American Journal of Pathology, Vol. 168, No. 5, May 2006

DOI: 10.2353/ajpath.2006.051071

Epithelial and Mesenchymal Cell Biology

Delineation of Matriptase Protein Expression by Enzymatic Gene Trapping Suggests Diverging Roles in Barrier Function, Hair Formation, and Squamous Cell Carcinogenesis

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The membrane serine protease matriptase is required for epidermal barrier function, hair formation, and thymocyte development in mice, and dysregulated matriptase expression causes epidermal squamous cell carcinoma. To elucidate the specific functions of matriptase in normal and aberrant epidermal differentiation, we used enzymatic gene trapping combined with immunohistochemical, ultrastructural, and barrier function assays to delineate the spatiotemporal expression and function of matriptase in mouse keratinized tissue development, homeostasis, and malignant transformation. In the interfollicular epidermis, matriptase expression was restricted to postmitotic transitional layer keratinocytes undergoing terminal differentiation. Matriptase was also expressed in keratinizing oral epithelium, where it was required for oral barrier function, and in thymic epithelium. In all three tissues, matriptase colocalized with profilaggrin. In staged embryos, the onset of epidermal matriptase expression coincided with that of profilaggrin expression and acquisition of the epidermal barrier. In marked contrast to stratifying keritinized epithelium, matripase expression commenced already in undifferentiated and rapidly proliferating profilaggrin-negative matrix cells and displayed hair growth cycle-dependent expression. Exposure of the epidermis to carcinogens led to the gradual appearance of matriptase in a keratin-5-positive proliferative cell compartment during malignant progression. Combined with previous studies, these data suggest that matriptase has diverging functions in the genesis of stratified keratinized epithelium, hair follicles, and squamous cell carcinoma. ***(Am J Pathol 2006, 168:1513–1525; DOI: 10.2353/ajpath.2006.051071)***

The epithelial compartment of the skin is composed of a multilayered interfollicular epidermis and a follicular epidermis consisting of hair follicles with associated sebaceous glands.[1] During epidermal development and homeostasis, cell proliferation and differentiation are compartmentalized and tightly regulated processes. The interfollicular epidermis undergoes continuous renewal when proliferative cells residing in the basal layer commit to differentiation and move outwards to give rise to the spinous layer, the granular layer, the transitional layer, and the terminally differentiated cornified layer, the stratum corneum.[2] The interfollicular epidermis serves a critical function as a first line of defense against the external environment by providing a protective barrier against mechanical, chemical, and biological insults.[3] The epidermis also provides a water-impermeable barrier that prevents excessive loss of body fluids, a function that is critical for the survival of all terrestrial vertebrates. The epidermal barrier function resides in the stratum corneum and consists of an interlocking meshwork of flattened terminally differentiated keratinocytes in which the plasma membrane is replaced by a highly cross-linked, insoluble cornified envelope of the corneocytes. The corneocytes are connected by desmosomes and are embedded in a specialized intercorneocyte lipid matrix. This unique structure provides the skin with a mechanically robust, water-impermeable epithelium.[4,5] Keratinized ep-

Supported by the National Institutes of Health Intramural Program and by a grant from the Department of Defense (DAMD-17-02-1-0693 to T.H.B.).

Accepted for publication January 24, 2006.

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ithelium is not restricted to the epidermis and is found in several other tissues, including the oral epithelium and the Hassal's corpuscles of the thymic medulla.[2,6–8]

The hair follicle is a complex, dynamic organ consisting of multiple keratinocyte populations. Rapidly proliferating transient amplifying cells located in the follicular matrix, a zone located at the proximal end of the hair surrounding the dermal papilla, give rise to the cortex, medulla, and cuticle of the hair shaft and to the inner root sheath cells, which continuously move upwards. The outer root sheath of the hair follicle is continuous with the basal layer of the interfollicular epidermis. The matrix transient amplifying cells are relatively undifferentiated and periodically withdraw from the cell cycle and commit to terminal differentiation. As the matrix cells lose their proliferative capacity, the hair follicle growth subsides, and the follicular regression phase progresses. After a rest period, the hair follicle receives growth initiation stimuli involving the dermal papilla, which gives rise to a new cycle of active hair growth (anagen), hair follicle regression (catagen), and rest (telogen).[9–11]

We have previously reported that the type II transmembrane serine protease matriptase (also known as MT-SP1, epithin, ST14, and TAGD-15[12–15]) has pivotal roles in epidermal and thymic development. The membrane protease was required for completion of a terminal differentiation program in the interfollicular epidermis that included cornified envelope and lipid lamellar granule formation. The defects caused by matriptase deficiency included severely impaired formation of extracellular skin lipids, abnormal cornified envelope formation, and loss of epidermal barrier function. They correlated at the molecular level with an impaired proteolytic processing of profilaggrin, leading to a complete loss of both filaggrin monomer and filaggrin S-100 protein in the absence of alterations in the expression of other major differentiation markers.[16,17] Loss of matriptase also seriously affected hair follicle development and resulted in generalized follicular hypoplasia, absence of erupted vibrissae, and lack of vibrissal hair canal formation.[16] Furthermore, the thymus, an organ rich in keratinized epithelium,[7,8] displayed pronounced hypoplasia caused by dramatically increased thymocyte apoptosis, leading to depletion of thymocytes.[16]

Matriptase was identified in part through its consistent overexpression in human carcinoma and has been causally linked to epithelial carcinogenesis in a large number of studies.[18–29] Most recently, modest matriptase overexpression in transgenic mice was found to suffice to initiate multistage squamous cell carcinogenesis and to promote strongly carcinogen-induced epidermal tumor formation.[29]

Expression studies of normal human tissues have shown that matriptase is widely expressed in epithelia including the epidermis.[30] To provide a sensitive and specific method for studying matriptase expression in the mouse, we have now generated two transgenic mouse strains in which the endogenous *matriptase* gene is fused to a bacterial *β-galactosidase* marker gene under transcriptional control of the endogenous matriptase locus. We used these transgenic mice to delineate matriptase expression and function in normal and aberrant keratinized epithelia by combining the enzymatic detection of β-galactosidase with immunohistochemistry, electron microscopy, and epidermal barrier function assays. We show that matriptase is expressed in the keratinized part of the oral and thymic epithelium and that the requirement of matriptase expression for acquisition of barrier formation extends to the oral cavity. Moreover, we show that matriptase is expressed in three functionally very different keratinocyte populations: 1) keratinized stratified epithelium (terminally differentiating profilaggrin-processing cells), 2) hair follicles (rapidly proliferating, profilaggrin-negative, transit amplifying matrix cells), and 3) epidermal carcinomas (well-differentiated and keratin-5-expressing poorly differentiated proliferating cells). Together with previous studies of the effects of epidermal ablation and overexpression of matriptase, these data suggest divergent roles of the membrane protease in the three tissues and provide a platform for further exploration of matriptase molecular functions in the development and malignant transformation of keratinized epithelium.

Materials and Methods

Mice

ES cell lines XM184 and RST485 with a gene trap insertion into introns 1 and 16, respectively, of the mouse *matriptase* gene were obtained from Bay Genomics (baxgenomics.ucsf.edu, San Francisco, CA). The embryonic stem cells (ES) cell lines were generated using a gene trap protocol with the trapping construct vectors pGT0pfs and pGt1TM, respectively, containing the intron from the engrailed 2 gene upstream of the gene encoding the β-galactosidase/neomycin-phosphotransferase fusion protein (Bay Genomics). The 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 offspring carrying one β-galactosidase-targeted matriptase allele. Conventional matriptase gene-targeted mice were generated as described previously.[16]

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Northern Blot Analysis

The gene trap insertion sites in the mouse matriptase gene were confirmed by the identification of truncated matriptase-β-galactosidase fusion mRNA species in ES cell clones XM184 and RST485 by RT-PCR and Northern blot. Total RNA from ES cells was prepared by extraction in Trizol reagent (Gibco-BRL), as recommended by the manufacturer. RNA from ES cells was amplified by reverse transcription followed by PCR amplification using Ready-to-go RT-PCR beads (Amersham Pharmacia Biotech Inc., Piscataway, NJ), as recommended by the manufacturer. First-strand cDNA synthesis was performed using gene-trap-specific primers (clone XM184, 5′-TGGGTAACGCCAGGGTTT-3′, and clone RST485, 5′-GGTTGCTAGTAGACTTCTGCAC-3′). The subsequent

PCR amplification (annealing temperature, 55°C; denaturation temperature, 92°C; extension temperature, 72°C; 40 cycles) was performed with the first-strand primer in combination with a matriptase exon 1-specific primer (5′-AGCAATCGGGGCCGCAAGGCCG-3′) or a matriptase exon 16-specific primer (5′-CCAGGGCCACTTGTGTGGGGCCT-3′). The RT-PCR products were excised from agarose gels after electrophoresis, purified, and subsequently analyzed by DNA sequencing. For Northern blot analysis, total RNA (1 μg) from ES clones XM184 and RST485 were fractionated electrophoretically on formaldehyde agarose gels, blotted onto Nytran SuperCharge nylon membranes (Schleicher and Schuell, Keene, NH), and hybridized to a ^{32}P-labeled 3.5-kb matriptase expressed sequence tag probe (I.M.A.G.E. ID 2609399) that contains the complete full-length murine matriptase cDNA or a trap-specific probe amplified by PCR using primers 5′- GCTGGCTGGAGTGCGATCTT-3′ and 5′-ACTGTCCTGGCCGTAACCGA-3′. The membranes were subjected to PhosphorImage analysis using ImageQuant software from Molecular Dynamics (Sunnyvale, CA).

