EDNRA variants associate with smooth muscle mRNA levels, cell proliferation rates, and cystic fibrosis pulmonary disease severity

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Darrah R, McKone E, O’Connor C, Rodgers C, Genatossio A, McNamara S, Gibson R, Elborn JS, Ennis M, Gallagher CG, Kalsheker N, Aitken M, Wiese D, Dunn J, Smith P, Pace R, Londono B, Goddard K, Knowles MR, Drumm ML. EDNRA variants associate with smooth muscle mRNA levels, cell proliferation rates, and cystic fibrosis pulmonary disease severity. Physiol Genomics 41: 71–77, 2010. First published December 22, 2009; doi:10.1152/physiolgenomics.00185.2009.—Airway inflammation and pulmonary disease are heterogeneous phenotypes in cystic fibrosis (CF) patients, even among patients with the same cystic fibrosis transmembrane conductance regulator (CFTR) genotype. Endothelin, a proinflammatory peptide and smooth muscle agonist, is increased in CF airways, potentially contributing to the pulmonary phenotype. Four cohorts of CF patients were screened for variants in endothelin pathway genes to determine whether any of these variants associated with pulmonary function. An initial cohort of 808 CF patients homozygous for the common CF mutation, ΔF508, showed significant association for polymorphisms in the endothelin receptor A gene, EDNRA (P = 0.04), but not in the related endothelin genes (EDN1, EDN2, EDN3, or EDNRB) or NOS1, NOS2A, or NOS3. Variants within EDNRA were examined in three additional cohorts of CF patients, 238 patients from Seattle, WA, 303 from Ireland and the U.K., and 228 from Cleveland, OH, for a total of 1,577 CF patients. The three additional groups each demonstrated a significant association between EDNRA 3′-untranslated region (UTR) variant rs5335 and pulmonary function (P = 0.002). At the molecular level, single nucleotide primer extension assays suggest that the effect of the variants is quantitative. EDNRA mRNA levels from cultured primary tracheal smooth muscle cells are greater for the allele that appears to be deleterious to lung function than for the protective allele, suggesting a mechanism by which increased receptor function is harmful to the CF airway. Finally, cell proliferation studies using human airway smooth muscle cells demonstrated that cells homozygous for the deleterious allele proliferate at a faster rate than those homozygous for the protective allele.

polymorphism; modifier genes; genetic association study; endothelin receptor type A

* R. Darrah and E. McKone contributed equally to this article.

THE UNDERLYING CAUSE of cystic fibrosis (CF), mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, has been known for 20 years (19, 29, 31), but an explanation for the variation in disease course has been more difficult to elucidate. The overall influence of genetics on airway disease has been estimated to account for 50% or more of the phenotypic variance (23, 37), although it remains unclear as to how many genes may be acting as modifiers. To identify genetic modifiers of CF pulmonary disease, the CF Gene Modifier Study (GMS) was initiated and includes a cohort of CF patients homozygous for the common CF mutation, ΔF508, each of whom has lung function in the upper and lower quartiles for his or her age (10). Genotype frequencies of candidate modifier genes are compared between the phenotypically distinct groups of CF subjects, and those genes with discordant allele and/or genotype frequencies are selected for further study (10). This approach has resulted in identification of transforming growth factor-β1 (TGFβ1), interleukin 8 (IL8) (16), and interferon-related developmental regulator 1 (IFRD1) as pulmonary disease-modifying genes. The genes showing association with lung function appear to account for a relatively small amount of the variance (10) of this phenotype, implying there are other modifying genes yet to be identified. In fact, other studies have identified modifiers for CF, such as mannose-binding lectin (9, 11).

