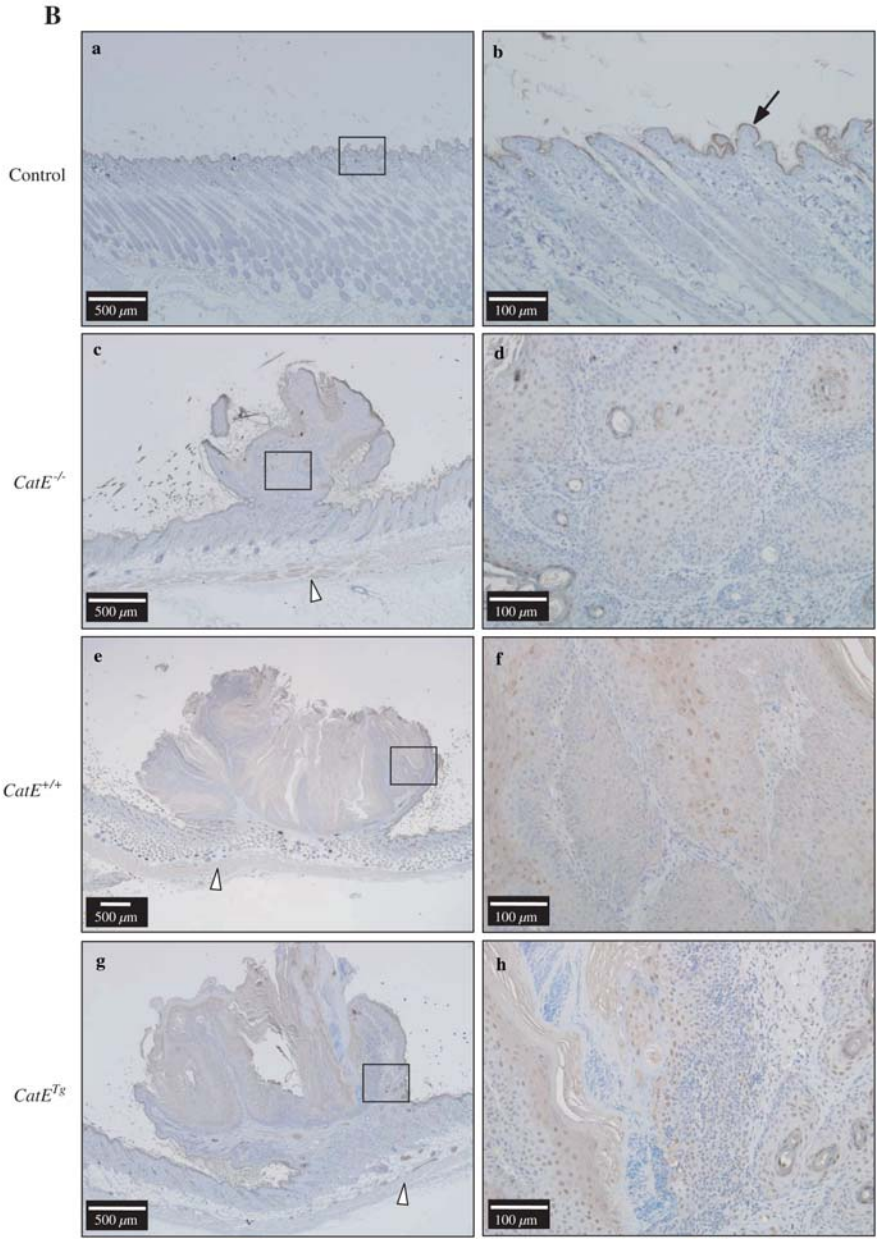

Figure 4 (Continued)

apoptosis. We next examined the correlation between CatE expression and keratinocyte differentiation in TPA treated cultured keratinocytes from each genotype. TPA is known to act as an efficient agent inducing homogenous proliferation and differentiation of mouse keratinocytes that are not initiated (Leder et al., 1990; Dlugosz and Yuspa, 1993). Quantitative RT-PCR analysis using TPA treated primary cultured keratinocytes revealed that the expression levels of *K1* and *loricrin* were relatively low in untreated $CatE^{-/-}$ keratinocytes, the mRNA expression of both genes was strongly stimulated in $CatE^{+/+}$ keratinocytes 48 h and 72 h after treatment with TPA (Table 2). A strong stimulation of the expression of *K1* and *loricrin* was also detected in cultured $CatE^{Tg}$ keratinocytes 48 h and 72 h after treatment with TPA. Notably, the mRNA expression of *loricrin* as a marker of terminal epidermal differentiation or keratinization at 48 h and 72 h after treatment with TPA was significantly higher in $CatE^{Tg}$ keratinocytes than in $CatE^{+/+}$ keratinocytes, whereas the mRNA expression of *K1* as a marker of the spinous and granular layers of the epidermis in $CatE^{Tg}$ keratinocytes was comparable to that in $CatE^{+/+}$ keratinocytes. It is worth emphasizing that the expression of both genes is strongly inhibited in TPA treated $CatE^{-/-}$ keratinocytes, although their expression profiles in themselves are similar to those of other genotypes of mice. To further prove the correlation between CatE expression and keratinocyte differentiation, we deter-

