Gene expression profiling of mammary glands of cathepsin E-deficient mice compared with wild-type littermates

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Gene expression profiling of mammary glands of cathepsin E-deficient mice compared with wild-type littermates

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Abstract

Cathepsin E is an endolysosomal aspartic proteinase predominantly expressed in cells of the immune system and has been implicated in various physiological and pathological processes. Because of physiological substrates of cathepsin E have not yet been identified, however, the physiological significance of this protein still remains speculative. To better understand the physiological significance of cathepsin E in the mammary gland, we investigated the effect of the deficiency of this protein on the gene expression profile of the tissue. Here we used mammary glands derived from multiparous and non-pregnant 11-month-old syngenic wild-type (*CatE*$^{+/+}$) and cathepsin E-deficient (*CatE*$^{-/-}$) mice for extraction of total RNA from each tissue and subsequent mRNA amplification, DNA fragmentation, and hybridization with cDNA mixroarray

chips. A total of 654 genes were identified as overexpressed (>2-fold) in *CatE*$^{-/-}$ mammary glands compared with *CatE*$^{+/+}$ counterparts. These included genes related to signal transduction, immune responses, growth factor activity, and milk proteins, which occupied a large portion of the gene fragments identified as overexpressed. In contrast, a total of 665 known genes were identified as underexpressed in the mammary gland of *CatE*$^{-/-}$ mice compared with *CatE*$^{+/+}$ counterparts. These included genes related to cytoskeleton, cell differentiation, cell cycle arrest and apoptosis, which occupied the majority of the gene fragments identified as underexpressed. The results thus suggest that cathepsin E in mammary glands plays a crucial role in the regulation of proteins involved in signaling, development, differentiation and proliferation in the mammary gland.

Key words: aspartic proteinase, cathepsin E, mammary gland, gene expression profiling

Introduction

Tissue remodeling is a key process involved normal development. The mammary gland undergoes extensive tissue remodeling during each lactation cycle. During pregnancy, the epithelial compartment of the gland is vastly expanded [1]. At the end of lactation the epithelial cells undergo apoptosis and adipocyte differentiation is induced [2]. Previous studies have demonstrated that ductal and alveolar growth during puberty and pregnancy, and the involution process are mediated by the action of proteases, including, matrix metalloproteinases (MMPs), plasminogen, membrane-peptidases, and cathepsin D, and the corresponding genes are activated during these periods [1, 3, 4]. MMPs are expressed in several cell types of the mammary gland, including stromal fibroblasts (e.g., MMP3, MMP2), epithelial cells (e.g. MMP7, MMP9), adipocytes (e.g., MMP2) and lymphoid cells (e.g. MMP9) [5, 6]. Several knockout mouse strains, including MMP2-, MMP3- or plasminogen-deficient mice, have displayed alterations to mammary gland structure and impairment of lactation [5, 6] and provided important insight into their functions in the mammary gland.

Cathepsin E is an intracellular aspartic proteinase of the pepsin superfamily, which is expressed predominantly in cells of the immune system [7-10] and highly secreted by activated phagocytes [11]. Like other pepsin family members, cathepsin E requires an acidic environment for optimal activity and is believed to be involved in unspecific bulk proteolysis in endolysosomal compartments, but there is growing evidence that this enzyme has specific, non-redundant functions [12, 13]. On the other hand, cathepsin E

possesses several unique properties not shown by other pepsin family members. Unlike other intracellular aspartic proteinases, cathepsin E forms a homodimer that is not necessarily required for expressing activity, correct intracellular localization, and carbohydrate modification, but is essential to structural stabilization of the molecule [14, 15]. Besides their structural and immunological distinction [16-22], cathepsin E is different from other pepsin family members in tissue distribution and intracellular localization [7, 8, 10, 23]. Differing from the definite localization of the analogous lysosomal aspartic proteinase cathepsin D, the intracellular localization of cathepsin E appears to vary with cell types [8, 10, 23]. In antigen presenting cells such as dendritic cells, microglia, and macrophages, cathepsin E is mainly found in endosomal structures mainly as a mature enzyme which is N-glycosylated mostly with complex-type oligosaccharides [8]. In some types of cells, including erythrocytes, renal proximal tubule cells, and osteoclasts, cathepsin E is exclusively confined to the plasma membrane [23, 24] mainly as a proenzyme having complex-type oligosaccharides. In a variety of other cell types, cathepsin E is also detected in the endoplasmic reticulumn and Golgi complex [10, 23, 25]. Cathepsin E has been implicated in various physiological and pathological processes [see for review refs. 26, 27], the precise role of this protein remains largely unknown, because the physiological substrates of this protein have not yet been identified. Recent genetic approaches using mice lacking cathepsin E have provided important insight into their biological functions [28-30]. Cathepsin E-deficient (*CatE*$^{-/-}$) mice were shown to spontaneously develop atopic

dermatitis-like skin lesions when reared under conventional conditions [28] and exhibit the increased susceptibility to bacterial infection accompanied by a marked decrease in killing of intracellular bacteria by macrophages [29]. Subsequent analysis of macrophages derived from *CatE*$^{-/-}$ mice revealed that the deficiency of this protein induced a novel form of lysosomal storage disorder manifesting the accumulation of major lysosomal membrane sialoglucoproteins such as LAMP-1 and LAMP-2 and the elevation of lysosomal pH [30]. Based on these observations, it has strongly been suggested that cathepsin E contributes to the maintenance of homeostasis by participating in host defense mechanisms.

. The aim of the present study was to gain further insight into the mechanisms underlying cathepsin E functions in mammary glands in vivo, because this protein also exists in the reproductive system including mammary glands, besides the immune system. To better understand the physiological significance of cathepsin E in mouse mammary gland, the strategy was to take advantage of *CatE*$^{-/-}$ mice. We thus performed global gene expression profiling of mammary glands and conducted a screen that select specifically for genes that were significantly altered by cathepsin E deficiency.

Materials and Methods

Animals

Wild-type and *CatE*$^{-/-}$ mice with C57BL/6 genetic background were housed as described previously [28]. All animals were maintained under specific pathogen-free conditions at

Kyushu University Station of Collaborative Research animal facilities according to the guidelines of the Japanese Pharmacological Society. All animal experiments were approved with the Animal Research Committee of Graduate School of Dental Science, Kyushu University.