Endothelin receptor type A (EDNRA) was chosen as a candidate modifier gene based on its role in pulmonary pathophysiology. The product of EDNRA, endothelin receptor A, is a G protein-coupled receptor that causes cellular proliferation and contraction (26, 27) of smooth muscle when ligated to endothelin. Polymorphisms in this gene have been associated with variation in vascular phenotypes stemming from vasoconstriction (4, 15, 17, 20, 25, 38), and thus this gene was considered a candidate for our studies as we speculated that airway smooth muscle may react in a manner similar to vascular tissue. Variation in smooth muscle physiology as a modifier of CF lung disease was suggested by our previous observation (13) that CF patients show differential responses to the bronchodilator albuterol and that this variation associates with alleles of the β2-adrenergic receptor, similar to that shown for asthma.
The identification of these modifying genes has indicated that common population variants are likely to have relatively small effects, and thus obtaining the statistical power to detect them will be challenging. Assessing multiple study populations is a useful strategy to determine whether an association can be replicated, but when large numbers of variants are tested even this strategy may suffer from power limitations. Because true modifying alleles must impart functional differences on the gene or the encoded protein, another method to support an association is functional studies. Here we begin by employing replication in four groups of CF subjects, each of which show reproducible association with variants at the 3’ end of EDNRA. This gene encodes a G protein-coupled receptor found on smooth muscle cells, and functional assessment of the EDNRA alleles shows a strong association with mRNA expression and cell proliferation.

MATERIALS AND METHODS

Four CF populations were used for the genetic association studies. All patients and parents of minors provided written informed consent for each of the individual studies. Protocols were submitted to and approved by institutional review boards. The multicenter study was approved by the Biomedical Institutional Review Board of the University of North Carolina and the institutional review board of each participating institution. The Case Western Reserve University studies were approved by University Hospitals Case Medical Center Institutional Review Board. The Seattle study was approved by the Institutional Review Board at Children’s Hospital and Regional Medical Center/University of Washington. The Irish study was approved by the Research Ethics Committees at St Vincent’s University Hospital and Queens University Belfast.

Multicentered U.S. cohort. This cohort was comprised of 808 ΔF508 homozygous CF patients representing the upper and lower quartiles of pulmonary function and survival. Forced expiratory volume in 1 s (FEV1) values were calculated with previously published methods (33). Detailed descriptions of this study population have been previously published (10, 33). Each of these individuals provided a blood sample from which lymphocytes were transformed and DNA extracted with the Epicentre MasterPure DNA purification kit.

Seattle cohort. Patients were prospectively recruited from the adult and pediatric CF clinics at the University of Washington Medical Center and Seattle Children’s Hospital and Regional Medical Center. All participants supplied a blood sample from which DNA was extracted from buffy coat with a Qiagen DNA Extraction Kit (Qiagen, Chatsworth, CA). Patients who had participated in an earlier study of modifier genes in CF with limited amounts of genomic DNA underwent whole genome DNA amplification (GenomiPhi DNA Amplification Kit, GE Healthcare). Clinical data for each Seattle patient were abstracted from local CF clinical databases (Table 1).

Ireland cohort. DNA samples were obtained from banked samples stored at Queens Medical Center, Nottingham, U.K. as part of an existing Ireland/U.K. collaborative study of genetic modifiers in CF. All CF patients were recruited from the adult CF clinics in St Vincent’s University Hospital, Dublin, Ireland and Belfast City Hospital, Belfast, UK. Clinical data for each patient were abstracted from medical charts (Table 1) DNA samples were shipped to University of Washington Medical Center for whole genome amplification and preparation for genotyping.

Cleveland cohort. The Cleveland CF population was comprised of 228 subjects who were cared for at the Rainbow Babies and Children’s Hospital Cystic Fibrosis Center in Cleveland, OH from 1998 through 2001. These patients were either homozygous for ΔF508 or carried one ΔF508 mutation and one nonsense mutation. Each participant provided a cheek swab from which DNA was extracted with a common NaOH cell lysis method (32). Clinical data were obtained from the local patient database (Table 1).

Genotyping (multicenter U.S./Cleveland cohort). Genotyping for all single nucleotide polymorphisms (SNPs) described was performed by either TaqMan assay (Applied Biosystems) or Illumina. TaqMan assays were performed according to manufacturer’s instructions. Illumina assays were performed as previously reported (10).

Genotyping (Seattle/Ireland cohort). Four micrograms of DNA from each patient was extracted and concentrated at 50 ng/μl with PicoGreen confirmation and shipped to Illumina (San Diego, CA). All DNA samples were genotyped with Illumina Beadarray platform.

Cell culture of airway smooth muscle cells. Airway smooth muscle cells were cultured from tracheas obtained from autopsy. Cells were isolated from human tracheal muscle by digesting with collagenase and elastase in the presence of soybean trypsin inhibitor at 37°C as previously described (35). The cells were harvested by centrifugation after filtering through Nytec and grown in Dulbecco’s modified Eagle’s medium (DMEM-F-12 1:1) containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO2 and 95% air.

