The Biology of Cystatin M/E and its Cognate Target Proteases

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Cystatin M/E is a member of a superfamily of evolutionarily-related cysteine protease inhibitors that provide regulatory and protective functions against uncontrolled proteolysis by cysteine proteases. Although most cystatins are ubiquitously expressed, high levels of cystatin M/E expression are mainly restricted to the epithelia of the skin (epidermis, hair follicles, sebaceous glands, and sweat glands) and to a few extracutaneous tissues. The identification of its physiological targets and the localization of these proteases in skin have suggested a regulatory role for cystatin M/E in epidermal differentiation. *In vitro* biochemical approaches as well as the use of *in vivo* mouse models have revealed that cystatin M/E is a key molecule in a biochemical pathway that controls skin barrier formation by the regulation of both cross-linking and desquamation of the stratum corneum. Cystatin M/E directly controls the activity of cathepsin V, cathepsin L, and legumain, thereby regulating the processing of transglutaminases. Misregulation of this pathway by unrestrained protease activity, as seen in cystatin M/E-deficient mice, leads to abnormal stratum corneum and hair follicle formation, as well as to severe disturbance of skin barrier function. Here, we review the current knowledge on cystatin M/E in skin barrier formation and its potential role as a tumor suppressor gene.

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INTRODUCTION

Skin is our largest organ and plays an important protective and esthetic role. It is the interface of the body with the environment and consists of a stratified, keratinizing epithelium anchored to a basement membrane. The underlying connective tissue layer, the dermis, contains the vascular network, the hair follicles, the sweat glands, and the sebaceous glands. To protect the body against the invasion of microorganisms, penetration of toxic agents, and loss of essential body fluids, the skin has evolved an intricate differentiation process that results in a tough, water-impermeable outer covering that is constantly renewing. The outcome of this epidermal differentiation process is an organized tissue in which morphologically distinguishable cells are arranged in discrete layers of basal, spinous, granular, and cornified cells (Archer, 1998; Eady *et al.*, 1998).

As keratinocytes terminally differentiate, rupture of the stratum granulosum cell integrity results in the release of lysosomal proteases into the cytoplasm that eventually cause cell death. These cells that have lost their nuclei and cytoplasmic organelles are now called corneocytes and form the stratum corneum. The corneocyte contains a stable and insoluble cornified envelope that was synthesized just beneath the disintegrating plasma membrane. This cornified envelope consists of several specific proteins that are cross-linked together in an orchestrated way by Ca^{2+}-dependent transglutaminases (TGMs). The terminal stage of the differentiation process is desquamation, which involves degradation of lipids in the intercellular spaces and loss of residual intercellular desmosomal connections. Cells reaching the skin surface, which consist largely of dead proteinaceous sacs of keratin intermediate filament cables, are continuously sloughed and replaced by inner cells differentiating and moving outward (Reichert *et al.*, 1993; Fuchs and Raghavan, 2002; Candi *et al.*, 2005; Segre, 2006).

A model for the assembly of the epidermal cornified envelope

Biochemical studies have led to a model for cornified envelope assembly that consists of three principal stages: (i) initiation of cornified envelope assembly; (ii) formation of the corneocyte lipid envelope; and (iii) reinforcement of the cornified envelope (Nemes and Steinert, 1999; Kalinin *et al.*, 2001, 2002). As intracellular Ca^{2+} concentrations increase in suprabasal cells, envoplakin, periplakin, and involucrin are expressed, which then associate with keratin intermediate filaments and desmosomal junctions. Nearly at the same time, TGM1 is expressed, which docks into the plasma membrane where it catalyzes the intrachain head-to-head and head-to-tail crosslinking of involucrin, as well as interchain crosslinking between both plakins and involucrin. These events first occur at interdesmosomal sites on the cell

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Abbreviations: CTSC, cathepsin C; CTSD, cathepsin D; CTSL, cathepsin L; CTSV, cathepsin V; HI, harlequin ichthyosis; IV, ichthyosis vulgaris; LGMN, legumain; LI, lamellar ichthyosis; SCC, squamous cell carcinoma; TGM, transglutaminase

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membrane, and eventually, involucrin and the plakin proteins (and perhaps other proteins) develop a monomolecular layer along the entire inner surface of the plasma membrane, including the desmosomes, thereby forming a scaffold. The second step in this model is the formation of the corneocyte lipid envelope. At a particular time point, perhaps coincident with the initiation of scaffold assembly, lamellar granules are thought to fuse with the plasma membrane of the granular cell and discharge their lipid membranes into the intercellular space to form a water-repelling envelope around the cornified envelope. Finally, other structural proteins, including loricrin and small proline-rich proteins, which together comprise about 80% of the total mass of the cornified envelope, are crosslinked onto the scaffold to reinforce the initial structure. The cytosolic, activated TGM3 enzyme catalyzes the homodimerization of loricrin and the heterotrimerization of loricrin and small proline-rich proteins, which are subsequently translocated to the border of the cell, where TGM1 crosslinks them onto the preexisting scaffold. Minor amounts of other proteins (cystatin A, SKALP/elafin, LEP/XP5 proteins, repetin, and trichohyalin) also become crosslinked to the cornified envelope. The last stage of the cornified envelope assembly is characterized by rupture of the cell integrity, which results in degradation of most cell organelles and internal structures. Finally, the dead cornified cells are shed from the skin by a process called desquamation. Taken together, the complete cornified envelope, consisting of the protein and lipid envelope, and the intercellular lipid lamellae provide the vital mechanical and water barrier functions for the skin and entire organism.

Lysosomal proteases and their inhibitors in skin

The analysis of the pathophysiology of inflammatory diseases, genetic diseases, the invasive and metastatic mechanism of malignant neoplasms, and the course of infectious diseases has revealed that fundamental knowledge of the regulatory mechanisms of proteases and their inhibitors is crucial for our understanding of these diverse problems (Turk *et al.*, 2002b; Mohamed and Sloane, 2006; Lopez-Otin and Bond, 2008). Regulation of proteolytic enzyme activity is essential for cells and tissues at many levels (activation, compartmentalization, and specific inhibition) because proteolysis at a wrong time and location may have disastrous consequences.

Lysosomal cysteine proteases were long considered to be primarily responsible for intralysosomal protein degradation, mediating ''housekeeping'' functions in the cell. However, numerous investigations have shown that classical cathepsins are involved in a variety of physiological processes, such as proenzyme activation, enzyme activation, antigen presentation, hormone maturation, tissue remodeling, and bone matrix resorption (Dickinson, 2002). Recent studies also suggest that cathepsins act as mediators of programmed cell death, and have shown apoptosis pathways to be dependent on lysosomal cathepsin activity (Stoka *et al.*, 2001; Lippens *et al.*, 2005). Cathepsins are synthesized as pre-proenzymes that are post-translationally glycosylated and phosphorylated to active enzymes. Disturbance in their transport to lysosomes could lead to cytosolic protease activity or secretion of the proenzyme to the extracellular matrix. Although an acidic pH is optimal for the activity of lysosomal cysteine proteases, individual enzymes are active over different pH ranges (Chapman *et al.*, 1997; Honey and Rudensky, 2003).