Real time RT-PCR

Total RNA was isolated with the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The yield and quality of RNA was evaluated by measuring its absorbance at A260/A280 and gel electrophoresis. cDNA synthesis was performed using an Ready-to-Go RT-PCR Beads (Amersham Biosciences Co., NJ, USA). A total of 1 μg of each sample was incubated in a 50 μl reaction mixture containing first-strand primers, forward and reverse primers (20 pmol), and RNase/DNase free water. RT was performed using a thermal program of 45 °C for 15 min, and 95 °C for 5 min. The specificity of each primer set was first tested by RT-PCR, followed by melting curve analysis using DyNAmo^{TM} HS SYBR$^{®}$ Green qPCR Kit (Finnzymes, Espoo, Finland) with an Rotor-Gene^{TM} 3000 (NIPPN TechnoCluster, Inc., Tokyo, Japan) and gel electrophoresis of the PCR products. Gene-specific forward and reverse cathepsin E primers were 5'-GTGCCCCTCAGAAGACATCA-3' and 5'-GTATCCCAGACCCAGAATCC-3', respectively. G3PDH expression was monitored as an endogenous control, during each PCR reaction. The forward and reverse primers for G3PDH were 5'-TCCACCACCCTGTTGCTGTA-3' and 5'-ACCACAGTCCATGCCATCAC-3', respectively. Each PCR reaction was carried

out in triplicate in a total volume of 20 μl, containing master mix which contains hot start version of a modified *Tbr* DNA polymerase, SYBR Green I, optimized PCR buffer, 5 mM $MgCl_2$, dNTP mix including dUTP, forward primer, reverse primer, water, and cDNA. Thermocycling conditions were as follows: 95 °C for 15 min, followed by 45 cycles at 94 °C for 10 s, 57 °C for 30 s, and 72 °C for 30 s for cathepsin E, 95 °C for 15 min, followed by 35 cycles at 94 °C for 10 s, 60 °C for 20 s, and 72 °C for 30 s for G3PDH, with fluorescent readings at the end of each cycle. Total RNA from wild-type mouse stomach was used as a positive control.

Immunoblot analysis

SDS-PAGE and immunoblot analysis were performed as described previously [31]. After transfer of proteins fractionated by SDS-PAGE to a nitrocellulose membrane and blocking with 5% nonfat dried milk in Tris-buffered saline, the membrane was incubated overnight at 4 °C with primary antibody to cathepsin E (diluted 1/4000). After washing, the membrane was incubated overnight at 4 °C with horseradish peroxidase-conjugated secondary antibody to rabbit immunoglobulin G (diluted 1/2000; Biosource International, Camarillo, CA). The membrane was washed several times and immune complexes were detected with chemiluminescence reagents (GE Healthcare UK Ltd., Buckinghamshire, England). Densitometry was performed with a LAS1000 analyzer (FUJIFILM Corp., Tokyo, Japan).

Acid treatment

Fifty μg of the tissue extract from wild-type mouse mammary gland was incubated at

37 °C for 10 min in 0.1 M sodium acetate buffer, pH 3.5, and then neutralized by addition of 0.1 M Tris-HCl buffer, pH 9.0. Then the samples were analyzed by SDS-PAGE under reducing conditions and immunoblotting.

Whole mount

Whole mounts were prepared following protocols described by the Laboratory of Genetics and Physiology at the National Institute of Diabetes, Digestive and Kidney Diseases within the National Institutes of Health (http://mammary.nih.gov), and Thompson et al. [32]. The mammary glands from wild-type and *CatE*$^{-/-}$ mice were removed and spread on a glass slide, fixed overnight in 10% phosphate-buffered formalin, dehydrated in 70, 95, and 100% alcohols, cleared in toluene, rehydrated and stained with Carmine Alum (Sigma-Aldrich Japan) overnight. After staining, the whole mounts were dehydrated as described above, cleared in xylene for 30 min, and mounted. The whole mounts were analyzed under a stereomicroscope for microscopic lesions.

Microarray studies

Mammary glands from multiparous and non-pregnant wild-type and *CatE*$^{-/-}$ mice at 11 months of age were excised, and immediately immersed in RNAlater RNA Stabilization Reagent (QIAGEN Sciences, Inc., Chatsworth, MD). Total RNA was isolated from each of the mammary glands using an RNA purification kit (RNeasy Mini Kit; QIAGEN Sciences, Inc., Chatsworth, MD). The total RNA was checked its qualities by the Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA) and a spectrometer (NanoDrop Technologies, Inc, Wilmington, DE), followed by mRNA

amplification using a T7 global amplification method (Two-Cycle Target Labeling kit; Affymetrix), DNA fragmentation, biotinylation, and hybridization with cDNA microarray chips, GeneChip® Mouse Genome 430 2.0 Array (Affymetrix, Inc., CA) containing probes representing more than 45000 mouse genes and expressed sequence tags. The scanned arrays passed the quality control standards such as visual inspection and eukaryotic control performance standards suggested by the manufacturer (Affymetrix). Gene expression data were analyzed using the software Signet Viewer (BIO MATRIX RESEARCH, Inc., Chiba, Japan; the authorized provider by Affymetrix). For genotypic comparisons (*CatE*$^{-/-}$ vs. wild-type), we initially excluded the data included more over 3 merginal or absent signals on the hybridization pattern with Perfect match (PM) and Mismatch (MM) probes, and the probe sets identified from the analysis at a false discovery rate (FDR) cut-off of 0.05 are listed in.

Results

Characterization of cathepsin E in mouse mammary gland

Previous studies in our laboratory have shown that cathepsins E and D are detected differentially in various exocrine glands including submandibular gland and lachrymal gland [7], we thus sought the expression levels of cathepsin E in mouse mammary gland using a quantitative RT-PCR technique. The mammary glands as well as brain, pancreas, spleen and stomach derived from multiparous and non-pregnant C57BL/6 mice at 11 months of age were isolated after perfusion with saline. Total RNA was extracted and

cDNA synthesized. Real-time PCR amplification and subsequent quantitation revealed that the level of cathepsin E mRNA in the mammary gland was higher than that in brain and pancreas but lower than that in spleen and stomach (Fig. 1A). Western blot analysis of the cell lysate of mammary glands under reducing conditions revealed a major 42-kDa band and a minor 46-kDa band corresponding to the monomeric mature and precursor forms of cathepsin E, respectively (Fig. 1B), indicating that cathepsin E in the mammary gland is largely expressed as the mature enzyme. This was further confirmed by acid treatment of the lysate at pH 3.5 and 37 ° for 10 min, because the proenzyme is rapidly converted to the mature form at pH values below pH 4 [33]. The electrophoretic mobility of the 42-kDa form of cathepsin E was not changed by acid treatment, indicating that this is the processed mature enzyme.

