Epithelial Integrity Is Maintained by a Matriptase-Dependent Proteolytic Pathway

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A pericellular proteolytic pathway initiated by the transmembrane serine protease matriptase plays a critical role in the terminal differentiation of epidermal tissues. Matriptase is constitutively expressed in multiple other epithelia, suggesting a putative role of this membrane serine protease in general epithelial homeostasis. Here we generated mice with conditional deletion of the *ST14* gene, encoding matriptase, and show that matriptase indeed is essential for the maintenance of multiple types of epithelia in the mouse. Thus, embryonic or postnatal ablation of *ST14* in epithelial tissues of diverse origin and function caused severe organ dysfunction, which was often associated with increased permeability, loss of tight junction function, mislocation of tight junction-associated proteins, and generalized epithelial demise. The study reveals that the homeostasis of multiple simple and stratified epithelia is matriptase-dependent, and provides an important animal model for the exploration of this membrane serine protease in a range of physiological and pathological processes. (Am J Pathol 2009, 175:1453–1463; DOI: 10.2353/ajpath.2009.090240)

The trypsin-like serine proteases are a particularly large group of proteolytic enzymes (approximately 200 in mammals) that reside in the pericellular space or on the cell surface and regulate multiple cellular processes in the context of embryonic development, tissue homeostasis, tissue repair, immunity, fertility, and more.^{1,2} Specific catalytic functions include activation of hormones, growth factors, and cytokines, activation of signaling receptors, regulation of ion fluxes, and regulation of paracellular permeability.^{3–18}

The surface of metazoan organs is lined by specialized epithelia that are endowed with multiple general and organ-specific functions. These include the maintenance of ion gradients, macromolecular transport, absorption, secretion of lipids, mucins, hormones and growth factors, and exclusion of pathogens. In its most simple form, an epithelium is a single layer of keratin-expressing cells attached to a basement membrane. Cells maintain close contact with each other through four types of specialized cell junctions termed desmosomes, gap junctions, adherens junctions, and tight junctions. These cell junctions are composed of an organized assembly of numerous integral and peripheral membrane proteins that are stabilized by anchorage to the cytoskeleton. The polarity of an epithelial sheath and prevention of paracellular diffusion is conferred by the tight junctions, which form a continuous belt encircling the apical end of the lateral membrane.¹⁹ However, how epithelial tight junctions are established and maintained is not fully understood. In particular, the specific contribution of extracellular serine proteases and their cognate inhibitors to epithelial tight junction formation, maintenance, and regulation of barrier function is unclear.^{3–6,20,21}

Matriptase is a trypsin-like transmembrane serine protease encoded by the *ST14* gene that belongs to the recently emerged type II transmembrane serine protease family. Orthologs of matriptase have been found in all vertebrate species analyzed to date, and homologs of the type II transmembrane serine proteases are present in bees and fruit flies.²² Mouse reverse genetics and clinical examination of humans homozygous for mutations in the *ST14* gene have linked matriptase to terminal epidermal

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differentiation processes, including lipid extrusion, profilaggrin processing, and cornified envelope formation.^{23–25} However, matriptase is also constitutively expressed in simple, stratified, transitional, and pseudostratified epithelia throughout the body that do not undergo cornification, secrete specialized lipids, or express profilaggrin. Moreover, this pattern of matriptase expression in epithelial cells is highly conserved between mice and humans.^{26,27} These observations all suggest a wider role of the matriptase proteolytic cascade in epithelial physiology. Indeed, when we generated mice with a conditional *St14* allele and performed tissue-specific, embryonic-onset or postnatal, acute *St14* gene ablation, we found that loss of matriptase from mouse epithelia caused severe organ dysfunction, which was often associated with increased paracellular permeability, loss of tight junction function, mislocation of tight junction-associated proteins, and generalized epithelial demise. The study reveals a global role of matriptase in the maintenance of epithelial homeostasis and provides a useful animal model for the further exploration of matriptase in multiple physiological and pathophysiological processes.

Materials and Methods

Mice

All procedures involving live animals were performed in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited vivarium following institutional guidelines and standard operating procedures. *St14*^{−/−} mice have been described previously.²⁴ *β-actin-Cre-ER*^{tm+/0} mice [B6.Cg-Tg(Cre/Esr1)5AMC/J],²⁸ *MMTV-Cre*^{+/0} mice [Tg(MMTV-Cre)4Mam/J],²⁹ and *villin-Cre*^{+/0} [B6.SJL-Tg(Vil-Cre)997Gum/J]³⁰ were purchased from The Jackson Laboratory (Bar Harbor, ME) and genotyped as described.^{28–30} The targeting vector used to generate a floxed *St14* allele was constructed using the pKO ScramblerV924 vector (Stratagene, La Jolla, CA). A 4.5-kb BamHI-SmaI mouse genomic DNA fragment containing part of intron 1 of the *St14* gene was isolated from a 129/SvJ bacteriophage library and inserted between the BglII and XhoI sites in the vector after insertion of a Sall linker into the SmaI site. Thereafter, an XhoI-Sall fragment containing a PGK-Neo cassette and a LoxP site was cloned into the Sall site of the vector. A PCR-generated (Platinum Pfx DNA polymerase, Invitrogen, Carlsbad, CA) 1.7-kb Sall-Sall fragment including exon 2 through exon 4 was next inserted into the Sall site of the pKO ScramblerV924. A second LoxP site was inserted into the AflII site of intron 2. The targeting vector was introduced into 129/SvEv/TAC embryonic stem (ES) cells by electroporation and ES cell clones were selected in neomycin-containing medium. Resistant ES cell clones were isolated, expanded, and screened for homologous recombination of the targeting vector into the *St14* locus and the presence of the intron 2 LoxP site by PCR using a composite primer complementary to a 10-bp sequence of intron 2 and 10 bp of the LoxP sequence (p01, 5'-GAGGGACATGATAACTTCGT-3') and an *St14* gene primer located in