The regulation of the activity of cysteine proteases for correct functioning is a fragile balance of many factors, one of the most crucial being their cognate protease inhibitors, which include members of the cystatin gene family. Cystatins are small proteins that are widely distributed in several human tissues and body fluids. They are generally tight-binding inhibitors of cysteine proteases, such as cathepsins B, L, H, K, and S (Abrahamson *et al.*, 2003), and should therefore serve a protective function to regulate the activities of such endogenous proteases. It is thought that cysteine proteases and their inhibitors play an important role in pathophysiological processes, such as invasion of normal tissues by tumors or microorganisms, viral infections, and inflammation (Korant *et al.*, 1986; Bjorck, 1990; Calkins and Sloane, 1995; Henskens *et al.*, 1996). Cystatins are members of a superfamily of evolutionarily-related proteins and can be divided into three major families, namely family-1 cystatins (A and B), family-2 cystatins (C, D, F, G, M/E, S, SN, and SA), and the kininogens (L- and H-kininogens), which belong to family-3 cystatins. In general, cystatins are competitive, reversible, tight-binding proteins that inhibit cysteine proteases in a micromolar to picomolar range (Turk *et al.*, 1997). Cystatins are viewed as ''emergency'' inhibitors, which mean that they rapidly trap a protease without delay and keep it in a stable complex, preventing any additional proteolysis (Bode and Huber, 2000; Turk *et al.*, 2002b). In normal situations, such an inhibitor would have a physiological concentration in large excess over the putative protease target(s). These inhibitors are in general physically separated from their target proteases and primarily act on escaped endogenous proteases or exogenous proteases of invading microorganisms.

Interestingly, an increase in lysosomal cysteine protease activity has been observed in the terminal differentiation process of keratinocytes (Tanabe *et al.*, 1991; Kawada *et al.*, 1997). Recent studies have reported that lysosomal proteases play important roles in physiological processes not restricted to lysosomes only (Turk *et al.*, 2002a). For example, protease activity and regulation outside lysosomes potentially contribute to the propagation of apoptosis, a process that is distinct from terminal differentiation of the epidermis but nevertheless shares some molecular and cellular features. The importance of regulated proteolysis in epithelia (Zeeuwen, 2004a) is well shown by the identification of the *SPINK5* serine protease inhibitor as the defective gene in Netherton syndrome (MIM 256500) (Chavanas *et al.*, 2000), cathepsin C (*CTSC*) mutations in Papillon–Lefevre syndrome (MIM 245000) (Toomes *et al.*, 1999), matriptase deficiency owing to mutations in the *ST14* gene in ichthyosis with hypotrichosis syndrome (MIM 610765) (Basel-Vanagaite *et al.*, 2007), cathepsin L (*CTSL*) deficiency in mice (Roth *et al.*, 2000; Benavides *et al.*, 2002; Tobin *et al.*, 2002), targeted ablation of cathepsin D (*CTSD*) in mice (Egberts *et al.*, 2004), and the phenotype of targeted epidermal overexpression of the stratum corneum chymotryptic enzyme in mice (Hansson *et al.*, 2002).

To obtain a systematic and quantitative overview of genes expressed by human epidermal cells, our group has

previously established transcriptomes of cultured human keratinocytes and purified epidermal cells through Serial Analysis of Gene Expression and microarrays (GEO (Gene Expression Omnibus) Series accession nos. GSE31 and GSE6601). These analyses revealed quantitative expression data on numerous proteases and protease inhibitors (Jansen *et al.*, 2001; van Ruissen *et al.*, 2002a, b). Cystatin M/E, a protein not known earlier to be expressed in human keratinocytes, was found at considerable levels in these analyses. Cystatin M was initially identified by differential display as a downregulated mRNA in metastatic breast tumor cells when compared with normal and primary breast tumor cells (Sotiropoulou *et al.*, 1997). Independently, the same molecule was found by others through expressed sequence tag sequencing in cDNA libraries derived from epithelial cells, and was designated cystatin E (Ni *et al.*, 1997). It was shown that it was really a protease inhibitor, as the recombinant protein was able to inhibit the plant protease papain. However, *in vivo* target proteases and the function of this protease inhibitor were unknown. Since then, investigations have been performed to study: (i) the molecular and biochemical characteristics of cystatin M/E gene and protein; (ii) the cellular aspects of cystatin M/E, for example, tissue localization, distribution, and compartmentalization; (iii) the cell biological and physiological functions of cystatin M/E; and (iv) the role of cystatin M/E in epidermal homeostasis and in disease. These investigations are reviewed here.

STRUCTURE AND EXPRESSION OF HUMAN CYSTATIN M/E

Cystatin M/E is a 14 kDa secreted protein that shares only 35% homology with the human family-2 cystatins. Cystatin M/E has an overall structure similar to other family-2 cystatins, such as a signal peptide and two intrachain disulfide bonds, but possesses the unusual characteristic of being a glycoprotein. This protein is only distantly related to the other known family members as reflected by the genomic position of the cystatin M/E gene on chromosome 11q13 (Stenman *et al.*, 1997), whereas all other family-2 cystatin genes are clustered in a narrow region on chromosome 20p11.2 (Schnittger *et al.*, 1993). The full-length cystatin M/E gene was cloned and sequenced, and deposited as *CST6* in the GenBank database (accession no. AY145051) (Zeeuwen *et al.*, 2003). The cystatin M/E gene is organized in three exons and two introns, and from the translation start site to the translation termination site it spans 1,354 bp of genomic DNA. Characterization of the genomic sequence of cystatin M/E shows some differences compared with that of the other type 2 cystatins. The size of both introns was remarkably smaller compared with that of other cystatin family-2 members (for example, cystatin S, SA, SN, C, and D). These cystatins harbor introns of 1.1–2.2 kb, whereas the introns of the cystatin M/E gene are only 542 and 365 bp long. Besides, these family-2 cystatins contain an identifiable TATA box in their 5′-flanking sequence, whereas cystatin M/E lacks this conserved sequence. Database analysis predicted three transcription start sites at −75, −42, and −34 bp, respectively, upstream to the translation initiation codon. This is in accordance with the average length of the expressed sequence tags found in the public database, in which the start positions vary between approximately −60 and −20 bp.