In Situ *Hybridization*

In situ hybridization on tissues from newborn mice was performed as previously described.[29] In brief, an expressed sequence tag containing the full-length murine matriptase cDNA (I.M.A.G.E. ID 2609399) was used as a template to generate two nonoverlapping PCR fragments for transcription of antisense and sense probes. Riboprobes were labeled with [^{35}S]UTP (NEN, Boston, MA) by *in vitro* transcription using T7 and T3 RNA polymerases (Roche, Basel, Switzerland) and approximately 1 μg of template. Paraffin sections were deparaffinized in xylene and hydrated with graded ethanol solutions. Sections were incubated at 99°C for 2 minutes in Tris EGTA buffer (10 mmol/L Tris, pH 9.0, 0.5 mmol/L EGTA) using a T/T Micromed microwave processor (Milestone, Sorisol, Italy). After an additional 20 minutes at room temperature, the sections were dehydrated with graded ethanol, and the ^{35}S-labeled probes (2×10^6 cpm in 20 μl of hybridization mixture per slide) were incubated overnight at 55°C in a humidified chamber. Sections were washed with standard saline citrate (SSC) buffers containing 0.1% sodium dodecyl sulfate and 10 mmol/L dithiothreitol at 150 rpm at 55°C for 10 minutes in 2× SSC, 10 minutes in 0.5× SSC, and 10 minutes in 0.2× SSC. Sections were then RNase A treated for 10 minutes to remove nonspecifically bound riboprobe. Subsequent washes were performed in 0.2× SSC as specified above. Sections were dehydrated and soaked in an autoradiographic emulsion, exposed for 10 to 14 days, developed, and counterstained with hematoxylin and eosin.

Whole-Mount Histological Analysis and β-Galactosidase Staining

Skin samples were fixed for 1 hour in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), rinsed in PBS, and stained with a β-galactosidase staining kit (Roche, Indianapolis, IN) overnight at 37°C. The tissues were post-fixed for 1 hour in 4% PFA, embedded in paraffin, and sectioned. Sections were counterstained with nuclear fast red or hematoxylin.

Immunohistochemistry

Tissues were fixed with 4% PFA, X-gal-stained, processed into paraffin, and sectioned as described above. Antigens were retrieved by incubation with 5 μg/ml proteinase K, blocked with 2% bovine serum albumin, and incubated overnight at 4°C with rabbit antibodies to mouse keratin 5, keratin 6, or filaggrin (Covance, Richmond, CA). The polyclonal antibody mIRSa used to visualize type I inner root sheath keratin mIRSa3.1 was a kind gift from Dr. Rebecca M. Porter.[38] The antibody AE13 (Abcam, Cambridge MA) was used to detect hair cortex keratins. Bound antibodies were visualized using Envision system HPRT secondary antibodies (DAKO, Carpentaria, CA) and a NovaRED substrate kit (Vector Laboratories, Burlingame, CA). Cell proliferation was visualized by intraperitoneal injection of 100 μg/g bromodeoxyuridine (BrdU) (Sigma Chemical Co., St. Louis, MO) 2 hours before euthanasia. BrdU incorporation was detected with a mouse anti-BrdU antibody (Accurate Chemical & Scientific Corporation, Westbury, NY), and bound antibodies were visualized with a Vectastain ABC peroxidase kit (Vector Laboratories). Cell proliferation was also visualized using a rabbit polyclonal Ki67 antibody (NovoCastra, Newcastle, United Kingdom) as described above.

Enzyme Electron Microscopy

The procedure described by Aoyama et al[32] was followed. Skin samples were trimmed into 1-mm^3 pieces and fixed in 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4, for 1 hour at 4°C. The tissues were then incubated in 0.1% 5-bromo-3-indolyl-D-galactoside (Bluo-gal) (Sigma-Aldrich, St. Louis, MO) with 5 mmol/L potassium ferricyanide, 5 mmol/L potassium ferrocyanide, and 2 mmol/L $MgCl_2$ overnight at 37°C. At the end of incubation, the tissues were briefly rinsed in PBS and postfixed in 2% paraformaldehyde and 2% glutaraldehyde in sodium cacodylate buffer for 12 hours. The samples were further fixed in 1% OsO_4 followed by ethanol dehydration, embedding, and staining with uranyl acetate and lead citrate. The samples were examined with a JEOL-1010 transmission electron microscope operated at 80 kV.

Epidermal and Oral Epithelium Permeability Assay

Newborn mice were euthanized and subjected to methanol dehydration and subsequent rehydration as described previously.[33] Whole pups or dissected tongues and hard palates were then stained for 1 hour at room

temperature in 0.1% toluidine blue/PBS (Fisher Scientific, Pittsburgh, PA), destained for 15 minutes in PBS at room temperature, and examined with a dissection microscope for epidermal dye penetration.

Chemical Two-Stage Carcinogenesis

The dorsal skin of 6- to 8-week-old matriptase$^{+/\beta\text{-gal}}$ and wild-type littermate mice was shaved and treated 2 days later with a single topical application of 25 μg of 7,12-dimethylbenzanthracene (DMBA) (Sigma) in 200 μl of acetone, followed 2 weeks later by weekly applications of 12 μg of phorbol 12-myristate 13-acetate (PMA) (Sigma) for up to 30 weeks.

Results

Generation of Mice with a β-Galactosidase Gene Inserted into the Matriptase Locus

Previous *in situ* hybridization studies of mouse tissues did not enable a detailed high-resolution analysis of matriptase expression,[29] and immunohistochemical screening of a series of commercial and in-house-generated matriptase antibodies using matriptase-deficient mouse tissue sections as specificity control revealed prohibitively high cross-reactivity in keratinized tissues (K. List and T.H. Bugge, unpublished data). We therefore used the technology of enzymatic gene trapping to study matriptase expression in the mouse. A search of the BayGenomics Web site revealed two ES cell clones in which a promoterless β-galactosidase-neomycin gene trap was inserted between exons 1 and 2 (ES clone XM184) and 16 and 17 (ES clone RST485) of matriptase. These insertions would give rise to a soluble intracellular matriptase-β-galactosidase fusion protein (gene trap insertion between exons 1 and 2) or a membrane-anchored fusion protein (gene trap insertion between exons 16 and 17) anchored via the CD4 transmembrane segment provided by the gene trap and the matriptase signal anchor. Both fusion proteins would be expressed under the control of the endogenous matriptase promoter. Northern blot (data not shown) and RT-PCR analysis combined with DNA sequencing (Figure 1) confirmed the expression of the predicted matriptase-β-galactosidase fusion mRNA in both ES cell clones. The two ES cell lines were microinjected into blastocysts, and breeding of the ensuing chimeras for germline transmission gave rise to two mouse strains carrying a gene trapped matriptase allele (hereafter referred to as matriptase$^{+/E1\beta\text{-gal}}$ and matriptase$^{+/E16\beta\text{-gal}}$). Expression analysis of tissues from both matriptase$^{+/E1\beta\text{-gal}}$ and matriptase$^{+/E16\beta\text{-gal}}$ mice showed X-gal staining in the same cell populations in all epithelia studied, including vibrissae, follicular epidermis, interfollicular epidermis, thymic and oral epithelium (Figure 2, A and B; data not shown). The only difference detected was that the staining intensity tended to be higher in the matriptase$^{+/E16\beta\text{-gal}}$ strain. The analysis in all cases also included matriptase$^{+/+}$ littermate controls, which were negative for X-gal staining in the tissues that were examined in this study (Figures 2C and 6D, data not shown). Furthermore, to confirm that the expression pattern of both the matriptase E1-β-galactosidase and E16-β-galactosidase fusion proteins properly reflected that of the wild-type matriptase transcript, parallel *in situ* hybridizations of vibrissal hair follicles were performed (Figure 2, D and E). Vibrissal hair follicles were suitable for this comparative analysis because of their large size and high matriptase expression level, which enabled relatively high resolution by *in situ* hybridization. The expression of matriptase as detected by X-gal staining and by *in situ* hybridization was specifically detected in follicular epithelial cells, including matrix cells, of the vibrissal hair follicles with minimal staining of the dermal papillae. This confirms that the expression patterns of the two different matriptase β-galactosidase fusion proteins expressed under the endogenous matriptase promoter are indistinguishable from the matriptase mRNA expression pattern in wild-type mice.

