Title

The Endoplasmic Reticulum Resident Protein AGR3. Required for Regulation of Ciliary Beat Frequency in the Airway

Journal

American Journal of Respiratory Cell and Molecular Biology, 53(4)

ISSN

1044-1549

Authors

Bonser, Luke R
Schroeder, Bradley W
Ostrin, Lisa A
et al.

Publication Date

2015-10-01

DOI

10.1165/rcmb.2014-0318oc

Peer reviewed

ORIGINAL RESEARCH

The Endoplasmic Reticulum Resident Protein AGR3
Required for Regulation of Ciliary Beat Frequency in the Airway

Luke R. Bonser[1], Bradley W. Schroeder[1], Lisa A. Ostrin[2], Nathalie Baumlin[3], Jean L. Olson[4], Matthias Salathe[3], and David J. Erle[1]

[1]Lung Biology Center and [4]Department of Pathology, University of California San Francisco, San Francisco, California; [2]College of Optometry, University of Houston, Houston, Texas; and [3]Division of Pulmonary, Allergy, Critical Care and Sleep Medicine, University of Miami, Miami, Florida

Abstract

Protein disulfide isomerase (PDI) family members regulate protein folding and calcium homeostasis in the endoplasmic reticulum (ER). The PDI family member anterior gradient (AGR) 3 is expressed in the airway, but the localization, regulation, and function of AGR3 are poorly understood. Here we report that AGR3, unlike its closest homolog AGR2, is restricted to ciliated cells in the airway epithelium and is not induced by ER stress. Mice lacking AGR3 are viable and develop ciliated cells with normal-appearing cilia. However, ciliary beat frequency was lower in airways from AGR3-deficient mice compared with control mice (20% lower in the absence of stimulation and 35% lower after ATP stimulation). AGR3 deficiency had no detectable effects on ciliary beat frequency (CBF) when airways were perfused with a calcium-free solution, suggesting that AGR3 is required for calcium-mediated regulation of ciliary function. Decreased CBF was associated with impaired mucociliary clearance in AGR3-deficient airways. We conclude that AGR3 is a specialized member of the PDI family that plays an unexpected role in the regulation of CBF and mucociliary clearance in the airway.

Keywords: protein disulfide isomerase; AGR2; AGR3; ciliary beat frequency; mucociliary clearance

Clinical Relevance

Our study provides the first evidence of a specific physiological function for anterior gradient (AGR) 3. We show that AGR3 is expressed selectively in multiciliated cells of the airway, is required for regulation of ciliary beat frequency, and contributes to mucociliary clearance. Hence, we report a completely novel role for a protein disulfide isomerase family member and identify a new molecule that regulates a process crucial for lung health and disease.

Effective mucociliary clearance protects the lungs from the deleterious effects of inhaled pollutants, allergens, and pathogens (1). Secretions from secretory cells within the surface epithelium and submucosal glands contribute to the respiratory mucus gel, which traps inhaled particulates (2). The gel, in turn, is cleared by motile cilia located on the apical surface of multiciliated airway epithelial cells (2, 3). Genetically determined or acquired defects in any of the components of the mucociliary system can lead to impaired mucociliary clearance. Impaired mucociliary clearance is a cardinal feature of chronic airway diseases, including cystic fibrosis, primary ciliary dyskinesia (PCD), asthma, and chronic obstructive pulmonary disease (1, 4).

The molecular basis of specialized epithelial functions required for mucociliary clearance is a subject of active investigation. Recent genomic and proteomic studies have revealed numerous genes that are selectively expressed in airway epithelial cells (5–7), but the functions of many of these genes remain obscure. We recently showed that the anterior gradient protein 2

(Received in original form August 11, 2014; accepted in final form February 19, 2015)

This work was supported by National Institutes of Health grant HL099101. Human airway cell models were provided by the Cystic Fibrosis Cell Models Core supported by grant DK072517 from the National Institutes of Health and by grant DR613-CR11 from the Cystic Fibrosis Foundation.

Author Contributions: Conception and design: B.W.S., L.R.B., M.S., and D.J.E. Acquisition, analysis, and interpretation: B.W.S., L.R.B., L.A.O., N.B., J.L.O., M.S., and D.J.E. Manuscript preparation: L.R.B. and D.J.E.

Correspondence and requests for reprints should be addressed to David J. Erle, M.D., University of California San Francisco Mail Code 2922, San Francisco, CA 94143-2922. E-mail: david.erle@ucsf.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 53, Iss 4, pp 536–543, Oct 2015

Originally Published in Press as DOI: 10.1165/rcmb.2014-0318OC on March 9, 2015
Internet address: www.atsjournals.org

homolog (AGR2) is expressed selectively in mucus cells and plays a role in the processing of mucins (8, 9). AGR2 is a member of the protein disulfide isomerase (PDI) family. PDI family members are characterized by the presence of at least one thioredoxin (TRX) domain together with an endoplasmic reticulum (ER) retention signal (10). TRX domains of some PDI family members have been shown to mediate formation, shuffling, or reduction of disulfide bonds in client secretory proteins transiting through the ER. AGR2, for example, can form mixed disulfide bonds with mucins in the ER, and loss of AGR2 has led to impaired mucin production and the induction of epithelial ER stress (9). In addition to roles in oxidative protein folding, some PDI family members have been shown to participate in ER-associated degradation, trafficking, and calcium homeostasis (11–13). The potential contributions of PDI family members other than AGR2 to mucociliary function are largely unknown.

Anterior gradient protein 3 homolog (*AGR3*), also known as *AG3*, *HAG3*, *hAG-3*, *BCMP11*, or *PDIA18*, is the PDI family member that is most similar to AGR2 (12, 14). The *AGR3* gene is located at chromosome 7p21.1, adjacent to the *AGR2* gene, and encodes a 166–amino acid protein. AGR3 shares 71% sequence identity with AGR2 and, like AGR2, comprises an amino terminal signal peptide, a carboxy terminal ER retention sequence, and a nonclassical TRX core fold (CXXS motif) (12, 15). These features suggest that AGR2 and AGR3 arose from duplication of an ancestral gene. Gene duplications can contribute to evolution through the development of paralogs with divergent functions (16). Like AGR2, AGR3 is expressed in the lung (14). However, the function of AGR3 and its functional relationship to AGR2 have not been previously described.