The expression pattern of human cystatin M/E is unique among cystatins, as high expression levels are largely confined to cutaneous epithelia, whereas other cystatins are mainly ubiquitously expressed. In human skin, cystatin M/E is expressed in the differentiating and cornifying layers of the epidermis (Figure 1) (Zeeuwen *et al.*, 2001, 2007). This is in accordance with the observation that, *in vitro*, cystatin M/E is expressed only in differentiating but not in proliferating keratinocytes (Zeeuwen *et al.*, 2001). Cystatin M/E, which has a putative signal peptide, is indeed a secreted protein and is found *in vitro* in culture supernatant and *in vivo* in human sweat and semen (Zeeuwen *et al.*, 2001; Tjabringa *et al.*, 2005). Furthermore, cystatin M/E is also highly expressed in the appendages of the skin, such as the secretory coils of the eccrine sweat glands, the sebaceous glands, and the hair follicles (Cheng *et al.*, 2008). Immunohistochemical data have also shown a moderate expression of cystatin M/E in the bronchi of the lung, the proximal tubuli of the kidney, and in the cornea of the eye (Zeeuwen *et al.*, 2004b). Recently, the expression of cystatin M/E was analyzed in a large panel of human tissues at the mRNA level by real-time quantitative PCR, which is currently the most accurate method to detect quantitative gene expression levels. These experiments revealed that high cystatin M/E mRNA expression was seen in the skin, whereas moderate-to-low levels were observed in the esophagus, oropharyngeal tissues, bronchus, lung, and kidney (Cheng *et al.*, in press). Cystatin M/E protein expression was also reported recently in normal human breast tissue (Rivenbark *et al.*, 2007), breast ductal epithelium (Ai *et al.*, 2006), and in oligodendrocyte-like cells and astrocyte-like cells of normal human brain (Qiu *et al.*, 2008).

CYSTATIN M/E-DEFICIENT MICE

Analysis of adult mouse tissues revealed an expression pattern that is less restricted than that observed earlier in humans (Zeeuwen *et al.*, 2002b). Relatively high levels of

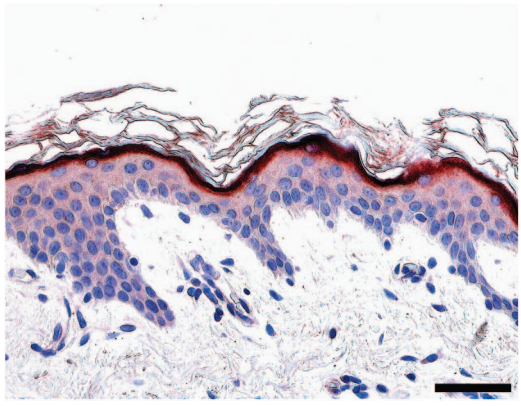

Figure 1. Cystatin M/E expression in normal human skin. Formalin-fixed section of normal human skin was stained by rabbit anti-cystatin M/E antiserum (Zeeuwen *et al.*, 2001). Staining is limited to the stratum granulosum. Bar = 100 μm.

cystatin M/E mRNA expression were found in the skin, ileum, stomach, eye, and cerebellum; moderate-to-low levels of expression were found in the tongue, palatum, nasal cavity, colon, bladder, skeletal muscle, placenta, and thymus. During embryogenesis, mRNA expression of cystatin M/E was observed from day 16 onward, which is a developmental stage where epidermal stratification is completed. Cystatin M/E protein was found to be highly expressed in the ciliated tracheal epithelium and in bronchial epithelium. Similar to the expression pattern in humans, cystatin M/E was found in the infundibular epithelium of hair follicles and in the stratum granulosum of interfollicular epidermis. The mouse cystatin M/E gene, which was subsequently cloned and characterized (deposited as *Cst6* in the GenBank database, accession no. AY093591), has an exon–intron structure identical to that of all other type 2 cystatins and encodes a 149-amino-acid protein (Zeeuwen *et al.*, 2002b). Alignment with its presumed human ortholog revealed 69% amino-acid identity and 82% amino-acid conservation. The chromosomal localization of mouse cystatin M/E was determined by fluorescence *in situ* hybridization and mapped to the proximal end of chromosome 19, a region that is syntenic to human chromosome 11q13 where the human cystatin M/E gene resides.

With the knowledge of the cystatin M/E tissue expression and the locus of the gene in mice, it was hypothesized that cystatin M/E could be a candidate gene for the *ichq* mouse, a naturally occurring mutant laboratory mouse (Figure 2). This mouse had morphological and biochemical similarities to a human form of ichthyosis caused by an unknown gene defect (Sundberg *et al.*, 1997). This hypothesis was confirmed by the identification of a mutation in the cystatin M/E gene of these *ichq* mice (Zeeuwen *et al.*, 2002b). Homozygosity for a single nucleotide deletion in exon 1 of the cystatin M/E gene was identified in these mutant mice. This 42delG deletion alters the reading frame, resulting in a premature stop codon at amino acid position 20. Immunohistochemistry confirmed the absence of cystatin M/E at the protein level in *ichq* mice. Mice that were homozygous for two null alleles displayed a hyperplastic, hyperkeratotic epidermis and abnormal hair follicles, and died between 6 and 12 days of age. Heterozygous mice and wild-type littermates displayed a normal cystatin M/E expression pattern, suggesting that cystatin M/E functions as an inhibitor of hitherto unidentified proteases. The mouse *ichq* mutant is another example of a disturbed protease–antiprotease balance causing faulty differentiation processes in the epidermis and hair follicle. Other examples of dysregulated proteases and protease inhibitors leading to abnormal cornification include SPINK5, matriptase, cathepsin C, caspase 14, and CTSD (Toomes *et al.*, 1999; Chavanas *et al.*, 2000; Egberts *et al.*, 2004; Basel-Vanagaite *et al.*, 2007; Denecker *et al.*, 2007).

Figure 2. Phenotype of *ichq* mice. Cystatin M/E-deficient mice (9 days of age) are covered with white scales that start to appear at day 5 on the dorsal skin, just behind the neck. The mice die between days 6 and 12 from dehydration.

At this point, the next goal was to identify the putative target protease(s) for cystatin M/E and to investigate the mechanism by which the absence of cystatin M/E causes epidermal abnormalities in *ichq* mice. The additional questions that arose from these investigations were (i) which proteins are processed by the target protease(s)?; (ii) which biological processes are regulated by the target protease(s)?; and (iii) which processes are disturbed by their unrestricted protease activity as was probably the case in cystatin M/E-deficient mice?