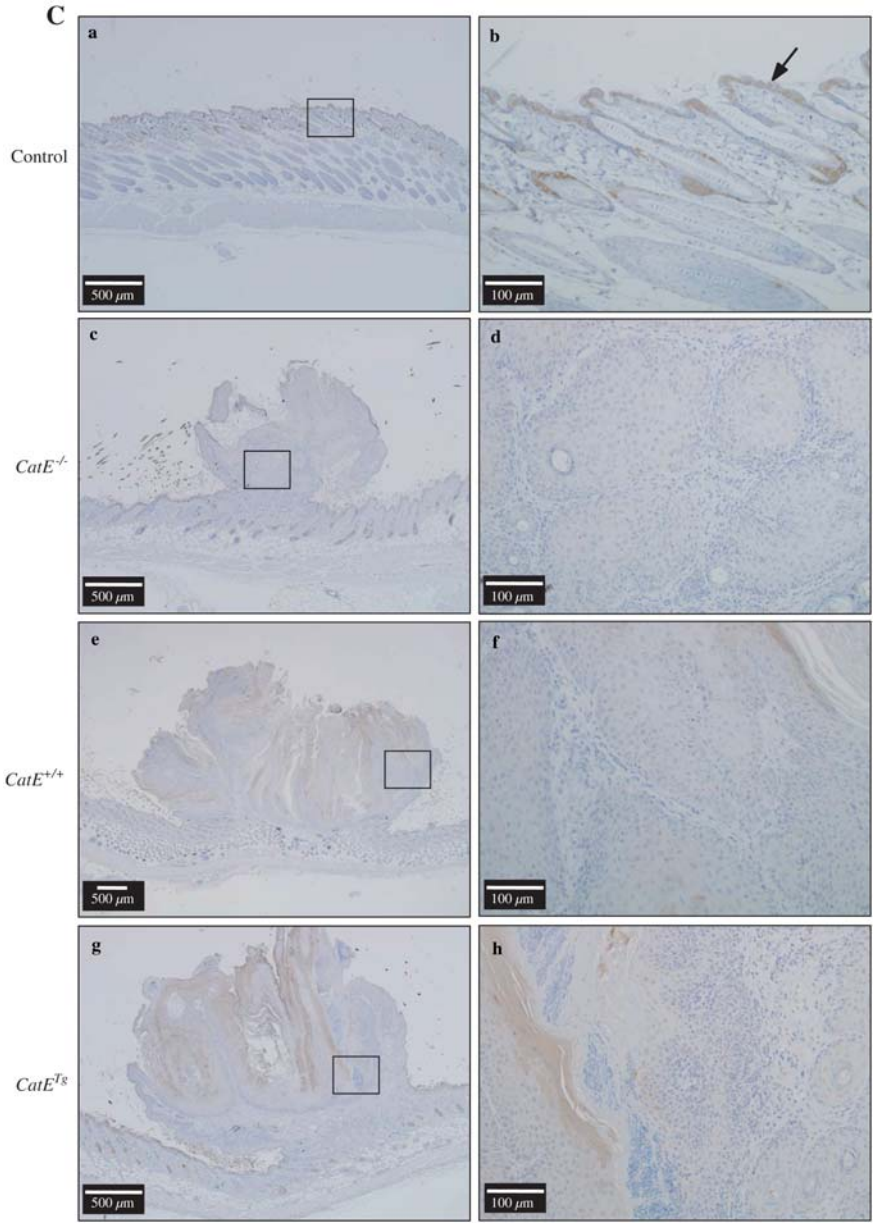

Figure 4 Immunohistochemical detection of K1 (A), loricrin (B) and K13 (C) in sections of papillomas generated in each genotype of mice initiated with DMBA and promoted with twice weekly treatment of TPA for 14 weeks.
Sections were stained by the avidin-biotin complex method with primary antibodies directed to each marker protein and by nuclei staining hematoxylin. Data are representative of results obtained using eight animals in each group. Control indicates the sections from the wild-type mice treated with acetone without DMBA and TPA. The photographs in each right column represent higher magnification views of the images (squares) in the corresponding left column. Arrows and white arrowheads indicate epidermis and muscle layer, respectively.

mined the expression of *K13*, a TPA mediated epidermal differentiation marker expressed in differentiating and/or differentiated keratinocytes, in each genotype of the cells. *K13* expression was significantly increased in TPA treated $CatE^{+/+}$ keratinocytes in a time dependent manner and reached a plateau value at 48 h after the treatment. A strong stimulation of *K13* expression was induced in $CatE^{Tg}$ keratinocytes compared with $CatE^{+/+}$ keratinocytes, with the effect of TPA on *K13* expression in the former cells at 48 h and 72 h after the treatment being approximately 18 times more apparent for the latter cells. Although the terminal expression level of *K13* mRNA in TPA treated $CatE^{-/-}$ keratinocytes was comparable to that in $CatE^{+/+}$ keratinocytes, the rate of terminal differentiation of the former cells was apparently delayed compared with that of the latter cells, indicating that the keratinocyte differentiation process is exacerbated in the absence of CatE. These abnormal expression patterns of keratinocyte differentiation markers, K1 and loricrin, were also found to be induced when the cells were treated with the classical differentiation inducing reagent, Ca^{2+} (data not

Table 1 Expression profiles of molecular markers of keratinocyte proliferation and differentiation in skin neoplasias of three different genotypes of syngeneic mice formed by local application of DMBA/TPA.

	Horny layer		Epidermis		Sebum/hair follicle	Muscle layer
	Normal	Papilloma	Normal	Papilloma		
K1						
CatE$^{+/+a}$	+++	No	+++	No	++	+
CatE$^{+/+}$	+++	+++	+++	+++	++	++
CatE$^{-/-}$	+++	+++	+++	+++	++	+++
CatE^{Tg}	+++	+++	+++	+++	++	++
Loricrin						
CatE$^{+/+a}$	+++	No	–	No	–	–
CatE$^{+/+}$	+	+++	+	+++	++	–
CatE$^{-/-}$	±	++	++	++	++	+++
CatE^{Tg}	++	+++	++	+++	++	+
K13						
CatE$^{+/+a}$	±	No	±	No	+	–
CatE$^{+/+}$	+	+++	+	+	++	–
CatE$^{-/-}$	++	±	+	+	++	–
CatE^{Tg}	+	+++	+	±	++	–

[a]*CatE*$^{+/+}$ mice treated with acetone as a control.

Expression and localization of K1, loricrin and K13 in mouse skin neoplasias formed by local application of DMBA/TPA on dorsal skin was determined by immunohistochemical analysis. The expression density was defined as follows: –, absent; ±, boundary between absent and present; +, slight; ++, moderate; +++, intense.

shown). In agreement with RT-PCR results, Western blot analysis data revealed that CatE, particularly the mature enzyme, increased in a time dependent fashion in *CatE*$^{+/+}$ keratinocytes treated with TPA (Figure 6). Given that epidermal homeostasis depends on a balance of proliferation and differentiation of keratinocytes, we further assessed whether or to what extent the observed effect of TPA on primary cultures of keratinocytes from each animal group is affected by their TPA induced proliferation using papillomas from each animal group after twice weekly treatment of TPA for 14 weeks. Immunohistochemical staining of papilllomas of each genotype with the monoclonal antibody Ki-67, which reacts with a nuclear antigen present only in proliferating cells (Gerdes et al., 1984), revealed that the number of Ki-67 positive cells was most abundant in *CatE*$^{-/-}$ papillomas (Figure 7). The number of Ki-67 positive cells in papillomas of other genotypes was apparently less than that of *CatE*$^{-/-}$ mice. These results strongly suggest that the relative proliferation/differentiation ratio of keratinocytes after treatment with TPA decrease in proportion to the expression of CatE. We thus concluded that the altered expression and localization of the keratinocyte differentiation markers observed in a model of skin papilloma by DMBA/TPA treatment was related to the altered expression of CatE in the epidermis.

