Matrilysin-1 Mediates Bronchiolization of Alveoli, a Potential Premalignant Change in Lung Cancer

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Matrilysin-1 (also called matrix metalloproteinase-7) is expressed in injured lung and in cancer but not in normal epithelia. Bronchiolization of the alveoli (BOA), a potential precursor of lung cancer, is a histologically distinct type of metaplasia that is composed of cells resembling airway epithelium in the alveolar compartment. We demonstrate that there is increased expression of matrilysin-1 in human lesions and BOA in the CC10-human achaete-scute homolog-1 transgenic mouse model. Forced expression of the matrilysin-1 gene in immortalized human normal airway epithelial BEAS-2B and HPLD1 cells, which do not normally express matrilysin-1, promoted cellular migration, suggesting a functional link for BOA formation via bronchiolar cell migration. In addition, matrilysin-1 stimulated proliferation and inhibited Fas-induced apoptosis, while a knockdown by RNA interference decreased cell growth, migration, and increased sensitivity to apoptosis. Western blotting demonstrated increased levels of phospho-p38 and phospho-Erk1/2 kinases after matrilysin-1 expression. Gene expression analysis uncovered several genes that were related to cell growth, migration/movement, and death, which could potentially facilitate bronchiolization. *In vivo*, the formation of BOA lesions was reduced when CC10-human achaete-scute homolog-1 mice were crossed with matrilysin-1 null mice and was correlated with reduced matrilysin-1 expression in BOA. We conclude that matrilysin-1 may play an important role in the bronchiolization of alveoli by promoting proliferation, migration, and attenuation of apoptosis involving multiple genes in the MAP kinase pathway. (*Am J Pathol* 2009, 175:592–604; DOI: 10.2353/ajpath.2009.080461)

Lung cancer is the leading cause of cancer deaths in both men and women in the United States. Despite advances in therapy, the overall 5-year survival rate for lung cancer patients remains only 16%.¹ The poor survival is largely due to the advanced stage of disease at diagnosis. To date, screening trials have had no significant impact on survival.² If lung cancer could be identified earlier, at a pre-malignant stage before angiogenesis, invasion, and micrometastases occur, it would improve survival. Unfortunately, we have limited understanding on the pathobiology of progenitor lesions that relate to lung cancers.

Bronchiolization of the alveoli (BOA) is a metaplastic lesion characterized by cells resembling bronchiolar epithelium lining normal or thickened alveolar walls, often in acinar formation. BOA occurs in a variety of pathological conditions, including inflammation, chemical irritation and exposure to carcinogens.^{3–5} Up to 12% of non-small cell lung cancer resection specimens contain BOA. Our previous studies showed focal p53 expression in advanced human BOA lesions, while *c-myc* mRNA and c-Jun protein were observed in all grades suggesting that BOA may be a potential pre-malignant lesion in lung.⁵ Subsequent to these findings, we established a transgenic BOA mouse model in which constitutive ex-

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Table 1. List of Antibodies for Immunohistochemistry and Western Blot

Antibody M = monoclonal, P = polyclonal	Source	Dilution		Positive control (IHC)
		IHC	WB	
CC10 (P)	Dr. Franco DeMayo	1:100,000		Normal airway
Cytokeratin 8 (P)	The Binding Site	1:100		Normal airway
Cytokeratin 14 (P)	The Binding Site	1:100		Squamous cell carcinoma
β -Tubulin (P)	Dr. Charles Bevins	1:1000		Normal airway
SPC (P)	Chemicon	1:1000		alveoli
Fas (P)	Santa Cruz	1:200	1:500	Jurkat cell
CEA (P)	Dako		1:1000	Colon cancer
β -Actin (M)	Sigma		1:10000	
Matrilysin-1 (M)	Dr. Barbara Fingleton	1:500	1:2000	Lung cancer
Matrilysin-1 (M)	NeoMarker	1:200		Lung cancer
Phospho-P38 MAPK (P)	Cell Signaling Tech		1:1000	
Phospho-Erk1/Erk2 (P)	Cell Signaling Tech		1:1000	
Phospho-SAPK/JNK (P)	Cell Signaling Tech		1:1000	
WASF3 (P)	Santa Cruz		1:200	
MGMT (M)	Chemicon		1:1000	
AKT3 (P)	Upstate		1:1000	

pression of human achaete-scute homolog-1 (hASH1) under the Clara cell specific CC10 promoter leads to airway cell dysplasia and BOA.^{6,7} Further evidence that BOA is a pre-malignant change came from studies showing that bronchiolization was associated with increased airway cell proliferation and marked reduction in apoptosis involving multiple genes implicated in lung carcinogenesis.⁸

To elucidate the mechanism of BOA formation we focused on matrilysin-1. Matrilysin-1, which is also called matrix metalloproteinase (MMP)-7, is the smallest of the known MMPs.⁹ Typically undetectable in normal or resting tissues, MMP production and activity are up-regulated during repair or remodeling. Matrilysin-1 was of interest because of its unique expression pattern almost exclusively in the BOA lesions of transgenic mice. Furthermore, unlike many other MMPs that are highly expressed in the stroma, matrilysin-1 is expressed in epithelial cells, which are the targeted tissue in our model containing little inflammation or extracellular matrix. We showed constitutive expression of matrilysin-1 in both human and mouse BOA lesions. *In vitro* matrilysin-1 promoted cell migration, growth, and resistance to apoptosis when transfected into immortalized normal human airway epithelial cells. This was associated with a parallel gene expression profile of cell growth, migration/mobility, and death. Finally *in vivo*, BOA lesions were diminished in the lungs of CC10-hASH1 transgenic mice crossed with matrilysin-1-null mice. Our results indicate that matrilysin-1 plays an important role in BOA formation. Our study also contributes to further understanding of the mechanism of BOA.

Materials and Methods

Animals, Immunohistochemistry, and Laser Capture Microdissection

CC10-hASH1 (mouse model of BOA), CC10-SV40-Tag (mouse model of lung adenocarcinoma), and CC10-hASH1/SV40-Tag (mouse model of lung neuroendocrine

carcinoma) transgenic mice were generated, and genotyped as previously described.⁷ Matrilysin-1-null mice were purchased from Jackson Laboratories, Bar Harbor, ME and crossed with CC10-hASH1 mice. All animals were housed disease free and handled in a humane manner at a facility accredited by, and following the guidelines of a protocol approved by the National Cancer Institute Animal Care and Use Committee.

Human and mouse BOA lesions were identified as previously described.^{5,8} Patients were enrolled in Institutional Review Board-approved clinical protocols.

Immunohistochemistry (IHC) on paraffin-embedded tissues was done using avidin-biotinylated peroxidase method and Vectastain ABC kits (Vector, Burlingame, CA). The primary antibodies (Table 1) were incubated overnight at 4°C and signals were visualized using 3, 3'-diaminobenzidine tetrahydrochloride, as previously described.¹⁰ Tissue sections were counterstained with Mayer's hematoxylin or Light Green.