PHYSIOLOGICAL TARGETS OF CYSTATIN M/E

Biochemical studies have indicated that cystatin C and cystatin M/E *in vitro* bind with high affinity to the asparaginyl endopeptidase legumain (LGMN) (Alvarez-Fernandez *et al.*, 1999). This was an unexpected finding, as cystatins were primarily regarded as inhibitors of papain-like cysteine proteases in the unrelated family C1 (Abrahamson *et al.*, 2003). LGMN belongs to the family C13 of cysteine proteases and is strictly specific for hydrolysis of asparaginyl bonds (Chen *et al.*, 1997). Furthermore, this lysosomal protease was shown to be involved in the processing of other lysosomal proteases, such as cathepsins B, H, and L (Shirahama-Noda *et al.*, 2003; Maehr *et al.*, 2005). Although cystatin M/E contains a predicted binding site for papain-like cysteine proteases, the first reports only showed moderate-to-low inhibition activity toward human cathepsin B (Ni *et al.*, 1997; Zeeuwen *et al.*, 2001). Recently it was shown that cystatin M/E is a high-affinity inhibitor of cathepsin V (CTSV) and CTSL by a reactive site that is distinct from the LGMN-binding site (Cheng *et al.*, 2006). These kinetic studies have shown that K_i values of cystatin M/E for the interaction with CTSV and CTSL are 0.47 and 1.78 nM, respectively.

CTSV is a recently discovered protease, also known as CTSL2 or stratum corneum thiol protease. CTSV is closely related to CTSL, and these proteins share ~80% sequence identity (Adachi *et al.*, 1998; Santamaria *et al.*, 1998; Bromme *et al.*, 1999; Watkinson, 1999). Their encoding genes are located on adjacent sites on chromosome 9q22.2, suggesting that CTSV and CTSL have evolved by duplication from an ancestral gene. However, although CTSL is ubiquitously expressed, human CTSV expression is more restricted and predominantly found in the thymus, testis, cornea of the eye, and epidermis. Besides the general function of lysosomal cathepsins, that is bulk proteolysis, studies using a knockout mouse model have suggested that CTSL has specific functions in keratinocyte proliferation and hair follicle morphogenesis and cycling (Roth *et al.*, 2000; Benavides *et al.*, 2002; Tobin *et al.*, 2002). Recently, the phenotype of CTSL

knockout mice was rescued by transgenic epidermal re-expression of either murine CTSL or human CTSV, indicating that human CTSV can compensate for murine CTSL (Hagemann *et al.*, 2004). No ortholog of CTSV in mice has been discovered yet, but it is likely that murine CTSL is actually the ortholog of human CTSV, in view of the fact that human CTSV is phylogenetically more closely related to murine CTSL than to human CTSL (Reinheckel *et al.*, 2005).

On the basis of the analogous sites in cystatin C (Alvarez-Fernandez *et al.*, 1999), site-directed mutagenesis was used to identify the presumed binding sites of these cathepsins and LGMN in human cystatin M/E. Substitution of a conserved amino acid by alanine in the two reactive sites resulted in the loss of inhibitory activity. It was shown that the W135A cystatin M/E mutant was rendered inactive against CTSV and CTSL but retained LGMN-inhibiting activity. Conversely, the N64A mutant lost LGMN-inhibiting activity but remained active against the papain-like cysteine proteases, which means that LGMN and both papain-like cysteine proteases are inhibited by two distinct non-overlapping sites (Cheng *et al.*, 2006). The presumed role of LGMN, CTSV, and CTSL in terminal differentiation of keratinocytes and hair follicle morphogenesis is that these proteases process and activate other enzymes in pathways that lead to skin barrier formation (see the section Cystatin M/E: function and regulation).

CYSTATIN M/E: FUNCTION AND REGULATION

Cystatin M/E-deficient mice show defects in epidermal cornification and die between 6 and 12 days of age (Sundberg *et al.*, 1997; Zeeuwen *et al.*, 2002b). Autopsy on neonatal *ichq* mice strongly suggested that these mice died of dehydration, probably owing to a disturbed skin barrier function (Zeeuwen *et al.*, 2004a). It was shown that the transepidermal water loss through the skin of the cystatin M/E-deficient mice is increased when compared with phenotypically normal littermates. In addition, a progressive weight loss was observed in the cystatin M/E-deficient mice starting at the time point at which transepidermal water loss was apparent. At day 11, body weights were ~50% compared with normal littermates, and most mice died shortly thereafter (Zeeuwen *et al.*, 2004a).

Morphological and functional analysis has indicated a compromised stratum corneum and aberrant cornification of the hair follicles in cystatin M/E-deficient mice, which could be the result of abnormal expression or processing of structural components of the cornified envelope. In two earlier studies, no abnormalities were found in structural proteins such as cytokeratins, involucrin, and filaggrin (Sundberg *et al.*, 1997; Dunnwald *et al.*, 2003). This was investigated again by studying the expression and distribution of loricrin, involucrin, and filaggrin, which are important stratum corneum proteins that are involved in skin barrier function. Immunohistochemical analysis showed that all three proteins are normally expressed in the skin of cystatin M/E-deficient mice. However, western blot analysis revealed that loricrin monomers and loricrin dimers are strongly diminished in skin extracts of cystatin M/E-deficient mice (Zeeuwen *et al.*, 2004a). Collectively, these findings suggest misregulated crosslinking of loricrin monomers.

As described earlier in the Introduction section, it is known that the cytosolic, activated TGM3 enzyme is responsible for the crosslinking of loricrin molecules. The resulting homodimers and heterodimers are subsequently translocated to the periphery of the cell, where TGM1 crosslinks them onto the preexisting scaffold. Proteolytic cleavage of TGM3 is required to achieve maximal-specific activity of the enzyme (Kim *et al.*, 2001), and one important question was how TGM3 could be processed into its active form. TGM3 activation during keratinocyte differentiation involves cleavage of the 77 kDa zymogen by an unknown protease resulting in the release of 30 and 47 kDa fragments, which then associate and form the active enzyme (Ahvazi *et al.*, 2002). Western blot analysis revealed an abundant presence of proteolyzed (and hence activated) TGM3 in skin extracts of cystatin M/E-deficient mice at day 6, whereas no appreciable levels of proteolyzed TGM3 could be detected in skin extracts of wild-type littermates (Zeeuwen *et al.*, 2004a). This indicated that premature, high levels of activated TGM3 are present at the time when skin lesions develop in cystatin M/E-deficient mice. It was hypothesized that cystatin M/E controls the activity of an unknown protease that is responsible for TGM3 activation, which leads to crosslinking of loricrin molecules and skin barrier formation. If cystatin M/E is missing in the skin, unrestrained protease activity is followed by too much, and too early activity of TGM3, probably leading to pathology as seen in cystatin M/E-deficient mice.