Single base extension. RNA was isolated from airway smooth muscle cells with the Qiagen RNEasy kit. One microgram of RNA was then reverse transcribed with Invitrogen MMLV (Moloney murine leukemia virus) First Strand Synthesis. The resulting cDNA was then diluted 10-fold, and 2% of that was used in the following PCR: 0.2 μM 5’ primer (5’-CATGCTCTGCTGCTGCTGTTA-3’), 0.2 μM 3’ primer (5’-AACAGACTCTACTCTGCGG-3’), 1.5 mM MgCl2, 0.25 mM dNTPs, 1 U of Taq polymerase. Genomic DNA was extracted from each cell line with the Epicentre MasterPure DNA purification kit. One hundred nanograms of DNA was used in the following PCR: 0.1 μM 5’ primer (5’-ATGCCCTCTGCTGCTGTTAC-3’), 0.1 μM 3’ primer (5’-CCTTGGTGTCTGTGGAAAAGC-3’), 3 mM MgCl2, 0.2 mM dNTPs, 1 U of Taq polymerase. Both reactions were amplified on an MJ Thermocycler with a standard low touchdown protocol. The ABI SnapShot Multiplex kit (Applied Biosystems) was then used according to manufacturer’s instructions to perform single base extension on each template with the following primer: 5’-TCTTCTCG-GAGAAAAAATCACAAGGCAACTGTTA-3’. After the SnapShot sequencing reaction, results were analyzed on an ABI 310 genetic analyzer. GeneScan analysis software was used to determine the area under each fluorescent peak (C and G), and a ratio of the two areas was determined. A standard curve was generated by mixing known amounts of homozygous genomic DNA as the template. Three standard curve runs were averaged, and the resulting equation \( y = 2.4291e^{0.0457x} \) was determined to define the standard curve. Three separate SnapShot reactions were performed on genomic DNA and cDNA from each cell line, and results were averaged. The resulting averaged C-to-G ratio was plotted on the curve to determine relative percentage of C expression (12).

# Table 1. Clinical characteristics of three cystic fibrosis patient cohorts

<table>
<thead>
<tr>
<th></th>
<th>Cleveland</th>
<th>Seattle</th>
<th>Ireland</th>
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</thead>
<tbody>
<tr>
<td>n</td>
<td>228</td>
<td>238</td>
<td>305</td>
</tr>
<tr>
<td>Sex, % male</td>
<td>54.8</td>
<td>52.1</td>
<td>51.2</td>
</tr>
<tr>
<td>Age, yr</td>
<td>19.2 (11.5)</td>
<td>20.8 (8.94)</td>
<td>25.9 (6.83)</td>
</tr>
<tr>
<td>FEV1, liters</td>
<td>2.11 (1.00)</td>
<td>2.42 (1.04)</td>
<td>2.33 (1.03)</td>
</tr>
<tr>
<td>FVC, liters</td>
<td>3.01 (1.19)</td>
<td>3.34 (1.27)</td>
<td>3.28 (1.20)</td>
</tr>
<tr>
<td>Height, cm</td>
<td>156 (19)</td>
<td>162 (16)</td>
<td>164 (9)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>49.1 (17.3)</td>
<td>54.8 (15.4)</td>
<td>58.4 (11.6)</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa infection, %</td>
<td>65.1</td>
<td>79.6</td>
<td>72</td>
</tr>
<tr>
<td>delFS508 homozygotes, %</td>
<td>46</td>
<td>57</td>
<td>51</td>
</tr>
</tbody>
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Values are means (SD) or percentages. FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity.
**Cell proliferation studies.** Human tracheal airway smooth muscle cells were cultured as described above. Cells were plated in 96-well culture plates at a starting density determined by cell counting with a hemocytometer. Plates were then allowed to incubate for 72 h, in DMEM medium with or without BQ-123 (10^{-6} M). Medium was changed every 24 h. Cell proliferation was analyzed with the CyQuant Cell Proliferation Assay Kit per manufacturer’s instructions. Three cell lines of each genotype were assayed, with each cell line assayed three independent times. Therefore, the graphs presented represent an average of nine measurements.