Matriptase Expression Coincides with Profilaggrin Expression and Epidermal Barrier Formation during Embryonic Development

The onset of matriptase expression in the developing epidermis was determined by whole-mount X-gal staining of E12.5, E14.5, E15.5, and E16.5 embryos extracted from matriptase$^{+/+}$ females crossed to matriptase$^{+/\beta\text{-gal}}$ males (Figure 3, top row; data not shown). In E14.5 embryos, weak staining was present in the head area, because of expression of matriptase in the developing oral cavity and vibrissal hair follicle primordia (see below), and in the developing ear (data not shown). Matriptase expression at these sites was stronger at E15.5; at this time point, a weak matriptase expression was also detected in the dorsal area of the developing epidermis. At E16.5, strong and uniform matriptase expression was observed at all epidermal surfaces. E17.5 and later stage embryos could not be analyzed for matriptase expression by this method because the epidermal barrier prevented penetration of X-gal into the epidermis (data not shown). Histological analysis showed that matriptase expression was exclusively localized to the outermost layer of the developing epidermis. Inspection of a number of epidermal sections at different stages of differentiation showed that the onset of matriptase expression tightly correlated with the emergence of flattened corneocyte-like cells in the outermost epidermal layer (Figure 3C, top panels; data not shown). Interestingly, profilaggrin expression coincided temporally with matriptase, being undetectable at E15.5 and strong at E16.5 as determined by immunohistochemistry (Figure 3C, bottom panels). Littermate matriptase$^{+/\beta\text{-gal}}$ embryos were next subjected to a toluidine blue dye-penetration assay to compare the onset of matriptase expression with the acquisition of epidermal barrier function (Figure 3B). E14.5 and E15.5 embryos displayed complete dye penetration, indicating the absence of an epidermal barrier at this embryonic stage as reported previously.[33] At E16.5, a functional epidermal barrier was present in the dorsal

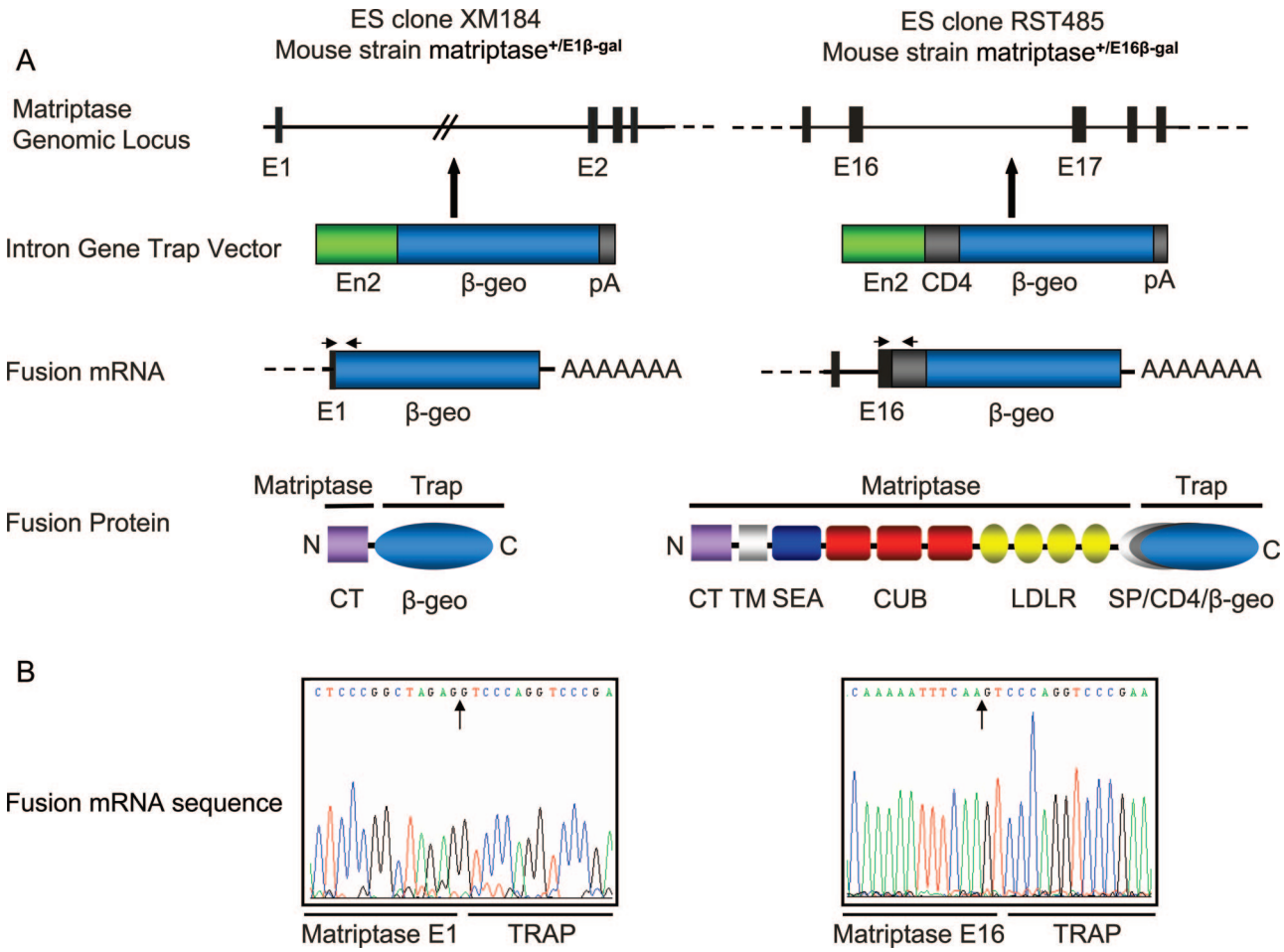

Figure 1. Generation of mouse strains with a β-galactosidase-tagged matriptase locus. **A:** Schematic structure of the mouse matriptase gene locus. ES cell clone XM184 (giving rise to mouse strain Matriptase$^{+/E1\beta\text{-gal}}$) has a gene trap consisting of the engrailed-2 (En2) splice acceptor site-β-galactosidase-neomycin phosphotransferase fusion gene (β-geo) and an SV40 polyadenylation site (pA) inserted between exons 1 and 2 (E1 and E2). ES cell clone RST485 (mouse strain Matriptase$^{+/E16\beta\text{-gal}}$) has a gene trap consisting of the En2 splice acceptor site, a CD4 transmembrane domain-β-galactosidase-neomycin fusion gene, and an SV40 polyadenylation site inserted between exons 16 and 17 (E16 and E17). Clone XM184 gives rise to an mRNA that generates a protein that consists of the cytoplasmic tail (CT) of matriptase fused to the β-galactosidase-neomycin fusion protein. Clone RST485 gives rise to an mRNA that generated a protein that replaces most of the serine protease domain of matriptase with a CD4 transmembrane domain-β-galactosidase-neomycin phosphotransferase fusion protein. SA, signal anchor; SEA, sea urchin sperm protein; enterokinase, agrin domains; CUB, complement factor 1R-urchin embryonic growth factor-bone morphogenetic protein domains; LDLR, low-density lipoprotein receptor domains; SP, serine protease domain. **B:** RT-PCR amplification followed by DNA sequencing of matriptase fusion mRNAs confirming the matriptase exon 1-engrailed-2 splice event in clone XM184 and the matriptase exon 16-engrailed-2 splice event in clone RST485. The primer pairs used for the analysis are depicted above the respective fusion mRNAs in **A**.

and tail area of the epidermis, and less extensive dye penetration was observed in the remaining part of the embryo. A fully functional barrier covering the entire epidermal surface was evident in newborn mice (Figure 3B, right). The data show a temporal coincidence between matriptase expression, profilaggrin expression, and acquisition of epidermal barrier function in the developing mouse embryo.