Subsequently, it was investigated whether human LGMN could process human recombinant TGM3 *in vitro* into its active form. LGMN was the first candidate because localization of free LGMN activity was found in the stratum granulosum and in the infundibular part of the hair follicle in cystatin M/E-deficient mice. This matches the reported localization of tissue pathology in these mice, such as excessive cornification in hair follicles and epidermis, leading to plugging of the hair follicle and ichthyosis. No LGMN activity could be shown in the skin of wild-type littermates, which suggested that the presence of cystatin M/E normally regulates LGMN activity in skin *in vivo* (Zeeuwen *et al.*, 2004a). It was found that TGM3 was slowly processed into its active form by a human kidney extract as detected by mAbs directed against the human zymogen and the 30 and 47 kDa fragments. As no active recombinant LGMN was available at that time, human kidney extract was used as a source of LGMN activity. Proteolytic processing of recombinant TGM3 by the human kidney extract could be completely inhibited by a specific LGMN inhibitor Cbz-Ala-Ala-Aasn-EP-COOEt. As it is known that LGMN affects processing of lysosomal cathepsins (Shirahama-Noda *et al.*, 2003; Maehr *et al.*, 2005), it was considered that LGMN possibly mediates processing of TGM3 in an indirect manner. Indeed, using compound E-64, a synthetic broad-spectrum inhibitor of lysosomal cysteine proteases that is not effective against LGMN, a complete inhibition of TGM3 processing was found. This finding was addressed in further detail by investigating the *in vitro* processing of TGM3 by a panel of purified lysosomal cysteine proteases (Cheng *et al.*, 2006). It was shown that CTSL is the elusive protease that can process

and activate TGM3, whereas other cathepsins (B, D, S, and V) were not able to cleave the TGM3 zymogen. It was shown that cleavage by CSTL causes a biologically active TGM3 molecule, whereas unprocessed TGM3 did not show any activity. N-terminal sequencing of the 30 kDa TGM3 fragment revealed that the CTSL cleavage site in TGM3 is between amino acids Ala^{466} and Ala^{467}. As CTSL preferentially cleaves peptide bonds with aromatic residues in the P2 and hydrophobic residues in the P3 position (Barrett *et al.*, 2004), this is most likely the exact cleavage site of human TGM3. This conclusion was based on the fact that, in the P2 and P3 positions of the TGM3 cleavage site, an aromatic residue (Phe^{465}) and a hydrophobic residue (Pro^{464}), respectively, are found. Future studies should clarify whether this is the physiological cleavage site *in vivo* as well.

To further address the possible role of cystatin M/E and its physiological target proteases in epidermal differentiation, the exact localization and compartmentalization of these proteins was investigated by immunofluorescence microscopy (Zeeuwen *et al.*, 2007). Although CTSL and LGMN were broadly expressed in the cytoplasm of the epithelial cells of the skin, a specific colocalization of cystatin M/E and CTSV was found in the stratum granulosum and in the root sheath of the hair follicles, showing a typical granular pattern (Figure 3). Immunoelectron microscopy revealed that cystatin M/E and CTSV are separately transported within the lamellar granules. Recently, it was shown that epidermal lamellar granules transport different cargoes (for example, CTSD, glucosylceramides, corneodesmosin, and KLK7) as distinct aggregates, which were delivered to the apical region of granular keratinocytes (Ishida-Yamamoto *et al.*, 2004). One could speculate that separate transport of cystatin M/E and CTSV by lamellar granules may prevent interaction within the cells. Moreover, cystatin M/E and CTSV are detected in the extracellular spaces of the upper stratum corneum layers where both proteins are associated with desmosomes, suggesting that these molecules could have an important role in desquamation, which is the final event in terminal differentiation of the epidermis. During desquamation, breakdown of lipids in the intercellular spaces and loss of residual intercellular desmosomal connections lead to cell shedding of the dead corneocytes from the stratum corneum surface (Elias, 2005). A number of studies have shown the involvement of different epidermal proteases in the progressive degradation of the desmosomal proteins desmocollin-1, desmoglein-1, plakoglobin, and corneodesmosin (Lundstrom and Egelrud, 1990; Simon *et al.*, 2001; Caubet *et al.*, 2004). As an acidic pH dominates in the outer stratum corneum layers (Elias, 2004), it is conceivable that CTSV activity in these layers fulfills a significant function in the desquamation process (for example, degradation of desmosomal constituents), as opposed to serine proteases that mainly act in the neutral pH environment of the lower stratum corneum layers. This was supported by *in vitro* experiments that showed degradation of corneodesmosomal proteins by CTSV at an acidic pH, whereas no degradation products were observed, as reactions were performed at a neutral pH of 7 (Cheng *et al.*, in press).

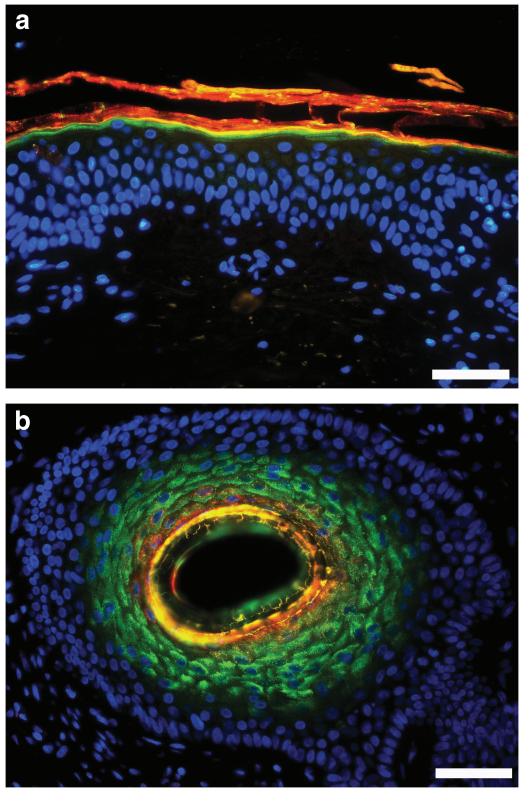

Figure 3. Colocalization of cystatin M/E and CTSV in human epidermis and hair follicle. Immunofluorescence double staining (yellow-orange merge color) for cystatin M/E (green) and CTSV (red) in the (**a**) epidermis and a transversal section of the (**b**) hair follicle. DNA staining by DAPI (blue). Bar = 50 μm.