**Statistical analysis.** Single-marker tests of association were conducted by using the \( \chi^2 \) goodness of fit test and comparing to the method of genomic control (2, 8) to account for the possibility of spurious associations because of undetected population stratification within Caucasian populations (3). The genomic control parameter \( \lambda \) was calculated from 300 markers typed in the multicenter U.S. cohort. The test statistic for each marker is adjusted by dividing by \( \lambda \), and the \( P \) value is obtained from the \( \chi^2 \) distribution with this adjusted test statistic. If population stratification is present, the test statistics will be inflated for many markers, and the distribution of the test statistic will be shifted. The value of \( \lambda \) was found to be 1.0 for females and 1.14 for males (where 1.0 represents no evidence of population stratification), and the \( \lambda \)-adjusted \( P \) values are given in Fig. 1. We have adjusted the \( P \) values for population stratification, but no other corrections were made. Of the markers examined in this group of genes, only SNPs in or near EDNRA did not exceed our threshold of \( P = 0.05 \). Thus only EDNRA was pursued further in this study.

For the Seattle, Ireland, and Cleveland cohorts, ANOVA was used to test the relationship between lung disease severity (outcome variable) and the EDNRA genotypes (exposure variable). Severity of lung disease was estimated by using the highest FEV1 in a 1-yr period, which was analyzed as a continuous variable after log transformation. Sex, age, and height (including higher-order terms) were also included in a multivariate model as possible confounders or effect modifiers. Two-sided \( P \) value \(< 0.05 \) was considered statistically significant. All analyses were carried out with Stata 8.0.

**RESULTS**

**Replication of genetic association.** Eight hundred and eight \( \Delta F508 \)-homozygous subjects were genotyped for 14 SNPs spanning the EDNRA gene, and nominal associations were
detected in two distinct regions of the gene, corresponding to the 5'-untranslated region (UTR) and the 3'-UTR (Fig. 1).

As an effort to determine whether the associations between EDNRA variants and CF phenotype identified by the GMS were biologically based or statistical artifacts, we initiated a second screen of EDNRA variants in three additional cohorts of CF patients (Fig. 1).

Two additional, independently ascertained cohorts of CF subjects, from the Seattle area (n = 238) and Ireland (n = 303), were genotyped with eight SNPs representing the five linkage blocks surveyed in the GMS cohort. These populations were sampled from the entire phenotype distribution rather than from the extremes as in the GMS project. A SNP in intron 2 and one marking the 3' end of the gene showed significant associations with lung severity (Fig. 1). The intron 2 SNP (rs10305895) displayed a significant association in the Seattle and Ireland cohorts (P = 0.006 and 0.03, respectively) but showed no significant association in the GMS cohort (Fig. 1). In contrast, the 3' SNP rs5335 showed remarkable concordance in all three groups.

The 3'-UTR SNP rs5335 associated with pulmonary phenotype in each of the three independent studies. The final cohort analyzed was a group of CF patients from the Cleveland area who, like the Seattle and Ireland cohorts, were not stratified by pulmonary phenotype. Descriptions of the demographics of these three CF cohorts can be found in Table 1. The Cleveland cohort was genotyped for rs5335 and, like the Seattle and Ireland cohorts, showed a significant association between the rs5335 genotype and pulmonary function (P = 0.04). Finally, the Seattle, Ireland, and Cleveland populations were all similarly ascertained and shared similar clinical demographics (Table 1) and thus were pooled to determine the combined significance of the association in these three populations (P = 0.002) and to estimate effect size. Thus all four independent CF populations show that homozygosity for the C allele at rs5335 is associated with poorer pulmonary function than homozygosity for the G allele (Fig. 2) and that the GG genotype at rs5335 appears to play a protective role in CF.