Gene expression differences in mammary glands between *CatE*$^{-/-}$ and wild-type mice

To determine the common molecular alterations induced by cathepsin E deficiency in the mammary gland, we performed global gene expression profiling of the mammary gland from wild-type and *CatE*$^{-/-}$ mice at 11 months of age. Allowing for a false discovery rate of 0.05, of the 45101 genes detected on the array, a total 654 gene fragments were identified as overexpressed (>2-fold) in the mammary gland of *CatE*$^{-/-}$ mice compared with the normal mammary gland of wild-type littermates (Table 1), whereas a total 665 gene fragments were identified as underexpressed in the mammary gland of *CatE*$^{-/-}$ mice compared with the normal mammary gland of wild-type

littermates (Table 2). Genes related to signal transduction and immune response, including aurora kinase C, chemokine ligands, defensin beta, and tumor necrosis factor family members, and genes related to growth factor activity such as nodal and insulin-like growth factor binding protein (IGFBP) 2 occupied a large portion of the 654 gene fragments identified as overexpressed in the mammary gland of *CatE*$^{-/-}$ mice (Table 3). In addition, genes associated with milk proteins, including whey acidic protein (WAP) and lactalbumin, comprised a significant part of the overexpressed genes by cathepsin E deficiency (Table 1). On the other hand, genes related to cytoskeleton proteins including myosin, toroponin, actinin, troponin, toropomyosin, titin, and actin occupied the majority of the 665 gene fragments identified as underexpressed in the mammary gland of *CatE*$^{-/-}$ mice (Table 4). Furthermore, genes related to cell differentiation, cell cycle arrest and apoptosis comprised a significant part of the underexpressed genes by cathepsin E deficiency.

Whole mount of mammary gland

To gain more information about the influence of cathepsin E deficiency on phenotypic differences between wild type and *CatE*$^{-/-}$ mice, the mammary gland whole-mount preparations were carried out. The morphology of the mammary tree of *CatE*$^{-/-}$ mice appeared to be grossly abnormal compared with that of the wild-type littermates (Fig. 2). The mammary tree of *CatE*$^{-/-}$ mice showed more enlarged ducts having multiple hyperplastic alveolar nodules compared with that of wild-type animals. Furthermore, the mammary gland of *CatE*$^{-/-}$ mice had supernumerary budding along the primary ducts

compared with the wild-type one.

Discussion

Cathepsin E is one of the most enigmatic aspartic proteinases in the pepsin family members. This enzyme can be detected in both intracellular and extracellular spaces under various physiological and pathological conditions. Its expression at both mRNA and protein levels is significantly increased in antigen presenting cells upon stimulation with interferon-γ and lipopolysaccharide [10, 11] and inversely decreased in these cells by treatment with IL-10 [11]. Cathepsin E expression is also increased in vivo in both glial cells and neurons in the vulnerable regions of rat brain to transient forebrain ischemia [34] and in the senescent rat brain [35], and at the advancing margins of gastric carcinomas and in inflammatory cells within and nearby carcinomas [36]. Its cleavage specificity toward certain peptide substrates also changes with pH [37]. Therefore, these unique properties and flexibilities may confer pleiotropic nature and manifold functions on cathepsin E. Furthermore, recent gene or protein expression profiling of cartain cancers suggests the clinical utility of cathepsin E as a potential cancer biomarker [38-42], although the views on its role in carcinogenesis are not necessarily consistent among the literature.

The present study was undertaken to gain further insight into the physiological significance of cathepsin E in the mammary gland and the identification of candidate proteins regulated by this protein. We first analyzed the gene expression profiles of

CatE$^{-/-}$ mammary gland and compared them with those of normal mammary gland of wild-type mice. Strikingly, a total of 654 known genes were increased (>2-fold) in *CatE*$^{-/-}$ mammary glands compared with *CatE*$^{+/+}$ counterparts. These included genes related to signal transduction and immune responses, growth factor activity, and milk proteins, which comprised a large portion of the gene fragments identified as overexpressed. Like aurora kinase A and B, aurora kinase C identified as an overexpressed signal transduction-related gene in *CatE*$^{-/-}$ mammary glands is involved in various biological processes, including cytokinesis and dysregulated chromosome segregation [43]. Although the precise role and regulation of aurora kinase C is largely unknown, the expression level of this kinase is increased in several human cancer cell types [44], and inhibitors of these kinases are expected to be novel antioncogenic agents. Chemokines such as CXCL12 were also identified as overexpressed by cathepsin E deficiency. These chemokines are also known to up-regulate in tumor myoepithelial cells and bind to their receptors on epithelial cells and thereby enhance the proliferation, migration, and invasion of these cells [45], implying that the increased expression of these chemokines may be associated with tumorigenesis of the mammary gland. Up-regulation of the growth factor activity-related genes such as nodal and IGFBP2 are known to be associated with cell proliferation [46]. IGFBP2 has shown to play a role in the normal development of mammary gland tissue or the mammary gland tumor etiology [47]. The mitogenic signals of IGFBP2 through autocrine/paracrine regulatory mechanisms are believed to be responsible for the growth of mammary gland

carcinomas and to serve as an indicator of malignancy [46]. Thus, the present results suggest that up-regulation of the signal transduction/immune response-related genes and the growth factor activity-related genes by cathepsin E deficiency may be associated with the induction of hyperplastic or carcinogenic alterations in mouse mammary gland.

In contrast, a total of 665 known genes were decreased in the mammary gland of *CatE*$^{-/-}$ mice compared with *CatE*$^{+/+}$ counterparts. Of these genes identified as underexpressed, 31 genes were related to cytoskeleton/smooth muscle-related molecules (>2-fold decrease). It is well known that the most important portion of the mammary gland development occurs postnatally, with distinct periods of intensive morphogenesis taking place between birth and puberty and during pregnancy and lactation. A mammary epithelium bilayer is organized by luminal secretory and basal myoepithelial cells. During postnatal development, mammary myoepithelial cells progressively acquire a differentiated phenotype as revealed by the expression of various smooth muscle-related markers [48, 49]. Therefore, mammary gland differentiation is more likely to be closely related to gene expression of cytoskeleton/smooth muscle-associated molecules. Myogenic factor 6 identified as underexpressed in *CatE*$^{-/-}$ mammary gland is known to be specifically involved in development of muscle [50]. Interestingly, morphological analysis revealed that the *CatE*$^{-/-}$ mammary gland appears to lead to aberrant myoepithelial cell differentiation and abnormal mammary gland development. In addition, there were genes related cell cycle arrest and anti-apoptosis identified as underexpressed by cathepsin E deficiency. Given that the growth and development of

mammary gland are regulated by a balance between positive and negative factors of cell proliferation, these genes identified as overexpressed by cathepsin E deficiency are likely to contribute to the growth and development of this tissue.