Laser capture microdissection (LCM) was performed using frozen sections as previously described.⁸ The LCM samples for quantitative real-time polymerase chain reaction (qRT-PCR) were stored at -80°C until used.

Cell Culture, Analysis of Growth, Apoptosis, Cell Cycle, and Expression of FAS

The immortalized human normal bronchial epithelial cell line BEAS-2B and human lung adenocarcinoma cell lines NCI-H23 and A549 were purchased from the American Type Culture Collection (Manassas, Virginia). The immortalized human normal bronchiolar epithelial cell line HPL1D was a gift from Dr. Takashi Takahashi (Aichi Cancer Center Research Institute, Division of Molecular Oncology, Nagoya, Japan). The small cell lung cancer cell line DMS53 was a gift from Dr. Douglas Ball (Johns Hopkins University, Baltimore, MD) and the lung carcinoma cancer cell line NCI-H727 was a gift from Dr. Adi Gazdar (University of Texas Southwestern Medical Center, Dallas, TX).

The BEAS-2B and HPL1D cells were cultured in bronchial epithelial cell growth medium with “bullet kit” additives (Cambrex Bio-science, Walkersville, MD) composed of growth factors and antibiotics. Both H23 and H727 cell lines were grown in RPMI 1640 (Invitrogen Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum, DMS53 was cultured in Waymouth's medium (Invitrogen Life Technologies, Rockville, MD) with 15% fetal bovine serum and A549 was grown in DMEM with 10% fetal bovine serum.

To establish growth curves, a total of 1×10^5 /dish or 0.5×10^5 /well BEAS-2B cells were plated in triplicate in 60-mm tissue culture dishes or in six well plates in complete medium. Viable cells were determined via trypan blue exclusion every 24 hours by hemocytometer after cells were seeded.

5-bromo-2'-deoxyuridine (BrdU) flow kit (BD Pharmingen, San Diego, CA) was used for cell cycle analysis and the detection of BrdU incorporation following manufacturer's instructions. For the negative control, the primary antibody was omitted. The stained cells were analyzed by fluorescence-activated cell sorting (FACS) scan (BD, Franklin Lakes, NJ), using the FlowJo 7.1.3 software (Tree Star, Inc. Ashland, OR).

Flow cytometry analysis of apoptosis was performed as described elsewhere.¹¹ Gating was performed on blinded samples. Experiments were repeated three times.

To analyze Fas expression, the cell suspension was adjusted to a concentration of $1-5 \times 10^6$ cells/ml in ice cold 2% BSA/PBS/0.02% sodium azide. $2 \mu\text{g}/10^6$ cells, Fas primary antibody conjugated to FITC or control isotypic antibody (BD) were added to cell suspension and incubated for at least 30 minutes on ice in dark. The cells were washed three times by centrifugation at 400 g for 5 minutes and resuspended in ice cold 2% BSA/PBS/0.02% sodium azide. The stained cells were analyzed by FACS scan (BD, Franklin Lakes, NJ), using the FlowJo program.

Matrilysin-1 Transfection and RNA Interference Using Lentiviruses

A pcDNA 3.1/myc-His Expression Kit (Invitrogen) was used for construction of the plasmid. For the transfection of human immortalized bronchial epithelial cell line BEAS-2B and bronchiolar (small airways) epithelial cell line HPL1D, the full length of matrilysin-1 gene, which expresses pro-matrilysin-1, was inserted into pcDNA 3.1/myc-His. For stable expression of matrilysin-1, following G418 (Invitrogen) $150 \mu\text{g}/\text{ml}$ treatment in the BEAS-2B cells derived from a single colony were cultured at least 4 weeks with selective antibiotic. The expression of the gene was tested by IHC, confocal microscopy, enzyme-linked immunosorbent assay (ELISA), qRT-PCR, and western blotting.

To reduce Matrilysin-1 expression a short hairpin (sh)RNA mir GIPZ lentiviral vector targeting matrilysin-1 was used (Open Biosystems, Huntsville, AL). TLA-HEK293T cells (Open Biosystems) were transfected with

the *Trans*-Lentiviral Packaging Mix and pGIPZ transfer vector at 50% confluence using Arrest-In transfection reagent (Open Biosystems) according to the manufacturer's instructions. BEAS-2B/Matrilysin-1 clone 6 (BEAS-2B/C6) cells with the matrilysin-1 specific shRNA or a non-silencing shRNA (negative control) were selected for stable integration.

ELISA, Zymography, Migration Assays

Protein concentrations of MMPs released into the conditioned media of cultured cells were measured by ELISA using commercially available ELISA kits (Amersham Biosciences, Piscataway, NJ).¹² Assays were performed as described in the manufacturer's protocol. The ELISA kit for pro-matrilysin-1 (28 kDa, latent form) does not cross-react with MMP-1, -2, -3, -8, -9, or MT1-MMP. All experiments were done at least twice in triplicate.

Zymography was used to measure the activity of MMPs in conditioned media. Protein concentration was measured by the Bradford method (Pearce, Rockford, IL). Proteolytic activity of matrilysin-1 and MMP-9 was evaluated by β -casein zymography.

For migration assay 1×10^5 cells/well were plated onto 24-well uncoated inserts ($8 \mu\text{m}$ pore size) (BD Biosciences Discovery Labware, Bedford, MA) and incubated at 37°C , 5% CO_2 atmosphere for 24 to 40 hours. 10% fetal bovine serum and 20 ng/ml IGF were added into the bottom of the wells as chemoattractants. Active rhMat at concentrations of 1 nmol/L, 5 nmol/L, and 25 nmol/L, was added to the upper inserts 2 hours after plating of untransfected cells. Non-migrative cells were removed from the top of the wells with a cotton swab, followed by fixation and staining with 0.25% crystal violet in 25% ethanol. All experiments were completed in triplicate and at least four fields per insert were counted. In addition, cell migration was also assessed using an *in vitro* wound scratch assay as described elsewhere.¹³

qRT-PCR and Western Blot

Total RNA was extracted from LCM samples using RNeasy Micro Kit (Qiagen, Valencia, CA) and from cultured cells using RNeasy Minikit followed the manufacturer's protocol. Quantitative RT-PCR was performed as previously described⁸ (Table 2). The PCR reactions for the target gene and GAPDH or β -actin were performed in separate tubes to avoid possible competition and/or interference in a single reaction tube.^{14,15}

Western blotting was performed using the Criterion system (Bio-Rad, Hercules, CA). Cells were lysed in ice-cold lysis buffer. Primary antibodies (Table 1) were detected using horseradish peroxidase-linked secondary antibodies and visualized with chemiluminescence (Amersham Biosciences).