Discussion

It has long been considered that the functions of CatE are confined to terminal and nonspecific degradation of proteins in endosomal and/or lysosomal compartments. Accumulating evidence, however, suggests that this enzyme has additional more specific functions *in vivo* (Lees et al., 1990; Athauda et al., 1991; Kageyama, 1993; Kageyama et al., 1995; Henningsson et al., 2005; Shin et al., 2007). To identify the specific *in vivo* functions of this enzyme, CatE deficient mice were generated by gene targeting and their phenotypic alterations were analyzed (Tsukuba et al., 2003, 2006; Kakehashi et al., 2007; Kawakubo et al., 2007; Yanagawa et al., 2007). Based on previous studies, it has been suggested that CatE contributes to the maintenance of homeostasis through more specific functions in host defense mechanisms. CatE is known to be predominantly expressed in the rapidly regenerating gastric mucosal cells and epidermal keratinocytes, in addition to the immune system cells. However, the specific functions of this enzyme in these cells are still speculative. In this study, we first demonstrate that CatE deficiency leads to some remarkable alterations in the epidermis and hair follicle, which were characterized by the moderately expanded corium, the reduced subcutaneous tissue, the reduced hair follicle and the dry skin. Pathological inflammatory responses were excluded as a putative cause of the observed skin and hair disorder. It has previously been shown that defects in the cornified cell envelop proteins such as transglutaminase 1 are found in human cornification disorders (Huber et al., 1995; Russel et al., 1995). In addition, various mouse mutants with defects in not only growth factors such as keratinocyte growth factor (Hébert et al., 1994; Guo et al., 1996) but also proteases such as cathepsins L (Roth et al., 2000) and D (Egberts et al., 2004) have been shown to display the spontaneous development of skin and hair disorders. However, the phenotypic alterations found by mutations with these genes were likely to be different from those by CatE deficiency. Therefore, the phenotypic alterations

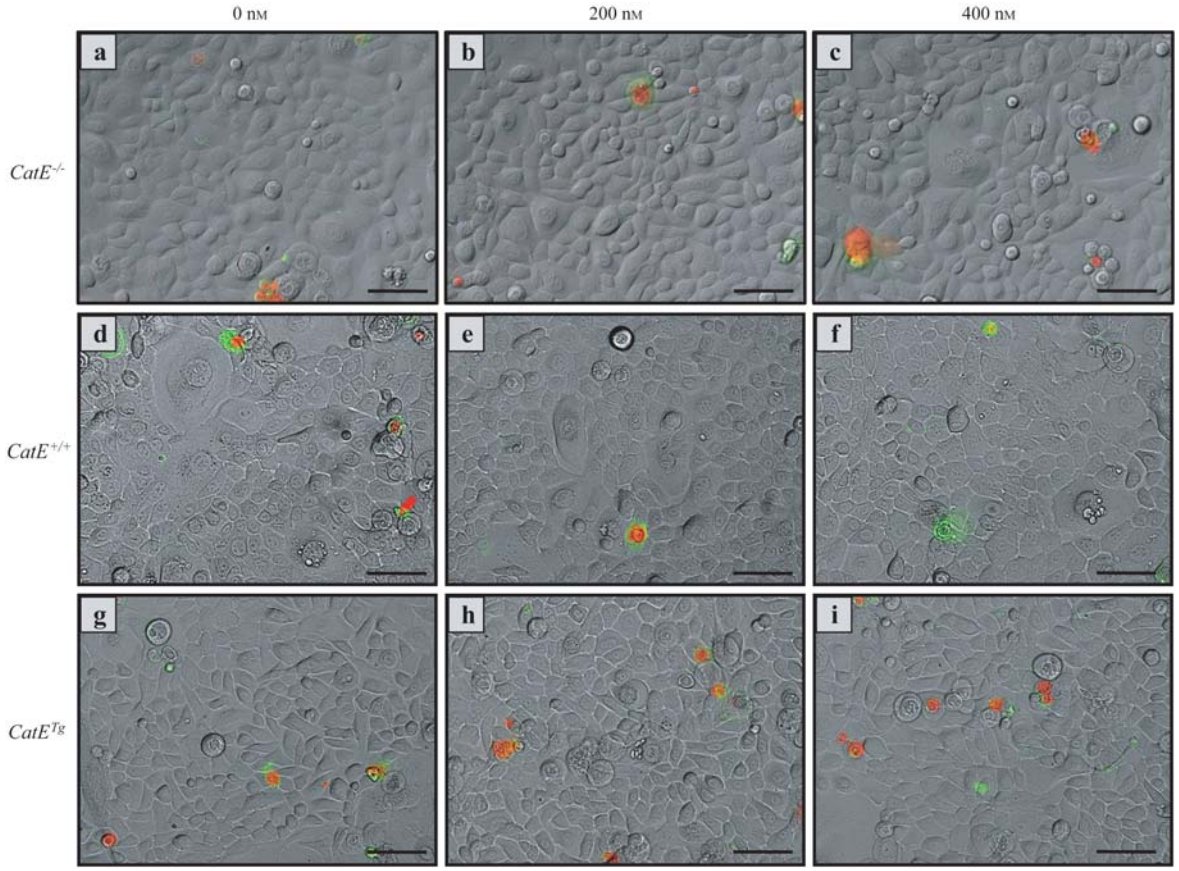

Figure 5 Effect of DMBA on primary cultures of keratinocytes prepared from the three different genotypes of syngeneic mice used in this study.
Cultured keratinocytes from each genotype were incubated with acetone alone and DMBA (200 and 400 nM) for 24 h and then subjected to an apoptosis/necrosis assay with Annexin-V-FLUOS (green) and PI staining kits (red), which represent apoptosis and necrosis, respectively. Scale bars, 50 μm.

observed in the epidermis and hair follicles of *CatE*$^{-/-}$ mice prompted us to explore the specific functions of CatE in epidermal differentiation processes.