intron 4, downstream of the short arm of the targeting vector (p02, 5'-CCTGGGGCCAGCTCAAATTT-3'). *St14* gene targeting was further verified by Southern blot hybridization of XbaI-digested genomic DNA using a ³²P-labeled 700-bp XhoI-BamHI probe that was upstream from the targeting vector sequences. *St14*-targeted ES cells were injected into the blastocoel cavity of C57BL/6J-derived blastocysts and implanted into pseudopregnant females. Chimeric male offspring were bred to NIH Black Swiss females (Taconic Farms, Germantown, NY) to generate *St14*^{LoxP/+} offspring. Genotyping of mice was performed by PCR amplification of DNA from ear or tail biopsies with the primers p03 (5'-GCATGCTCCAGACTGCCTTG-3') and p05 (5'-GTGGAGGTGGAGTTCTCATACG-3') to detect the presence of the targeted *St14* allele and p04 (5'-CAGTGCTGTTCAGCTTCCTCT-3') and p05 (5'-TGGAGGTGGAGTTCTCATACG-3') to detect the wild-type *St14* allele and the floxed *St14* allele.

Real-Time Quantitative PCR Analysis

Total RNA from mouse organs was prepared by extraction in TRIzol reagent (Gibco-Invitrogen, Carlsbad, CA), as recommended by the manufacturer. First-strand cDNA synthesis was performed using oligo(dT) primers with the RETROscript reverse transcription kit (Applied Biosystems/Ambion, Foster City, CA). An iCycler, gene expression analysis software, and IQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) were used for real-time PCR in accordance with the manufacturer's directions, using a primer complementary to a sequence in matriptase exon 1, 5'-AACCATGGGTAGCAATCGGGG-3', and matriptase exon 2, 5'-A-CTCCACACCCTCCTCAAAGC-3' (annealing temperature 60°C, denaturation temperature 95°C, 40 cycles). Matriptase expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase levels in each sample. Glyceraldehyde-3-phosphate dehydrogenase mRNA was amplified with the primers 5'-GTGAAGCAGGCATCTGAGG-3' and 5'-CATCGAAGGTGGAAGAGTGG-3'. Mucosal cytokine mRNA expression was detected with the following primer pairs: tumor necrosis factor- α : 5'-CAGCCTCTTCTCATTCCTGC-3' and 5'-AGGGTCTGGGCCATAGAAGT-3'; interferon- γ : 5'-AGCTCTTCCTCATGGCTGTTTC-3' and 5'-ATGTTGTTGCTGATGGCCTGA-3'; interleukin-6: 5'-TCAATCCAGAAACCGCTATGA-3' and 5'-CACCAGCATCAGTCCCAAGA-3'; and interleukin-17A: 5'-TCCAGAAGGCCCTCAGACT-3' and 5'-CTTCCCAGATCACAGAGGGA-3'.

Tamoxifen-Induced Matriptase Ablation

Eight- to 28-week-old *β-actin-CreER*^{tm+/0}; *St14*^{LoxP/−} and littermate control mice (*β-actin-CreER*^{tm+/0}; *St14*^{LoxP/+}, *β-actin-CreER*^{tm0}; *St14*^{LoxP/+}, and *β-actin-CreER*^{tm0}; *St14*^{LoxP/−}) received a daily installation of sterilized tamoxifen (4 mg/mouse) in corn oil (Sigma-Aldrich, St. Louis, MO) by oral gavage for up to 9 days.

Intestinal Permeability Assays

In vivo intestinal permeability was determined as described previously.³¹ In brief, mice were gavaged with 10

ml/kg of a solution of 22 mg/ml fluorescein isothiocyanate (FITC)-dextran (molecular mass 4 kDa, Sigma) in PBS, pH 7.4. Blood was collected from the inferior vena cava 3 hours after gavage, and plasma was collected by centrifugation at 3000 rpm for 20 minutes at 4°C. Fifty microliters of plasma in 50 μ l of PBS was added to a 96-well enzyme-linked immunosorbent assay plate. The concentration of fluorescein was determined by using a VICTOR plate reader (PerkinElmer, Wellesley, MA) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm using serially diluted samples of the tracer as a standard.

Epidermal Tight Junction Assay

Epidermal tight junction integrity was measured as described.³² In brief, 50 μ l of 10 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific, Waltham, MA) in PBS containing 1 mmol/L CaCl₂ was injected into the interscapular region of the dermis of newborn mice. After 30 minutes of incubation, the mice were euthanized by decapitation, and the skin was excised and embedded in OCT. Five-micrometer-thick frozen sections were fixed in 95% ethanol at 4°C for 30 minutes and in 100% acetone at room temperature for 1 minute. Sections were blocked for 30 minutes with 2% bovine serum albumin in PBS, incubated with ZO-1 rabbit polyclonal antibody (diluted 1:25 in blocking solution, Zymed Laboratories, South San Francisco, CA) O/N at 4°C, then washed three times with PBS and incubated 60 minutes at room temperature with a mixture of tetramethylrhodamine B isothiocyanate goat anti-rabbit IgG conjugate (diluted 1:100 in blocking solution, Zymed Laboratories) and streptavidin Alexa Fluor 448 conjugate (5 μ g/ml in blocking solution, Invitrogen). Sections were washed three times with PBS and mounted with VECTASHIELD HardSet Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA).