Gene Microarray Analysis

Total RNA was prepared from BEAS-2B vector control and matrilysin-1-expressing clone 6 (C6) cells using

Table 2. Sequences of the Oligonucleotide Primers and Probes Used for Quantitative RT-PCR

Sequences of the oligonucleotide (Genebank Access Number)	Location (bp)
Mouse GAPDH (NM-008084)	
Forward: 5'-CTCAACTACATGGTCTACATGTTCCA-3'	158 to 183
Reverse: 5'-CCATTCTCGGCCTTGACTGT-3'	234 to 215
Probe: 5'-FAM-TGACTCCACTCACGGCAAATTCAACGT-TAMRA-3'	187 to 212
Mouse matrilysin-1 (NM_010810)	
Forward: 5'-TAGGCGGAGATGCTCACTTT-3'	606 to 625
Reverse: 5'-TTCTGAATGCCTGCAATGTC-3'	816 to 797
Probe: 5'-FAM-CACTCTCTGGGTCTGAGTCACTCTTCTGTT-TAMRA-3'	701 to 730
Human β -Actin (CR624852)	
Forward: 5'-TGAGCGCGGTACAGCTT-3'	657 to 673
Reverse: 5'-TCCTTAATGTCACGCACGATT T-3'	715 to 694
Probe: 5'-FAM-ACCACCACGGCCGAGCGG-TAMRA-3'	675 to 692
Human matrilysin-1 (BC003635)	
Forward: 5'-TGGTAGCAGTCTAGGGATTAACCTCCT-3'	631 to 657
Reverse: 5'-CATAGGTTGGATACATCACTGCATTA-3'	740 to 715
Probe: 5'-FAM-TGCTGCAACTCATGAACCTGGCCATT-TAMRA-3'	661 to 686
Human AGTPBP1 (XM_520100)	
Forward: 5'-TGTGATTATCATGGCCATTCC-3'	3008 to 3028
Reverse: 5'-TCCTCCACAACATCACATGAA-3'	3120 to 3100
Human AKT3 (NM_005465)	
Forward: 5'-TGGACAAGATGGCCACATA-3'	917 to 936
Reverse: 5'-ACTGCTCGCCATAGTCATT-3'	1061 to 1042
BNIP3 (BC009603)	
Forward: 5'-GCTGGAACACGTACCATCCT-3'	260 to 279
Reverse: 5'-ATCTGCCATCTTCTTGTTGG-3'	403 to 384
Human FBN1 (NM_000138)	
Forward: 5'-CAAGAGGATGGAAGGAGCTG-3'	7757 to 7776
Reverse: 5'-GGTAAATCCGGGAGGACATT-3'	7876 to 7857
GSPT1 (BC009503)	
Forward: 5'-CCGTTTGTGCAACAAGATCAA-3'	1892 to 1911
Reverse: 5'-GCAATGGTCTTACCCTCATCTC-3'	2020 to 2001
Human HRASLS (NM_020386)	
Forward: 5'-AGAGACCCAGGACACACAC-3'	342 to 361
Reverse: 5'-TAGCCAGGACGGAACACTTC-3'	488 to 469
Human KRT14 (NM_000526)	
Forward: 5'-TGAGAGGCCAGGTGGGTG GAG AT-3'	840 to 862
Reverse: 5'-CCAGCTGCATGCAGTAGCGACCTT TG-3'	1172 to 1147
Human MGMT (NM_002412)	
Forward: 5'-GCAATGAGAGGCAATCCTGT-3'	532 to 561
Reverse: 5'-CAGTCCTCCGGAGTAGTTGC-3'	621 to 602
Human PRKDC (NM_006904)	
Forward: 5'-TCCTTGCCACACAACATCAT-3'	2260 to 2279
Reverse: 5'-GCATTCAAGGCCTACTTCTGC-3'	2372 to 2353
Human RRAGD (NM_021244)	
Forward: 5'-AGGAGGAGGAGGAGGATGAG-3'	119 to 138
Reverse: 5'-GGATTCTCGGCTTCACTTCA-3'	262 to 243
Human SERPINA3 (NM_001085)	
Forward: 5'-CGTGGTGGAGCTGAAGTACA-3'	865 to 884
Reverse: 5'-GAGTCTCTCCACCGCTTCAG-3'	984 to 865
SLITRK5 (NM_015567)	
Forward: 5'-GCTCTTGAGCAGCATGGATA-3'	876 to 895
Reverse: 5'-GATGCTGTCCAACCAATCCT-3'	978 to 959
Human SMARCA2 (BC040029)	
Forward: 5'-GAAGAGATTCAAGCCAGCACAC-3'	29 to 49
Reverse: 5'-CGTGGACATCTACCTCTCCTG-3'	108 to 89
Human WASF3 (NM_006646)	
Forward: 5'-TGACGGTAGGAATGTGAGCA-3'	3237 to 3256
Reverse: 5'-GCCACTCGAGACACACACAC-3'	3341 to 3322
GOS2 (NM_015714)	
Forward: 5'-AGGAGGAGAACGCTGAGGTC-3'	37 to 56
Reverse: 5'-CGTACAGCTTCACCATCTTCC-3'	244 to 224
Human PHLDA1 (AF220656)	
Forward: 5'-CTTGGAGAATAAAAAAGGGCGG-3'	586 to 607
Reverse: 5'-GCTGGCACAACAATGAAAGTGTC-3'	687 to 665
SNN (AF070673)	
Forward: 5'-TTGTTCATCCTCATTTGCCATCCTGACT-3'	222 to 241
Reverse: 5'-GCTCTCCTCGTCTCTGACT-3'	322 to 303
Mouse MMP-2 (NM_008610)	
Forward: 5'-GCTGATACTGACACTGGTACTG-3'	1648 to 1669
Reverse: 5'-CAATCTTTTCTGGGAGCTC-3'	1864 to 1846
Mouse MMP-9 (NM_013599)	
Forward: 5'-CTTTGAGTCCGGCAGACAAT-3'	1583 to 1602
Reverse: 5'-TTCCAGTACCAACCGTCCCTT-3'	1682 to 1663

Trizol according to the manufacturers' instructions (Invitrogen Corp., Carlsbad, CA). Twenty-five ng of total RNA was double amplified using the two-cycle amplification method (Affymetrix, Santa Clara, CA) for linear amplification of total RNA. A 15 μ g aliquot of labeled cRNA was fragmented by heat and ion-mediated hydrolysis, and subsequently hybridized to a Human U133A oligonucleotide array (ca. 283000 probe sets) (Affymetrix). The arrays were washed and stained in a Fluidics Station 450 (Affymetrix), and then scanned using a confocal laser GeneChip Scanner 3000 (Affymetrix) and GeneChip Operating Software (Affymetrix). Biometric Research Branch (BRB) ArrayTools version 3.2.2 software developed by Dr. Richard Simon and Amy Peng Lam (NCI/NIH, Bethesda, MD) (<http://linus.nci.nih.gov/BRBArrayTools.html>) was used to compute the Robust Mathematical Analysis expression values.¹⁶ 11141 probe sets whose expression variances were in the top 50th percentile were retained. A list of 2050 probe sets containing <10% false positives at 80% confidence with a differential gene expression significance at $P < 0.001$ was generated using the Exact Multivariate Permutation test in BRB ArrayTools (NCI/NIH, Bethesda, MD).