Matriptase Is Expressed in Terminally Differentiating Cells of the Interfollicular Epidermis, Oral Epithelium, and Thymic Epithelium and Colocalizes with Profilaggrin

The skin, the hard palate and tongue of the oral cavity, and parts of the thymus all contain keratinized stratified epithelium. The expression of matriptase in the three tissues was determined by X-gal staining of matriptase$^{+/\beta\text{-gal}}$ mice combined with immunohistochemistry and by electron microscopy after visualization of matriptase expression with the electron-dense β-galactosidase substrate Bluo-gal (Figure 4). In the interfollicular epidermis, matriptase expression was exclusively confined to the transitional cell/stratum corneum interface (Figure 4A). High-resolution analysis by enzyme electron microscopy localized the bulk of matriptase expression to transitional layer cells and the first corneocyte layer (Figure 4, B and C). Profilaggrin is sequestered in large insoluble granules that are easily visible by light microscopy in the granular and transitional cell layers. After proteolytic processing of profilaggrin in the transitional layer during terminal differentiation, the liberated filaggrin monomers are incorporated into the cornified envelopes of maturating corneocytes.[31,34,35] Interestingly, combined X-gal

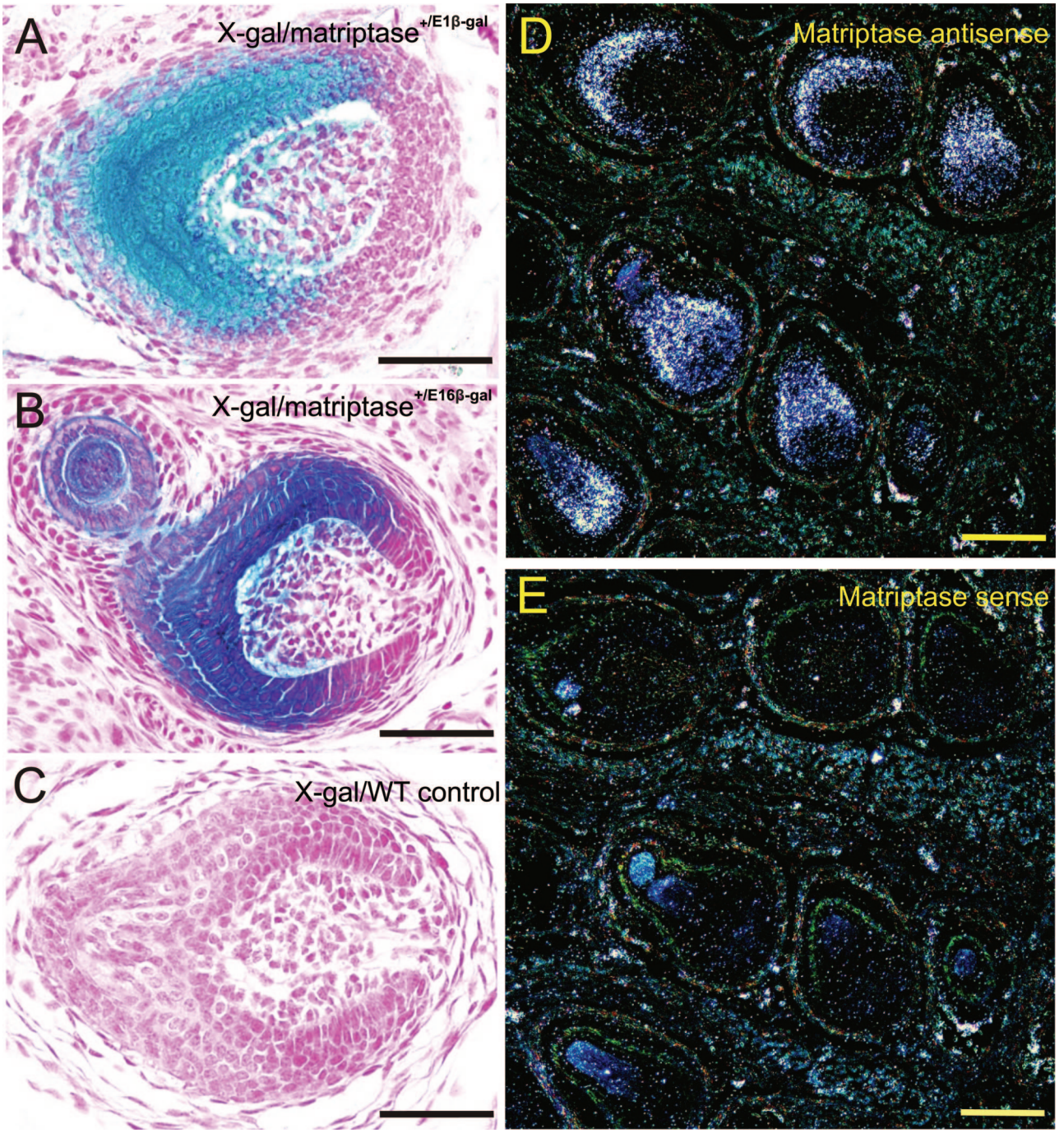

Figure 2. Expression of matriptase β-galactosidase fusion proteins corresponds to endogenous matriptase mRNA expression in vibrissal hair follicles. **A–C:** Representative histological sections showing X-gal staining of matriptase fusion proteins (cyan) in vibrissae from newborn matriptase$^{+/E1\beta\text{-gal}}$ (**A**), matriptase$^{+/E16\beta\text{-gal}}$ mice (**B**), and wild-type mice (**C**). **D–E:** *In situ* hybridization of endogenous matriptase mRNA expression in vibrissae from newborn wild-type mice using ^{35}S-labeled antisense probes (**D**) and sense probes (**E**). Identical results were obtained with two nonoverlapping pairs of antisense and sense probes. Scale bars, 50 μm (**A–C**); 100 μm (**D**, **E**).

staining and immunohistochemical staining with profilaggrin/filaggrin antibodies revealed that filaggrin immunoreactive material located below matriptase-expressing cells was almost exclusively in profilaggrin granules (Figure 4D). Conversely, filaggrin immunoreactive material located above matriptase was almost exclusively present in the plasma membrane/cornified envelope of emerging and mature corneocytes. This shows that matriptase expression defines a distinct boundary between granule-located profilaggrin and proteolytically liberated filaggrin monomers and underscores the close association between matriptase expression and profilaggrin processing. Furthermore, enzyme electron microscopy revealed that, besides the plasma membrane, matriptase was consistently located in intracellular structures that were in close prox-