Histological Analysis

Tissues were fixed for 24 hours in 4% paraformaldehyde in PBS or 10% neutral-buffered zinc formalin (Z-fix, Anat-ech, Battle Creek, MI), processed into paraffin, sectioned, and stained with H&E or PAS staining.

Immunofluorescent Staining

Mice were euthanized by CO₂ inhalation. Tissues were fixed overnight in Z-fix, embedded in paraffin, and cut into 5- μ m sections. Antigens were retrieved by incubation for 10 minutes at 37°C with 10 μ g/ml proteinase K (Fermentas, Hanover, MD) for ZO-1 and occludin staining or by 20 minutes of boiling in 0.01 M sodium citrate buffer, ~pH 6 for cadherin staining. Sections were blocked for 30 minutes with 3% BSA in PBS, incubated with primary antibodies (ZO-1 rabbit polyclonal antibody [1:25 in blocking solution, Zymed Laboratories], occludin rabbit polyclonal antibody [1:25 in blocking solution, Zymed Laboratories], and pan-cadherin rabbit polyclonal antibody [1:100 in blocking solution, Invitrogen]) O/N at

4°C, and incubated 60 minutes at room temperature with FITC goat anti-rabbit IgG conjugate (diluted 1:100 in blocking solution, Zymed Laboratories). Sections were mounted with VECTASHIELD HardSet Mounting Medium with DAPI. All microscopic images were acquired on an Axio Imager.Z1 microscope using an AxioCam HRC/MRM digital camera system (both Carl Zeiss Ltd., Jena, Germany) or an IX81 inverted confocal microscope (Olympus Inc., Center Valley, PA).

Immunohistochemistry

Mice were euthanized by CO₂ inhalation. Tissues were fixed overnight in Z-fix solution, embedded in paraffin, and cut into 5- μ m-thick sections. Antigens were retrieved by heating in epitope retrieval buffer-reduced pH (Bethyl Laboratories, Montgomery, TX). The sections were blocked for 1 hour at room temperature with 10% normal rabbit serum in PBS and incubated overnight at 4°C with a sheep anti-matriptase antibody (R&D Systems, Minneapolis, MN). Bound antibodies were visualized using a biotin-conjugated rabbit anti-sheep secondary antibody (Thermo Scientific, Rockford, IL) and a VECTASTAIN ABC kit (Vector Laboratories) using 3,3'-diaminobenzidine substrate (Sigma-Aldrich). 5-Bromo-2'-deoxyuridine (BrdU) staining was performed as described previously.³³

Intestinal Epithelial Turnover Assay

Intestinal epithelial turnover was determined essentially as described.³⁴ In brief, mice were injected i.p. with 0.1 mg/g b.wt. BrdU 1 or 12 hours before sacrifice. BrdU staining was performed as described above. Fifty colonic crypts per mouse were scored for BrdU-labeled cells, and their position was recorded relative to the base of the crypt.

Western Blot Analysis

Fresh tissues were homogenized and lysed in buffer containing 25 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS and subjected to a Western blot analysis using primary anti-rat AQP5 antibody (Alomone Laboratories, Jerusalem, Israel) and secondary anti-rabbit antibody conjugated to alkaline phosphatase (DakoCytomation, Glostrup, Denmark).

Saliva Collection

Mice were anesthetized by intramuscular injection of an anesthetic combination (ketamine [100 mg/ml]/xylazine [20 mg/ml], 3:2), 10 to 15 μ l/mouse. Whole saliva was collected using microhematocrit capillary tubes for 20 minutes after stimulation of saliva flow by s.c. injection of 0.5 mg/kg b.wt. pilocarpine (Sigma-Aldrich) and frozen at -80°C before analysis by SDS-polyacrylamide gel electrophoresis and Coomassie Brilliant Blue staining.

Figure 1. Generation of a conditional *St14* allele in mice. **A:** Schematic drawing of the targeting vector, *St14* gene, floxed *St14* gene, and floxed *St14* gene after Cre-mediated recombination. Cre-mediated recombination leads to the excision of exon 2, which was previously shown to generate a null allele.³⁶ **Red arrowheads** indicate the position of LoxP sites. The black bar and P01 and P02 **arrows** show the location of the probe and the primers used, respectively, for Southern blot hybridization and PCR screening of ES cells. P03, P04, and P05 show the location of primers used for identification of *St14*⁺, *St14*^{loxP}, and recombined *St14*^{loxP} alleles. **B:** PCR analysis of DNA from *St14*^{+/+} (lanes 1 and 4), *St14*^{loxP/loxP} (lanes 2 and 5), and β -actin-CreER^{tm1.0}; *St14*^{loxP/loxP} (lanes 3 and 6) mice, using the P04/P05 primer pair (lanes 1–3) or the P03/P05 primer pair (lanes 4–6) shows Cre-dependent excision of the floxed exon 2. Lane 7 is a molecular weight marker.

Results

Tissue-Specific Embryonic Deletion of Matriptase Results in Loss of Organ Function