Genes identified by BRB Array Tools were used for network and gene ontology analyses. Gene accession numbers were imported into the Ingenuity Pathway Analysis version 3.1 software (Ingenuity Systems, Mountain View, CA), and the gene products were categorized based on location, cellular components, and reported or suggested biochemical, biological, and molecular functions using the software. Mapping to genetic networks available in the Ingenuity database was also performed with ranking by score, the probability that a collection of genes equal to or greater than the number in a network could be achieved by chance alone. A score of three indicates that there is a 1/1000 chance that the focus genes are in a network due to random chance. Therefore, scores of three or higher have a 99.9% confidence level of not being generated by random chance alone. This score was used as the cut-off for identifying gene networks. Eighteen genes were validated using qRT-PCR and three genes were chosen for further validation by Western blotting and one gene by IHC.

Results

BOA and Lung Tumors Express Increased Levels of Matrilysin-1

By definition, BOA is a metaplastic lung lesion where airway cells can be detected in the alveolar compartment normally containing only type I and type II epithelial cells.⁴ Figure 1A (left panel) is a photomicrograph of a normal terminal bronchiolus (TB) whose epithelium terminates promptly at the bronchioalveolar duct junction (BADJ), which represents the border between airways and alveoli. In wild-type mice the demarcation was sharp. The transgenic CC10-hASH1 mice with BOA featured progressive growth of airway epithelial cells at the BADJ and expansion to the alveolar compartment (Figure 1A,

right panel). By 16 months of age, many of the cells in BOA lesions appeared ciliated (inset) and bronchiolar type epithelium was seen lining normal or slightly thickened alveolar walls as described by Nettekoven and Szakal.⁴

To verify the cellular differentiation patterns of BOA lesions we used IHC to detect bronchiolar markers (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). Both mouse and human BOA lesions demonstrated strong immunoreactivity for the markers of airway epithelial cells such as cytokeratin 8 and 18 (not shown), CC10 in Clara cells, and β -tubulin in ciliated cells, while they were in general negative for the type II cell marker SP-C.

The epithelial specific matrilysin-1 is typically expressed at low levels in normal airways, but its expression is up-regulated under a variety of pathological conditions in the lung.^{17–19} To measure the amount of matrilysin-1 in tissues we used qRT-PCR. Highest levels of matrilysin-1 mRNA were detected in the microdissected BOA specimens from CC10-hASH1 transgenic mice (Figure 1B). In addition, we were able to measure increased levels in 5 out of 5 lung specimens from CC10-hASH1 transgenic mice with BOA and CC10-hASH1/SV40-Tag mice with pulmonary neuroendocrine carcinomas.⁶ Only 2 out of 5 wild-type lungs or lungs from CC10-SV40-Tag mice containing pulmonary adenocarcinomas expressed low levels of matrilysin-1 (Table 3). Well-characterized human pulmonary epithelial cell lines expressed variable levels of matrilysin-1 mRNA (Table 4). While the relative expression in the immortalized normal human bronchial (BEAS-2B) and bronchiolar (HPL1D) epithelial cells was low (1.0 and 0.75, respectively), the expression in the lung adenocarcinoma cell line A549 was elevated.³⁵ In a parallel fashion, increased levels of pro-matrilysin-1 protein were also detected in the culture media of A549 cells by ELISA. As seen in Figure 1, mouse (Figure 1C) and human (Figure 1D) BOA lesions as well as lung tumors revealed increased expression of matrilysin-1 while normal airway epithelium was negative. Matrilysin-1 immunoreactivity, which was cytoplasmic, was observed mainly in the epithelial cells and scattered macrophages, while the stroma was negative. This distribution confirms that matrilysin-1 is primarily an epithelial metalloproteinase.^{17–19}

Taken together our results show that matrilysin-1 is low in normal lung and airway epithelial cells, but becomes elevated during remodeling such as BOA and carcinogenesis, and is released to the media.

Matrilysin-1 Promotes Migration and Proliferation of Airway Epithelial Cells in Vitro

The proposed mechanism of BOA in CC10-hASH1 transgenic mice involves proliferation and migration of distal airway epithelial cells into the alveolar compartment at the BADJ.⁸ To examine the functional impact of increased matrilysin-1 expression *in vitro*, we expressed matrilysin-1 in the immortalized normal human bronchial (BEAS-2B) and bronchiolar (HPLD-1) epithelial cells that normally are void of matrilysin-1. Following a stable trans-

Figure 1. Morphology of BOA and expression of matrilysin-1 during lung tumorigenesis. **A:** Normal (NL; **left** panel) peripheral airway epithelium in wild-type mouse composed of a single layer of Clara cells protruding to the lumen. The demarcation toward alveoli at BADJs (**arrows**) is sharp. Prominent BOA formation (**right** panel) extends from the BADJ toward the alveoli composed of bronchiolar-type cells in a 16 month old CC10-hASH1 transgenic mouse. **Inset** shows ciliated cells of BOA. (H&E stain, scale bar = 50 μ m, inset by $\times 60$ objective). **B:** Bar graph of relative expression of matrilysin-1 mRNA by qRT-PCR following microdissection of the epithelium. Expression is highest in BOA from the transgenic (H) mice. BL = bronchiolus, TB = terminal bronchiolus, BOA = bronchiolization of alveoli, BADJ = bronchioalveolar duct junctions. No matrilysin-1 was seen in normal mouse (**C; top**) or human (**D; bottom**) airways. Scattered matrilysin-1-containing cells in mouse and human BOA. Tumors from mouse and human lung were diffusely positive for matrilysin-1. (**C** and **D**: immunoperoxidase stain; scale bar = 50 μ m).

fection of the full length matrilysin-1 gene in BEAS-2B cells, one clone with high level (C6) and another clone with low level (C2) of matrilysin-1 mRNA expression were

chosen for further experiments. BEAS-2B/C6 cells demonstrated over tenfold increase in the expression of matrilysin-1 mRNA compared with that in C2 cells, which

Table 3. Matrilysin-1 mRNA Expression in Wild-Type and Transgenic Mice

Genotype	Lung phenotype	n	Matrilysin-1 mRNA by qRT-PCR (relative level)		
			Positive samples	Mean $\pm$ SD*	Range
Wild-type	Normal	5	2/5	2.19 $\pm$ 1.24	0~3.07
CC10-hASH1	BOA	5	5/5	89.09 $\pm$ 147.09	6.36~350.61
CC10-SV40	Adenocarcinoma	5	2/5	0.07 $\pm$ 0.01	0~0.08
CC10-hASH1/SV40	Neuroendocrine cancer	5	5/5	2.31 $\pm$ 2.78	0.06~6.81

*Only matrilysin-1 positive samples were calculated.