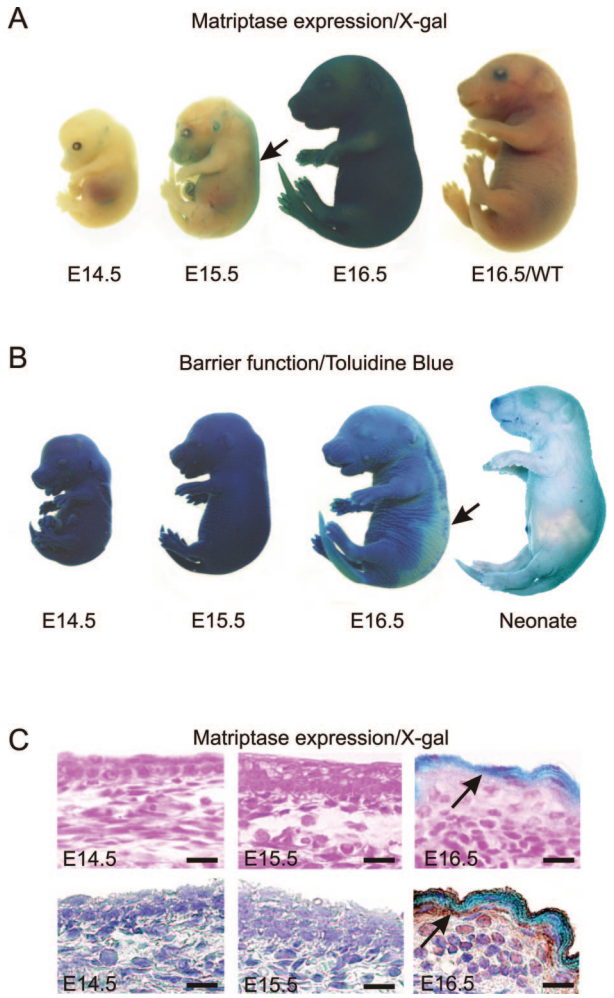

Figure 3. Onset of epidermal matriptase expression during development correlates with profilaggrin expression and the acquisition of barrier function. **A:** Whole-mount X-gal staining of matriptase$^{+/\beta\text{-gal}}$ embryos at E14.5–16.5 and a control wild-type E16.5 embryo. **B:** Toluidine-blue dye penetration assay of matriptase$^{+/\beta\text{-gal}}$ embryos at E14.5–16.5 and a control wild-type newborn pup (the latter is shown at a lower magnification than the embryos). **C:** Matriptase expression (cyan) in histological sections of developing epidermis of X-gal-stained embryos (**top panel**). Immunostaining of profilaggrin/filaggrin in parallel X-gal stained sections (**bottom panel**). Expression of matriptase in the developing epidermis commences at E15.5 in the dorsal area of the embryo (**arrow** in **A**) and correlates with the patterned acquisition of barrier function (**arrow** in **B**). Matriptase expression in the developing epidermis is confined to flattened corneocyte-like cells (**arrow** in **C**, **top panel**). Profilaggrin/filaggrin can readily be detected in the developing epidermis in E16.5 embryos (**arrow** in **C**, **bottom panel**), whereas E14.5 and E15.5 displayed no detectable expression. In **C**, **top panels** were counterstained with nuclear fast red and **bottom panels** with hematoxylin. Scale bars, 20 μm.

imity to profilaggrin granules in various stages of dissolution, suggesting a close physical proximity (Figure 4C). However, extreme caution must be taken when interpreting the latter finding, because the serine protease domain-substituted matriptase-β-galactosidase fusion protein may not display the same subcellular localization as the unmodified matriptase.

In the oral cavity, the filiform papillae that cover the surface of the tongue display a stratification that is comparable with the interfollicular epidermis. This includes the presence of a granular layer and a stratum corneum-like layer that provides the oral epithelium with a permeability barrier.[2,6] X-gal staining of the filiform papillae of the tongue revealed expression of matriptase only in the uppermost terminally differentiating layers of the epidermis, analogs to the expression in the interfollicular epidermis (Figure 4E). Furthermore, combined X-gal and immunohistochemical staining again demonstrated colocalization of matriptase and profilaggrin/filaggrin (Figure 4F).

Hassal's corpuscles of the thymic medulla are formed by concentrically arranged epithelioreticular cells that show histological evidence of keratinization as well as the expression of several late epidermal differentiation-associated proteins, including profilaggrin.[7,8] The critical role of matriptase in thymocyte development[16] prompted the examination of matriptase expression in the thymus. X-gal staining revealed matriptase expression in both cortical and medullary epithelioreticular cells and in Hassal's corpuscles (Figure 4G). In contrast, no matriptase staining was found in the thymocytes. Interestingly, coexpression of matriptase and profilaggrin could again be demonstrated in Hassal's corpuscles of the thymic medulla (Figure 4H).

Matriptase Is Required for Oral Epithelial Barrier Function

The expression analysis performed above revealed that matriptase was prominently expressed in the terminally differentiating layers of the keratinized part of the oral epidermis and was coexpressed with profilaggrin. The close similarity of interfollicular epidermal and oral epithelial matriptase expression prompted the examination of a possible extended role of matriptase in the acquisition of barrier formation in the oral cavity. Tongues (Figure 5) and hard palates (data not shown) from newborn matriptase-sufficient and -deficient littermate mice were excised and subjected to the dye penetration assay. Tongues (Figure 5, left panel) and hard palates from matriptase-sufficient mice displayed a functional barrier, as revealed by the limited penetration of toluidine blue. In contrast, extensive dye penetration was observed in both tongues (Figure 5, right panel) and hard palates of matriptase-deficient mice. These data demonstrate that matriptase expression in the oral cavity is functionally relevant to terminal differentiation of oral epithelium and is required for the acquisition of barrier function in the oral cavity.

Matriptase Is Expressed in Proliferative Cells in the Hair Follicle Matrix Compartment

Hair follicles are complex, dynamic structures that undergo cycles of active hair growth (anagen), regression (catagen), and rest (telogen). To study the expression of matriptase during hair follicle development and during the various stages of the hair cycle, mouse skin from matriptase$^{+/\beta\text{-gal}}$ mice of different ages was stained with X-gal and examined histologically. Matriptase expression was detected in hair germ ker-