Matriptase is constitutively expressed in epithelia of the integumentary system, digestive, urogenital, and respiratory tracts.^{27,35} To query the possible function of matriptase in global epithelial homeostasis, we created mice carrying a conditional *St14* allele by flanking exon 2 with LoxP sites (hereafter termed *St14*^{LoxP} allele) (Figure 1, A and B). This strategy was chosen because we have previously shown that the deletion of exon 2 of the *St14* gene gives rise to an *St14* null allele.³⁶ We first examined the effect of embryonic *St14* ablation from the gastrointestinal tract where matriptase is highly expressed in the intestinal epithelium. Interbreeding of mice carrying the *St14*^{LoxP} allele with mice carrying an *St14* null allele (*St14*^{+/-}) and mice carrying a Cre transgene under the control of the intestine-specific villin promoter (*villin-Cre*^{+/-})³⁰ resulted in the generation of *villin-Cre*^{+/-}; *St14*^{LoxP/-} and their associated *villin-Cre*^{+/-}; *St14*^{LoxP/+}, *villin-Cre*⁰; *St14*^{LoxP/-}, and *villin-Cre*⁰; *St14*^{LoxP/+} littermates (hereafter termed *St14*⁺). Quantitative PCR and immunohistochemical staining showed that *St14* mRNA and matriptase protein were efficiently ablated from the intestinal tract of the *villin-Cre*^{+/-}; *St14*^{LoxP/-} mice (Figure 2, A and B). Interestingly, *villin-Cre*^{+/-}; *St14*^{LoxP/-} mice were easily distinguishable from their *St14*⁺ littermates by their persistent diarrhea, which could be detected in the preweaning period, by their short lifespan of usually just a few weeks to up to 2 months (data not shown), and by their markedly smaller size (Figure 2C). Gross inspection of the intestinal tract of *villin-Cre*^{+/-}; *St14*^{LoxP/-} mice revealed a dramatic enlargement of the colon (Figure 2, D and E), which was not caused by obstruction, as evidenced by the diarrhea. Histological examination of colonic tissues showed gross disruption of the tissue archi-

ture, edema, and pervasive inflammation (Figure 2, F and G), epithelial hyperproliferation, and cessation of mucin production as revealed by PAS staining (data not shown). Other parts of the gastrointestinal tract of *villin-Cre*^{+/-}; *St14*^{LoxP/-} mice, such as the small intestine, were outwardly and histologically normal (Figure 2, H and I; data not shown). We next addressed the developmental requirement of matriptase in an anatomically very different organ, the salivary gland, which expresses matriptase predominantly in its ductal epithelium.³⁵ For this purpose, we used a mouse mammary tumor virus (MMTV)-Cre transgenic mouse strain that directs high-level Cre activity in salivary ductal epithelium.²⁹ With use of the breeding strategy described above, *MMTV-Cre*^{+/-}; *St14*^{LoxP/-} mice and their associated *MMTV-Cre*^{+/-}; *St14*^{LoxP/+} and *MMTV-Cre*⁰; *St14*^{LoxP/+}, *MMTV-Cre*⁰; *St14*^{LoxP/-} littermates (hereafter termed *St14*⁺) were generated. Ablation of about 80% of the *St14* mRNA from the salivary glands was confirmed by quantitative PCR (Figure 3A). Matriptase protein levels could not be assessed by immunohistochemistry because of high nonspecific background in this tissue (data not shown). To access salivary function in matriptase-ablated salivary glands, *MMTV-Cre*^{+/-}; *St14*^{LoxP/-} and *St14*⁺ littermates were subjected to pilocarpine-induced salivation, and total salivary gland secretion was recorded over a 20-minute period (Figure 3B). This analysis revealed that the loss of matriptase caused a near-complete loss of saliva production and, thus, that the membrane protease was critical for salivation. However, unlike the large intestine, this loss of organ function was not associated with acute changes in salivary gland architecture, as judged by the protein composition of the residual saliva secretion (Figure 3C), the expression of the major salivary gland water transporter, aquaporin-5 (Figure 3D), expression and localization of several of tight junction and cell polarity markers (data not shown), or histological appearance (Figure 3E).

Figure 2. Tissue-specific intestinal ablation of matriptase causes growth retardation, megacolon, and loss of intestinal tissue integrity. **A:** Quantitative PCR determination of *St14* mRNA levels in large intestine (left panel) and small intestine (right panel) of weaning age *St14*^{+/+} (left bars, *N* = 3) and littermate *villin-Cre^{+/0};St14^{loxP/}* (right bars, *N* = 3) mice shows efficient Cre-mediated ablation of *St14* mRNA in intestinal tissues. mRNA levels are normalized to *St14*^{+/+} and shown as mean ± SEM. **B:** Representative example of matriptase immunohistochemical analysis of epithelium from large intestine of weaning age *St14*^{+/+} (left panel) and a littermate *villin-Cre^{+/0};St14^{loxP/}* mouse (right panel) showing loss of matriptase (example with arrow) from the basolateral membranes of colonic epithelial cells. Scale bar = 20 μm. **C:** Outward appearance of 8-week-old *St14*^{+/+} (left) and littermate *villin-Cre^{+/0};St14^{loxP/}* (right) mouse showing growth retardation associated with intestinal matriptase ablation. **D** and **E:** Representative example of the gross appearance of the large intestine of weaning age *St14*^{+/+} (**D**) and littermate *villin-Cre^{+/0};St14^{loxP/}* (**E**) mice showing pronounced enlargement of the distal portion of the large intestine caused by matriptase ablation. Scale bar = 1 cm. **F** and **G:** Representative example of the histological appearance of the large intestine of a weaning age *St14*^{+/+} (**F**) and littermate *villin-Cre^{+/0};St14^{loxP/}* (**G**) mouse. Matriptase ablation is associated with gross mucosal disruption, with reparative changes in the epithelium and evident lack of mucin-secreting goblet cells and focal loss of intercellular adhesions and apoptosis (arrow in inset in **G**). Focal ulcerations were also evident (not shown). Chronic inflammatory infiltration is evident in the submucosa, extending beyond the muscularis mucosae to reach the parietal smooth muscle layer (arrowheads). **H** and **I:** Representative example of the histological appearance of the small intestine of a weaning age *St14*^{+/+} (**H**) and littermate *villin-Cre^{+/0};St14^{loxP/}* (**I**) mouse. Scale bar = 50 μm.