We hypothesized that AGR3, like its paralog AGR2, would play a specialized role in airway epithelial cell function. To test this hypothesis, we investigated the localization of AGR3 in human and mouse airway, analyzed whether AGR3 was induced by ER stress, and used an AGR3-deficient mouse to investigate AGR3 function in the airway. Our findings indicate that AGR3 has a specialized function in the mucociliary apparatus that is distinct from the function of its closely related paralog, AGR2. Some of the results of these studies have been previously reported in the form of an abstract (17).

Materials and Methods

Human Airway Tissue

Dr. Walter Finkbeiner (Department of Pathology, San Francisco General Hospital) generously provided deidentified sections of human trachea.

Immunofluorescence

Immunofluorescence was performed as described in the online supplement. Images were captured using a confocal scanner (CSU-22; Yokagawa, Tokyo, Japan) and a microscope (Ti-E; Nikon, Tokyo, Japan) (Nikon Imaging Center at the University of California San Francisco [UCSF]) and processed with ImageJ (National Institutes of Health, Bethesda, MD).

Human Bronchial Epithelial Cell Culture

The primary human bronchial epithelial (HBE) cell air–liquid interface (ALI) system was described previously (6). To model mucus metaplasia, cultures were treated with IL-13 (10 ng/ml) for 14 days. To examine the effects of ER stress, HBE cells were cultured at ALI for 28 days and then treated with tunicamycin (1 μM for 8 h) or thapsigargin (2 μM for 4 h), and RNA was harvested and analyzed by quantitative RT-PCR.

Gene Expression Analysis

Methods for real-time quantitative PCR and PCR-based analysis of *Xbp1* splicing have been previously described (9). Transcript copy number was determined relative to the mean cycle threshold for three housekeeping genes (Actin, GADPH, and Cyclophilin A).

Mouse Experiments

Mouse experiments were approved by the UCSF Committee on Animal Research and performed in accordance with NIH guidelines. $Agr2^{-/-}$ mice (JAX Stock No. 025630) (8), $Agr3^{-/-}$ mice (Taconic), and wild-type littermates were bred at UCSF. Methods for ovalbumin sensitization and challenge and airway brushing were described previously (9).

RNA Sequencing

The TruSeq Stranded mRNA kit (Illumina, San Diego, CA) was used to analyze transcripts from lungs of $Agr3^{-/-}$ and $Agr3^{+/+}$ littermates. Details are provided in the online supplement.

Electron Microscopy

For scanning electron microscopy, tracheas were excised, postfixed in 1% osmium tetroxide (3 h), washed in sodium cacodylate, and then dehydrated through a graded ethanol series. After critical point drying, the tissue samples were mounted on aluminum stubs and viewed with a scanning electron microscope (TM-1000; Hitachi, Tokyo, Japan). For transmission electron microscopy, tracheal rings were cut from dissected trachea before freeze substitution and resin embedding. Sections were stained, imaged, and scored as previously described (18).

Ciliary Beat Frequency

Ciliary beat frequency (CBF) was recorded as described in the online supplement. CBF was measured after perfusion with buffer containing 1 mM Ca^{2+}, buffer containing ATP, or calcium-free buffer (0 mM Ca^{2+} with EGTA).

Mucociliary Transport

Tracheas were opened along the trachealis muscle and pinned to Sylgard plates (Dow Corning Corporation, Midland, MI). After applying 2-μm Fluospheres (Invitrogen, Eugene, OR) to the trachea, images were acquired at 0.5-second intervals for 30 seconds using a wide-field epifluorescence microscope. Bead trajectory and speed were analyzed with ImageJ.

Intracellular Calcium Measurements

Tracheal epithelial cells from $Agr3^{-/-}$ and control ($Agr3^{+/+}$ littermate) mice were cultured at an ALI (19) and then loaded with fura-2-acetoxymethyl ester (Life Technologies, Carlsbad, CA). Intracellular calcium was determined from the ratio of the fluorescence at 340- and 380-nm excitations as previously described (20, 21).

Statistical Analyses

Data are reported as mean ± SEM. Significance testing was performed by Student's *t* test or by ANOVA and Tukey-Kramer post-test for multiple groups. RNA sequencing data were analyzed using DESEquation (22).

Results

AGR3 Is Expressed in the ER of Human Airway Ciliated Cells

We first examined the localization of AGR3 within the human airway epithelium. AGR3 staining was distinct from immunostaining for AGR2 within tracheal epithelial cells: using Manders' colocalization coefficient, we calculated that 97.7% of AGR3 staining was found in regions that did not stain for AGR2 (Figure 1A). Staining for the ER-resident proteins GRP78/GRP94 revealed that 99.5% of AGR3 localized to the ER (Figure 1B). Although AGR2 is largely confined to mucus cells (9), AGR3 was restricted to cells containing DNAI1, a component of motile cilia (Figure 1C). We conclude that AGR3 is restricted to the ER of ciliated cells within human airway epithelium.

We next investigated the expression of *AGR3* during differentiation of HBE cultures grown at ALI (Figure 1D). Between 7 and 21 days of culture, expression of the ciliated cell genes *DNAI1* and forkhead box protein J1 (*FOXJ1*) increased whereas expression of the mucus cell gene *MUC5AC* did not change significantly. The increase in ciliated cell genes was associated with a large increase in *AGR3* expression. To examine the relationship between mucus metaplasia and *AGR3* expression, we treated HBE cells with IL-13 for 14 days. As expected, IL-13 strongly induced *MUC5AC* and reduced expression of *FOXJ1* and *DNAI1*. *AGR3* expression was also decreased after IL-13 treatment, consistent with its expression in ciliated cells rather than mucus cells.