In vivo experiments using a model of skin papilloma provides evidence that the CatE expression is correlated positively with the number of papillomas per mouse. These results indicate that the sensitivity of DMBA to keratinocytes is comparable among the three different genotypes of mice suggesting that CatE had little effect on the initiation step in papillomagenesis. Therefore, the remarkable resistance of *CatE*$^{-/-}$ mice to papilloma development is related to the altered TPA mediated differentiation and proliferation processes. In an attempt to identify the role of CatE in epidermal differentiation, we first determined whether the expression of the differentiation specific marker proteins was altered in papillomas of the respective genotypes of mice. The expression and localization of K1, a major protein to be expressed during an earlier cornification stage in the horny layer and epidermis, was independent of the expression levels of CatE, except that the ectopic expression and localization in the muscle layer were induced by CatE deficiency. These results strongly suggest that CatE participates in epidermal differentiation at a more advanced stage. By contrast, the expression and localization of loricrin, a major cornified cell envelope protein of terminally differentiated epidermal cells in papillomas in the horny layer and epidermis, of *CatE*$^{-/-}$ mice were significantly reduced compared with those of other genotypes. Concurrently, a stimulation of the ectopic expression and localization in the muscle layer of K1 and loricrin was detected in *CatE*$^{-/-}$ mice. It is also noteworthy that the expression and localization of the terminal keratinocyte differentiation marker K13 were more intensely reduced in papillomas of *CatE*$^{-/-}$ mice compared with those of other genotypes. Given that the expression of K13 is restricted to differentiating and differentiated keratinocytes, the reduced expression and localization of K13 in *CatE*$^{-/-}$ papillomas shows the functional link between CatE expression and epidermal terminal differentiation. In addition, our previous study demonstrated that the overexpression of CatE in mice resulted in a marked increase in the number and extent of tumor infiltrating effector cells such as macrophages within or close to the tumors formed by injection of tumor cells, thereby leading to apoptosis in tumor cells (Kawakubo et al., 2007). In accordance with these findings, we found that anti-Gr1-positive neutrophils were the most abundant in papillomas of *CatE*^{Tg} mice, whereas papillomas

Table 2 Expression profiles of molecular markers of keratinocyte differentiation in primary cultures of keratinocytes from three different genotypes of syngeneic mice.

	0 h	8 h	24 h	48 h	72 h
CatE					
CatE$^{+/+}$	1	1.34*,†	2.17*,†	1.47*	7.38*,†
CatE$^{-/-}$	0	0	0	0	0
CatE^{Tg}	9.62×10^5	13.7×10^5*,†	12.5×10^5*	41.8×10^5*,†	42.1×10^5*
K1					
CatE$^{+/+}$	1	0.59	1.26*,†	122*,†	213*,†
CatE$^{-/-}$	0.43	0.09	0.21	11.8*,†	30.4*,†
CatE^{Tg}	2.64	1.19	2.10	152*,†	152*
Loricrin					
CatE$^{+/+}$	1	0.25	1.29	24.3*,†	138*,†
CatE$^{-/-}$	0.62	0.16	0.39	5.68*,†	54.6*,†
CatE^{Tg}	0.26	0.22	0.96*,†	83.1*,†	261*,†
K13					
CatE$^{+/+}$	1	1.54*,†	2.93*,†	14.3*,†	11.5*
CatE$^{-/-}$	0.94	1.59	1.39	7.59*,†	11.8*,†
CatE^{Tg}	5.42	2.81*,†	7.23*,†	254*,†	199*

Primary cultures of keratinocytes from *CatE*$^{+/+}$, *CatE*$^{-/-}$ and *CatE*^{Tg} mice were treated with TPA (40 nM) for the time indicated. Total RNA was then extracted from keratinocytes of each genotype and subjected to quantitative RT-PCR analysis for *CatE*, *K1*, *loricrin*, and *K13*. The relative expression levels of each gene, normalized to the amount of each transcript in *CatE*$^{+/+}$ keratinocytes at 0 h is indicated (*$p<0.001$ and $^{\dagger}p<0.001$ was considered statistically significant when compared with each expression quantity at 0 h and the last period, respectively). Data are mean values from two independent experiments performed in triplicate for each group.

of *CatE*$^{-/-}$ mice had only a small amount of anti-Gr1-positive cells. Accordingly, it is more likely that an increased number of tumor infiltrating effector cells in papillomas of *CatE*^{Tg} mice serve to eliminate transformed cells induced by DMBA/TPA treatment and concurrently the enhanced CatE expression forced keratinocytes to develop benign papillomas. This could be one of the reasons for the appearance of a small size of papillomas in *CatE*^{Tg} mice. Papillomas generated in each genotype of mice under the conditions used were shown to be premalignant and there were little papillomas which progressed to squamous carcinomas. However, the incidence of conversion of papillomas to squamous carcinomas was increased by prolonged application of TPA (data not shown). This was consistent with a previous report showing that a significant portion of papillomas generated by the initiation or promotion protocols progressed to squamous cell carcinoma 24 weeks after twice weekly topical application of TPA (Dhawan et al., 1999; Fu et al., 2009). The *in vivo* studies with different genotypes of mice strongly suggest that CatE is essential for keratinocyte terminal differentiation required for the promotion step of papillomas.

The functional link of CatE to the expression of terminal differentiation marker proteins *in vivo* was also corroborated by *in vitro* studies with primary cultures of keratinocytes from the three different genotypes of mice. TPA treatment of primary cultures of keratinocytes of each genotype

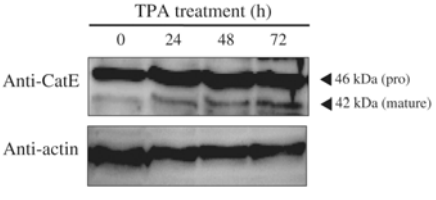

Figure 6 Western blot analysis of primary cultures of keratinocytes from *CatE*$^{+/+}$ mice treated with TPA.
The cell extract of keratinocytes at the indicated times after TPA treatment were subjected to SDS-PAGE under reducing conditions, followed by immunoblotting with antibodies against CatE or actin.