Figure 3. Tissue-specific salivary gland ablation of matriptase causes loss of saliva production. **A:** Quantitative PCR determination of *St14* mRNA levels in salivary glands (submandibular and sublingual) of weaning age *St14*^{+/+} (left bars, *N* = 3) and littermate *MMTV-Cre^{+/0};St14^{loxP/}* (right bars, *N* = 3) mice shows Cre-mediated ablation of *St14* mRNA in salivary gland tissues. mRNA levels are normalized to *St14*^{+/+} mice and shown as mean ± SEM. **B:** Total salivary gland secretion from young adult pilocarpine-treated *St14*^{+/+} (left bar, *N* = 3) and littermate *MMTV-Cre^{+/0};St14^{loxP/}* (right bar, *N* = 5) mice over a 20-minute period reveals a dramatic reduction of the saliva flow of matriptase-ablated salivary glands. **C:** Protein content of saliva from three individual pilocarpine-treated *St14*^{+/+} (lanes 1–3) and four individual littermate *MMTV-Cre^{+/0};St14^{loxP/}* (lanes 4–7) mice analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie Brilliant Blue staining shows normal secretion of SDS-polyacrylamide gel electrophoresis-resolvable proteins. **D:** Western blot analysis of salivary gland protein extracts for aquaporin-5 expression in parotid (left panel), sublingual (middle panel), and submandibular (right panel) from *St14*^{+/+} (lanes 1, 2, 5, 6, 9, and 10) and *MMTV-Cre^{+/0};St14^{loxP/}* littermates (lanes 3, 4, 7, 8, 11, and 12), showing unaltered levels of aquaporin-5. Position of the molecular weight markers is indicated at the left. **E:** Representative histological appearance of submandibular (top panels) and sublingual (bottom panels) salivary glands from young adult *St14*^{+/+} (left panels) and *MMTV-Cre^{+/0};St14^{loxP/}* littermates (right panels) showing the overall preservation of tissue integrity after matriptase ablation. Scale bar = 50 μm.

Sustained Matriptase Expression Is Essential for Epithelial Homeostasis of Adult Tissues

The above studies demonstrated that ablation of *St14* in intestinal and salivary gland epithelium disrupts organ function. However, both the villin and the MMTV promot-

Figure 4. Loss of tissue integrity after tamoxifen-induced ablation of matriptase from adult mouse tissues. **A:** Quantitative PCR determination of *St14* mRNA levels in large intestine (top left), small intestine (top right), kidney (bottom left), and lung (bottom right) of young adult *St14⁺* mice (left bars, $N = 3$) and littermate β -actin-Cre-ER^{tm+/0}; *St14^{LoxP/+}* (right bars, $N = 3$) mice 10 days after the beginning of tamoxifen administration shows that efficient Cre-mediated ablation of *St14* mRNA. mRNA levels are normalized to *St14⁺* mice and are shown as mean $\pm$ SEM. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. **B:** Representative examples of matriptase immunohistochemical analysis of epidermis (top panels), small intestine (middle panels), and large intestine (bottom panels) of young adult *St14⁺* (left panels) and littermate β -actin-Cre-ER^{tm+/0}; *St14^{LoxP/+}* (right panels) mice 10 days after the beginning of tamoxifen administration show efficient Cre-mediated ablation of matriptase protein. Residual staining of the stratum corneum and the keratinized portion of the hair follicles of β -actin-Cre-ER^{tm+/0}; *St14^{LoxP/+}* epidermis is revealed as nonspecific by the identical staining pattern of *St14^{-/-}* epidermis (inset in top right panel). Scale bar = 20 μ m. **C:** Body weight of young adult *St14⁺* (black squares, $N = 7$) and littermate β -actin-Cre-ER^{tm+/0}; *St14^{LoxP/+}* (open circles, $N = 7$) mice after daily administration of tamoxifen by oral gavage. Data are shown as mean $\pm$ SEM. Reduction of body weight of β -actin-Cre-ER^{tm+/0}; *St14^{LoxP/+}* mice was first apparent at day 6, became significant at day 7, and approached 25% at the termination of the experiment at day 10. * $P < 0.05$. Statistical significance was calculated by Student's *t*-test (two-tailed). **D** and **E:** Representative examples of the outward appearance (top left panels) and gross appearance of the gastrointestinal tract (right panels) of adult *St14⁺* (**D**) and β -actin-Cre-ER^{tm+/0}; *St14^{LoxP/+}* (**E**) littermate mice after daily administration of tamoxifen by oral gavage for 9 days. **Bottom left panels** are higher magnification of boxed areas of the right panels. Dry and scaly skin with pervasive hair loss and edema of the small and large intestine caused by matriptase ablation are seen. Scale bar = 1 cm.

ers direct expression of Cre during prenatal and postnatal development.^{29,30} This raised the question of whether matriptase was required only for embryonic epithelial development or whether the constitutive presence of the protease in adult epithelia was required to maintain tissue homeostasis. To address this important issue, we acutely ablated the *St14* gene in adult mice by using a tamoxifen-inducible Cre. β -actin-Cre-ER^{tm+/0} mice express a Cre protein that is fused to a tamoxifen-sensitive version of the estrogen receptor.²⁸ In the presence of tamoxifen, the Cre-ERtm protein is released from chaperoning heat shock proteins, translocates to the nucleus, and mediates efficient recombination in most adult tissues. Young adult β -actin-Cre-ER^{tm+/0}; *St14^{LoxP/+}* mice and their associated β -actin-Cre-ER^{tm+/0}; *St14^{LoxP/+}*, β -actin-Cre-ER^{tm0}; *St14^{LoxP/+}*, and β -actin-Cre-ER^{tm0}; *St14^{LoxP/+}* littermates (hereafter *St14⁺*) were generated by interbreeding. The mice were treated daily with tamoxifen by oral gavage to activate the Cre fusion protein. This treatment resulted in the efficient ablation of the *St14* gene, as assessed by quantitative PCR analysis of *St14* mRNA of large intestine, small intestine, kidney, and lungs (Figure 4A) 10 days after initiation of tamoxifen administration and as assessed by immunohistochemical staining for matriptase protein in skin, small