AGR3 Is Expressed in Ciliated Cells in the Murine Airway

We next explored AGR3 expression in the murine respiratory tract. As in the human airway, AGR3 staining was limited to ciliated cells (Figure 2A). In the absence of allergen challenge, few mucus cells were apparent, and AGR2 staining was rarely observed. To induce mucus metaplasia, we sensitized and challenged mice with ovalbumin (Figure 2B). In allergen-challenged mice, AGR2 strongly associated with MUC5AC-containing mucus cells as previously reported (9), whereas AGR3 localized to ciliated cells and was not evident in mucus cells. To determine whether AGR3 compensated for loss of AGR2, we analyzed AGR3 localization in $Agr2^{-/-}$ mice. Even in the complete absence of AGR2, AGR3 was only present in ciliated cells and was not detected in mucus cells.

AGR3 Expression Is Independent of ER Stress

AGR2 and several other PDI family genes are induced by ER stress (9). To examine the response of *AGR3* to ER stress, we challenged differentiated HBE cell cultures with tunicamycin, an inhibitor of N-glycosylation, and thapsigargin, a selective sarcoplasmic/endoplasmic reticulum calcium pump inhibitor (Figure 3A). Tunicamycin strongly induced classical markers of the ER stress pathway (*BIP*, *CHOP*, and *ATF4*) and increased *AGR2* mRNA levels 13-fold. Tunicamycin, however, did not increase *AGR3* mRNA expression. A similar response was observed with thapsigargin (Figure 3B). These data indicate that AGR3, unlike AGR2, is not induced as a part of the cellular response to ER stress.

AGR3 Is Not Essential for Viability or Fertility or for Formation of a Ciliated Airway Epithelium

To elucidate the function of AGR3 *in vivo*, we used AGR3-deficient ($Agr3^{-/-}$) mice. Male and female $Agr3^{-/-}$ mice were viable and fertile. Blinded inspection of tracheal sections from five control and five $Agr3^{-/-}$ mice revealed no apparent differences (Figures 4A and 4B). Scanning electron microscopy confirmed that the apical surface of the trachea was lined with ciliated cells in both control and $Agr3^{-/-}$ mice (Figures 4C and 4D). We used transmission electron microscopy to examine ciliary ultrastructure (Figures 4E and 4F). A pathologist with experience in diagnosing ciliopathies (J.L.O.) used transmission electron microscopy to examine the ultrastructure of 50 cilia from each of three control and three $Agr3^{-/-}$ mice. There were no obvious defects in the appearance of cilia in $Agr3^{-/-}$ mice compared with control mice: all mice had the normal 9 + 2 microtubule arrangement, and dynein arms were present and had

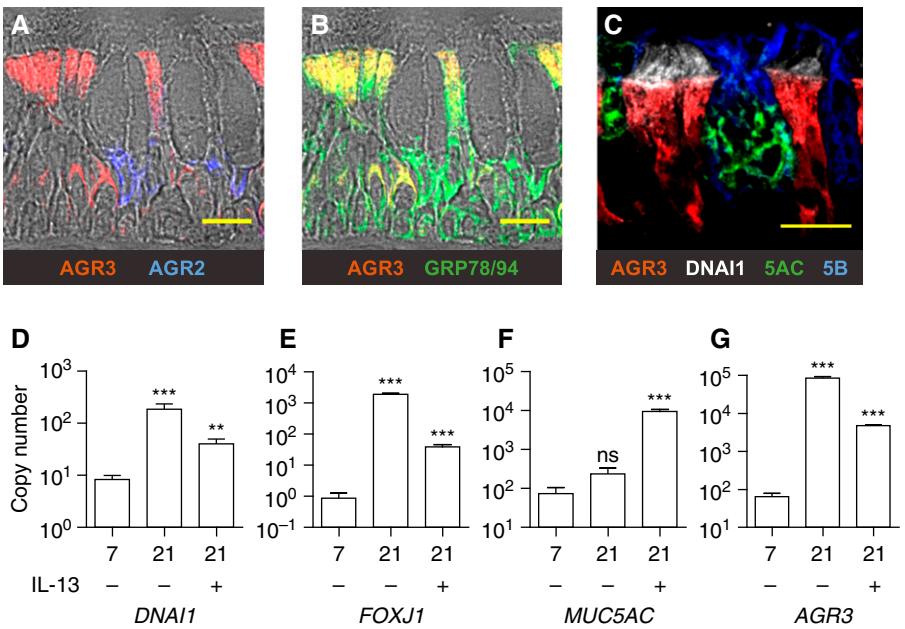

Figure 1. Anterior gradient 3 (*AGR3*) is expressed in human airway ciliated cells, and expression increases with mucociliary differentiation *in vitro*. (*A–C*) Human tracheal sections were stained with antibodies against AGR3 (*red*) and AGR2 (*blue*) (*A*), AGR3 (*red*) and the endoplasmic reticulum marker GRP78/GRP94 (*green*) (*B*), and AGR3 (*red*), DNAI1 (*white*), MUC5AC (5AC, *green*), and MUC5B (5B, *blue*) (*C*). *Scale bars*: 20 μm. (*D*) Analysis of *AGR3* messenger RNA (mRNA) during mucociliary differentiation. Human bronchial epithelial cells were cultured at air–liquid interface for 7 to 21 days without cytokine stimulation (−) or in the presence of IL-13 (10 ng/ml from Days 7–21, +), and levels of the ciliated cell mRNAs *FOXJ1* and *DNAI1*, the mucin mRNA *MUC5AC*, and *AGR3* mRNA were analyzed by quantitative RT-PCR. Values represent mean ± SEM ($n = 3$). $^{**}P < 0.01$; $^{***}P < 0.001$. ns, not significant.