Taken together, these data suggest a mechanism in which cystatin M/E is a key molecule in a biochemical pathway that controls skin barrier formation by the regulation of both crosslinking and desquamation of the stratum corneum (Figure 4). First, cystatin M/E could have a role in desquamation by the regulation of CTSV protease activity, which is involved in the degradation of corneodesmosomal components. Second, cystatin M/E could regulate crosslinking of structural proteins by TGM3 in the cornification process of the epidermis and the hair follicle by controlling CTSL and LGMN activities. Furthermore, cystatin M/E might also be involved in the regulation of TGM1 activity, as it is known that CTSL can process and activate CTSD (Wille *et al.*, 2004; Laurent-Matha *et al.*, 2006). It has been reported that CTSD, an aspartate protease, is involved in the regulation of TGM1 (Higuchi *et al.*, 2001; Egberts *et al.*, 2004). *In vitro* experiments have shown that TGM1 activity is stimulated by exogenous CTSD in cultured keratinocytes, and that in CTSD-deficient mice, a reduced TGM1 enzymatic activity and defective TGM1 processing were observed. The regulatory role of cystatin M/E in the cornification process of the epidermis was further supported by the observation that, in skin extracts of 6- to 12-day-old cystatin M/E-deficient mice, relatively high levels of proteolyzed CTSD and TGM1 are found, whereas no appreciable levels of these proteolyzed proteins could be detected in skin extracts of wild-type

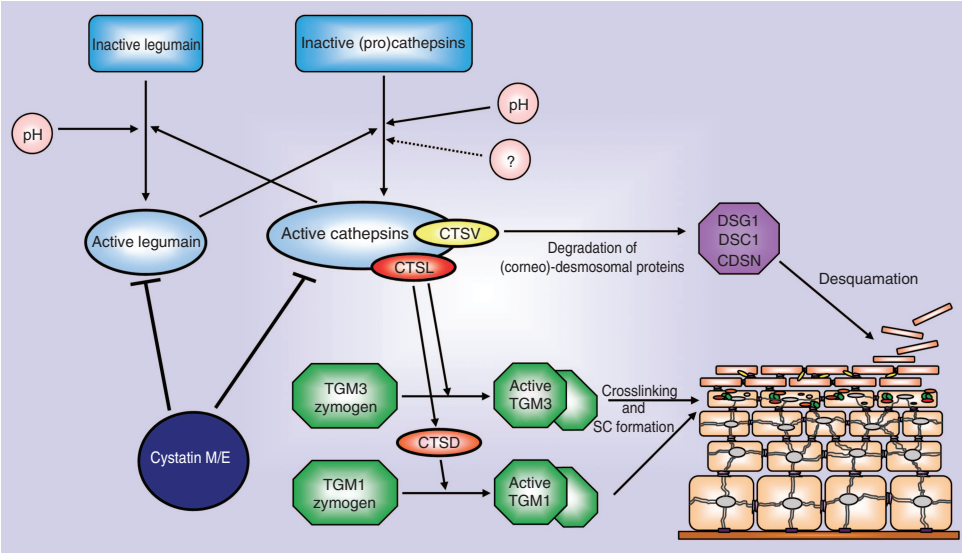

Figure 4. Regulation of epidermal protease activity by cystatin M/E. A simplified scheme of the presumed regulatory role of cystatin M/E in processes that control epidermal cornification and desquamation. Cystatin M/E is an inhibitor of the asparaginyl endopeptidase LGMN and the cysteine proteases CTSL and CTSV. Inhibition of LGMN might regulate the processing of (pro)-cathepsins, a process that is also under the control of intra- and extracellular pH changes. Inhibition of CTSV putatively regulates desquamation, as CTSV is able to degrade (corneo)-desmosomal proteins such as desmoglein-1 (DSG1), desmocollin-1 (DSC1), and corneodesmosin (CDSN). Inhibition of CTSL activity by cystatin M/E is thought to be important in the cornification process, as CTSL is the elusive processing and activating enzyme for TGM3. CTSL is also able to process CTSD, an aspartate protease that can regulate TGM1 activity.

littermates (Zeeuwen *et al.*, 2004b). Furthermore, increased levels of mainly membrane-bound TGM activity (*in situ*) were observed in 6- to 12-day-old cystatin M/E-deficient mice, suggesting that increased TGM1 activity could be involved in the pathology of these mice, which is clearly a direction for future research. In addition, regulation of CTSD activity is also important in the desquamation process of the skin, as the mature active form of CTSD is able to degrade desmosomal components (Horikoshi *et al.*, 1999).

Recent epigenetic studies have provided evidence that regulation of cystatin M/E gene expression is regulated by methylation of the CpG islands present in the cystatin M/E proximal promoter region and exon 1 (Ai *et al.*, 2006; Rivenbark *et al.*, 2006; Schagdarsurengin *et al.*, 2007; Qiu *et al.*, 2008). Methylation of CpG islands is a postreplicative modification thought to play a role in gene transcription in eukaryotes, either through interference with transcription factor binding or through the recruitment of repressors that specifically bind sites containing methylated CpG. A growing number of human diseases have been found to be associated with aberrant DNA methylation (Robertson, 2005; Feinberg, 2007). It was reported that cystatin M/E expression is frequently silenced in breast carcinomas by epigenetic inactivation related to DNA methylation (see section Cystatin M/E and cancer for details).

CYSTATIN M/E AND DISEASE

Ichthyosis

The *ichq* mouse phenotype includes hyperkeratosis, abnormally large mitochondria, and absence of lamellar granules (Sundberg *et al.*, 1997); some of these morphological and biochemical features are similar to human type 2 harlequin ichthyosis (HI, MIM 242500), suggesting that the *ichq* mouse might be a good model for the human disorder. The involvement of cystatin M/E in HI in humans was evaluated by sequencing the entire coding region and intron–exon boundaries for mutations in 11 sporadic HI patients (Zeeuwen *et al.*, 2003). No cystatin M/E mutations were detected in this group, which comprised types 1 and 2 HI patients. Disturbed transcription/translation owing to mutations in regulatory and non-coding regions of cystatin M/E was unlikely because cystatin M/E protein expression was observed in all patients examined, as assessed by immunohistochemistry. These data have indicated that cystatin M/E is unlikely to be a major gene responsible for types 1 and 2 HI. Recently, it was discovered that mutations in *ABCA12*, a member of the ATP-binding cassette transporter superfamily, underlie congenital HI (Kelsell *et al.*, 2005). It was reported that *ABCA12* works as an epidermal keratinocyte lipid transporter and that defective *ABCA12* results in a loss of the skin lipid barrier, leading to HI (Akiyama *et al.*, 2005). However, cystatin M/E and the genes that have a role in biological pathways that are controlled by cystatin M/E should be considered as candidate genes for human disorders of cornification of unknown etiology, especially for lamellar ichthyosis (LI, MIM 242300), ichthyosis vulgaris (IV, MIM 146700), and other ichthyosiform conditions. An immunohistochemical evaluation for cystatin M/E protein expression, followed by mutation analysis of all cystatin M/E exons including exon/intron boundaries, was performed on a number of cornification disorders (LI $n=15$; IV $n=5$; HI $n=3$, Netherton syndrome $n=2$) (Oji *et al.*, 2005). Cystatin M/E expression was observed in the stratum granulosum in most cases of LI, including those showing TGM1 deficiency.