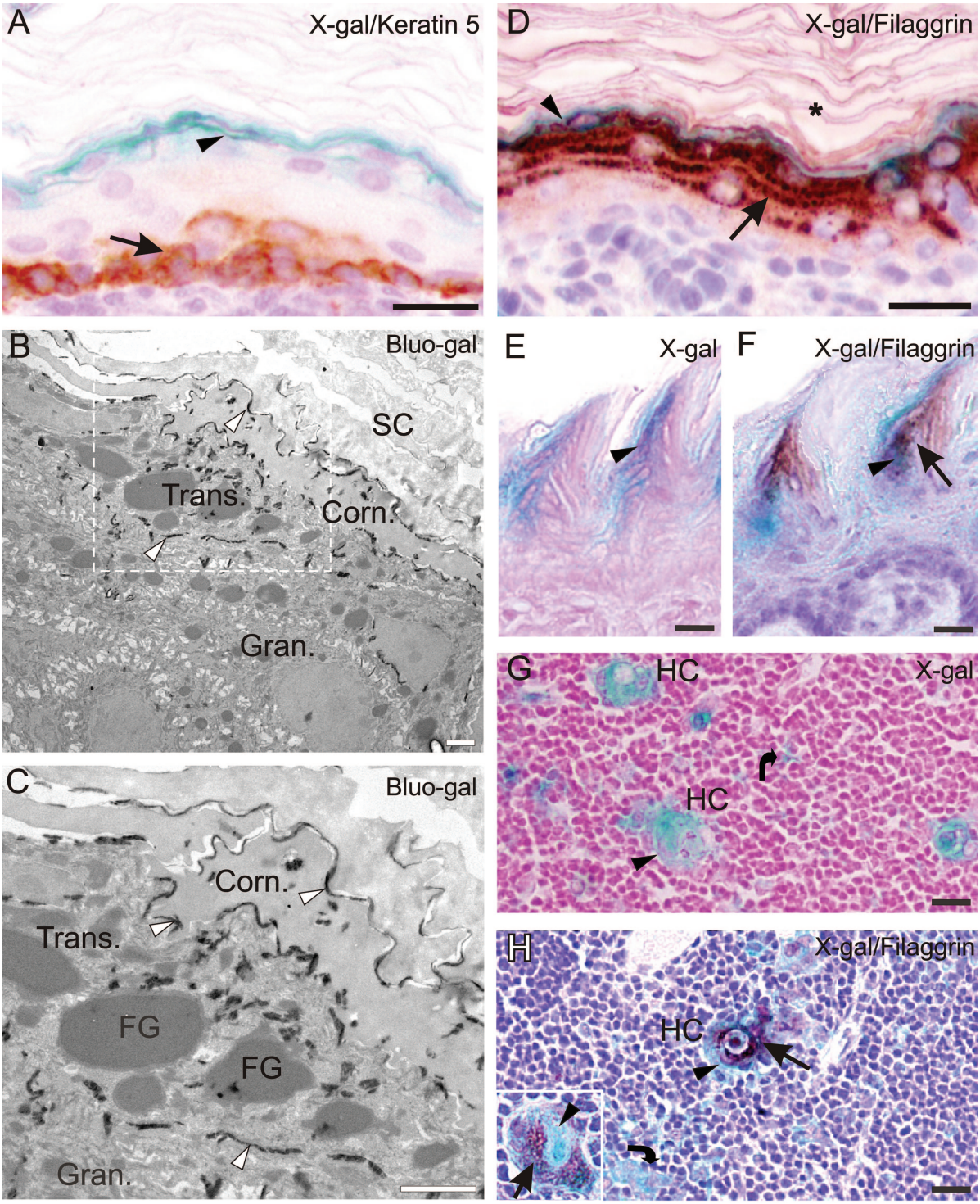

Figure 4. Matriptase and profilaggrin colocalize in interfollicular epidermis, keratinized oral epithelium, and the thymic epithelioreticular cells. **A**, **D:** Histological sections of combined X-gal staining of matriptase (cyan, example with **arrowheads**) and immunohistochemistry (brown, example with **arrows**). Section in **A** was stained with keratin-5 antibodies to visualize basal keratinocytes (**arrow**), and the section in **D** was stained with antibodies against profilaggrin/filaggrin. Matriptase is expressed in terminally differentiating transitional layer cells at the intersection between profilaggrin granules and proteolytically processed filaggrin incorporated into cornified envelopes. **B**, **C:** Bluo-gal-stained electron microscopy sections (black precipitate, examples with **arrowheads**) of matriptase expression in the interfollicular epidermis of newborn matriptase$^{+/\beta\text{-gal}}$ mice. Matriptase expression in terminally differentiating transitional layer cells (Trans.) and maturing corneocytes (Corn.) in the stratum corneum (SC). Location of the granular layer is indicated (Gran.). **C:** High magnification of **inset** in **B** showing the close proximity of matriptase and profilaggrin granules (FG). X-gal staining (cyan, example with **arrowheads**) (**E**) and combined X-gal staining of matriptase and immunohistochemical staining of profilaggrin/filaggrin (brown, example with **arrow**) (**F**) of filiform papillae of the tongue from 9-day-old mice showing expression of matriptase in terminally differentiating cells and colocalization with profilaggrin/filaggrin. X-gal staining (cyan, example with **arrowheads**) (**G**) and combined X-gal staining of matriptase (cyan) and immunohistochemical staining of profilaggrin/filaggrin (brown) (**H**) of the thymus from 21-day-old mice. Expression of matriptase in epireticular cells (example with curved **arrows**) and Hassal's corpuscles (HC) (examples with **arrowheads**) of the thymic medulla. Colocalization of matriptase with profilaggrin/filaggrin is observed in Hassal's corpuscles (examples with **arrows** including **boxed area**). Sections in **A**, **D**, **F**, and **H** were counterstained with hematoxylin, and sections in **E** and **G** were counterstained with nuclear fast red. Scale bars, 20 μm (**A**, **D–F**); 2 μm (**B, C**).

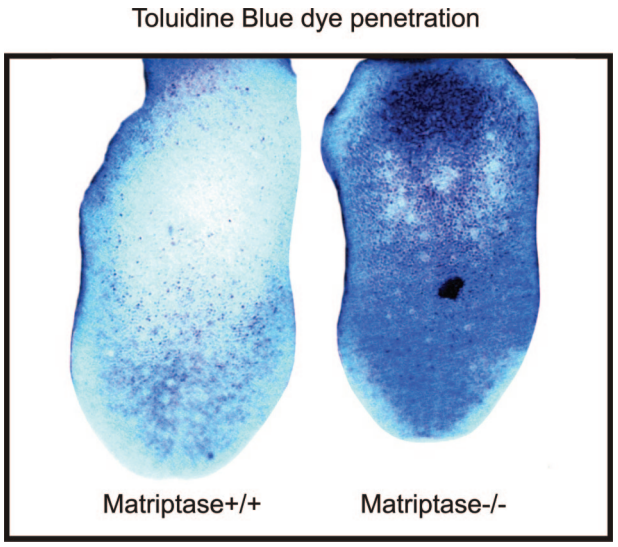

Figure 5. Matriptase is required for barrier function of keratinized oral epithelium. Tongues were excised from newborn matriptase-sufficient (**left**) and matriptase-deficient (**right**) mice. The tongues were submerged in toluidine blue, briefly destained with PBS, and photographed. A representative experiment is presented. Matriptase-deficient, but not matriptase-sufficient, tongues display extensive dye penetration throughout the surface.

atinocytes as early as E14.5 in vibrissal follicle primordia (Figure 6A) and E16.5 pelage hair follicle primordia (data not shown). In both cases, the expression was localized to a population of primitive epithelial cells known as hair germ keratinocytes.[36] In early anagen pelage hair follicles of newborn pups and anagen hair follicles of 9-day-old mice, matriptase was expressed in the hair matrix, continuing into the precortex and cortex of the developing hair shaft (Figure 6, B, C, and G). Matriptase was also expressed in sebocytes of the hair follicle sebaceous gland (Figure 6, C and F). To delineate the precise cell populations expressing matriptase in the anagen hair follicle, combined X-gal staining and immunohistochemical staining of hair follicle differentiation markers were performed. Matriptase and the outer root sheath marker keratin-5 were clearly expressed in separate keratinocyte populations of the hair follicle (Figure 6G). Likewise, no colocalization of matriptase and keratin-6 was detected in the follicular companion layer,[37] which is located between the outer root sheath and the inner root sheath (data not shown). Costaining of matriptase and the inner root sheath marker showed some areas of possible overlap, suggesting that matriptase could be expressed by some inner root sheath keratinocytes[38] (Figure 6H). Interestingly, many matriptase-expressing cells in the hair matrix were positive for Ki67 (Figure 6I) and displayed incorporation of BrdU (Figure 6J), demonstrating that the matriptase is expressed in hair matrix cells. Moreover, matriptase expression colocalized with hair keratins in the precortex and cortex that are specifically recognized by the antibody AE13[39] (Figure 6K). This pattern of matriptase expression in the hair follicle persisted throughout the anagen phase. In catagen and telogen phase hair follicles, matriptase expression was limited to club hair and occasionally to cells in the remnant hair bulb (Figure 6, E and F).

Carcinogen Exposure of Epidermis Causes Aberrant Expression of Matriptase to Include Proliferating Keratin-5-Positive Cells

Several studies causally link matriptase to epithelial carcinogenesis.[18–29] To delineate the pattern of matriptase expression during multistage squamous cell carcinogenesis, the skin of matriptase$^{+/\beta\text{-gal}}$ and wild-type littermate mice was initiated with DMBA followed by weekly promotion by PMA. The ensuing lesions were excised at various stages of progression and examined for matriptase expression by X-gal staining (Figure 7). Hyperplastic lesions retained a matriptase expression pattern that was very similar to that of normal interfollicular epidermis (Figure 7A). Expression was predominantly observed in the uppermost aberrantly differentiated layer. Occasionally, weak expression in suprabasal cells could be appreciated. Papillomatous lesions with clear stratification generally presented with expression of matriptase in multiple suprabasal layers of well-differentiated cells (data not shown). Dysplastic lesions, however, typically presented matriptase expression in both well-differentiated cells that displayed pronounced keratinization and in basal cells in direct apposition to the underlying dermal connective tissue (Figure 7B). Squamous cell carcinoma lesions displayed patchy expression of matriptase in both well-differentiated and poorly differentiated tumor cells (Figure 7D). To identify the specific cell populations expressing matriptase during malignant progression, combined X-gal staining and immunohistochemical staining for keratin-5 and BrdU incorporation were performed. Interestingly, during the transition from pre-neoplastic lesions to squamous cell carcinoma, matriptase expression was increasingly observed in a population of cells that also expressed keratin-5 (Figure 7, E–G). This keratinocyte population, which includes epidermal stem cells, basal cells of the interfollicular epidermis, and outer root sheath cells of the hair follicle, was never observed to express matriptase in the normal follicular or interfollicular epidermis (see above). Furthermore, a gradual increase in the number of proliferating cells expressing matriptase was observed during malignant progression, as assessed by BrdU incorporation (Figure 7H). This shows that matriptase undergoes a shift in expression during epidermal carcinogenesis to include premalignant and malignant proliferating keratinocytes that express keratin-5.