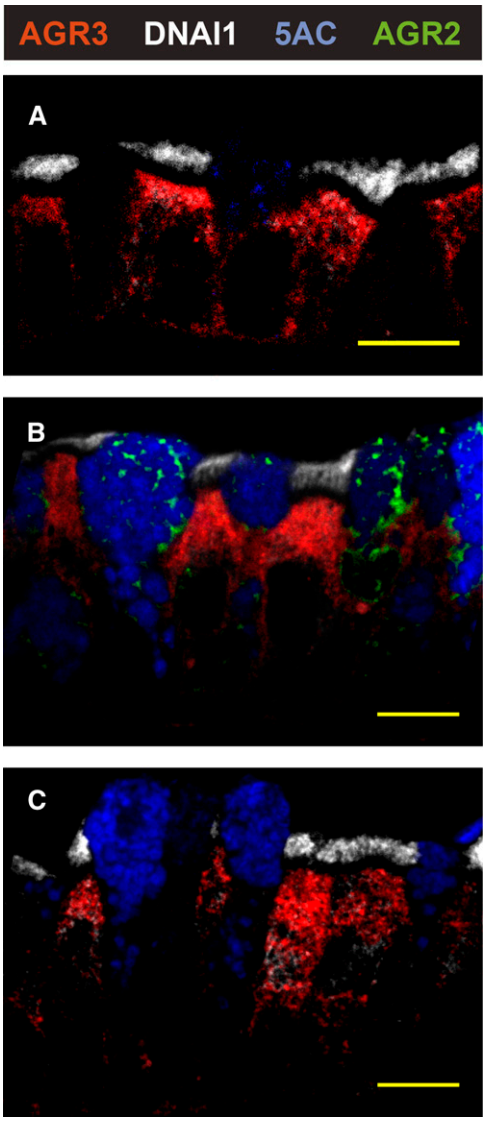

Figure 2. AGR3 localizes to ciliated cells in mouse airway epithelium. Airway sections from saline-challenged wild-type mice (*A*), allergen-challenged wild-type mice (*B*), and allergen-challenged *Agr2*$^{-/-}$ mice (*C*) were stained with antibodies against AGR3 (*red*), DNAI1 (*white*), MUC5AC (*blue*), and AGR2 (*green*). *Scale bars*: 20 μm.

a similar appearance in the two genotypes. Massively parallel RNA sequencing showed that ciliated cell– and secretory cell–specific transcripts were present at similar levels in lungs from wild-type and homozygous null mice (Figure 5A).

Agr3$^{-/-}$ Mice Do Not Develop Detectable ER Stress

Mice deficient in AGR2 develop an ER stress response (9, 23). To determine whether lack of AGR3 induces a similar response, we examined the expression of ER stress–associated genes. Massively parallel RNA sequencing (Figure 5A) and quantitative RT-PCR (Figures 5B and 5C) revealed no differences in expression of ER stress response genes in the lung or trachea of *Agr3*$^{-/-}$ mice compared with control mice. We previously used PCR analysis of *Xbp-1* splicing, which is induced by ER stress, to demonstrate that lack of AGR2 is associated with ER stress in epithelial cells recovered by airway brushing (9). Using the same approach, we were unable to detect changes in *Xbp-1* splicing in epithelial cell brushings from *Agr3*$^{-/-}$ mice (Figure 5D). We were also unable to detect differences in the levels of *Agr2* or other PDI family member transcripts between *Agr3*$^{-/-}$ and wild-type mice (Figure 5A). We conclude that loss of AGR3 does not induce a detectable ER stress response or lead to a compensatory increase in expression of its *Agr2* or other PDI family members.

CBF Is Reduced in *Agr3*$^{-/-}$ Mice

The specific localization of AGR3 in human and mouse ciliated cells raised the question of whether ciliary function was affected by loss of AGR3. Microscopic analysis of freshly isolated tracheal tissue revealed that cilia in the trachea of *Agr3*$^{-/-}$ mice were motile. We therefore analyzed CBF in tracheas from *Agr3*$^{-/-}$ mice and *Agr3*$^{+/+}$ littermates. In the presence of 1 mM extracellular calcium, tracheas from *Agr3*$^{+/+}$ mice exhibited a CBF of 18.4 ± 3.9 Hz, comparable to previously published data (24). In tracheas from *Agr3*$^{-/-}$ mice, however, ciliary beat frequency was reduced by 20% (Figure 6A). Extracellular ATP, an important regulator of ciliary function, increases CBF by activating purinergic receptors and increasing intracellular calcium concentration (25). After ATP stimulation, CBF was 35% lower in *Agr3*$^{-/-}$ tracheas compared with controls (Figure 6B).

To determine if the defect in CBF in *Agr3*$^{-/-}$ mice was calcium dependent, we perfused tracheal rings with a calcium-free solution (Figure 6C). In the absence of extracellular calcium, there was no apparent difference in CBF in tracheas from *Agr3*$^{-/-}$ and *Agr3*$^{+/+}$ mice. These data indicate that AGR3 influences CBF in a calcium-dependent manner. Analysis of cultured tracheal epithelial cells showed that intracellular calcium was lower in cells from *Agr3*$^{-/-}$ mice compared with cells from *Agr3*$^{+/+}$ littermates (139.7 ± 98.2 nM versus 362.1 ± 55.0 nM [mean ± SEM]; $n = 6$; $P < 0.001$), implicating AGR3 in the regulation of intracellular calcium in tracheal epithelial cells.

Mucociliary Clearance Is Reduced in *Agr3*$^{-/-}$ Mice

CBF is a primary determinant of mucociliary clearance in the airway (1). To investigate mucociliary function in *Agr3*$^{-/-}$ mice, we measured the rate of particle transport in the trachea *ex vivo* (Figure 6D). Particle speed was reduced by 35% in tracheas from *Agr3*$^{-/-}$ mice. These data indicate that AGR3 is required for normal ciliary activity and for optimal mucociliary clearance in the airway.

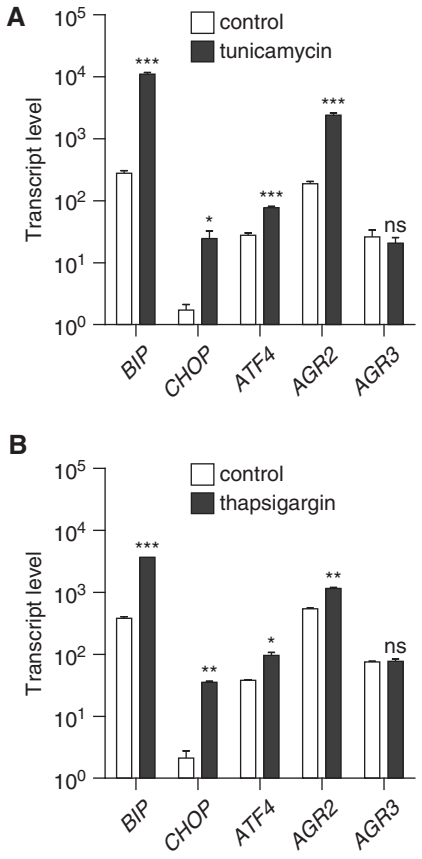

Figure 3. *AGR3* expression is not induced by endoplasmic reticulum (ER) stress. Differentiated human bronchial epithelial cultures were grown at air–liquid interface and cultured in the presence or absence of the classic ER stress inducers tunicamycin (*A*) and thapsigargin (*B*). The mRNA levels of *AGR2* and *AGR3* and markers of the ER stress pathway (*BIP*, *CHOP*, and *ATF4*) were analyzed. Values represent mean ± SEM ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Discussion