Three patients with LI had a faint staining, whereas in HI protein expression was normal. In contrast, skin sections of IV also displayed a signal of cystatin M/E in the upper layer of the epidermis including the stratum corneum. Interestingly, one individual with LI presenting an enhanced epidermal expression of cystatin M/E showed a mutation on one allele in exon 1 (AA110TT) resulting in an amino-acid change from glutamic acid to valine (E37V) next to the reactive site of the proteinase inhibitor, which makes it plausible that the cystatin M/E inhibitory activity is reduced in this patient. This mutation was absent in 90 different control chromosomes. Interestingly, this patient was also heterozygous for a filaggrin mutation, which is the major predisposing factor for IV and atopic dermatitis (Palmer *et al.*, 2006; Smith *et al.*, 2006). Patients heterozygous for a filaggrin mutation normally show a mild skin phenotype; however, this patient was characterized by a severe ichthyosis phenotype. This suggested that the heterozygous E37V mutation in the cystatin M/E gene in combination with heterozygosity for filaggrin might be responsible for the severe skin phenotype. Therefore, site-directed mutagenesis was performed to produce a recombinant cystatin M/E variant in which the Glu^{37} residue was substituted for a Val residue. However, no reduced cystatin M/E inhibitory activity against CTSV and CTSL was found compared with wild-type cystatin M/E. This indicates that the heterozygous E37V mutation found in the LI patient does not disturb the presumed binding site of cathepsins (Zeeuwen *et al.*, 2004b).

Hair follicle-related diseases

Cystatin M/E-deficient mice show a severe skin and hair phenotype characterized by defects in cornification processes, suggesting that regulation of protease activity by cystatin M/E is important not only in the skin but also in terminal differentiation of the hair follicle, and that disturbance could lead to abnormal development of this skin appendage. Therefore, heritable genetic skin diseases that are characterized by hyperkeratinization of hair follicles are candidate diseases for cystatin M/E mutations. Cystatin M/E was found to be normally expressed at the protein level in two patients with ichthyosis follicularis with atrichia and photophobia (MIM 308205) (Cambiaghi *et al.*, 2002). No mutations were found in any of the cystatin M/E exons or exon/intron boundaries. This study, and that of Oji *et al.* (2005), who examined another case of ichthyosis follicularis with atrichia and photophobia, excluded cystatin M/E as the causative gene for this disease (Zeeuwen *et al.*, 2004b). Keratosis pilaris defines a group of cutaneous disorders characterized by follicular hyperkeratosis and frequently occurring with atopy or ichthyosis (MIM 604093). Keratosis pilaris is a very common skin/hair follicle disease that can range from minimal to severe forms. It is characterized by hyperkeratinization of hair follicles, and as a result, the hair is prevented from exiting the skin. Keratosis follicularis spinulosa decalvans is a rare disorder affecting also the eye (MIM 308800). Although most affected families are compatible with X-linked inheritance, this disease appears to be genetically heterogeneous. We speculate that the group of recessive keratosis pilaris disorders is a possible candidate for cystatin M/E mutations, and as such they are currently under investigation.

Inflammatory skin diseases

Atopic dermatitis and psoriasis are two common chronic inflammatory skin diseases in which the expression of many genes and the formation of the epidermal barrier are altered. Although both diseases are generally regarded as immune-mediated conditions, recent genetic studies have indicated the importance of abnormalities in epithelium-expressed genes as a primary cause. Loss-of-function alleles of the skin barrier protein, filaggrin, were found to be a major predisposing factor for atopic dermatitis (Palmer *et al.*, 2006), and it was recently shown that a copy number polymorphism of a β-defensin gene cluster and the deletion of the late cornified envelope 3B and 3C genes were associated with increased risk for psoriasis (Hollox *et al.*, 2008; de Cid *et al.*, 2009). In view of the fact that the cystatin M/E–cathepsin pathway is important for correct epidermal differentiation, the expression of all players of this biochemical pathway in these two major inflammatory skin diseases was investigated (Cheng *et al.*, in press). Generally, mRNA expression levels of cystatin M/E and CTSV are decreased in inflamed skin, whereas the levels of CTSL and TGM3 are increased. These findings are in accordance with microarray analyses in patients with atopic dermatitis or psoriasis (GEO profiles GDS2381 and GDS2518, accessible at the NCBI Gene Expression Omnibus http://www.ncbi.nlm.nih.gov). Decreased protein expressions of cystatin M/E and CTSV in atopic dermatitis and psoriasis were confirmed by the quantification of immunofluorescent stainings, but the elevated transcriptional levels of CTSL and TGM3 were not matched by increased protein expression (Cheng *et al.*, in press). It was concluded that disturbance of the cystatin M/E–cathepsin pathway could contribute to dysregulated skin barrier function as observed in these inflammatory dermatoses.

Cystatin M/E and cancer

Several studies have shown that cysteine proteases such as cathepsin B and CSTL are involved in cancer through their role in proteolytic pathways that enable tumor growth, migration, invasion, and metastasis. This is supported by increased cysteine protease expression in a wide variety of cancers (Mohamed and Sloane, 2006). The importance of the fine balance between cysteine proteases and their inhibitors in cancer is further underlined by the abnormal expression of cystatins in many types of tumors (Keppler, 2006).

Cystatin M/E was initially identified by differential display as a downregulated mRNA in metastatic breast tumor cells when compared with normal and primary breast tumor cells, suggesting that loss of the expression of cystatin M/E is likely associated with the progression of a primary tumor to a metastatic phenotype (Sotiropoulou *et al.*, 1997). This hypothesis was tested *in vitro* by transfection of the highly tumorigenic and metastatic human breast cancer cell line, MDA-MB-435S, which normally does not express cystatin M/E, with a cystatin M/E cDNA expression vector. The constitutive cystatin M/E expression in transfected cells resulted in a significant reduction of cell proliferation, migration, Matrigel invasion, and endothelial cell adhesion