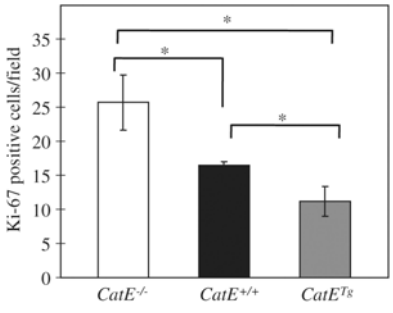

Figure 7 Immunohistochemical detection of Ki-67 in sections of papillomas generated from each genotype of mice initiated with DMBA and promoted with twice weekly treatment of TPA for 14 weeks.
After IHC for Ki-67, using a grid at a magnification of 20×, we selected five fields at random in the base (non-cornificated) regions of each papilloma and the number of positive cells was counted and then calculated. Data are the means±SD of values from five independent experiments. *$p<0.001$ vs. the values for the corresponding counterparts.

induced terminal differentiation of $CatE^{+/+}$ keratinocytes accompanying the expression of *CatE* and terminal differentiation marker genes in a manner associated with differentiation. The maximal expression of *CatE* was observed at 72 h after TPA treatment, suggesting its predominant expression in differentiating and/or differentiated keratinocytes. The message levels of CatE in $CatE^{Tg}$ keratinocytes without TPA addition were shown to be approximately 100 000-fold those in $CatE^{+/+}$cells, which were further increased approximately four times at 72 h after TPA treatment. We have previously reported that the pCAGGS vector used has a CAG promoter (CMV-IE enhancer/β-actin/β-globin poly A signal) (Kawakubo et al., 2007). Given that neither the CMV-IE (Hertzel et al., 2000) nor the β-actin (Pirkkala et al., 1999) promoter is directly affected by TPA, it seems unlikely that the CAG promoter of pCAGGS has a significant influence on TPA-induced CatE expression. However, because we have no conclusive data to establish a direct association between the CAG promoter and TPA induced CatE expression at present, this issue remains to be answered in future studies. The expression profiles of *K1*, *Loricrin* and *K13* were similar to that of *CatE*. Together, these results strongly suggest that CatE is positively involved in keratinocyte differentiation. The over expression of CatE led to a significant shortening of the time required to reach the maximal expression of *K1* and *K13*. By contrast, CatE deficiency resulted in a marked reduction in the expression of *K1*, *loricrin* and *K13*, accompanying the impaired differentiation of keratinocytes. The present data thus indicate that CatE is functionally linked to the expression of such TPA mediated keratinocyte terminal differentiation markers as K1, loricrin and K13 and thereby regulates the terminal regulation of keratinocytes.

Materials and methods

Chemicals and reagents

DMBA and TPA were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Rabbit polyclonal antibodies against K1 (ab24643), K13 (ab58744) and loricrin (ab24772) was purchased from Abcam Inc. (Cambridge, MA, USA). Rat monoclonal antibody against Ki-67 (TRC-3) and Gr-1(Ly-6G) were products from Dako (Glostrup, Denmark) and BioLegend (San Diego, CA, USA), respectively. CnT-07 was from CELLnTEC Advanced Cell Systems (Bern, Switzerland). TrypLE Express was from GIBCO (Gaithersberg, MD, USA). Annexin V-FLUOS Staining Kit and High Pure RNA Isolation Kit were from Roche (Basel, Switzerland). High Capacity cDNA Reverse Transcription Kit was from Applied Biosystems (Foster City, CA, USA).

Mice and cells

All animals were maintained according to the guidelines of the Japanese Pharmacological Society in a specific pathogen free facility at the Kyushu University Station for Collaborative Research. All animal experiments were approved by the Animal Research Committee of the Graduate School of Dental Science, Kyushu University. Wild-type and $CatE^{-/-}$ mice of the C57BL/6 genetic background were used as described previously (Tsukuba et al., 2003). Transgenic mice overexpressing CatE ($CatE^{Tg}$) of the C57BL/6 genetic background were generated according to the method as described (Kawakubo et al., 2007). Three different genotypes of the syngeneic mice were fertile and exhibited normal bleeding behavior and no obvious phenotypes when raised under specific pathogen free conditions.

Skin treatments

Each genotype of mice at eight to nine weeks of age was assigned to two treatment groups of eight mice each. Only those mice that were in the resting phase of hair cycle were used. The lower dorsal skin of each genotype was shaved with a hair clipper and then DMBA (400 nmol) in acetone was applied on an approximately 2 cm area. The control group received acetone alone. After one week, twice weekly topical application of 20 nmol TPA in 0.1 ml of acetone was begun and continued for 14 weeks. Tumors were defined as raised lesions of at least 1 mm diameter that had been present for at least one week. The first tumors that appear during a two stage mouse skin tumorigenesis protocol using DMBA and TPA as the initiator and promoter, respectively, are premalignant lesions called papillomas (reviewed in Klein-Szanto, 1989). The papillomas that initially develop during mouse skin initiation promotion protocols have long been considered heterogeneous in that some will persist, some will disappear or regress and only a small portion will progress to an invasive squamous cell carcinomas (reviewed in DiGiovanni, 1992). Indeed, the conversion of papillomas to carcinomas was too infrequent in any genotype to allow meaningful comparisons under the conditions used. Papillomas formed were thus mapped and counted weekly.

CatE gene specific ISH

Skin and stomach of each genotype of C57BL/6 mice at 12 weeks of age were dissected, fixed with tissue fixative (Genostaff Co., Ltd., Tokyo, Japan), embedded in paraffin and then sectioned at 4 μm. *In situ* hybridization was performed on these sections using digoxigenin labeled RNA probes (Roche) under contract by Genostaff. Probes for a 360 base pair fragment were designed from position 176–535 of the open reading frame of mouse *CatE*. Hybridization was carried out as described previously (Fujiwara et al., 2004). For probe preparation, a mouse cDNA for CatE was amplified by RT-PCR using a gene specific primer set. Digoxigenin labeled antisense and sense RNA probes were prepared by *in vitro* transcription with T7 RNA polymerase (DIG RNA Labeling Kit, Roche) using amplified cDNA of the gene as a template. Targeted mRNA sequence position for mouse CatE (NM_00779) was 176–535 (360 nucleotides). The probes were hybridized at 60°C with 60% formamide. Sections were treated with anti-digoxigenin antibody at room temperature and visualized with diaminobenzidine.