Our findings add to an emerging understanding of the multifaceted roles of PDI family members in the lung and other organs. Some PDI family members are widely expressed and have more generalized roles in ER function, but others, including AGR2 and AGR3, are restricted to specific cell types and play more specialized roles. Our previous work established a specialized role for AGR2 in mucus cells in the airway and the intestine (8, 9, 23). In the present study, we analyzed the function of the AGR2 paralog, AGR3, in the airway. Despite their strong structural similarity, we found dramatic differences in the expression, regulation, and biological function of these two PDI family members in airway epithelial cells. Unlike AGR2, which is required for normal mucin processing in mucus cells (9), AGR3 is required for the regulation of CBF in multiciliated cells.

We found that AGR3 is localized to the ER of ciliated cells in the airway epithelium in humans and mice. ER localization is a defining feature of PDI family members, although some PDI family members have been reported to localize in other compartments, including the cytoplasm, the nucleus, and the extracellular space in certain systems (12). Our finding that endogenous AGR3 is confined to the ER of ciliated cells complements previous reports of ER localization in nonciliated cells transfected with epitope-tagged AGR3 (26).

AGR3 expression increases dramatically with differentiation of airway epithelial cells (27): in our experiments, *AGR3* mRNA increased approximately 1,000-fold between Day 7 and Day 21. Consistent with this finding, publicly available microarray data show that *AGR3* transcripts were at or near the limit of detection in purified human basal cells (28) (NCBI GEO dataset GSE24337) and became detectable at Day 4 of *in vitro* differentiation, concurrent with the onset of expression of the ciliated cell transcription factor *FOXJ1* (29) (GDS2615).

AGR3 expression in cells with motile cilia is apparently not confined to the airway epithelium; expression was also detected in ciliated cells in the oviduct (30), and *AGR3* was identified as a component of the transcriptome of ciliated cells using an

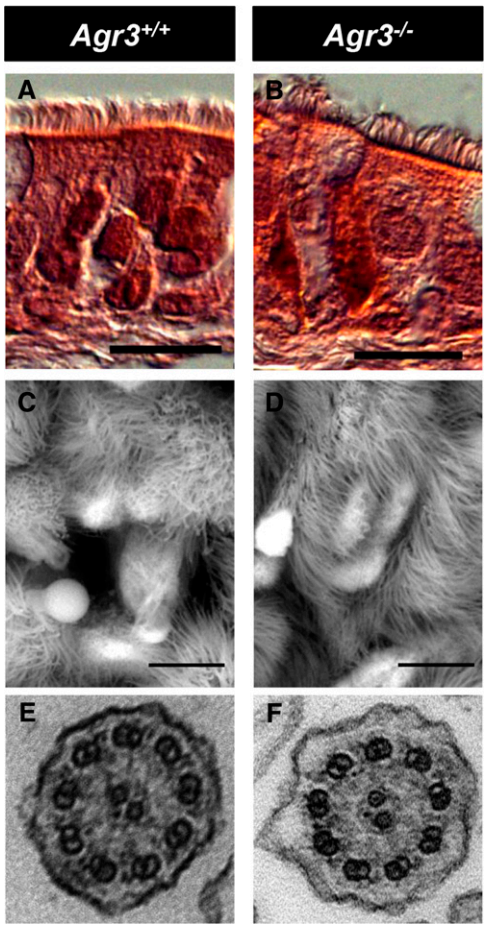

Figure 4. Multiciliated cells are present in airways of AGR3-deficient mice. (*A* and *B*) Histological sections of airways from wild-type ($Agr3^{+/+}$) (*A*) and $Agr3^{-/-}$ (*B*) mice stained with hematoxylin and eosin. *Scale bars*: 10 μm. (*C* and *D*) Scanning electron microscopy of cilia from $Agr3^{+/+}$ (*C*) and $Agr3^{-/-}$ (*D*) mice. *Scale bars*: 5 μm. (*E* and *F*) Transmission electron microscopy of airways from $Agr3^{+/+}$ (*E*) and $Agr3^{-/-}$ (*F*) mice.