Table 4. Matrilysin-1 Level in Conditioned Media and mRNA Expression in Immortalized Normal Airway Epithelium and Lung Cancer Cell Lines

Cells	Type	Matrilysin-1 mRNA (relative expression)	Pro-Matrilysin in culture media (ng/ml)*
BEAS-2B	Immortalized normal bronchial epithelial cell	1	0.06 ± 0.02
HPL1D	Immortalized normal bronchiolar epithelial cell	0.75	0.13 ± 0.07
NCI-H727	Lung carcinoid	0.21	0.23 ± 0.04
DMS53	SCLC	0.80	0.24 ± 0.07
NCI-H23	Lung adenocarcinoma	1.73	0.66 ± 0.28
A549	Lung adenocarcinoma	35.22	11.53 ± 0.51

*Pro-matrilysin detected by ELISA.

was associated with a comparable increase in the pro-matrilysin-1 protein detected in the culture media (Figure 2A). Both pro- and active matrilysin-1 were increased in concentrated conditioned media from BEAS-2B/C2 and C6 cells. By IHC we detected matrilysin-1 in cytoplasmic location in BEAS-2B/C6 cells (Figure 2A). There was up to threefold increase in migration of BEAS-2B/C6 cells (Figure 2B), when BEAS-2B/V cells, which were transfected with the empty vector (V) were used as control. Similar increase in migrating cells was noted when increasing concentrations of enzymatically active rhMat were added into the culture medium of parental BEAS-2B cells (Figure

2C). Moreover, the cellular migration doubled in the immortalized human bronchiolar HPLD-1 cells following transient expression of matrilysin-1 (Figure 2, D and E). To confirm the specific contribution of matrilysin-1 to cellular migration needed for bronchiolization, we knocked down matrilysin-1 expression fivefold by specific shRNA in BEAS-2B/C6 cells (Figure 2F, left panel) and studied the impact on cell migration using the scratch wound assay (Figure 2F middle panel). As a consequence, the closure of the wound field by the cells was impaired almost threefold when compared with the cells transfected with non-silencing shRNAs

Figure 2. Matrilysin-1 promotes migration of human airway epithelial cells *in vitro*. **A:** Stable expression of matrilysin-1 in immortalized normal human bronchial epithelial BEAS-2B cells was established using a pcDNA 3.1/myc-His vector. Two clones were used for experiments. pcDNA3.1-MatC2 (C2) is a low expressor, and pcDNA3.1-MatC6 (C6) a high expressor. The **left** panel shows relative mRNA level by qRT-PCR, and the **middle** panels show matrilysin-1 protein in 10× concentrated conditioned media by ELISA and Western blotting. The **right** panel reveals increased cytoplasmic matrilysin-1 using fluorescence immunostaining in C6 cells viewed by confocal microscopy. Nuclei were counterstained by DAPI (blue). V = cells transfected with empty vector (control). **B:** Cell migration was examined using uncoated chambers. A two to three-fold increase of migrative ability was found in matrilysin-1 expressing clones C2 and C6 compared with BEAS-2B/vector only cells (V). **C:** In the **top** panel, a zymograph assay demonstrated increasing proteolytic activity of the conditioned media at 19 kDa when a BEAS-2B cell line was treated by active human recombinant matrilysin-1 (rhMat) for 48 hours at 0, 1, 5, 25 nmol/L concentrations. In the **bottom** panel, the migration of BEAS-2B cells was increased significantly depending on the concentration of rhMat. **D:** Matrilysin-1 was transfected into an immortalized human bronchiolar epithelial HPL1D cells. Matrilysin-1 protein level in the media was significantly increased. **E:** Cell migration was increased in HPL1D-matrilysin-1 containing cells (Mat). **F:** Knockdown of matrilysin-1 expression by interfering shRNA in BEAS-2B/C6 cells (**left** panel). In a wound closure assay the knockdown of matrilysin-1 also impaired migration (**middle** and **right** panels; Mat shRNA = matrilysin-1 shRNA; NS shRNA = non-silencing shRNA). **G:** Pro- and active MMP-2 and MMP-9 were increased in BEAS-2B-MatC6 conditioned media by WB and zymograph, respectively. MMP-2 was also increased in the low expressor C2. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Figure 3. Expression of matrilysin-1 in human bronchial epithelial BEAS-2B cells promotes proliferation. **A:** Transfection of matrilysin-1 into the normal immortalized human bronchial epithelial BEAS-2B cells was associated with significantly increased BrdU incorporation in clones C6 ($P < 0.001$) and C2 ($P = 0.003$) compared with empty vector (V) measured by flow cytometry. Treatment with MMP inhibitors (GM6001 and MMP inhibitor III) decreased BrdU incorporation in the cells. **B:** Growth curve of clones C2, C6, and V of BEAS-2B cells. **C:** Cell cycle analysis demonstrates a low population of cells in G0/G1, but the population is higher in S phase in clone 6. **D:** No changes in BrdU incorporation was seen in BEAS-2B cells treated by rhMat. **E:** Transfection efficiency of GFP-labeled shRNAs in BEAS-2B/C6 cells (top panel). Growth curve showing reduced growth by the matrilysin-1 shRNA (Mat shRNA) interference in the cells (bottom panel). (NS shRNA = non-silencing shRNA). ** $P < 0.01$, *** $P < 0.001$.

(Figure 2F, right panel). The results further support the concept that cell migration contributes to BOA formation. Furthermore, overexpression of matrilysin-1 increased both pro- and active MMP-2 and MMP-9 levels in conditioned media, indicating that other MMPs may also be involved (Figure 2G).

To examine the contribution of matrilysin-1 expression to proliferation we studied BrdU incorporation and growth of transfected immortalized human bronchial epithelial BEAS-2B cells. We were able to show small but statistically significant increases in BrdU labeling rates in BEAS-2B/C2 and C6 cells that expressed matrilysin-1 when compared with BEAS-2B/V cells containing only the empty vector (Figure 3A). When the cells were treated with the MMP inhibitors GM6001 or MMP inhibitor III, BrdU incorporation decreased in C2 and C6 cells, but no significant changes were seen in BEAS-2B/V cells carrying the empty vector. Growth curves showed enhanced growth of both BEAS-2B/C2 and C6 cells over the control BEAS-2B/V cells, and the high matrilysin-1 expressing C6 cells grew faster than C2 cells with lower matrilysin-1 levels (Figure 3B). Moreover, cell cycle analysis revealed a statistically significantly increased population of BEAS-2B/C6 cells in S-phase (Figure 3C). Cell cycle analysis was consistent with BrdU incorporation indicating that matrilysin-1 drives DNA synthesis. However, the treatment of BEAS-2B cells with rhMat had no effect on cell proliferation (Figure 3D). To show that the increase in cell growth was particular of matrilysin-1, we knocked down its expression in BEAS-2B/C6 cells using specific shRNAs (Figure 3E). It reduced the growth by half in 5 days when compared with that in the cells transfected by non-silencing shRNAs.