Immunohistochemistory

Surgical specimens were fixed in 10% neutral formalin for 24 h at room temperature, embedded in paraffin at 55°C and cut into parallel consecutive 3 μm thick sections for the subsequent immunohistochemical study using Simple Stain Mouse MAX-PO (Nichirei Biosciences Inc., Tokyo, Japan). Briefly, following deparaffinized in xylene, the sections were pretreated in an autoclave for antigen retrieval in TE buffer (10 mM Tris HCl, 1 mM EDTA pH 9.0). After washing with PBS, endogenous peroxidase activity was then blocked with 3% H_2O_2 in methanol for 10 min, and following several washes, blocking reagent was applied for 60 min to prevent nonspecific reactions. Sections were successively incubated with primary antibodies against K1, K13, and Loricrin, Ki-67 and Gr-1

(1:100 dilution) at 4°C overnight. For K13, the CSA II (Dako, Glostrup, Denmark), biotin free catalyzed amplification system was performed before DAB color development. Following another wash, the bound primary antibodies were visualized by peroxidase detection using MAX-PO (Rabbit) or MAX-PO (Rat) reagent and DAB reagent. All sections were counterstained with hematoxylin.

Primary cultures of keratinocytes

Primary cultures of keratinocytes were generated from each genotype of newborn mice. Neonatal skin was taken from each genotype and incubated in CnT-07 supplement with 5 mg/ml dispase at 4°C overnight. Following several washes with CnT-07, the epidermis was gently separated from the dermis, and then placed on a 500 μl drop of TrypLE Express (Gibco, Gaithersberg, MD, USA) in petri dishes at room temperature for 25 min. After several washes with CnT-07, keratinocytes were collected and centrifuged and then seeded in dishes at 4.0×10^4 cells/cm^2 and cultured in a humidified 5% CO_2 environment. Unattached cells were subsequently removed by washing with PBS and attached cells were further cultured in fresh medium, which was replaced every three days. For analysis of cell proliferation, primary cultures of keratinocytes from each genotype were seeded at 3×10^5 cells in 35 mm dish (ibidi GmbH, Martinsried, Germany) and cultured to reach 80% confluence and then incubated in the presence or absence of DMBA (200 and 400 nM) at 37°C for 24 h in a humidified 5% CO_2 environment. After complete removal of the culture medium, the attached cells were washed with PBS. The extent of viability of the remaining cells was assessed by an apoptosis/necrosis assay with an Annexin-V-FLUOS Staining Kit (Roche). The cells treated with the kit in HEPES buffer containing propidium iodide (PI) in the dark for 10 min at room temperature were analyzed by fluorescence microscopy. For analysis of cell differentiation, primary cultures of keratinocytes from each genotype were incubated with TPA (40 nM) for various time intervals at 37°C in a humidified 5% CO2 environment. The extent of differentiation was assessed by the expression profiles of various differentiation markers by quantitative RT-PCR.

Real-time RT-PCR

Total RNA was extracted by High Pure RNA Isolation Kit (Roche) and two step real time qPCR was carried out using the Taqman® real time PCR system on Light Cycler 480 instrument (Roche). cDNA was prepared from 500 ng total RNA in a 20 μl reaction volume using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Amplicon sizes were confirmed using RT-PCR [GAPDH (75 bp), CatE (61 bp), K1 (127 bp), K13 (99 bp), and Loricrin (76 bp)]. Primers used to amplify the following transcripts are as follows: GAPDH, TGT CCG TCG TGG ATC TGA C (forward), CCT GCT TCA CCA CCT TCT TG (reverse); cathepsin E, AAC CTC TGG GTC CCT TCT GT (forward), GGA ATA CTG GGT GTG CCT TG (reverse); Keratin1, TGA GCT GAA GAA CAT GCA AGA (forward), CAT GTA AGC TGA ATC CAC ATC C (reverse); Keratin13: GGA GCT CCG GAT CAA GAT T (forward), TTG AGC CTG AAG TCA TCT GC (reverse), and Loricrin: GGT TGT GGA AAG ACC TCT GG (forward), AGC CGC CAC CGC TAT AAT (reverse). A Light Cycler Universal Probe Master (Roche) specific for each sequence was applied to number 80 (for GAPDH and K13), number 26 (for CatE), number 31 (for K1), and number 63 (for Loricrin), respectively.

Immunoblot analysis

SDS-PAGE and immunoblot analysis were performed as described previously (Yamamoto et al., 1985).

Statistical analysis

Data was analyzed using an unpaired Student's *t*-test. A *p*-value of <0.05 was considered statistically significant.

Acknowledgments

This study was supported in part by a Grant in Aid for Scientific Research (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Aldaz, C.M., Conti, C.J., Klein-Szanto, A.J.P., and Slaga, T.J. (1987). Progressive dysplasia and aneuploidy are hallmarks of maouse skin pappilomas: relevance to malignancy. Proc. Natl. Acad. Sci. USA *84*, 2029–2032.

Athauda, S.B.P., Takahashi, T., Inoue, H., Ichinose, M., and Takahashi, K. (1991). Proteolytic activity and cleavage specificity of cathepsin E at the physiological pH as examined towards the B chain of oxidized insulin. FEBS Lett. *292*, 53–56.

Blanpain, C. and Fuchs, E. (2009). Epidermal homeostasis: a balancing act of stem cells in the skin. Nat. Rev. Mol. Cell Biol. *10*, 207–217.

Burns, F.J., Vanderlaan, M., Sivak, A., and Albert, R.E. (1976) Regression kinetics of mouseskin papillomas. Cancer Res. *36*, 1422–1427.

Candi, E., Schmidt, R., and Melino, G. (2005). The cornified envelope: a model of cell death in the skin. Nat. Rev. Mol. Cell Biol.*6*, 328–340.

Chavanas, S., Bodemer, C., Rochat, A., Hamel-Teillac, D., Ali, M., Irvine, A.D., Bonafe, J.-L., Wilkinson, J., Taieb, A., Barrandon, Y., et al. (2000). Mutations of SPINKS5, encoding a serine protease inhibitor, cause Netherton syndrome. Nat. Genet. *25*, 141–142.

Demerjian, M., Hachem, J.P., Tschachler, E., Denecker, G., Declercq, W., Vandenabeele, P., Mauro, T., Hupe, M., Crumrine, D., Roelandt, T., et al. (2008). Acute modulations in permeability barrier function regulate epidermal cornification. Am. J. Pathol. *172*, 86–97.

Dhawan, D., Balasubramanian, S., Amonkar, A.J., and Singh, N. (1999). Chemopreventive effect of 4-methyl epipodophyllotoxin on DMBA/TPA-induced mouse skin carcinogenesis. Carcinogenesis *20*, 997–1003.

DiGiovanni, J. (1992). Multistage carsinogenesis in mouse skin. Pharmac. Ther. *54*, 63–128.