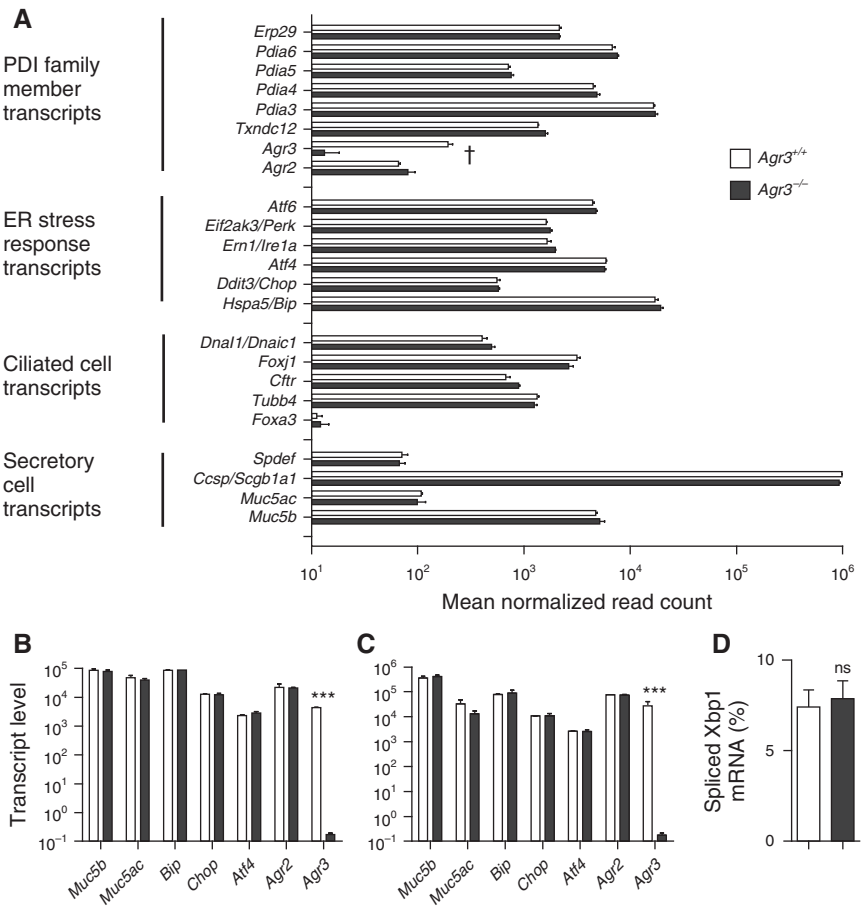

Figure 5. AGR3-deficient mice do not develop detectable ER stress. (*A*) Transcript levels were measured by RNA sequencing analysis of lungs from mice lacking AGR3 ($Agr3^{-/-}$) and wild-type ($Agr3^{+/+}$) littermates. Values represent mean ± SEM ($n = 3$). †False discovery rate $q = 3 \times 10^{-18}$; $q > 0.9$ for other transcripts. (*B* and *C*) Quantitative PCR analysis of transcripts in lungs (*B*) and tracheas (*C*) from wild-type ($Agr3^{+/+}$) and $Agr3^{-/-}$ mice. Values represent mean ± SEM ($n = 3$). ***$P < 0.001$; other differences were not statistically significant. (*D*) Spliced *Xbp1* mRNA (as a percentage of total *Xbp1* mRNA) was determined from epithelial brushings from wild-type and $Agr3^{-/-}$ mice. Values represent mean ± SEM ($n = 6$). PDI, protein disulfide isomerase.

approach that relied on coexpression with known ciliated cell genes across multiple human tissues (5). *AGR3* transcripts have also been detected in stomach, lung, colon, and prostate (14) and in liver (31). Because not all *AGR3*-expressing organs contain multiciliated cells, AGR3 likely has other functions in these organs. Further studies are required to determine whether AGR3 regulates ciliary function in cells outside the lung, identify functions for AGR3 in nonciliated cells, and analyze the significance of increased *AGR3* expression in certain cancers, including liver (31), prostate (32), and breast (15) cancer.

Unlike AGR2 and several other PDI family members, AGR3 is not induced during ER stress responses. Increases in levels of PDI family members with roles in ER protein processing are believed to contribute to restoration of ER proteostasis by promoting proper oxidative folding or removal of unfolded or misfolded proteins (33, 34). Here we confirmed that ER stress induces expression of *AGR2* in airway epithelial cells but found no induction of *AGR3* under identical conditions. Further evidence for the role of AGR2 in maintaining ER proteostasis came from the observation that $Agr2^{-/-}$ mice develop ER stress (9, 23). In contrast, we were unable to find evidence of an ER stress response in $Agr3^{-/-}$ mice.

The observation that AGR3 is present in multiciliated airway epithelial cells led us to ask whether AGR3 is required for the development of these cells. $Agr3^{-/-}$ mice had ciliated cells, and we were unable to detect abnormalities in the histologic appearance of the airway, the ultrastructure of motile cilia, or expression of cilia-specific genes in these mice, demonstrating that AGR3 is not essential for the formation of multiciliated cells. This is consistent with phylogenetic studies showing that AGR3 is present only in amphibians and higher vertebrates, although multiciliated cells are present in nonvertebrates (14, 35). Hence, the specialized function of AGR3 in the airway epithelium apparently evolved after the origin of multiciliated cells.

Although ciliated cells developed in the absence of AGR3, we did detect differences in CBF in airways from $Agr3^{-/-}$ mice. CBF was similar in $Agr3^{-/-}$ mice compared with control mice when cells were perfused with a calcium-free solution, arguing against a simple mechanical or structural defect in the ciliary apparatus or a nonspecific toxic effect on multiciliated cells. However, in the presence of extracellular calcium, CBF was increased in control mice but not in $Agr3^{-/-}$ mice. An even more marked defect in CBF was seen after ATP stimulation, which acts by increasing intracellular calcium concentration. These findings demonstrate that AGR3 is required for normal calcium-dependent CBF regulation in the airway.

Our studies suggest a mechanistic relationship between AGR3, intracellular Ca^{2+}, and regulation of CBF. Intracellular Ca^{2+} is a major regulator of CBF (25). AGR3 is localized in the ER, which is the single largest calcium store inside nonstriated muscle cells (36). Most Ca^{2+} in the ER is bound to proteins. Members of the PDI family have been shown to bind Ca^{2+} directly (36) and to associate with and modulate activity of major ER Ca^{2+}-buffering proteins, such as calreticulin (37). In addition, at least one PDI family member, ERp44, can also affect release of Ca^{2+} from the ER to the cytosol. ATP-induced Ca^{2+} release requires activation of inositol 1,4,5-trisphosphate receptors (25). ERp44 interacts with free cysteine residues in an ER luminal loop of the type 1 inositol 1,4,5-trisphosphate receptors to modulate cytoplasmic Ca^{2+} concentrations (38). Based on our finding of altered intracellular calcium levels in $Agr3^{-/-}$ airway epithelial cells, we speculate that AGR3 regulates CBF by binding to and modulating the activity of ER calcium buffering proteins or calcium channels. This binding would likely involve the conserved active site cysteine residue