Matrilysin-1 Inhibits Apoptosis in Vitro

We have previously discovered that cells in the BOA lesions of transgenic CC10-hASH1 mice show reduced apoptosis.⁸ Since one of the potential targets for matrilysin-1 is Fas/FasL-mediated apoptosis,^{20,21} we examined whether Fas pathway were activated in the airway cells and would be blocked by matrilysin-1. By IHC and Western blot we were able to show that both TBs and BOA lesions in the lung as well as BEAS-2B cells expressed Fas receptor, respectively (Figure 4A). To activate the Fas pathway BEAS-2B cells were incubated with different concentrations (0, 0.1, 1, 10, 100, 1000 ng/ml) of Fas agonist antibody (CH11) for 48 hours and subjected to FACS analysis to detect apoptosis by measuring the sub-G1 fraction. The treatment resulted in a threefold increase in the apoptotic rate of BEAS-2B/V which was blocked in the matrilysin-1 containing BEAS-2B/C2 and C6 cells, and the same pattern was seen when the results were normalized by using the isotypic IgM control (Figure 4B).

To examine if the effect of matrilysin-1 on apoptosis can be reversed, we incubated the BEAS-2B cells with the two general MMP inhibitors GM6001 and MMP inhibitor III. No significant changes were found after the treatment of MMP inhibitors compared with treatment of CH11 antibody alone (data not shown). To further address the particular impact of matrilysin-1 on apoptosis, we knocked down the expression of matrilysin-1 in BEAS-2B/C6 cells by specific shRNAs. The results are shown in Figure 4C. Following the incubation with the Fas agonist CH11 antibody at 100 ng/ml, there was a 40% decrease in the apoptotic rate in BEAS-2B/C6 cells compared with that of control (V) cells, while the basic fraction of apo-

Figure 4. Matrilysin-1 attenuates apoptosis in human bronchial epithelial cells. **A:** **Top** panel with intense immunoreactivity for Fas in the distal airway epithelium and BOA (red arrows) in the CC10-hASH1 transgenic mouse (Immunoperoxidase staining; scale bar = 50 μ m; TB = terminal bronchiolus). **Bottom** panel: Western blot analysis of Fas expression in the immortalized normal human bronchial epithelial BEAS-2B and Jurkat (positive control) cells. **B:** Following treatment by increasing doses of the Fas agonist antibody (CH11), the apoptotic rate in the BEAS-2B cells was obtained by determining the sub-G1 population via FACS analysis. **Top:** Both matrilysin-1 expressing C2 and C6 clones revealed a low apoptotic cell population (percent) compared with vector (V). No difference in apoptosis was seen by treatment with IgM isotypic control. **Bottom:** Apoptotic rate normalized by IgM isotype was low in clones C2 and C6. **C:** Apoptotic rate (percentage of sub-G1 population; mean $\pm$ SD) detected by FACS following the treatment by the Fas agonist antibody (CH11; 100 ng/ml). Expression of matrilysin-1 in C6 cells reduced apoptosis compared with V control. Knockdown of matrilysin-1 by shRNA increased apoptosis. (NS shRNA = non-silencing shRNA; Mat shRNA = matrilysin-1 shRNA).

ptotic cells was unchanged. However, the knockdown of matrilysin-1 in the BEAS-2B/C6 cells increased apoptosis twofold in both unstimulated and CH11-stimulated C6 cells when compared with the control cells. While these experiments confirmed that matrilysin-1 may protect airway cells from apoptosis, the molecular mechanism remained elusive. It has been previously reported that the decreased sensitivity to Fas-mediated apoptosis in tumor cells may be due to decreased expression of the cell surface Fas receptor via proteolytic activity of MMP-7.²¹ To test if matrilysin-1 prevented Fas-induced apoptosis through the cleavage of Fas, we measured Fas expression using FACS. BEAS-2B cells were treated with increasing concentrations of CH11, but there was no difference in the amount of FAS in the BEAS-2B/C2 and C6 cells containing matrilysin-1 compared with the negative BEAS-2B/V cells (see Supplemental Figure S2 at <http://ajp.amjpathol.org>).

Gene Microarray Analysis Uncovered the Target Genes of Matrilysin-1

To discover potential target genes and pathways for matrilysin-1 in the airway epithelium, we performed cDNA microarray analysis on BEAS-2B cells. The hybridization results with BEAS-2B/C6 with highest matrilysin-1 content were normalized by BEAS-2B/V cells that did not express matrilysin-1. Labeled cRNA was hybridized to an Affymetrix Human U133A oligonucleotide array. After normalization, 2050 probe sets were differentially expressed at a false discovery rate of less than 10% with 80% confidence. Out of 2050 probe sets, we identified 130 probe sets that were down-regulated and 143 probe sets that were up-regulated $\geq$ twofold and displayed at

$P < 0.0001$ (see Supplemental Table S1 at <http://ajp.amjpathol.org>). Using the Ingenuity Pathway Analysis tool, 21 networks were found associated with matrilysin-1 expressing BEAS-2B/C6 cells. Networks with high scores (>13) in which more than half of the genes were affected, are listed in the supplementary material (see Supplemental Table S2 at <http://ajp.amjpathol.org>). They were associated with cell growth and proliferation, cell movement, cell death, cell cycling, and cell-to-cell signaling. The colored genes are those identified by microarray analysis, red for down-regulated and blue for up-regulated (see Supplemental Table S2 at <http://ajp.amjpathol.org>). We also performed gene ontology analysis using the Ingenuity Pathway Analysis tool, which yielded 101 genes that were identified as cellular growth and proliferation associated genes, 60 genes were associated with cellular movement and 84 genes were related with cell death (Table 5).

Eighteen of the differentially expressed genes were validated with qRT-PCR in BEAS-2B cells and the results obtained from 16 out of the 18 genes concurred with the microarray data, yielding a 89% validation rate. The comparative mRNA expression data of the 16 genes that are related to cell migration (WASF3, KRT14), proliferation/apoptosis (AGTPBP1, MGMT, SERPINA3,

Table 5. Ontology Analysis of Proliferation, Cellular Movement, and Cell Death-Related Genes in BEAS-2B/Matrilysin-1 Cells

Related functions	Significance	Associated genes
Cellular growth and proliferation	4.05E-6~3.06E-2	101
Cell movement	2.18E-5~3.05E-2	60
Cell death	9.35E-4~2.91E-2	84

Table 6. Migration, Proliferation, and Apoptosis-Related Genes Differentially Expressed in BEAS-2B/V and BEAS-2B/ Matrilysin C6 and Validated by qRT-PCR