Discussion

Previous studies have demonstrated a critical role of matriptase in mouse epidermal development and malignant progression,[16,17,29] but the molecular mechanisms by which the membrane protease exerts its physiological and pathological functions in this tissue are currently

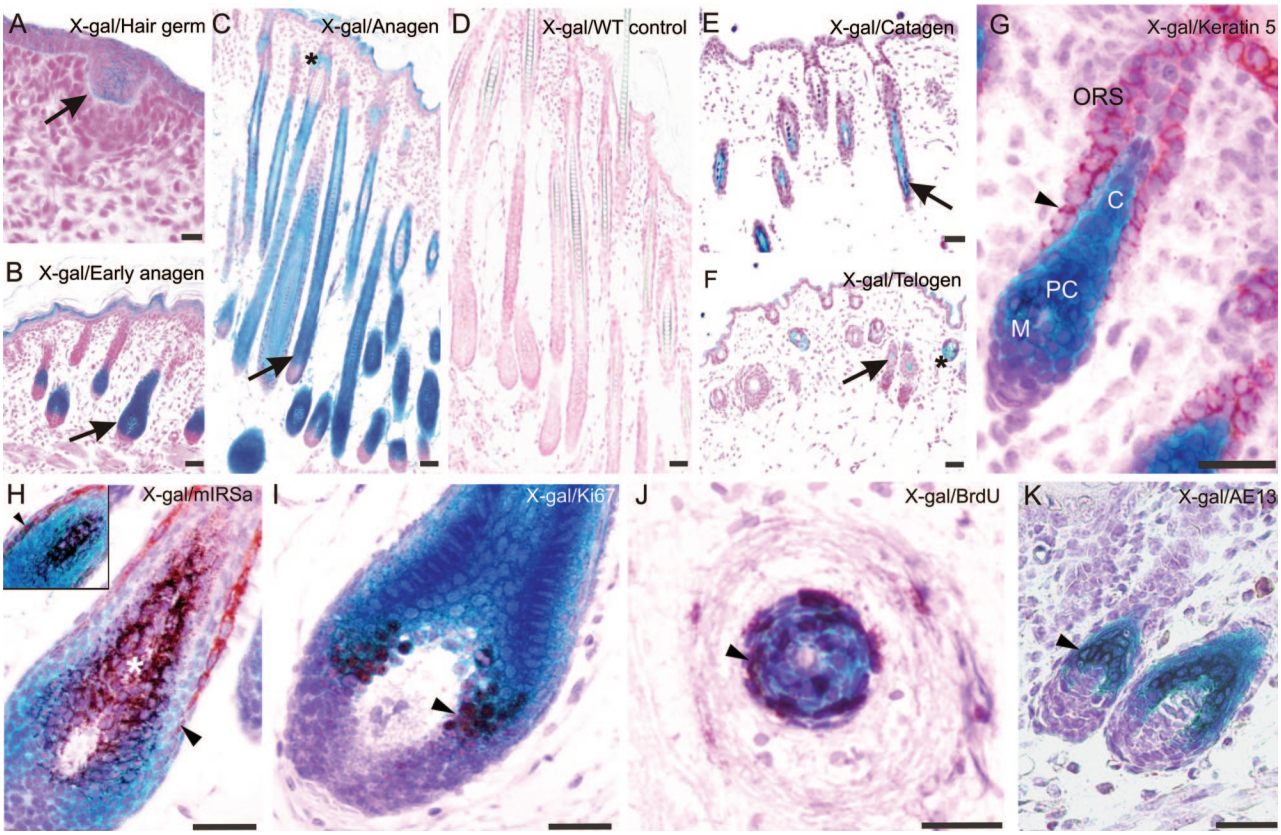

Figure 6. Matriptase is expressed during hair follicle development and in proliferating hair matrix cells in the anagen hair follicle. Histological sections of X-gal staining of matriptase (cyan, examples with **arrows**) (**A–F**) and combined X-gal staining of matriptase (cyan) and immunohistochemistry (brown, example with **arrowheads**) (**G–K**) of hair follicles in different stages of the hair cycle. **A–C** and **E–K** are skin from matriptase$^{+/\beta\text{-gal}}$ mice, and **D** is skin from wild-type littermate mice. **A:** Expression of matriptase in hair germ keratinocytes of the vibrissal follicle primordia at E14.5. In early anagen (**B**, **G**) and anagen (**C**) pelage hair follicles, matriptase is expressed in the hair matrix (examples with **arrows** in **B** and **C**, and "M" in **G**), continuing into the precortex (PC; **G**) and cortex (C; **G**) of the developing hair shaft. Matriptase expression in club hair (**arrow**) of catagen hair follicles (**E**). Minimal residual expression of matriptase in the club hair of catagen hair follicles (**F**, **arrow**). Matriptase was also expressed in sebocytes of the hair follicle sebaceous gland at all stages of the hair cycle (examples with **stars** in **C** and **F**). **G–K:** Delineation of the cell populations expressing matriptase in the anagen hair follicle. No overlap of matriptase expression with the outer root sheath marker keratin-5 (**G**, **arrowhead**) and possible partial overlap between matriptase and the inner root sheath marker mIRSa in cells of the hair bulb (**H**, **arrowheads**). **Star** in **H** indicates melanin deposits, and **boxed area** indicates anagen hair follicle in cross section. Matriptase expression colocalized with hair precortex and cortex keratins recognized by AE13 antibodies (**K**, **arrowhead**). Matriptase-expressing hair matrix cells were frequently proliferating as shown by Ki67 expression (**I**) and BrdU incorporation (**J**) (**arrowhead**). Sections in **A–F** were counterstained with nuclear fast red, and sections in **G–K** were counterstained with hematoxylin. Scale bars, 50 μm (**A–F**); 25 μm (**G–K**).

unknown. The generation of mice with a β-galactosidase-tagged matriptase gene described here permitted the sensitive, high-resolution mapping of matriptase expression in epithelial development, homeostasis, regeneration, and malignant transformation. The data generated here provide a step forward toward achieving a molecular understanding of matriptase in epidermal biology and pathology.

The epidermis is a complex and highly dynamic structure that consists of multiple types of keratinocytes and other cell types that together form two principal epidermal compartments: the interfollicular epidermis and the hair follicle (see the introduction). Our previous studies showed that matriptase is required for both interfollicular epidermis and hair follicle development.[16,17] The expression data presented here, combined with our previous observations of the diverging effects of epidermal matriptase ablation in the two epidermal compartments, would now suggest that the membrane protease could have distinct molecular functions in the two processes. In the interfollicular epidermis, matriptase expression is very narrowly confined to the uppermost layer of living cells, the transitional cell layer, and it correlates precisely with the onset of terminal differentiation. The transitional cell layer is a single postmitotic cell layer that is in the process of undergoing a rapid and dramatic series of events that lead to the complete transformation of living metabolically active cells into the flattened, lipid-embedded corneocytes that constitute the stratum corneum. These events include the disappearance of the nucleus and other intracellular organelles, the replacement of the plasma membrane with a thick, cross-linked, cornified envelope, the aggregation of cytoplasmic keratins, and the extrusion of large amounts of specialized epidermal lipids. The confinement of matriptase expression to this compartment of the interfollicular epidermis is in agreement with the functional requirement of matriptase for cornified envelope formation and epidermal lipid extrusion and with the lack of detectable alterations in the basal, suprabasal, and spinous layers of matriptase-deficient mice.[16,17] Thus, matriptase is essential for stratum corneum formation through the initiation of a localized series