Importantly, we also provide the first evidence that cathepsin E deficiency may disrupt the epithelial-mesenchymal transition in the mammary gland. In physiological conditions, the mammary gland is hormonally regulated through transition between glanular epithelium and adipose tissue. In virgin female mice, most of the resting gland consists of adipocytes, but during pregnancy and lactation ~ 90 % of the organ is occupied with secretory epithelial cells. After lactation, rapid adipogenesis is induced. This epithelial-mesenchymal transition is regulated a proper balance between differentiation and proliferation-related gene expression. In this study, the genes related to milk proteins such as WAP and lactalbumin alpha were identified as overexpressed by cathepsin E deficiency. WAP is a principal whey protein found in rodent milk that contains a cysteine-rich motif identified in some protease inhibitors and proteins involved in tissue modeling. The expression of the Wap gene is known to be increased more than 1,000-fold around mid-pregnancy [51]. Although WAP is not required for alveolar specification and functional differentiation, it is used as a marker for advanced differentiation of mammary epithelial cells. Furthermore, WAP plays a negative regulatory role in the cell cycle progression of mammary epithelial cells through an autocrine or paracrine mechanism [51]. Therefore, up-regulation of the Wap gene by cathepsin E deficiency is more likely to accelerate cell differentiation proliferation in

the mammary gland. Our results thus suggest that cathepsin E may play a crucial role in regulation of the epithelial-mesenchymal transition.

Conclusion

To better understand the physiological significance of cathepsin E in mouse mammary gland, we performed gene expression profiling of the tissues of syngenic wild-type and *CatE*$^{-/-}$ mice. Several genes involved in signal transduction/immune responses and growth were overexpressed, whereas many genes involved in cytoskeleton, cell differentiation, and cell cycle arrest/anti-apoptosis were underexpressed. Therefore, gene expression profiling revealed that cathepsin E might be involved in the regulation of signaling, development, and cell proliferation and differentiation in the mammary gland.

References

[1] C. Benaud, R. B. Dickson, E. W. Thompson, Roles of the matrix metalloproteinases in mammary gland development and cancer, Breast Cancer Res. Treat. 50 (1998) 97-116.

[2] J. Lilla, D. Stickens, Z. Werb, Metalloproteases and Adipogenesis: A Weighty Subject, Am. J. Pathol. 160 (2002) 1551-1554.

[3] C.M. Alexander, S. Selvarajan, J. Mudgett, Z. Werb, Stromelysin-1 Regulates Adipogenesis during Mammary Gland Involution, J. Cell. Biol. 152 (2001) 693-703.

[4] P.S. Rudland, R. Barraclough, D.G. Fernig, J.A. Smith, Growth and differentiation of the normal mammary gland and its tumors, Biochem. Soc. Symp. 63 (1998) 1-20.

[5] L.R. Lund, J. Rømer, N. Thomasset, H. Solberg, C. Pyke, M.J. Bissell, K. Danø, Z. Werb, Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways, Development 122 (1996) 181-193.

[6] B.S. Wiseman, M.D. Sternlicht, L.R. Lund, C.M. Alexander, J. Mott, M.J. Bissell, P. Soloway, S. Itohara, Z. Werb, Site-Specific inductive and inhibitory activities of MMP-2 and MMP-3 orchestrate mammary gland branching morphogenesis, J. Cell Biol. 162 (2003) 1123-1133.

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

[8] D.F. Sastradipura, H. Nakanishi, T. Tsukuba, K. Nishishita, H. Sakai, Y. Kato, T. Gotow, Y. Uchiyama, K. Yamamoto, Identification of cellular compartments involved in processing of cathepsin E in primary cultures of rat micriglia. J. Neurochem. 70 (1998) 2045-2056.

[9] P.J. Tatnell, S.D. Fowler, D. Bur, W.E. Lees, and J. Kay, Cathepsin E: the best laid plans of mice and men. Adv. Exp. Med. Biol. 436 (1998) 147-152.

[10] T. Nishioku, K. Hashimoto, K. Yamashita, S.Y. Liou, Y. Kagamiishi, H. Maegawa, N. Katsube, C. Peters, K. von Figura, P. Saftig, N. Katsunuma, K. Yamamoto, H. Nakanishi, Involvement of cathepsin E in exogenous antigen processing in primary cultured murine microglia, J. Biol. Chem. 277 (2002) 4816-4822.

[11] M. Yanagawa, T. Tsukuba, K. Okamoto, R. Takii, Y. Terada, T. Kadowaki, K.

Yamamoto, Up-regulation, enhanced maturation, and secretion of cathepsin E in mouse macrophages treated with interferon-γ or lipopolysaccharide, J. Oral Biosci. 48 (2006) 218-225.

[12] T. Kageyama, 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 (1993) 717-728.

[13] T. Kageyama, M. Ichinose, S. Yonezawa, Processing of the precursors to neurotensin and other bioactive peptides by cathepsin E. J. Biol. Chem. 270 (1995) 19135-19140.

[14] S.D. Fowler, J. Kay, B.M. Dunn, P.T. Tatnell, Monemeric human cathepsin E. FEBSLett. 366 (1995) 72-74.

[15] T. Tsukuba, H. Sakai, M. Yamada, H. Maeda, H. Hori, T. Azuma, A. Akamine, K. Yamamoto, Biochemical properties of the monomeric mutant of human cathepsin E expressed in Chinese hamster ovary cells: comparison with dimeric forms of the natural and recombinant cathepsin E. J. Biochem. 119 (1996) 126-134.

[16] T. Azuma, G. Pals, T.K. Mohandas, J.M. Couvreur, R.T. Taggart, Human gastric cathepsin E. Predicted sequence, localization to chromosome 1, and sequence homology with other aspartic proteinases. J. Biol. Chem. 264 (1989) 16748-16753.

[17] T. Kageyama, M. Ichinose, S. Tsukada, K. Miki, K. Kurokawa, O. Koiwai, M. Tanji, E. Yakabe, B.P. Athauda, K. Takahashi, Gastric procathepsin E and progastricsin from guinea pig. Purification, molecular cloning of cDNAs, and

characterization of enzymatic properties, with special reference to procathepsin E. J. Biol. Chem. 267 (1992) 16450-16459.

[18] K. Okamoto, H. Yu, Y. Misumi, Y. Ikehara, K. Yamamoto, Isolation and sequencing of two cDNA clones encoding rat spleen cathepsin E and analysis of the activation of purified procathepsin E. Arch. Biochem. Biophys. 322 (1995) 103-111.

[19] P.J. Tatnell, W.E. Lees, J. Kay, Cloning, expression and characterization of murine procathepsin E. FEBS Lett. 408 (1997) 62-66.

[20] N. Ostermann, B. Gerhartz, S. Worpenberg, J. Trappe, J. Eder, Cryasatl structure of an activation intermediate of cathepsin E. J. Mol. Biol. 342 (2004) 889-899.

[21] K. Yamamoto, O. Kamata, N. Katsuda, K. Kato. Immunochemical difference between cathepsin D and cathepsin E-like enx¥zyme from rat spleen. J. Biochem. 87 (1980) 511-516.