LocusLink ID	Gene symbol	Description	Microarray	qRT-PCR
204042	<i>WASF3</i>	WAS protein family, member 3	4.94	5.98
204500	<i>AGTPBP1</i>	ATP/GTP binding protein 1	2.95	12.65
204880	<i>MGMT</i>	O-6-methylguanine-DNA methyltransferase	3.25	101.14
202376	<i>SERPINA3</i>	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	3.31	17.27
221524	<i>RRAGD</i>	Ras-related GTP binding D	3.92	6.96
208694	<i>PRKDC</i>	Protein kinase, DNA-activated, catalytic polypeptide	2.55	3.48
217707	<i>SMARCA2</i>	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2	2.22	3.89
209351	<i>KRT14</i>	Keratin 14 (epidermolysis bullosa simplex, Dowling-Meara, Koebner)	-52.63	-46.27
212607	<i>AKT3</i>	Sv-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)	2.42	2.95
215438	<i>GSPT1</i>	G1 to S phase transition 1	2.00	4.19
217996	<i>PHLDA1</i>	Pleckstrin homology-like domain, family A, member 1	-3.91	-4.11
213524	<i>G0S2</i>	G0/G1switch 2	-3.01	-9.58
214930	<i>SLITRK5</i>	SLIT and NTRK-like family, member 5	2.33	6.11
201848	<i>BNIP3</i>	BCL2/adenovirus E1B 19kDa interacting protein 3	-2.20	-2.47
202766	<i>FBN1</i>	Fibrillin 1 (Marfan syndrome)	2.07	12.91
218032	<i>SNN</i>	Stannin	2.53	2.58

RRAGD, AKT3, SLITRK5, G0S2, SNN, PRKDC, PHLDA1, BNIP3, GSPT1), regulator of chromatin (SMARCA2) and to disorders of connective tissue (FBN1) are shown in Table 6. Twelve of the genes were up-regulated ranging from 2.6 to over 100 fold, and four of the genes down-regulated ranging from 2.5 to 46-fold. Increased expression of WASF3, MGMT, and AKT3 in BEAS-2B/C6 cells was also detected by Western blot (Figure 5A) and the decreased expression of KRT14 was confirmed by IHC (Figure 5B).

Figure 5. Validation of differentially expressed genes and pathway activation in normal human bronchial epithelial BEAS-2B cells. **A:** A Western blot showed that WASF3, MGMT and Akt3, phosphor-p38 MAP kinase, and pErk1/2 were increased in matrilysin-1 expressing clone C6 cells after matrilysin-1 transfection. A slight increase of p-SAPK/JNK was also noted. **B:** Photomicrographs of keratin 14 immunoreactivity. Control BEAS-2B cells containing the empty vector (v) were intensely positive. Note the darkly staining cytoplasmic filaments (left panel), while C6 cells expressing matrilysin-1 were negative (right panel) (Immunoperoxidase stain, scale bar = 25 μm).

Expression of Matrilysin-1 Activated MAP Kinase Pathway, not AKT/mTOR Pathway

Western blot (Figure 5A, right panel) showed that both phosphor-p38 MAP kinase (p-P38) and p-Erk1/2 were increased in BEAS-2B/C6 cells that contained matrilysin-1. There was a slightly increased expression of p-SAPK/JNK as well (Figure 5A). We also examined the levels phospho-AKT s473 and phospho-mTOR, but no changes were found in the AKT/mTOR pathway (data not shown).

Diminished BOA Formation in Vivo in CC10-hASH1/Matrilysin-1^{+/-} Mice

Previous animal models have supported a role for matrilysin-1 in early tumor growth. As an example, ablation of matrilysin-1 was shown to reduce tumor formation in the multiple intestinal neoplasia mouse model of intestinal cancer.²² To examine the impact of reduced-matrilysin-1 expression on BOA formation *in vivo*, we crossed the CC10-hASH1 transgenic mouse with the matrilysin-1 null mouse. The resulting CC10-hASH1/matrilysin-1^{+/-} mouse had one mutant and one normal matrilysin-1 allele. As shown by the photomicrographs, the sharp demarcation of normal BADJs (Figure 6A) was commonly replaced by the formation of BOA in the CC10-hASH1 mouse model (Figure 6B) at 3 months of age. In contrast, many of the BADJs in the lungs of CC10-hASH1/matrilysin-1^{+/-} mice had lost BOA lesions (Figure 6C). Moreover, a detailed histological analysis of over 1300 BADJ structures revealed that the relative number of BADJs harboring BOA lesions was decreased by 35% ($P < 0.001$; Figure 6D). To correlate matrilysin-1 levels with BOA formation *in vivo*, we obtained epithelial cells of BOA and terminal bronchioli from CC10-hASH1 and CC10-hASH1/matrilysin-1^{+/-} mice by LCM. By qRT-PCR we found a threefold decrease in matrilysin-1 mRNA from

Figure 6. Reduced BOA formation in matrilysin-1 haploinsufficient mice. **A:** Normal wild-type mouse lung with sharp demarcation between the airway and alveolar epithelium at BADJs (black arrows). **B:** Lung tissue from a 3-month-old CC10-hASH1 transgenic mouse with prominent BOA lesions (circles) located at BADJs. **C:** A CC10-hASH1/matrilysin-1 $^{+/+}$ mouse lung lacks BOA lesions (circle) at many BADJs (red arrows). (Photomicrographs **A–C:** H&E stain; Scale bar = 100 μ m; BL = bronchiolus; TB = terminal bronchiolus; AD = alveolar duct). **D:** Bar graph showing the percentage of BADJs with BOA formation in matrilysin-1 $^{+/+}$ (Mat $^{+/+}$), CC10-hASH1 (hASH1) and CC10-hASH1/matrilysin-1 $^{+/+}$ (hASH1/Mat $^{+/+}$) mice. Each group included data from six mice ($n = 6$). **E:** Bar graph showing the fold change of the various MMP mRNAs by qRT-PCR in the microdissected epithelia from CC10-hASH1/matrilysin-1 $^{+/+}$ ($n = 3$) mice compared with CC10-hASH1 ($n = 3$) mice. RNA from terminal bronchioles and BOA lesions was pooled. Compared with CC10-hASH1 mice, expression of matrilysin-1 was decreased threefold in CC10-hASH1/Mat $^{+/+}$ mice, while hASH1 mRNA level was similar in the two groups ($n = 3$). A modest increase in of MMP-2 and MMP-9 mRNA levels was found in CC10-hASH1/Mat $^{+/+}$ mice.

CC10-hASH1/matrilysin-1 $^{+/+}$ mice compared with that of CC10-hASH1 mice (Figure 6E). This was associated with a small increase in MMP-2 and MMP-9 mRNA levels, while the expression of hASH1 mRNA remained the same. The results suggest that reduction of matrilysin-1 (matrilysin-1 haploinsufficiency) impairs bronchiolization *in vivo*.

Discussion

We have discovered that human BOA lesions and those in the CC10-hASH1 animal model show similar patterns of airway epithelial differentiation and increased expression of matrilysin-1, which remained low in normal lung. Expression of matrilysin-1 in airway cells was associated with increased migration, proliferation and resistance to apoptosis via activation of p38 and Erk1/2 MAP kinase pathways. Notably, many differentially expressed genes in these cells were related to cell growth, movement, and death. Finally, CC10-hASH1 mice that were matrilysin-1 haploinsufficient demonstrated a significant reduction in BOA formation. Taken together, our results provide functional evidence that matrilysin-1 contributes to the process of bronchiolization.