intestine, and large intestine (Figure 4B). Interestingly, *St14* ablation in adult tissues was associated with a precipitous decline in mouse health. Weight loss became apparent 6 days after the initiation of tamoxifen treatment of β -actin-Cre-ER^{tm+/0}; *St14^{LoxP/+}* mice and reached 25% of initial body weight at day 10 (Figure 4C). In sharp contrast, the weight loss of *St14⁺* littermates was insignificant, showing that this weight loss was caused by *St14* ablation and not by the toxic effect of the tamoxifen or the expression and activation of a mutated Cre protein. All β -actin-Cre-ER^{tm+/0}; *St14^{LoxP/+}* mice became cachectic and typically entered a moribund state within 9 to 10 days of initiation of tamoxifen treatment and were subjected to detailed macroscopic and histological analysis (Table 1). Tamoxifen-treated β -actin-Cre-ER^{tm+/0}; *St14^{LoxP/+}* mice were outwardly distinguishable from their tamoxifen-treated *St14⁺* littermate mice by a marked loss of fur, by scaling of the skin, and by scaling and inflammation of orofacial surfaces (compare Figure 4, D and E, top left panels). Histologically, the skin displayed many of the features observed in transplanted *St14* null skin,²⁴ including retention and hyperproliferative ichthyosis and dermal fibrosis (Figure 5B, compare with Figure 5A). Inspection of the gastrointestinal tract of tamoxifen-treated β -actin-Cre-ER^{tm+/0}; *St14^{LoxP/+}* mice revealed edema of the

Table 1. Macroscopic and Histological Appearance of Tissues/organs from Tamoxifen-treated β -actin-Cre-ER^{tm+/0};St14^{LoxP/-} 10 days After the Initiation of Tamoxifen Administration

Tissue/organ	Phenotype
Epidermis	Dry and scaly, retention and hyperproliferative ichthyosis, dermal fibrosis, inflammation of orofacial surfaces
Hair follicles	Pervasive hair loss, follicular hyperplasia
Esophagus	Normal
Forestomach	Normal
Glandular stomach	Normal
Small intestine	Normal
Large intestine	Edema of the crypts and submucosa, gross architectural distortion, hyperproliferation of colonic crypt cells, cessation of mucin production
Kidney	Normal
Liver/bile ducts	Normal
Bladder	Normal
Lungs	Normal
Pancreas	Normal
Spleen	Normal

Organs/tissues from 8- to 28-week-old β -actin-Cre-ER^{tm+/0};St14^{LoxP/-} and littermate St14⁺ mice were examined by a pathologist unaware of animal genotype by routine necropsy and microscopic analysis of H&E- and PAS-stained tissue sections.

small and large intestines (Figure 4, D and E, right and bottom left panels). However, the tamoxifen-treated β -actin-Cre-ER^{tm+/0};St14^{LoxP/-} mice always presented with ingested food matter, showing that their pervasive weight loss was not caused by a cessation of food ingestion (data not shown). Identical results were observed when tamoxifen was administered by i.p. injection, showing that the gastrointestinal phenotype was unrelated to the oral administration of the drug (data not shown). Histologically, the large intestine displayed varying degrees of dissolution of tissue architecture and pronounced edema of the crypts and submucosa (Figure 5D, compare with Figure 5C) and increased expression of mucosal inflammatory cytokines (Supplemental Figure S1, see <http://ajp.amjpathol.org>). Other conspicuous features included the increased proliferation of colonic crypt cells (Figure 5H, compare with Figure 5G; Supplemental Figure S2, see <http://ajp.amjpathol.org>), possibly representing a compensatory response to the loss of the intestinal barrier (see below) and the almost complete cessation of mucin production, as revealed by PAS staining (Figure 5J, compare with Figure 5I). In contrast, the histological appearance of the small intestine was unremarkable (Figure 5, E and F). Pulse labeling with BrdU showed that the rate of transit of epithelial cells through the colonic crypt, as determined by their shift in position within an 11-hour period, was 1.5-fold increased in matriptase-ablated colon, indicating an increase in intestinal cell turnover (Supplemental Figure S2, see <http://ajp.amjpathol.org>). Taken together, these studies show that sustained expression of matriptase is required for the functional integrity of adult mouse epithelia.

Figure 5. Sustained matriptase expression is essential for epithelial integrity. Representative histological appearance of epidermis (A and B), large intestine (C and D, G–J), and small intestine (E and F) of young adult St14⁺ (A, C, E, G, and I) and littermate β -actin-Cre-ER^{tm+/0};St14^{LoxP/-} (B, D, F, H, and J) mice 10 days after initiation of tamoxifen administration. A–F: H&E staining. G and H: BrdU staining. I and J: PAS staining. Hyperkeratosis (arrow), hyperplasia (arrowhead), and dermal fibrosis (asterisk) in matriptase-ablated (B) but not matriptase-sufficient (A) skin. Gross disruption of tissue architecture (compare D with C), hyperproliferation of crypt cells (arrows, compare H with G), and cessation of mucin production (arrows, compare J with I) of matriptase-ablated large intestine. Scale bars = 20 μ m.