Dlugosz, A.A. and Yuspa, S.H. (1993) Coordinate changes in gene-expression which mark the spinous to granular-cell transition in epidermis are regulated by protein kinase-C. J. Cell Biol. *120*, 217–225.

Egberts, F., Heinrich, M., Jensen, J.M., Winoto-Morbach, S., Pfeiffer, S., Wickel, M., Schunck, M., Steude, J., Saftig, P., Proksch, E., et al. (2004). Cathepsin D is involved in the regulation of transglutaminase 1 and epidermal differentiation. J. Cell Sci. *117*, 2295–2307.

Fu, W., McCormick, T., Qi, X., Luo, L., Zhou, L., Li, X., Wang, B.C., Gibbons, H.E., Abdul-Karim, F.W., and Gorodeski, G.I. (2009). Activation of $P2X_7$-mediated apoptosis inhibits DMBA/TPA-induced formation of skin papillomas and cancer in mice. BMC Cancer 9, 114–133.

Fujiwara, K., Ochiai, M., Ohta, T., Ohki, M., Aburatani, H., Nagao, M., Sugimura, T., and Nakagama, H. (2004). Global gene expression analysis of rat colon cancers induced by a food-borne

carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. Carcinogenesis. *25*, 1495–1505.

Gerdes, J., Lemke, H., Baisch, H., Wacker, H.H., Schwab, U., and Stein, H. (1984). Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J. Immunol. *133*, 1710–1715.

Gimenez-Conti, I., Aldaz, C.M., Bianchi, A.B., Roop, D.R., Slaga, T.J., and Conti, C.J. (1990). Early expression of type I K13 keratin in the progression of mouse skin papillomas. Carcinogenesis. *11*, 1995–1999.

Guo, L., Degrenstein, L., and Fuchs, E. (1996). Keratinocyte growth factor is required for hair development but not for wound healing. Gene Dev. *10*, 165–175.

Hara, K., Fukuyama, K., Sakai, H., Yamamoto, K., and Epstein, W.L. (1993). Purification and immunohistochemical localization of aspartic proteinases in rat epidermis. J. Invest. Dermatol. *100,* 394–399.

Henningsson, F., Yamamoto, K., Saftig, P., Reinheckel, T., Peters, C., Knight, S.D., and Pejler, G. (2005). A role for cathepsin E in the processing of mast-cell carboxypeptidase A. J. Cell Sci. *118*, 2035–2042.

Hébert, J.M., Rosenquist, T., Götz, J., and Martin, G.R. (1994). FGF5 as a regulator of the hair growth cycle: evidence from targeted and spontaneous mutations. Cell *78*, 1017–1025.

Hertzel, A.V., Sanders, M.A., and Bernlohr, D.A. (2000). Adenovirus-mediated gene transfer in primary murine adipocytes. J. Lipid Res. *41,* 1082–1086.

Huber, M., Rettler, I., Bernasconi, K., Frenk, E., Lavvrijsen, S.P., Ponec, M., Bon, A., Lautenschlager, S., Schorderet, D.F., and Hohl, D. (1995). Mutations of keratinocyte transglutaminase in lamellar ichithyosis. Science *267*, 525–528.

Igarashi, S., Takizawa, T., Takizawa, T., Yasuda, Y., Uchiwa, H., Hayashi, S., Brysk, H., Robinson, J.M., Yamamoto, K., and Brysk, M.M. (2004). Cathepsin D, but not cathepsin E, degrades desmosomes during epidermal desquamation. Br. J. Dermatol. *151*, 355–361.

Ishimatsu-Tsuji, Y., Moro, I., and Kishimoto, J. (2005). Expression profiling and cellular localization of genes associated with the hair cycle induced by wax depilation. J. Invest. Dermatol. *125*, 410–420.

Kageyama, T. (1993). Rabbit procathepsin E and cathepsin E. Nucleotide sequence of cDNA, hydrolytic specificity for biologically active peptides and gene expression during development. Eur. J. Biochem. *216*, 717–728.

Kageyama, T., Ichinose, M., and Yonezawa, S. (1995). Processing of the precursors to neurotensin and other bioactive peptides by cathepsinE. J. Biol. Chem. *270*, 19135–19140.

Kakehashi, H., Nishioku, T., Tsukuba, T., Kadowaki, T., Nakamura, S., and Yamamoto, K. (2007). Differential regulation of the nature and functions of dendritic cells and macrophages by cathepsin E. J. Immunol *179*, 5728–5737.

Kartasova, T., Roop, D.R., Holbrook, K.A., and Yuspa, S.H. (1993). Mouse differentiation-specific keratin 1 and 10 require a preexisting keratin scaffold to form a filament network. J. Cell Biol. *120*, 1251–1261.

Kawakubo, T., Okamoto, K., Iwata, J., Shin, M., Okamoto, Y., Yasukochi, A., Nakayama, K.I., Kadowaki, T., Tsukuba, T., and Yamamoto, K. (2007). Cathepsin E prevents tumor growth and metastasis by catalyzing the proteolytic release of soluble TRAIL from tumor cell surface. Cancer Res. *67*, 10869–10878.

Kim, D.J., Kataoka, K., Sano, S., Connolly, K., Kiguchi, K., and DiGiovanni, J. (2009). Targeted disruption of Bcl-xL in mouse keratinocytes inhibits both UVB- and chemically induced skin carcinogenesis. Mol. Carcinog. *48*, 873–885.

Klein-Szanto, A.J.P. (1989). Pathology of human and experimental skin tumors. In: Skin Tumors: Experimental and Clinical Aspects, C.J. Conti, T.J. Slaga, and A.J.P. Klein-Szanto, eds. (New York, USA: Raven Press), pp. 19–53.

Leder, A., Kuo, A., Cardiff, R.D., Sinn, E., and Leder, P. (1990). V-Ha-Ras transgene abrogates the initiation step in mouse skin tumorigenesis – effects of phorbol esters and retinoic acid. Proc. Natl. Acad. Sci. USA *87*, 9178–9182.

Lees, W.E., Kalinska, S., Meech, J., Capper, S.J., Cook, N.D., and Kay, J. (1990). Generation of human endothelin by cathepsin E. FEBS Lett. *273*, 991–2.

List, K., Haudenschild, C.C., Szabo, R., Chen, W., Wahl, S.M., Swaim, W., Engelholm, L.H., Behrendt, N., and Bugge, T.H. (2002). Matriptase/MT-SP1 is required for postnatal survival, epidermal barrier function, hair follicle development, and thymic homeostasis. Oncogene *21*, 3765–3779.