Quantitative analysis of rs5335. SNP rs5335 is located in the 3'-UTR of exon 8, and variants to either side, in the coding region or 3' to the gene, but located only a few kilobases away show weaker association (Fig. 1). This positioning of rs5335 within the transcript, but outside of the coding sequence, suggests that this variant's effect is quantitative rather than qualitative, influencing mRNA levels. To test this idea, EDNRA mRNA levels were measured from non-CF tracheal smooth muscle cells of each rs5335 genotype. Primary smooth muscle cell cultures were genotyped, and EDNRA mRNA levels were compared by two methods (Fig. 3). First, a single nucleotide primer extension assay was utilized to compare relative expression of the C allele to the G allele in cell lines heterozygous at rs5335. Relative expression was quantified by interpolating sample values on a standard curve generated by mixing known amounts of homozygous DNA. In cells heterozygous at rs5335, the C-containing allele was expressed at a level 20% greater, on average, than the G allele (Fig. 3, A and B), while genomic DNA from the same samples showed the expected 50–50 ratio. The proportion of G allele expressed never exceeded the C allele in any of the cell lines tested. Real-time quantitative PCR was then used to examine the total amount of EDNRA mRNA present in the non-CF primary airway smooth muscle cells as a function of genotype. When stratified by genotype, cells carrying the CC genotype expressed on average approximately two times the amount of EDNRA mRNA compared with cells homozygous for the GG genotype at rs5335 (P = 0.03) (Fig. 3C).

Cell proliferation studies. Endothelin receptor A binds endothelin-1 (ET-1) in airway smooth muscle cells to stimulate smooth muscle proliferation (5). We compared cell proliferation rates from human tracheal airway smooth muscle cells homozygous for the C allele at rs5335 to those homozygous for the G allele at rs5335. After 72 h of growth, the cell lines homozygous for the C allele had 50% more cell growth than the cells homozygous for the G allele (Fig. 4). Receptor antagonism by endothelin receptor A blocker BQ-123 attenuates the growth difference, indicating that the observed differences in cell proliferation rates are due to EDNRA variation (Fig. 4).

In summary, the EDNRA C allele at rs5335 is found more commonly in severe CF patients in four separate cohorts of CF patients, correlates with increased EDNRA message levels, and is associated with increased smooth muscle proliferation.

DISCUSSION

As part of a multicenter study surveying candidate genes as modifiers of CF pulmonary disease, variants within EDNRA demonstrated a significantly different genotype frequency between phenotypically different groups of CF patients. EDNRA polymorphism rs5335, in the 3'-UTR of the gene, was examined in three additional populations of CF patients and associated similarly in all four groups. In all of the populations tested, CF patients homozygous for the C allele at rs5335 had pulmonary function levels interpolated or extrapolated for age 20 at levels ~10% lower than patients homozygous for the G allele at rs5335. This difference in pulmonary function would be
predicted to have significant clinical ramifications, such as differences in life expectancy (33).

Variants within EDNRA, and more specifically polymorphism rs5335, have been studied in relation to a number of diseases. The C allele has been associated with a risk for increased pulse pressure (25). The G allele has been associated with an increased risk for hypertension and a worsening of glaucoma symptoms (18, 28). Polymorphism rs5335 is located at nucleotide 211 of exon 8 of EDNRA, which is within the 3′-UTR. 3′-UTRs of eukaryotic mRNAs are often involved in posttranscriptional regulatory pathways and have been implicated in message stability (36). Genetic variation within the EDNRA 3′-UTR could have significant effects on mRNA message stability and thus the total amount of functional receptor within the airway.

We propose that the effect of EDNRA variants in CF is acting through smooth muscle. Studying this concept directly is impeded because primary smooth muscle tissue from CF subjects is not readily available. However, these variants are not exclusive to CF subjects, and thus non-CF cells may be suitable to determine the functional nature of these variants. Accordingly, non-CF samples suggest that the adverse genotypes express greater relative amounts of EDNRA mRNA than the genotypes associated with mild disease. In addition, smooth muscle cells containing the adverse genotypes appear to proliferate at a faster rate than cell lines with the protective genotype. Although the differences in EDNRA mRNA quantity and cell proliferation rates between the rs5335 genotypes are clearly detectable under acute culture conditions, it is not clear how they affect the CF airway chronically. Such effects would require in vivo studies to understand the functional consequences at the tissue level. Nonetheless, CF airway disease is a progressive process that occurs over years, so we expect that the effects in vivo are relatively subtle and that the differences could be amplified by stimuli that normally increase EDNRA expression. Because these EDNRA variants are common in the population and not suspected to be disease causing, one might expect their effects to be subtle under most conditions. The effect of the variants appears to be quantitative, influencing mRNA levels. However, while the genotypic groups appear to be different in EDNRA expression, there is clearly overlap between them, indicating that the genotypes we have examined are unlikely to be the causes of variation but rather markers of it.