Matriptase Sustains Tight Junction Function of Both Simple and Stratified Epithelia

Analysis of the basolateral marker, E-cadherin, by immunofluorescence did not reveal any dramatic defects in cell polarity of intestinal epithelial cells of tamoxifen-treated β -actin-Cre-ER^{tm+/0};St14^{LoxP/-} mice (Figure 6, A and B). However, a similar analysis of the tight junction-associated proteins, occludin (Figure 6D, compare with Figure 6C), ZO-1 (Figure 6F, compare with Figure 6E), and claudin-1 (Figure 6H, compare with Figure 6G) re-

Figure 6. Altered expression of tight junction-associated proteins after acute matriptase ablation in adult intestine. Representative examples of immunofluorescence staining of the large intestine for E-cadherin (A and B), occludin (C and D), ZO-1 (E and F), and claudin-1 (G and H) of young adult *St14*⁺ (A, C, E, and G) and littermate *β-actin-CreER*^{tm+/0}; *St14*^{LoxP/-} (B, D, F, and H) mice 10 days after initiation of tamoxifen administration. Matriptase-ablated intestinal epithelial cells retain their basolateral expression of E-cadherin (examples with arrowheads in A and B). Insets in A and B are cross sections of individual crypts. Longitudinal (C and D) and cross (E–H) sections of the epithelium show the focal loss of occludin, ZO-1, and claudin-1 from tight junctions (examples of tight junctions are indicated with arrows in C, E, and G). Scale bar = 20 μm.

vealed various degrees of tight junction disruption, ranging from decreased staining intensity to focal areas displaying a complete absence of immunofluorescence detection of the three proteins. A similar poor definition of the tight junctions of the granular layer of the epidermis was observed in *St14*^{-/-} mice after staining for ZO-1 (see below). Fluorescent tracer studies were next used to further explore the role of matriptase in epithelial physiology. Young adult *β-actin-CreER*^{tm+/0}; *St14*^{LoxP/-} and *St14*⁺ littermates were treated with tamoxifen for 9 days. FITC-dextran (4 kDa) then was instilled by oral gavage and the rate of diffusion of the tracer through the intestinal epithelium was determined by measuring the accumulation of FITC-dextran in the bloodstream (Figure 7). This tracer permeates the intestinal barrier by paracellular diffusion and, thus, can be used as a sensitive marker for tight junction function.³² This analysis showed a nearly 10-fold increase in the concentration of FITC-dextran in the serum of *β-actin-CreER*^{tm+/0}; *St14*^{LoxP/-} compared with *St14*⁺ littermates 3 hours after installation of the tracer, indicative of a marked increase in paracellular permeability.

Tight junctions confer barrier function not only to simple epithelium but also to multilayered squamous epithelium such as the epidermis, where tight junctions are formed between the keratinocytes forming the granular layer, and substantially contribute to the acquisition of the epidermal barrier.³⁷ Because matriptase is specifically located in the granular and transitional cell layers,³⁵ we determined the requirement of matriptase for epidermal tight junction formation. For this purpose, we used a

previously devised technique to determine the capacity of a reactive biotin tracer injected into the dermis to diffuse through the epidermal layers of newborn mice.^{33,37}

Figure 7. Matriptase ablation in young adult mice increases intestinal permeability. Four *St14*⁺ (closed circles) and six littermate *β-actin-CreER*^{tm+/0}; *St14*^{LoxP/-} (open circles) mice were treated with tamoxifen for 9 days. FITC-dextran (4 kDa) was instilled by oral gavage, and the concentration of FITC-dextran in the blood open circles was determined after 3 hours. Horizontal bars indicate averages. Significance was determined by the nonparametric Wilcoxon rank-sum test (two-tailed).

Figure 8. Loss of epidermal tight junction function in matriptase-deficient mice. Newborn *St14*^{+/+} (A, C, and E) and *St14*^{-/-} (B, D, and F) mice were injected intradermally with Sulfo-NHS-LC-Biotin. After 30 minutes the skin was excised, sectioned, and stained for biotin (green, A and B) and ZO-1 (red, C and D). E and F are merged images. Nuclei (blue) are stained with 4,6-diamidino-2-phenylindole. Arrows in A and B indicate the position of the basement membrane. Strong accumulation of the biotin tracer in the stratum corneum (examples with arrowheads in B and F) and poor morphological definition of tight junctions (examples with arrowheads in C) in matriptase-deficient epidermis (D) are seen. The basement membrane of *St14*^{-/-} epidermis is unremarkable.²⁴ The enhanced labeling of the *St14*^{+/+} epidermal basement membrane reflects an increased local concentration of the biotin tracer because of the intact epidermal tight junctions.

In *St14*^{+/+} mice, the biotin tracer diffused into the epidermis but did not exit the granular layer and accumulate in the stratum corneum (Figure 8, A, C, and E). In sharp contrast, the biotin tracer readily diffused through the granular layer of *St14*^{-/-} mice and accumulated in large quantities at the transitional/stratum corneum interface (Figure 8, B, D, and F). Taken together, these results show that the integrity of tight junctions of both simple and stratified mouse epithelia is matriptase-dependent.

Discussion

One unexpected outcome of the completion of the sequencing of the genomes of several mammalian species at the turn of the millennium was the unveiling of a large new family of membrane-anchored serine proteases with largely unknown functions in mammalian physiology.³⁸ One such protease was the complex modular type II transmembrane serine protease, matriptase. This protease has attracted particular attention because of its consistent overexpression in a variety of human epithelial tumors, its prognostic significance in cancer, and its ability to promote both early and late events of tumor progression.²² Although the critical role of matriptase in mouse epidermal development was recognized early,³⁶

this study now demonstrates a global and essential role of this protease in maintaining the homeostasis of multiple, functionally diverse epithelia. The loss of epithelial integrity after *St14* ablation was rapid, occurring within days of the depletion of *St14* mRNA and matriptase protein, uniform and obligate. These data all strongly argue for a primary role of matriptase in tissue homeostasis, rather than a role in the restoration of homeostasis after chance injury to epithelial tissues. It is noteworthy that both insufficient and excess matriptase proteolytic activity are equally detrimental to mouse epithelia. Thus, the loss of matriptase inhibition by its cognate inhibitor HAI-1 in developing embryos causes rapid dissolution of key epithelial features such as the basement membrane, E-cadherin junctions, and membrane-associated β -catenin.³⁹ Early studies of the effect of serine protease activity on the permeability of epithelial monolayers described a related biphasic effect. At low concentrations, serine protease activity strengthens tight junctions, whereas higher concentrations would lead to the loss of tight junction function.⁵ Whether these *in vivo* and *in vitro* phenomena are related remains to be established.