(Shridhar *et al.*, 2003). However, only cell migration and matrix invasion appeared to be controlled by the inhibition of cysteine protease activity, as the synthetic cysteine protease inhibitor E-64 showed no effect on cell proliferation and endothelial adhesion. This finding made the authors suggest that cystatin M/E may act as a tumor and metastasis suppressor through other mechanisms (than cathepsin inhibition), perhaps through its inhibitory effects on LGMN or even through modulation of gene transcription. Cystatin M/E expression in MDA-MB-435S cells significantly changed the expression of several genes involved in tumor growth, invasion, and angiogenesis, including downregulation of autotaxin, a signaling molecule that has been linked to breast cancer invasiveness (Song *et al.*, 2006). Subsequently, the transfected MDA-MB-435S cells were injected into *scid* mice to test whether cystatin M/E expression might have *in vivo* tumor-suppressing functions (Zhang *et al.*, 2004). The mice were analyzed for tumorigenesis and spontaneous metastasis in the lungs and compared with mice implanted with mock controls. During the first 45 days, *scid* mice bearing orthotopically injected cystatin M/E-expressing MDA-MB-435S cells developed significantly smaller tumors than mock-injected controls; however, no difference was found in the incidence of metastasis and the number of lesions in the lungs. Cystatin M/E expression showed no effect on seeding and survival of tumor cells, but reduced the growth of established lung tumors, which suggests that cystatin M/E has antiproliferative activity. One should keep in mind that there is some controversy about the origin of the MDA-MB-435S cancer cell line, as it was reported that these cells may be derived from melanocytes and as such cannot be considered with absolute certainty as a model for breast cancer (Ross *et al.*, 2000). Preliminary studies using laser-capture dissection of human breast cancer cells varied in outcome. Zhang *et al.* (2004) reported a downregulation of cystatin M/E mRNA and protein expression in invasive ductal carcinomas compared with normal breast tissue, which is in contrast with another study that showed cystatin M/E expression in most of the breast carcinomas, including primary and metastatic tumors (Vigneswaran *et al.*, 2005). Recently, epigenetic studies reported DNA methylation-dependent silencing of the cystatin M/E gene in breast cancer cell lines, primary breast tumors, and metastases. Hypermethylation, especially of the proximal promoter, was significantly associated with gene silencing and loss of cystatin M/E protein expression, whereas treatment with the demethylating agent, 5-aza-2′deoxycytidine, induced cystatin M/E mRNA expression in breast cancer cell lines (Ai *et al.*, 2006; Rivenbark *et al.*, 2006; Schagdarsurengin *et al.*, 2007). Cystatin M/E promoter methylation (and loss of cystatin M/E protein expression) occurred more frequently in lymph node metastases, whereas primary tumors were found to be negative for cystatin M/E promoter methylation. This suggests that epigenetic silencing of cystatin M/E is involved in breast cancer tumorigenesis and progression into metastasis (Rivenbark *et al.*, 2007). Furthermore, it was recently shown that epigenetic silencing of cystatin M/E is frequent in gliomas (Qiu *et al.*, 2008). The authors suggest that cystatin M/E methylation may therefore represent a new prognostic marker and therapeutic target specifically altered in glioma tumor-initiating cells.

Reduced or absent expression of cystatin M/E was also found in basal cell carcinomas of the skin, squamous cell carcinomas (SCCs) from the lung, and in lung cancer (Zeeuwen *et al.*, 2002a; Werle *et al.*, 2006), but in oropharyngeal SCCs, cystatin M/E expression was found to be increased with metastasis along with elevated cathepsin expression (Vigneswaran *et al.*, 2003). Silencing of cystatin M/E by small interference RNA in a metastatic oral cancer cell line increased *in vitro* cell proliferation, migration, and Matrigel invasion, emphasizing the tumor- and metastasis-suppressing abilities of cystatin M/E (Vigneswaran *et al.*, 2006). Gene expression profiling of cutaneous SCCs and psoriasis, a study designed to compare malignant versus benign keratinocyte hyperproliferation, revealed that cystatin M/E and several proteases were among the genes that were significantly upregulated in SCCs (Haider *et al.*, 2006).

Collectively these data suggest that cystatin M/E could act as a tumor suppressor through various mechanisms of action, including inhibition of cysteine protease activity and modulation of gene transcription. However, it is not yet clear what the exact role of cystatin M/E is in tumorigenesis and metastasis, as both down- and upregulation have been found depending on the type of cancer, suggesting that altered cystatin M/E expression could either have a causative effect or that it is alternatively a protective response. In an earlier study, we concluded that cystatin M/E expression was a not a useful marker to discriminate between benign (keratoacanthoma), premalignant (actinic keratosis), and malignant epidermal neoplasias (basal cell carcinomas and SCCs). Cystatin M/E expression in these skin-derived neoplasias appeared to be limited to the keratinized and well-differentiated cells. SCCs from the lung and the head and neck region were found to be negative for cystatin M/E. However, one cutaneous metastasis of a head and neck tumor was positive for cystatin M/E, suggesting that for these squamous neoplasias a cutaneous environment is necessary for cystatin M/E expression (Zeeuwen *et al.*, 2002a).

CONCLUSIONS

The past few years have taught us a great deal on the role of cystatin M/E and its cognate proteases in epidermal biology. Currently, no human disease is known that is caused by genetic alterations of the proteins in this newly discovered pathway. Nevertheless, all unresolved heritable human skin disorders that show faulty cornification, desquamation, and/or hair follicle morphogenesis are candidates for cystatin M/E deficiency, and the identification of such a disorder remains a major challenge. As CTSV and CTSD are involved in the breakdown of corneodesmosomal proteins, they could also be candidate genes for ichthyosiform skin diseases that show disturbed desquamation by retention of scales. In addition to excessive TGM activity owing to cystatin M/E deficiency, as seen in *ichq* mice, lack of TGM activity can also lead to disturbed cornification as witnessed by the autosomal-recessive ichthyosis LI in which mutations in TGM1 are responsible for the disease (Huber *et al.*, 1995). Although TGM3 accounts for more than 75% of the total TGM activity

in the epidermis (Kim *et al.*, 1990), it cannot compensate for cornified envelope formation in patients with LI in which TGM1 activity is absent (Candi *et al.*, 1998), probably because crosslinking by TGM1 precedes crosslinking by TGM3 according to the model for assembly of the cornified envelope (see Introduction). Although this might seem paradoxical at first glance, both loss and inappropriate gain of TGM activity could explain ichthyotic changes, although the mechanisms could be different. Excessive TGM1 and TGM3 activity could lead to retention of scales by hyper-crosslinked corneocytes, whereas deficiency for TGM1 could lead to irregular scaling owing to lack of attachment of involucrin to the ω-hydroxyceramides. There are, however, other forms that are clinically similar to LI but are not linked to the locus of the TGM1 gene. The redundancy of TGM3 is indicated by knockout mice that show no particular skin phenotype (John, 2006). This leaves open the possibility that mutations in genes that are involved in the processing of TGMs into the active form might be causative for other forms of ichthyosis. In conclusion, we suggest that cystatin M/E is a candidate gene for heritable human skin disorders that show faulty cornification and desquamation.

Recently, it was shown that human reconstructed skin is a valuable tool for future research as it correctly recapitulates the expression pattern of all players in the pathway (Cheng *et al.*, in press). Gene knockdown (by delivery of siRNA) or overexpression of certain genes by using lentiviral vectors would be an option to further delineate the role of cystatin M/E and its target proteases in the final steps of epidermal differentiation. Together with the use of knockout mouse models that we are currently generating (cystatin M/E–LGMN, cystatin M/E–CTSL, and cystatin M/E–TGM3 double knockout mice), this work extends our knowledge on the components that are involved in epidermal cornification and desquamation. In addition, we are rescuing the mutant cystatin M/E-deficient mice (neonatal lethal) by the introduction of a transgene that drives epidermis-specific cystatin M/E expression on a cystatin M/E null background. This will enable us to study the consequences of cystatin M/E deficiency on the physiology and maintenance of adult tissues. We expect that these data will lead to further understanding of human disorders characterized by disturbed skin barrier function.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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