[22] K. Yamamoto, E. Ueno, H. Uemura, Y. Kato, Biochemical and immunochemical similarity between erythrocyte membrane aspartic proteinase and cathepsin E. Biochem. Biophys. Res. Commun. 148 (1987) 267-272.

[23] T. Saku, H. Sakai, Y. Shibata, Y. Kato, K. Yamamoto, An immunocytochemical study on distinct intracellular localization of cathepsin E and cathepsin D in human gastric cells and various rat cells. J. Biochem. 110 (1991) 956-964.

[24] Y. Yoshimine, T. Tsukuba, R. Isobe, M. Sumi, A. Akamine, K. Maeda, K. Yamamoto, Specific immunocytochemical localization of cathepsin E at the ruffled border membrane of active osteoclasts. Cell Tissue Res. 281 (1995) 85-91.

[25] H. Sakai, Y. Kato, K. Yamamoto. Synthesis and intracellular distribution of cathepsins E and D in differentiating murine Friend erythroleukemia cells. Arch. Biochem. Biophys. 292 (1992) 412-417.

[26] K. Yamamoto, Cathepsin E and cathepsin D. in: V. Turk (ed.), Proteases: New Perspectives, Birkhäuser Verlag, Basel, Switzerland.

[27] T. Tsukuba, K. Okamoto, Y. Yasuda, W. Morikawa, H. Nakanishi, K. Yamamoto, New functional aspects of cathepsin D and cathepsin E. Mol. Cells 10 (2000) 601-611.

[28] T. Tsukuba, K. Okamoto, Y. Okamoto, M. Yanagawa, K. Kohmura, Y. Yasuda, H. Uchi, T. Nakahara, M. Furue, K. Nakayama, T. Kadowaki, K. Yamamoto, K.I. Nakayama, Association of cathepsin E deficiency with development of atopic dermatitis. J. Biochem. 134 (2003) 893-902.

[29] T. Tsukuba, S. Yamamoto, M. Yanagawa, K. Okamoto, Y. Okamoto, K.I. Nakayama, T. Kadowaki, K. Yamamoto, 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 (2006) 57-66.

[30] M. Yanagawa, T. Tsukuba, T. Nishioku, Y. Okamoto, K. Okamoto, R. Takii, Y. Terada, Y. K.I. Nakayama, T. Kadowaki, K. Yamamoto, 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 (2007) 1851-1862.

[31] K. Yamamoto, M. Ezaki, H. Yamamoto, M. Tatsumi, Y. Kato, Human erythrocyte membrane acid proteinase (EMAP): sidedness and relation to cathepsin D. J. Biochem. 97 (1986) 821-830.

[32] H. Thompson, Z. Zhu, S. Banni, K. Darcy, T. Loftus, C, Ip, Morphological and biochemical status of the mammary gland as influenced by conjugated linoleic acid: implication for a reduction in mammary cancer risk, Cancer Res. 57 (1997) 5067-5072.

[33] M. Takeda-Ezaki, K. Yamamoto, Isolation and biochemical characterization of procathepsin E from human erythrocyte membranes, Biochim Biophys Acta. 304 (1993) 352-358.

[34] H. Nakanishi, T. Tsukuba, T. Kondou, T. Tanaka, K. Yamamoto, Transient forebrain ischemia induces increased expression and specific localization of cathepsins E and D in rat hippocampus and neostriatum, Exp. Neurol. 121 (1993) 215-223.

[35] H. Nakanishi, K. Tominaga, T. Amano, I. Hirotsu, T. Inoue, K. Yamamoto, Age-related changes in activities and localizations of cathepsins D, E, B, and L in the rat brain tissues, Exp. Neurol. 126 (1994) 119-128.

[36] K. Matsuo, I. Kobayashi, T. Tsukuba, T. Kiyoshima, Y. Ishibashi, A. Miyoshi, K. Yamamoto, H. Sakai, Immunohistochemical localization of cathepsin D and E in human gastric cancer: a possible correlation with local invasive and metastatic activities of carcinoma cells, Hum. Pathol. 27 (1996) 184-190.

[37] S.B. Athauda, K. Takahashi, Distinct cleavage specificity of human cathepsin E at neutral pH with special preference for Arg-Arg bonds, Protein Pept. Lett. 9 (2002) 15-22.

[38] K. Uno, T. Azuma, M. Nakajima, K. Yasuda, T. Hayakumo, H. Mukai, T. Sakai, K. Kawai, Clinical significance of cathepsin E in pancreatic juice in the diagnosis of pancreatic ductal adenocarcinoma, J. Gastroenterol. Hepatol. 15 (2000) 1333-1338.

[39] R. Ullmann, P. Morbini, I. Halbwedi, M. Bongiovanni, M. Gogg-Kammerer, M. Papotti, S. Gabor, H. Renner, H.H. Popper, Protein expression profiles in adenocarcinomas of lung generated using tissue microarrays, J. Pathol. 203 (2004) 798-807.

[40] L. Busquets, H. Guillen, M.E. DeFord, M.A. Suckow, R.M. Navari, F.J. Castellino, M. Prorok, Cathepsin E is a specific marker of dysplasia in $APC^{min/+}$ mouse intestine, Tumor Biol. 27 (2006) 36-42.

[41] E. Blaveri, J.P. Simko, J.E. Korkola, J.L. Brewer, F. Baehner, K. Mehta, S. DeVries, T. Koppie, S. Pejavar, P. Carroll, F.M. Waldman, Bladder cancer outcome and subtype classification by gene expression, Clin. Cancer Res. 11 (2005) 4044-4055.

[42] P.J. Wild, A. Herr, C. Wissmann, R. Stoehr, A. Rosenthal, D. Zaak, R. Simon, R. Knuechel, C. Pilarsky, A. Hartmann, Gene expression profiling of progressive papillary noninvasive carcinomas of the urinary bladder, Clin. Cancer Res. 11 (2005) 4415-4429.

[43] P. Meraldi, R. Honda, E.A. Nigg, Aurora kinases link chromosome segregation and cell division to cancer susceptibility, Curr. Opin. Genet. Dev. 14 (2004) 29-36.

[44] J.J. Li, S.A. Li, Mitotic kinases: the key to duplication, segregation, and cytokinesis errors, chromosomal instability, and oncogenesis, Pharmacol Ther. 111 (2006)

974-984.

[45] M. Allinen, R. Beroukhim, L. Cai, C. Brennan, J. Lahti-Domenici, H. Huang, D. Porter, M. Hu, L. Chin, A. Richardson, S. Schnitt, W.R. Sellers, K. Polyak, Molecular characterization of the tumor microenvironment in breast cancer, Cancer Cell 6 (2004) 17-32.

[46] L.T. Busund, E. Richardsen, R. Busund, T. Ukkonen, T. Bjørnsen, C. Busch, H. Stalsberg, Significant expression of IGFBP2 in breast cancer compared with benign lesions, J. Clin. Pathol. 58 (2005) 361-366.