BOA is a histologically distinct metaplasia.^{3–5} It consists of airway cells lining alveolar walls in a variety of pathological conditions including lung cancer. It has been proposed that BOA cells are either transformed

from alveolar type II cells or derived from bronchiolar epithelial cells that colonize alveolar walls via migration.^{3–5} Our results support the latter hypothesis in the CC10-hASH1 transgenic mouse lung phenotype.⁸ In this model, constitutive expression of the transcription factor hASH1 resulted in increased proliferation and resistance to apoptosis in the most distal airway epithelium leading to BOA formation without alterations in alveolar type II cells, fibrosis, or accumulation of inflammatory cells. The epithelial cells in both mouse and human BOA lesions were positive for matrilysin-1 and the Clara cell marker CC10.

Matrilysin-1 is overexpressed in a variety of human cancer tissues and associated with cancer progression.^{9,23} Accordingly, we detected high to moderate levels of matrilysin-1 in human lung cancer cells, but low levels in normal, human immortalized airway epithelial cells.^{24–26} LCM studies indicated increased expression of matrilysin-1 mRNA in the epithelia of BOA and adjacent terminal bronchioles when compared with that of other airways in the CC10-hASH1 transgenic or wild-type mice. As BOA is a potential premalignant lesion, our results are in concert with the notion that matrilysin-1 is expressed and functioning during early carcinogenesis.^{22,27}

The main finding of our study was that matrilysin-1 contributes to the migration of bronchiolar cells that leads to BOA. Enforced expression of matrilysin-1 in immortalized human bronchial and bronchiolar cells resulted in a

significant increase in cellular migration, which was blocked by matrilysin-1 shRNA. Moreover, treatment of the bronchial cells by recombinant matrilysin-1 revealed a comparable, dose-dependent increase in migrating cells. Indeed, it has been previously reported that matrilysin-1 can drive migration of lung cancer cells in lung A549 cell line, which is potentially mediated by E-cadherin,¹⁸ and is involved in tracheal¹⁸ and airway re-epithelialization *in vivo*.¹³ Moreover, matrilysin-1 has been shown to activate other MMPs, such as proMMP-2 and proMMP-9, to facilitate tumor invasion.¹² These findings are consistent with our results.

Further evidence how matrilysin-1 promotes migration of bronchial epithelial cells came from cDNA microarray analysis, which resulted in 60 genes associated with cellular movement. One of them was WASF3, a member of the WASP/WAVE family,²⁸ which was over-expressed in the matrilysin-1-expressing BEAS-2B cells (C6) when validated by qRT-PCR and WB. Previous research suggests that WASF3 regulates actin cytoskeleton, cell motility, and invasion through the p38 MAP kinase pathway and MMP production including MMP-1, MMP-3, and MMP-9, but not MMP-2.²⁸ Sossey-Alaoui and colleagues reported that WAVE3-mediated cell migration and lamellipodia formation are regulated downstream of phosphatidylinositol 3-kinase.²⁹ Our study also showed that MAP kinase pathway was activated and Akt3 was up-regulated in matrilysin-1-expressing BEAS-2B cells. Based on the previously published findings and our results, we infer that matrilysin-1 drives Akt3 overexpression. Akt3 pathway promotes up-regulation of WASF3, which mediates MAPK pathway activation and results in increased BEAS-2B cell migration. This was potentially facilitated by the down-regulation of keratin 14, which may make it easier for the cells to change their shape and adapt for migration or undergo epithelial-to-mesenchymal transition.³⁰

Our microarray data also revealed 101 genes being associated with growth and proliferation. We showed by Western blotting that matrilysin-1 activated Erk1/2 and p38 MAP kinases. When MAP Erk1/2 is activated, Erk1/2 phosphorylate various downstream substrates involved in a multitude of cellular responses such as cell proliferation, cell differentiation, cell survival, and cell motility.³¹ Likewise, the effects of MAP p38 are dependent not only on the stimuli, but also on the cellular context, resulting in a large array of possibilities, often with opposing effects.³² It is clear that further studies need to be done for the functional assessment of the relevant MAP kinase pathways in human bronchial epithelial cells.

In progressive lesions such as BOA or tumorigenesis, cell number depends both on the rate of proliferation and apoptosis. We have previously demonstrated a relative sparsity of apoptosis in BOA in the CC10-hASH1 transgenic mouse model by the *in situ* terminal deoxynucleotidyl transferase dUTP nick-end labeling assay.⁸ An important effector of apoptosis mediated by matrilysin-1 is the Fas/Fas ligand system,³³ which may be significant during early tumor development. In fact, it has been shown that matrilysin-1 selects for apoptosis-resistant mammary cells *in vivo*, which may be one mechanism

how hyperplastic lesions and tumors form.³⁴ Transfection of matrilysin-1 into human BEAS-2B cells prevented apoptosis induced by the agonistic Fas antibody CH11 suggesting that matrilysin-1 contributes to the resistance to apoptosis in the BOA lesions. However, the exact mechanism remained elusive as the interference by matrilysin-1 shRNA also enhanced the overall apoptotic rate. In other cell lines, such as the matrilysin-1-expressing human HT-29 colon cancer cells, treatment with anti-sense matrilysin-1 oligonucleotides results in enhanced sensitivity toward Fas-mediated apoptosis.²¹ Using qRT-PCR and Western blot we validated the expression of several apoptosis-related genes such as PHLDA1, MGMT, and PRKDC obtained through the cDNA microarray analysis.

O-6-methylguanine-DNA methyltransferase (MGMT) and DNA-dependent protein kinase (PRKDC) were two DNA repair genes up-regulated by constitutive expression of matrilysin-1 in BEAS-2B cells. MGMT has been linked to increased resistance of cells to alkylating agents.³⁵ Lack of PRKDC causes defective DNA double-strand break repair and radiosensitization, associated with apoptosis.³⁶ Increased expression of MGMT and PRKDC potentially augments the resistance to cytotoxic agents in cells that overexpress of matrilysin-1.^{33,37}

An added confirmation that matrilysin-1 contributes to bronchiolar metaplasia *in vivo* came from a CC10-hASH1/matrilysin-1^{+/-} mouse. In this mouse, which is a cross between the CC10-hASH1 transgenic and matrilysin-1 null mouse and consequently matrilysin-1 haploinsufficient, the number of abnormal epithelial junctions or BADJs containing BOA was significantly reduced, and associated with marked decrease in matrilysin-1 mRNA levels in the epithelium. We propose that this was due to impaired migration of airway cells to the alveolar compartment due to disrupted matrilysin-1. The findings are comparable with other studies in which the removal of matrilysin-1 by genetic ablation reduced the number of intestinal tumors or early mammary tumors as well as precancerous pancreatic acinar-to-ductal metaplasia changes, indicating that matrilysin-1 is important for the genesis of neoplasia.^{22,27,38,39}

We conclude that BOA, a potential premalignant lesion, is derived from terminal bronchiolar epithelium through airway cell growth and migration mediated by matrilysin-1. Our results suggest a link between airway epithelium and glandular epithelium found in other organs where matrilysin-1 has been implicated in early neoplasia.

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