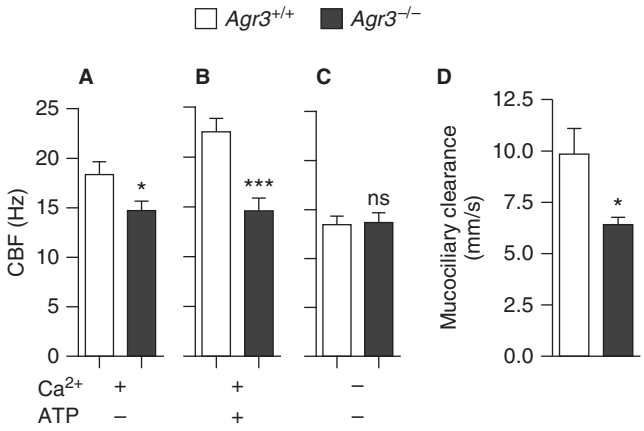

Figure 6. Lack of AGR3 leads to a reduction in ciliary beat frequency (CBF) and impaired mucociliary clearance. (*A–C*) CBF was measured in AGR3-deficient mice ($Agr3^{-/-}$) and wild-type control mice ($Agr3^{+/+}$) in buffer containing calcium (*A*), calcium and ATP (*B*), or calcium-free buffer without ATP (*C*). Values represent mean ± SEM ($n = 6$). $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with wild-type controls. (*D*) Tracheal clearance of fluorescent beads in mice lacking AGR3 ($n = 10$) and wild-type littermates ($n = 9$). $^{*}P < 0.05$.

found in AGR3 and all other PDI family members. Alternatively, our findings could be explained by a toxic effect of AGR3 deficiency on ER homeostasis, although the lack of an ER stress response makes this possibility less likely. Further studies are required to understand the mechanisms accounting for the functional effects of AGR3 in airway epithelial cells.

The functional abnormalities observed in $Agr3^{-/-}$ mice suggest that AGR3 could be relevant for human diseases with inherited or acquired defects in ciliary beat frequency and mucociliary clearance. For example, the reductions in CBF caused by AGR3 deficiency (20% reduction in the presence of extracellular calcium and 35% after ATP stimulation) are similar to the 25 to 29% reductions in CBF that have been described for some individuals with PCD (39, 40). Our findings therefore establish AGR3 as a logical candidate gene for PCD. No biallelic mutations in AGR3 were detected by exome sequencing of 72 subjects with PCD confirmed by clinical phenotyping, electron microscopy, and/or nasal nitric oxide analysis and 43 subjects with idiopathic bronchiectasis without confirmed PCD (M. B. Zariwala and M. R. Knowles, personal communication). Although this suggests that *AGR3* is unlikely to be a common cause of PCD, future studies could identify rare, disease-causing variants. In addition to PCD, mucociliary dysfunction is a characteristic feature of a broad spectrum of chronic airway diseases, including cystic fibrosis, chronic obstructive pulmonary disease, and asthma (1, 4). Consequently, a better understanding of the role of AGR3 and other molecules in regulation of these processes may also be relevant for these common disorders.

To our knowledge, our study provides the first evidence of a specific physiological function for AGR3: regulation of CBF in airway epithelial multiciliated cells. AGR3 and AGR2, which regulate mucus production in airway epithelial mucus cells, provide a striking example of how two paralogs expressed in different cell types make distinct contributions to the function of a single system, the mucociliary apparatus. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank Rebecca Barbeau, Andrea Barczak, and Joshua Pollack (UCSF SABRE Center Functional Genomics Core) for assistance with RNA sequencing; Lorna Zlock and Walter Finkbeiner (Department of Pathology, UCSF) for providing human cells and tissues; Kurt Thorn and DeLaine Larsen (UCSF Nikon Imaging Center) for assistance with microscopy; and Edwin Ostrin and Louis Nguyenvu for advice and technical assistance.

References

1. Mall MA. Role of cilia, mucus, and airway surface liquid in mucociliary dysfunction: lessons from mouse models. *J Aerosol Med Pulm Drug Deliv* 2008;21:13–24.
2. Thornton DJ, Rousseau K, McGuckin MA. Structure and function of the polymeric mucins in airways mucus. *Annu Rev Physiol* 2008;70:459–486.
3. Antunes MB, Cohen NA. Mucociliary clearance: a critical upper airway host defense mechanism and methods of assessment. *Curr Opin Allergy Clin Immunol* 2007;7:5–10.
4. Fahy JV, Dickey BF. Airway mucus function and dysfunction. *N Engl J Med* 2010;363:2233–2247.
5. Ivliev AE, 't Hoen PA, van Roon-Mom WM, Peters DJ, Sergeeva MG. Exploring the transcriptome of ciliated cells using in silico dissection of human tissues. *PLoS One* 2012;7:e35618.
6. Solberg OD, Ostrin EJ, Love MI, Peng JC, Bhakta NR, Hou L, Nguyen C, Solon M, Nguyen C, Barczak AJ, *et al*. Airway epithelial miRNA expression is altered in asthma. *Am J Respir Crit Care Med* 2012;186:965–974.
7. Steiling K, Kadar AY, Bergerat A, Flanigon J, Sridhar S, Shah V, Ahmad QR, Brody JS, Lenburg ME, Steffen M, *et al*. Comparison of proteomic and transcriptomic profiles in the bronchial airway epithelium of current and never smokers. *PLoS One* 2009;4:e5043.
8. Park SW, Zhen G, Verhaeghe C, Nakagami Y, Nguyenvu LT, Barczak AJ, Killeen N, Erle DJ. The protein disulfide isomerase AGR2 is essential for production of intestinal mucus. *Proc Natl Acad Sci USA* 2009;106:6950–6955.
9. Schroeder BW, Verhaeghe C, Park SW, Nguyenvu LT, Huang X, Zhen G, Erle DJ. AGR2 is induced in asthma and promotes allergen-induced mucin overproduction. *Am J Respir Cell Mol Biol* 2012;47:178–185.
10. Zhang K, Kaufman RJ. From endoplasmic-reticulum stress to the inflammatory response. *Nature* 2008;454:455–462.
11. Appenzeller-Herzog C, Ellgaard L. The human pdi family: versatility packed into a single fold. *Biochim Biophys Acta* 2008;1783:535–548.
12. Galligan JJ, Petersen DR. The human protein disulfide isomerase gene family. *Hum Genomics* 2012;6:6.
13. Koonin EV. Orthologs, paralogs, and evolutionary genomics. *Annu Rev Genet* 2005;39:309–338.
14. Persson S, Rosenquist M, Knoblach B, Khosravi-Far R, Sommarin M, Michalak M. Diversity of the protein disulfide isomerase family: identification of breast tumor induced Hag2 and Hag3 as novel members of the protein family. *Mol Phylogenet Evol* 2005;36:734–740.
15. Fletcher GC, Patel S, Tyson K, Adam PJ, Schenker M, Loader JA, Daviet L, Legrain P, Parekh R, Harris AL, *et al*. hAG-2 and hAG-3, human homologues of genes involved in differentiation, are associated with oestrogen receptor-positive breast tumours and