[47] M.A. Allar, T.L. Wood, Expression of the Insulin-Like Growth Factor Binding Proteins during Postnatal Development of the Murine Mammary Gland, Endocrinology 145 (2004) 2467-2477.

[48] F. Jolicoeur, Intrauterine breast development and the mammary myoepithelial lineage, J. Mammary Gland Biol. Neoplasia 10 (2005) 199-210.

[49] M.A. Deugnier, E.P. Moisevera, J.P. Thiery, M. Glukhova, Myoepithelial cell differentiation in the developing mammary gland: progressive acquisition of smooth muscle phenotype, Dev. Dyn. 204 (1995) 107-117.

[50] L.A. Sabourin, M.A. Rudnicki, The molecular regulation of myogenesis, Clin. Genet. 57 (2000) 16-25.

[51] N. Nukumi, K. Ikeda, M. Osawa, T. Iwamori, K. Naito, H. Tojo, Regulatory function of whey acidic protein in the proliferation of mouse mammary epithelial cells in vivo and in vitro, Dev. Biol. 274 (2004) 31-44.

Figure legends

Fig. 1. The expression of cathepsin E in mouse mammary gland. (A) Quantitative RT-PCR analysis of cathepsin E gene in the mammary gland of wild-type mice. The relative values were defined as the ratio of mRNA level of each tissue to that of brain. The data are the means of three independent experiments. (B) SDS-PAGE and immunoblot analysis of the cell extract of mouse mammary gland compared with purified rat spleen cathepsin E using antibodies specific to rat cathepsin E. (C) SDS-PAGE and immunoblot analysis of the cell extract of mouse mammary gland with or without acid treatment at pH 3.5 and 37 °C for 10 min.

Fig. 2. Carmine Alum stained sections of whole-mount preparations of mammary gland from multiparous and non-pregnant wild-type (the upper panel) and *CatE*$^{-/-}$ (the lower panel) mice at 11 months of age. The ducts of *CatE*$^{-/-}$ mammary gland were more profoundly enlarged than those of the normal gland, in which a lot of hyperplastic alveolar nodules were observed. Scale bar: 1 mm.

Table 1

Genes upregulated (>5-fold) in the mammary gland of *CatE*$^{-/-}$ mice (KO) compared with that of the wild-type littermates (WT).

GenBank	KO/WT	Gene
BC003207	80.33	Mus musculus cDNA clone IMAGE:3587523, partial cds.
BE686792	63.81	DNA segment, Chr 14, Wayne State University 89, expressed
NM_011709	32.60	whey acidic protein
AV376376	24.19	Transcribed sequences
NM_133892	22.31	L-amino acid oxidase 1
NM_013626	16.54	peptidylglycine alpha-amidating monooxygenase
NM_011315	13.45	serum amyloid A 3
AF128193	13.18	chemokine (C-C motif) ligand 7
BC021368	12.23	transmembrane protease, serine 4
NM_008108	11.85	growth differentiation factor 3
BB246912	11.49	RIKEN cDNA 1700112E06 gene
NM_010679	10.62	lactalbumin, alpha
BC003705	10.34	surfactant associated protein D
BB276544	10.01	BB276544 RIKEN full-length enriched, 10 days neonate cortex Mus musculus cDNA clone A830097P08 3', mRNA sequence.
BB200905	9.96	BB200905 RIKEN full-length enriched, 0 day neonate thymus Mus musculus cDNA clone A430031J12 3', mRNA sequence.
NM_009141	9.54	chemokine (C-X-C motif) ligand 5
M11024	9.24	envelope polyprotein; Mouse endogenous mammary tumor virus (MMTV) RNA, env gene and right LTR.
NM_010174	8.32	fatty acid binding protein 3, muscle and heart
BB280937	7.16	Adult retina cDNA, RIKEN full-length enriched library, clone: A930028O11 product:unclassifiable, full insert sequence
NM_009258	6.86	serine protease inhibitor, Kazal type 3
BB143568	6.19	orphan short chain dehydrogenase/reductase
AV333665	5.75	RIKEN cDNA 6330549D23 gene
BI658627	5.68	secreted frizzled-related sequence protein 1
BB386209	5.00	BB386209 RIKEN full-length enriched, 0 day neonate cerebellum Mus musculus cDNA clone C230045D21 3', mRNA sequence.

Table 2

Genes down-regulated (>6-fold) in the mammary gland of *CatE*$^{-/-}$ mice (KO) compared with that of the wild-type littermates (WT).

GenBank	KO/WT	Gene
AI595938	0.0363	RIKEN cDNA 6530403D07 gene
BM122177	0.0748	RIKEN cDNA 2310036G12 gene
AV005759	0.0749	Mus musculus C57BL/6J heart Mus musculus cDNA clone 1010001P05, mRNA sequence.
BC008538	0.0771	myosin, heavy polypeptide 2, skeletal muscle, adult
AI326984	0.0773	RIKEN cDNA 1110007C05 gene
AK009042	0.0784	creatine kinase, mitochondrial 2
BC025840	0.0801	titin
AV007148	0.0827	troponin I, skeletal, fast 2
AJ002522	0.0833	myosin, heavy polypeptide 1, skeletal muscle, adult
NM_021484	0.0843	titin immunoglobulin domain protein (myotilin)
NM_009813	0.0897	calsequestrin 1
AW108242	0.0946	RIKEN cDNA 8030451F13 gene
NM_007933	0.0981	enolase 3, beta muscle
NM_022314	0.0986	tropomyosin 3, gamma
AF223417	0.0995	triadin
NM_013456	0.101	actinin alpha 3
NM_016712	0.104	tropomodulin 4
BG794681	0.108	cDNA clone IMAGE:3488793
NM_010861	0.111	myosin, light polypeptide 2, regulatory, cardiac, slow
NM_029569	0.111	ankyrin repeat and SOCs box-containing protein 5
NM_011620	0.112	troponin T3, skeletal, fast
AK003186	0.113	tropomyosin 2, beta
NM_021508	0.114	myozenin 1
BG793713	0.115	ryanodine receptor 1, skeletal muscle
NM_011224	0.117	muscle glycogen phosphorylase
NM_013808	0.120	cysteine and glycine-rich protein 3
AF422244	0.124	RIKEN cDNA 2310001N14 gene
AI462244	0.124	c6.1a protein

(continued)

AJ278733	0.127	myosin heavy chain IIB
NM_007504	0.132	ATPase, Ca++ transporting, fast twitch 1
AK010167	0.132	titin-cap
NM_025357	0.134	small muscle protein, X-linked
NM_010243	0.134	fucosyltransferase 9
D61689	0.137	SRY-box containing gene 6
NM_011079	0.138	phosphorylase kinase gamma
NM_008733	0.144	nebulin-related anchoring protein isoform S; nebulin-related anchoring protein isoform C
NM_010473	0.146	histidine rich calcium binding protein
AK003186	0.153	tropomyosin 2, beta
BC025172	0.154	myoglobin
BQ031098	0.160	WD repeat and FYVE domain containing 1