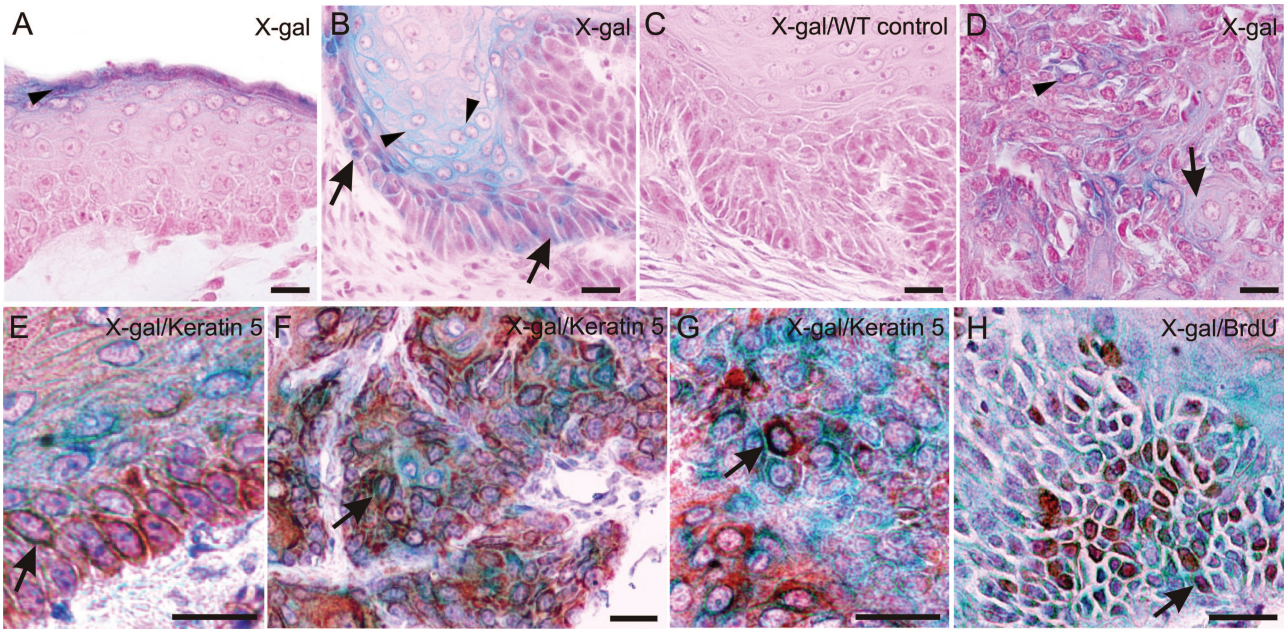

Figure 7. Matriptase expression redistributes during squamous cell carcinogenesis to include keratin-5-positive proliferating cells. Representative histological sections of X-gal staining of matriptase (cyan) (**A–D**) and combined X-gal staining of matriptase (cyan) and immunohistochemistry (brown, example with **arrows**) (**E–H**) of DMBA/PMA-induced epidermal neoplastic lesions at different stages of progression in matriptase$^{+/\beta\text{-gal}}$ mice (**A–C**, **E–H**) and wild-type littermate mice (**C**). **A:** Hyperplastic lesion showing matriptase expression in the uppermost aberrantly differentiating layer of the epidermis (**arrowhead**). **B:** Dysplastic lesion displaying expression of matriptase in large keratinizing well-differentiated cells (examples with **arrowheads**) as well as in small less differentiated cells in proximal to the underlying connective tissue (examples with **arrows**). **D:** Squamous cell carcinoma showing variable expression of matriptase in both poorly differentiated (examples with **arrowheads**) and well-differentiated (examples with **arrow**) tumor cells. **C:** X-gal-stained lesion from a wild-type mouse, demonstrating the specificity of the X-gal staining. **E–G:** Keratin-5 immunohistochemistry showing sporadic colocalization (examples with **arrows**) of matriptase with keratin-5-expressing cells in papillomatous (**E**) lesions, which become frequent in squamous cell carcinoma (**F**, **G**). **H:** Colocalization between matriptase and proliferating BrdU-incorporating cells in malignant lesion (example with **arrow**). Sections in **A–D** were counterstained with nuclear fast red, and sections in **E–H** were counterstained with hematoxylin. Scale bars, 25 μm.

of unknown molecular events in the transitional cell layer, the unraveling of which represents a future challenge. In this respect, it is highly noteworthy that epidermal deletion of prostasin, a membrane-anchored serine protease involved in epithelial water transport, produces a phenotype identical to matriptase, whereas deletion of the serine protease inhibitor SPINK-5 results in the opposite phenotype.[40–43] Furthermore, matriptase, prostasin, and SPINK-5 all appear to be coexpressed in the interfollicular epidermis. It is therefore thoroughly possible that matriptase is part of a proteolytic cascade that is activated during terminal differentiation of the interfollicular epidermis and involves several serine proteases and serine protease inhibitors.

The finding here that matriptase is expressed by proliferating hair matrix cells in the hair follicle growth phase and subsides during follicular regression and rest phases suggests that matriptase could play a direct role in hair follicle growth. This hypothesis is further supported by the previous findings of pelage hair follicle hypoplasia in matriptase-deficient skin and pelage hair follicle hyperplasia in matriptase-overexpressing skin.[16,29] It is tempting to speculate that matriptase expression in the hair follicle matrix serves to proteolytically activate a local reservoir of latent growth factors. In this respect, it should be noted that pro-hepatocyte growth factor, a potential matriptase substrate,[44] is produced by the dermal papillae and is located in direct apposition to the hair follicle matrix and regulates hair follicle growth.[45,46] However, the role of matriptase in pro-hepatocyte growth factor activation in the hair follicle in unclear, because we have found that matriptase-deficient and -sufficient hair follicles respond equally well to exogenous pro-hepatocyte growth factor when explanted *ex vivo* (R. Szabo and T.H. Bugge, unpublished data). Irrespectively, all data in this and previous studies taken together suggest that matriptase performs a molecular function in the hair matrix that is different from that of transitional cell matriptase in the interfollicular epidermis.

An important outcome of the current study was the expansion of the functional role of matriptase to keratinized tissue beyond the epidermis. Matriptase was found to be specifically expressed in the keratinized epithelium of the oral cavity and in keratinizing epithelioreticular cells of the thymus. We have previously shown that matriptase is critical for thymocyte survival,[16] and the analysis of matriptase-deficient mice presented here revealed that matriptase is critical for oral epithelial barrier function. The thymus is a lymphatic organ that exhibits certain unique structural features with similarities to the epidermis including keratinization and expression of late epidermal differentiation-associated proteins. The main cellular constituents of the thymus are thymocytes and epithelioreticular supporting cells. The epithelioreticular cells form a cytoplasmic framework for thymic lymphocytes, and interactions between these two cellular populations play a crucial role in T-cell development.[47] In the thymic medulla, Hassal's corpuscles are composed of

concentrically arranged epithelioreticular cells that form conspicuous keratin swirls.[7,8] Matriptase was expressed in both cortical and medullary epithelioreticular cells and in Hassal's corpuscles, but not in thymocytes. This suggests that matriptase acts in *trans* to promote thymocyte survival through the establishment or maintenance of a functional thymic epithelial microenvironment via as-yet-undefined molecular interactions. In light of the common ontogeny and the histological similarity of interfollicular epidermis and keratinized epithelium of the oral cavity, it is not entirely surprising that matriptase was also revealed here to be essential for the establishment of the oral epithelial barrier. Interestingly, however, whereas gross histological alterations are immediately evident in matriptase-deficient interfollicular epidermis,[16] no histologically apparent alterations in oral epidermis were observed in the original analysis of matriptase-deficient mice[16] or in subsequent studies (K. List and T.H. Bugge, unpublished data). This shows that the loss of the membrane protease can have histologically subtle but functionally important consequences for epithelial functionality. Matriptase is widely expressed in human[30] and mouse epithelium (K. List and T.H. Bugge, unpublished data). Thus, although major histological abnormalities were noted only in the epidermis, hair follicles, and thymus of matriptase-deficient embryos and newborn mice, the membrane protease may have important functions in epithelial development and homeostasis of other organs.

A substantial body of evidence now causally implicates matriptase in epithelial carcinogenesis.[18–29] The data presented here revealed that exposure of the epidermis to tumor-promoting agents caused a spatial dysregulation of matriptase expression that led to the appearance of the membrane protease in keratinocyte populations that do not express matriptase in either the hair follicle or the interfollicular epidermis. Most noteworthy was the emergence of matriptase in keratin-5-positive cells during the transition of neoplastic lesions from benign papilloma to malignant carcinoma. The specific cell type from which squamous cell carcinomas of the skin originate is being debated. However, there are multiple lines of evidence to show that these tumors arise from undifferentiated keratin-5-positive cells with high or unlimited self-renewal capacity that are located in the bulge of the hair follicle or within the basal layer of the epidermis.[48,49] We previously demonstrated that transgenic expression of matriptase in keratin-5-expressing cells sufficed to cause spontaneous multistage squamous cell carcinogenesis and strongly promoted DMBA-induced tumor formation.[29] Taken together, the data suggest that carcinogen-induced *de novo* expression of matriptase in a keratin-5-positive epidermal compartment with high self-renewal capacity may serve to unmask the oncogenic potential of the membrane protease. Studies are in progress to directly challenge this hypothesis.

In summary, the data presented in this paper show that matriptase displays restricted and distinct expression patterns in stratified keratinized epithelium, hair follicles, and squamous cell carcinoma and likely reflects diverging roles of the membrane protease in the three tissues.

Acknowledgments

We thank Dr. Rebecca M. Porter for providing the mIRSa antibody. We thank Drs. Robert Angerer, Silvio Gutkind, and Mary Jo Danton for critically reviewing this manuscript.

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