interact with metastasis gene C4.4a and dystroglycan. *Br J Cancer* 2003;88:579–585.
16. Zhang L, Gaut BS, Vision TJ. Gene duplication and evolution. *Science* 2001;293:1551.
17. Schroeder BW, Nguyenvu LT, Erle DJ. The specialized protein disulfide isomerase family members agr2 and agr3 have distinctive expression patterns in mucous and ciliated cells [abstract]. *Am J Respir Crit Care Med* 2012;185:A6344.
18. Shoemark A, Dixon M, Corrin B, Dewar A. Twenty-year review of quantitative transmission electron microscopy for the diagnosis of primary ciliary dyskinesia. *J Clin Pathol* 2012;65:267–271.
19. You Y, Brody SL. Culture and differentiation of mouse tracheal epithelial cells. *Methods Mol Biol* 2013;945:123–143.
20. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J Biol Chem* 1985;260:3440–3450.
21. Lieb T, Frei CW, Frohock JI, Bookman RJ, Salathe M. Prolonged increase in ciliary beat frequency after short-term purinergic stimulation in human airway epithelial cells. *J Physiol* 2002;538:633–646.
22. Anders S, Huber W. Differential expression analysis for sequence count data. *Genome Biol* 2010;11:R106.
23. Zhao F, Edwards R, Dizon D, Afrasiabi K, Mastroianni JR, Geyfman M, Ouellette AJ, Andersen B, Lipkin SM. Disruption of Paneth and goblet cell homeostasis and increased endoplasmic reticulum stress in Agr2-/- mice. *Dev Biol* 2010;338:270–279.
24. Lucas JS, Adam EC, Goggin PM, Jackson CL, Powles-Glover N, Patel SH, Humphreys J, Fray MD, Falconnet E, Blouin JL, *et al*. Static respiratory cilia associated with mutations in Dnahc11/DNAH11: a mouse model of PCD. *Hum Mutat* 2012;33:495–503.
25. Schmid A, Salathe M. Ciliary beat co-ordination by calcium. *Biol Cell* 2011;103:159–169.
26. Raykhel I, Alanen H, Salo K, Jurvansuu J, Nguyen VD, Latva-Ranta M, Ruddock L. A molecular specificity code for the three mammalian KDEL receptors. *J Cell Biol* 2007;179:1193–1204.
27. Hoh RA, Stowe TR, Turk E, Stearns T. Transcriptional program of ciliated epithelial cells reveals new cilium and centrosome components and links to human disease. *PLoS One* 2012;7:e52166.
28. Hackett NR, Shaykhiev R, Walters MS, Wang R, Zwick RK, Ferris B, Witover B, Salit J, Crystal RG. The human airway epithelial basal cell transcriptome. *PLoS One* 2011;6:e18378.
29. Ross AJ, Dailey LA, Brighton LE, Devlin RB. Transcriptional profiling of mucociliary differentiation in human airway epithelial cells. *Am J Respir Cell Mol Biol* 2007;37:169–185.
30. King ER, Tung CS, Tsang YT, Zu Z, Lok GT, Deavers MT, Malpica A, Wolf JK, Lu KH, Birrer MJ, *et al*. The anterior gradient homolog 3 (AGR3) gene is associated with differentiation and survival in ovarian cancer. *Am J Surg Pathol* 2011;35:904–912.
31. Brychtova V, Zampachova V, Hrstka R, Fabian P, Novak J, Hermanova M, Vojtesek B. Differential expression of anterior gradient protein 3 in intrahepatic cholangiocarcinoma and hepatocellular carcinoma. *Exp Mol Pathol* 2014;96:375–381.
32. Vaarala MH, Hirvikoski P, Kauppila S, Paavonen TK. Identification of androgen-regulated genes in human prostate. *Mol Med Rep* 2012;6: 466–472.
33. Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol* 2007;8:519–529.
34. Schröder M, Kaufman RJ. The mammalian unfolded protein response. *Annu Rev Biochem* 2005;74:739–789.
35. Ivanova AS, Tereshina MB, Ermakova GV, Belousov VV, Zaraisky AG. Agr genes, missing in amniotes, are involved in the body appendages regeneration in frog tadpoles. *Sci Rep* 2013;3:1279.
36. Prins D, Michalak M. Organellar calcium buffers. *Cold Spring Harb Perspect Biol* 2011;3:3.
37. Baksh S, Burns K, Andrin C, Michalak M. Interaction of calreticulin with protein disulfide isomerase. *J Biol Chem* 1995;270: 31338–31344.
38. Higo T, Hattori M, Nakamura T, Natsume T, Michikawa T, Mikoshiba K. Subtype-specific and ER lumenal environment-dependent regulation of inositol 1,4,5-trisphosphate receptor type 1 by ERp44. *Cell* 2005; 120:85–98.
39. Olm MA, Kögler JE Jr, Macchione M, Shoemark A, Saldiva PH, Rodrigues JC. Primary ciliary dyskinesia: evaluation using cilia beat frequency assessment via spectral analysis of digital microscopy images. *J Appl Physiol (1985)* 2011;111:295–302.
40. Horani A, Brody SL, Ferkol TW, Shoseyov D, Wasserman MG, Ta-shma A, Wilson KS, Bayly PV, Amirav I, Cohen-Cymberknoh M, *et al*. CCDC65 mutation causes primary ciliary dyskinesia with normal ultrastructure and hyperkinetic cilia. *PLoS One* 2013;8:e72299.