Table 3

Classification of genes identified as overexpressed (>2-fold) in the mammary gland of *CatE*$^{-/-}$ mice (KO) compared with that of the wild-type littermates (WT).

signal transduction, immune response	
NM_080843	suppressor of cytokine signalling 4
NM_013654	chemokine (C-C motif) ligand 7
NM_009141	chemokine (C-X-C motif) ligand 5
NM_020572	aurora kinase C
NM_030614	fibroblast growth factor 16
NM_009140	chemokine (C-X-C motif) ligand 2
NM_009843	cytotoxic T-lymphocyte-associated protein 4
NM_009425	tumor necrosis factor (ligand) superfamily, member 10
NM_011347	selectin, platelet
NM_008176	chemokine (C-X-C motif) ligand 1
NM_011067	period homolog 3
NM_008510	chemokine (C motif) ligand 1
NM_008998	RAB17, member RAS oncogene family
NM_133212	toll-like receptor 8
NM_008199	histocompatibility 2, blastocyst
NM_011331	chemokine (C-C motif) ligand 12
NM_010846	myxovirus (influenza virus) resistance 1
NM_007843	defensin beta 1
NM_145227	2'-5' oligoadenylate synthetase 2
NM_021274	chemokine (C-X-C motif) ligand 10
NM_023124	histocompatibility 2, Q region locus 8
NM_126166	toll-like receptor 3
NM_016719	growth factor receptor bound protein 14
NM_008331	interferon-induced protein with tetratricopeptide repeats 1
NM_008332	interferon-induced protein with tetratricopeptide repeats 2
NM_010501	interferon-induced protein with tetratricopeptide repeats 3
NM_011852	2'-5' oligoadenylate synthetase 1G
NM_008493	leptin

(continued)

growth factor activity	
NM_053087	epithelial mitogen
NM_008108	growth differentiation factor 3
NM_013611	nodal
NM_018865	WNT1 inducible signaling pathway protein 1
NM_008342	insulin-like growth factor binding protein 2
NM_009704	amphiregulin preproprotein
NM_022024	glia maturation factor, gamma

Cell cycle	
NM_010848	myeloblastosis proto-oncogene product
NM_011249	retinoblastoma-like protein 1 (cell cycle-negative)
NM_007891	E2F transcription factor 1
NM_011193	proline-serine-threonine phosphatase-interacting protein 1 (cytokinesis)
NM_010514	insulin-like growth factor 2
NM_010756	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein G

Table 4

Classification of genes identified as underexpressed (>2-fold) in in the mammary gland of *CatE*$^{-/-}$ mice (KO) compared with that of the wild-type littermates (WT).

cytoskeleton	
NM_025357	small muscle protein, X-linked
NM_009393	troponin C, cardiac/slow skeletal
NM_013456	actinin alpha 3
NM_021503	myozenin 2
NM_011618	troponin T1, skeletal, slow
NM_009416	tropomyosin 2, beta
NM_010867	myomesin 1
NM_008733	nebulin-related anchoring protein isoform S
NM_198059	nebulin-related anchoring protein isoform C
NM_024427	tropomyosin 1, alpha
NM_144961	myosin, heavy polypeptide 2, skeletal muscle, adult
AJ278733	myosin heavy chain IIB
BC025840	titin
AJ293626	myosin heavy chain IIX
NM_009606	actin, alpha 1, skeletal muscle
X65506	type I keratin 48kD (MHRa-1)
X67685	myosin, light polypeptide 3, alkali; ventricular, skeletal, slow
AJ002522	myosin heavy chain 2X
NM_008635	microtubule-associated protein 7
NM_016754	myosin light chain, phosphorylatable, fast skeletal muscle
NM_033268	actinin alpha 2
NM_010861	myosin, light polypeptide 2, regulatory, cardiac, slow
NM_019956	keratin complex 2, basic, gene 6g
NM_080728	myosin, heavy polypeptide 7, cardiac muscle, beta
NM_016712	tropomodulin 4
NM_022314	tropomyosin 3, gamma
NM_011620	troponin T3, skeletal, fast
NM_021467	troponin I, skeletal, slow 1
NM_021285	myosin, light polypeptide 1, alkali; atrial, embryonic

(continued)

NM_021508	myozenin 1
NM_021447	ring finger protein 30
cell differentiation	
AF100171	myeloid leukemia factor 1
NM_008657	myogenic factor 6
NM_011098	paired-like homeodomain transcription factor 2
NM_011445	SRY-box containing gene 6
NM_009379	thrombopoietin
immune response	
NM_008324	indoleamine-pyrrole 2,3 dioxygenase
NM_145581	sialic acid binding Ig-like lectin 5
NM_010391	histocompatibility 2, Q region locus 10
NM_009913	chemokine (C-C motif) receptor 9
cell cycle arrest, anti-apoptosis	
NM_021515	adenylate kinase 1
NM_021284	c-K-ras2 protein
NM_146066	G1 to phase transition 1
NM_011641	transcription factor
NM_007906	eukaryotic translation elongation factor 1 alpha 2
Signal Transduction	
NM_010600	potassium voltage-gated channel, subfamily H, member 1
NM_010207	fibroblast growth factor receptor 2 isoform IIIc
NM_023635	RAB27A protein
NM_016868	hypoxia inducible factor 3, alpha subunit
NM_011268	regulator of G-protein signaling 9
NM_010276	GTP binding protein (gene overexpressed in skeletal muscle)
NM_133485	PKC-potentiated PP1 inhibitory protein
NM_021411	RAB37, member of RAS oncogene family