Loss of matriptase in the epidermis is closely linked to a failure of specific processes that are unique to cornification, including epidermal lipid extrusion, profilaggrin processing, and cornified envelope formation. However, these processes do not take place in other matriptase-dependent epithelia such as the single-layered epithelium of the intestine and salivary glands. A molecular understanding of how matriptase maintains epithelial integrity in multiple diverse epithelia remains to be established. A potential key to this puzzle is our observation in this article that tight junction formation is compromised in matriptase-deficient epidermis. In the temporal sequence of events of terminal epidermal differentiation, tight junction formation immediately precedes cornification. It is thus quite plausible that the failure of adequate cornification of matriptase-ablated epidermis is a secondary consequence of the loss of epidermal tight junction formation and the inability to establish the requisite calcium and pH gradients that are essential to this process.⁴⁰ In this case, the absence of a single molecular event (such as cleavage of one particular substrate) may underlie the loss of the integrity of cornified, multilayered, and simple epithelia. In this regard, it is important to note that we have previously shown that matriptase promotes terminal epidermal differentiation by proteolytic activation of the glycosylphosphatidylinositol-anchored membrane serine protease prostaticin.⁴¹ Furthermore, we have shown that matriptase and prostaticin are constitutively expressed and colocalize in most simple, stratified, transitional, and pseudo-stratified epithelia, including all of the tissues affected by matriptase ablation in the current study.²⁶ It is also important to note that loss of epidermal tight junctions also has been reported in prostaticin-ablated epidermis,³ thus further strengthening a possible functional association between matriptase expression and prostaticin activation in the maintenance of global epithelial homeostasis.⁴¹ Alternatively, matriptase may have diverse and organ-specific molecular functions, cleaving different substrates at each location to maintain homeostasis.

Future studies will be required to definitively settle these issues.

Deletion of matriptase from mouse tissues generally was associated with either of two distinct outcomes: (i) loss of organ-specific function, which was quickly followed by generalized tissue degeneration or (ii) loss of organ-specific function in the absence of a subsequent major disruption of tissue integrity. It is noteworthy that the tissues that underwent an architectural demise after *St14* ablation (epidermis, orofacial tissues, and large intestine) are heavily colonized by a microbial flora, whereas the salivary gland and small intestine, which preserved overall tissue architecture after matriptase ablation, are more aseptic organs. Therefore, the pervasive organ-specific tissue destruction that is associated with the loss of matriptase in some epithelia may be caused by an environmental onslaught that is due to the initial loss of the permeability barrier.

The epidermal defects of matriptase-deficient mice and humans (autosomal ichthyosis with hypotrichosis syndrome/ichthyosis, follicular atrophoderma, and hypotrichosis) are fairly identical at the histological and biochemical level. Both include retention and hyperproliferative ichthyosis, impaired desquamation, hypotrichosis, lipid lamellar body defects, reduced prostasin activation, and loss of profilaggrin proteolytic processing.^{23–25,36,42,43}

Extra-epidermal manifestations, such as corneal opacity, photophobia, and abnormal permanent and deciduous teeth, have also been reported in matriptase-deficient humans. However, the pervasive loss of integrity and degeneration of simple epithelia is not a clinical consequence of matriptase deficiency in humans, and some affected individuals have reached adolescence.^{23,25,42} Although it is too early to provide a definitive explanation for these differences, some speculations can be made: We have previously shown that very low levels of matriptase (1 to 15%) are sufficient to maintain tissue homeostasis in mice,⁴³ which would be compatible with the human *ST14* mutations reported in the literature giving rise to hypomorphic alleles. The c.2672G→A (G827R)²³ and c.3G>A (M1I)^{23–25,36,42,43} alleles could be hypomorphic because of, respectively, a low residual catalytic activity of the mutated protease⁴³ and alternative translational start sites. However, it is more difficult to envision that the IVS17 + 1G>A splice site mutation²⁵ and, in particular, the c.2034delG (L678FfsX84) frameshift mutation²⁵ would give rise to a catalytically active protease. More likely, the less severe clinical manifestations of human matriptase deficiency compared with those of mouse matriptase deficiency are unrelated to the level of residual matriptase activity, but rather reflect physiological differences between primates and rodents. One difference, for example, could be the existence of a tissue-specific salvage pathway in humans that provides low-level residual prostasin activation or cleavage of other key matriptase proteolytic substrates. It is also likely that further clinical examination of individuals with autosomal ichthyosis with hypotrichosis syndrome/ichthyosis, follicular atrophoderma, and hypotrichosis will reveal additional epithelial defects. An illustrative example of such a defect is deficiency of the serine protease,

plasminogen, which was long described as a disease affecting only the eyes and the conjunctiva but now is well established as a systemic disease affecting multiple epithelia.⁴⁴

In summary, this study has revealed an essential role of matriptase in the maintenance of global epithelial homeostasis in the mouse and has provided an important animal model for the further exploration of matriptase function in multiple physiological and pathological processes.

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