The association between EDNRA variants and increased message levels and proliferation in non-CF airway smooth muscle cells could have implications for other diseases affecting smooth muscle. Genetic association studies have indicated that variation in EDNRA may play a role in asthma, cardiovas-

Fig. 3. The rs5335 C allele is expressed at a higher level than the G allele. A: a calibration curve was generated by mixing sequences containing either the C or G allele in varying ratios and carrying out the single base extension assay. The fluorescence signal for C:G (left) is plotted as a function of % G in the assay. B: relative expression of each allele was determined from 16 smooth muscle cell lines heterozygous at rs5335. A single base extension genotyping assay for rs5335 using fluorescently labeled CTP and GTP was carried out on reverse-transcribed RNA (cDNA) and genomic DNA. Fluorescence ratios (FC/FG) were quantified with the calibration curve shown in A and indicate that the C allele is expressed, on average, 20% higher than the G allele. C: EDNRA mRNA from primary cultures of non-cystic fibrosis (CF) tracheal smooth muscle from 15 individuals homozygous for rs5335 was quantified by quantitative RT-PCR and normalized to β-actin RNA. Shown are the normalized quantities stratified by rs5335 genotype.

Fig. 4. Cell proliferation is higher in tracheal smooth muscle cell lines with the rs5335 CC genotype (n = 3) compared with cell lines with the rs5335 GG genotype (n = 3). Addition of EDNRA antagonist BQ-123 shows a significant decrease (P = 0.02) in cell proliferation.
cular disease, and hypertension (7, 21, 25, 28). Variation in EDNRA signaling may explain these associations as well, offering exciting possibilities for therapeutic intervention.

In the lung, EDNRA activity is stimulated by ligation with ET-1, resulting in smooth muscle contraction, cell proliferation, and inflammation (5). Each of these events has been described as deleterious for the CF lung (22, 27). ET-1, reported to be elevated in CF lungs (6), combined with an EDNRA genotype that promotes increased expression, predicts an environment that would be detrimental to the CF airway. The data presented here indicate that mRNA levels are higher for those alleles associating with more severe disease, correlating with cellular proliferation rates, and that the proliferative differences are diminished by receptor antagonism. There are additional consequences of EDNRA stimulation that have yet to be explored, including apoptosis and cellular contraction. The relative contribution of each of the known EDNRA functions, cell proliferation, apoptosis, or contraction, to the pulmonary disease phenotype is not clear.

There is evidence to suggest that there are inherent differences in the regulation of tone in CF smooth muscle as well (30). The CFTR channel is present in airway smooth muscle cells and appears to modulate calcium release in response to contractile agents (24). Vascular smooth muscle from Cfrt−/− mice was not only more constricted than wild-type smooth muscle, it also did not respond as readily to induced vaso-relaxation (30). In humans, a similar pulmonary vascular dysfunction has been described in CF and is at least in part attributable to the endothelin pathway, because pharmaceutical blocking of this pathway leads to restored vasodilation (14).

The data presented here strongly support a role for EDNRA variants in modifying the severity of CF pulmonary disease. First, the association is reproducible, found in four independent groups of CF patients. Second, there are functional effects on gene expression associated with the alleles, providing a molecular mechanism for the associations. Third, the established role of EDNRA in smooth muscle physiology makes this gene biologically plausible, because differences in its activity would be predicted to influence airway smooth muscle proliferation and/or contractility. In sum, these data suggest a mechanism whereby a 3′-UTR variant is associated with increased EDNRA message levels, resulting in increased smooth muscle proliferation and a subsequent worsening of CF pulmonary phenotype. The prospect of EDNRA as a therapeutic target for CF is exciting, as therapies targeting endothelin signaling pathways have not yet been investigated. Antagonists to the receptor are already available and currently indicated for pulmonary hypertension (1, 34). Consequently, translational application of the findings reported here has potential that could be rapidly realized.

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DISCLOSURES

The authors are not aware of financial conflict(s) with the subject matter or materials discussed in this manuscript with any of the authors, or any of the authors’ academic institutions or employers.

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