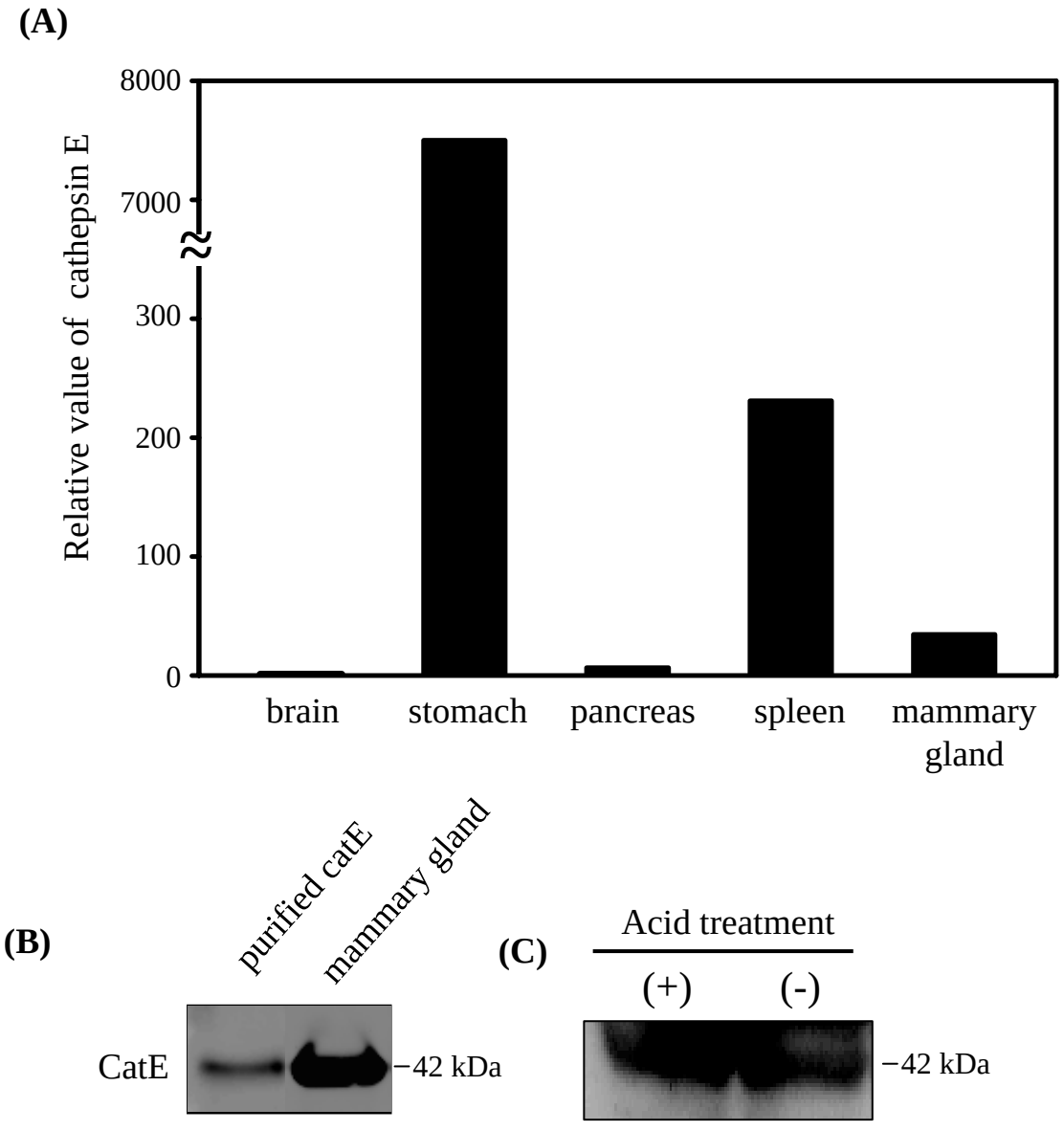
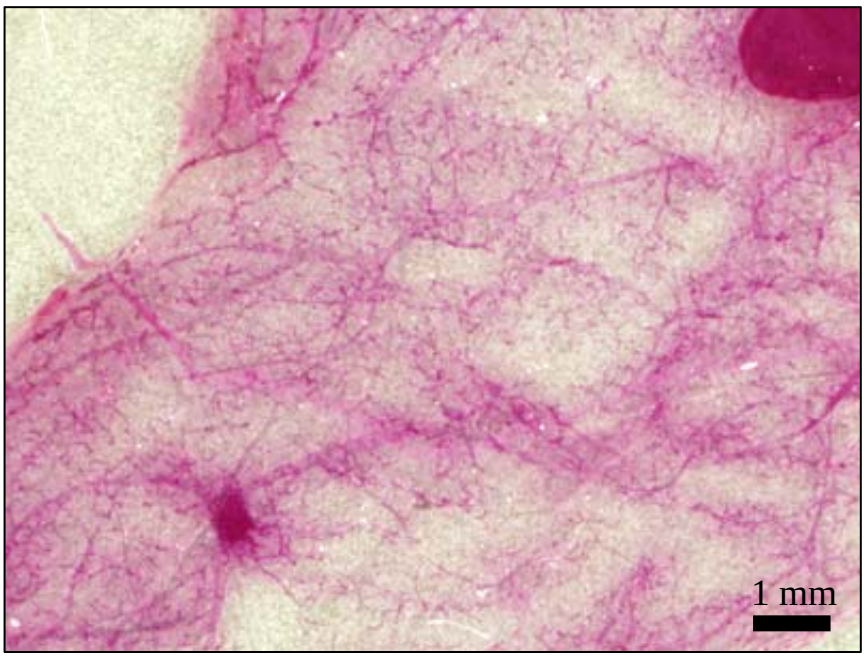


Fig. 1 Kawakubo et al.

Wild-type



CatE$^{-/-}$

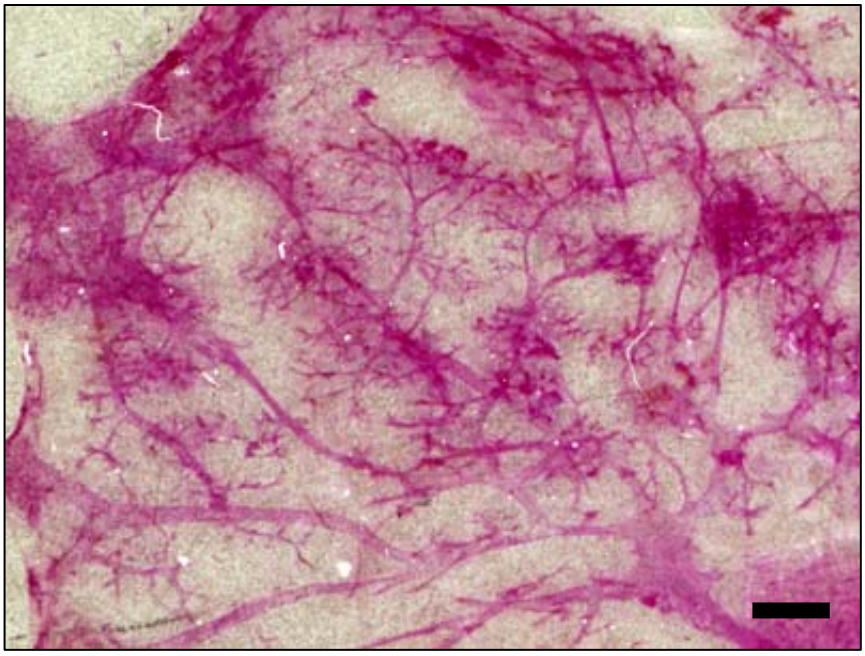


Fig. 2 Kawakubo et al.