Muller-Rover, S.B., Handjiski, C., van der Veen, S., Eichmuller, K., Foitzik, I.A., McKay, K.S., Stenn, K.S., and Paus, R. (2001). A comprehensive guide for the accurate classification of murine hair follicles in distinct hair cycle stages. J. Invest. Dermatol. *117*, 3–15.

Ovaere, P., Lippens, S., Vandennabeele, P., and Declercq, W. (2009). The emerging roles of serine protease cascades in the epidermis. Trends Biochem. Sci. *34*, 453–463.

Pirkkala, L., Alastalo, T.P., Nykänen, P., Seppä, L., and Sistonen, L. (1999). Differentiation lineage-specific expression of human heat shock transcription factor 2. FASEB J. *13,* 1089–1098.

Robles, A.I., Gimenez-Conti, I.B., Roop, D., Slaga, T.J., and Conti, C.J. (1993). Low frequency of codon 61 Ha-ras mutations and lack of keratin 13 expression in 7, 12-dimethylbenz[a]-anthracene-induced hamster skin tumors. Mol. Carcinog. *7*, 94–98.

Roth, W., Deussing, J., Botchkarev, V.A., Pauly-Eerts, M., Saftig, P., Hafner, A., Schmidt, P., Schmahl, W., Scherer, J., Anton-Lamprecht, I., et al. (2000). Cathepsin L deficiency as molecular defect of *furless*: hyperproliferation of keratinocytes and perturbation of hair follicle cycle. FASEB J. *14*, 2075–2086.

Russel, L.J., DiGiovanna, J.J., Rogers, G.R., Steinert, P.M., Hashem, N., Compton, J.G., and Bale, S.J. (1995). Mutations in the gene for transglutaminase 1 in autosomal recessive lamellar ichthyosis. Nat. Genet. *9*, 279–283.

Sakai, H., Saku, T., Kato, Y., and Yamamoto, K. (1989). Quantitation and immunohistochemical localization of cathepsins E and D in rat tissues and blood cells. Biochim. Biophys. Acta *991*, 367–375.

Shin, M., Kadowaki, T., Iwata, J., Kawakubo, T., Yamaguchi, N., Takii, R., Tsukuba, T., and Yamamoto, K. (2007). Association of cathepsin E with tumor growth arrest through angiogenesis inhibition and enhanced immune responses. Biol. Chem. *388*, 1173–1181.

Stenn, K.S. and Paus, R. (2001). Controls of hair follicle cycling. Physiol. Rev. *81*, 449–494.

Sundberg, J.P., Erickson, A.A., Roop, D.R., and Binder, R.L. (1994). Ornithine decarboxylase expression in cutaneous papillomas in SENCAR mice is associated with altered expression of Keratins 1 and 10. Cancer Res. *54,* 1344–1351.

Sundberg, J.P., Boggess, D., Hogan, M.E., Sundberg, B.A., Rourk, M.H., Harris, B., Johnson, K., Dunstan, R.W., and Davisson, M.T. (1997). Harlequin ichthyosis (ichq): a juvenile lethal mouse mutation with ichthyosiform dermatitis. Am. J. Pathol. *151*, 293–310.

Sutter, C., Nischt, R., Winter, H., and Schweizer, J. (1991). Aberrant *in vitro* expression of keratin K13 induced by Ca^{2+} and vitamin A acid in mouse epidermal cell lines. Exp. Cell Res. *195*, 183–193.

Tiano, H.F., Loftin, C.D., Akunda, J., Lee, C.A., Spalding, J., Sessoms, A., Dunson, D.B., Rogan, E.G., Morham, S.G., Smart, R.C., et al. (2002). Deficiency of either Cyclooxygenase (COX)-1 or COX-2 alters epidermal differentiation and reduces mouse skin tumorigenesis. Cancer Res. *62*, 3395–3401.

Toomes, C., James, J., Wood, A.J., Wu, C.L., McCormick, D., Lench, N., Hewitt, C., Moynihan, L., Roberts, E., Woods, G., et al. (1999). Loss-of-function mutations in the cathepsin C gene results in periodontal disease and palmoplantar keratosis. Nat. Genet. *23*, 421–424.

Tsukuba, T., Okamoto, K., Okamoto, Y., Yanagawa, M., Kohmura, K., Yasuda, Y., Uchi, H., Nakahara, T., Furue, M., Nakayama, K., et al. (2003). Association of cathepsin E deficiency with development of atopic dermatitis. J. Biochem. *134*, 893–902.

Tsukuba, T., Yamamoto, S., Yanagawa, M., Okamoto, K., Okamoto, Y., Nakayama, K.I., Kadowaki, T., and Yamamoto, K. (2006). Cathepsin E-deficient mice show increased susceptibility to bacterial infection associated with the decreased expression of multiple cell surface Toll-like receptors. J. Biochem. *140*, 57–66.

Yamamoto, K. (1999). Cathepsin E and cathepsin D. In: Proteases: New Perspectives, V. Turk, ed. (Basel, Switzerland: Birkhäuser Verlag), pp. 59–71.

Yamamoto, K., Takeda, M., Yamamoto, H., Tstsumi, M., Kato, Y. (1985). Human erythrocyte membrane acid proteinase (EMAP): sidedness and relation to cathepsin D. J. Biochem. *97*, 821–830.

Yanagawa, M., Tsukuba, T., Nishioku, T., Okamoto, Y., Okamoto, K., Takii, R., Terada, Y., Nakayama, K.I., Kadowaki, T., and Yamamoto, K. (2007). Cathepsin E deficiency induces a novel form of lysosomal storage disorder showing the accumulation of lysosomal membrane sialoglycoproteins and the elevation of lysosomal pH in macrophages. J. Biol. Chem. *282*, 1851–1862.

Zeeuwen, P.L., Vlijmen-Willems, I.M., Hendriks, W., Merkx, G.F., and Schalkwijk, J. (2002). A null mutation in the cystatin M/E gene of ichq mice causes juvenile lethality and defects in epidermal cornification. Hum. Mol. Genet. *11*, 2867–2875.

Zeeuwen, P.L.I.M. (2004). Epidermal differentiation: the role of proteases and their inhibitors. Eur. J. Cell Biol. *83*, 761–773.

Received December 20, 2010; accepted March 7, 2011