Candidate genes controlling pulmonary function in mice: transcript profiling and predicted protein structure

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1National Research Center for Environment and Health (GSF), Institute for Inhalation Biology, Neuherberg, Germany; 2Center for Environmental Genetics, Department of Environmental Health, University of Cincinnati, Cincinnati, Ohio; and 3Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh, Pennsylvania

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Ganguly K, Stoeger T, Wesselkamper SC, Reinhard C, Sartor MA, Medvedovic M, Tomlinson CR, Bolle I, Mason JM, Leikauf GD, Schulz H. Candidate genes controlling pulmonary function in mice: transcript profiling and predicted protein structure. Physiol Genomics 31: 410–421, 2007. First published September 5, 2007; doi:10.1152/physiolgenomics.00260.2006.—Impaired development and reduced lung capacity are risk factors of asthma and chronic obstructive pulmonary disease. Previously, we elucidated a genomewide linkage analysis of C3H/HeJ (C3H) and JF1/Msf (JF1) mouse strains identified quantitative trait loci (QTLs) associated with the complex traits of dead space volume (VD), total lung capacity (TLC), lung compliance (CL), and diffusing capacity for CO (DCO). We assessed positional candidate genes by comparing C3H with JF1 lung transcript levels by microarray and by comparing C3H, BALB/cByJ, C57BL/6J, A/J, PWD/PJ, and JF1 strains, using exon sequencing to predict protein structure. Microarray identified >900 transcripts differing in C3H and JF1 lungs related to lung development, function, and remodeling. Of these, three genes localized to QTLs associated with differences in lung function. C3H and JF1 strains differed in transcript and protein levels of superoxide dismutase 3, extracellular SOD3; mouse chromosome (mCh) 5; and transcript of trefoil factor 2 (TFF2; mCh 17: TLC and DCO), and eukaryotic pyrophosphate/phosphodiesterase 2 (ENPP2; mCh 15: TLC and CL). Nucleotide sequencing of Sod3, Tff2, and previously identified Relaxin 1 (Rln1; mCh 19: CL) uncovered polymorphisms that could lead to nonsynonymous amino acid changes and altered predicted protein structure. Gene-targeted Sod3+/− mice had increased conducting airway volume (Vb/VL) compared with strain-matched control Sod3+/+ mice, consistent with the QTL on mCh 5. Two novel genes (Tff2 and Enpp2) have been identified and two suspected genes (Sod3 and Rln1) have been supported as determinants of lung function in mice. Findings with gene-targeted mice suggest that SOD3 is a contributing factor defining the complex trait of conducting airway volume.

Increasing scientific interest has been focused on gene-environment interactions that determine human responses to cigarette smoke, metals, and ambient air pollution. Constant improvements in genetic analyses have enabled the acquisition of genomewide information on numerous species and the rapid determination of phenotype-genotype linkages that continue to provide a deeper understanding of common human diseases (i.e., complex traits). Respiratory diseases like chronic obstructive pulmonary disease (COPD) are characterized by an accelerated decline in lung function and have been associated with numerous environmental risk factors, especially cigarette smoking. Although smoking is declining in some countries, COPD continues to rise worldwide and is now the fourth leading cause of death. A striking reality of smoking is that the mass of irritants and particulate matter deposited in a two pack/day smoker’s lungs over a lifetime is staggering. Yet not everyone who smokes develops severe COPD, lung cancer, or other smoking-related diseases, which strongly supports a role for individual genetic susceptibility and resistance. For example, one study estimated that only 15% of the variation in lung function in smokers is conferred by the degree of smoking exposure (a primary environmental factor) (4), while another study estimated that >40% of the variation is conferred by heredity (genetic factors) (9).

Impaired embryonic and neonatal lung development may contribute to adolescent and adult lung diseases including asthma (45) and COPD (41). Shortened gestation is a proposed risk factor for adolescent asthma because it results in diminished airway diameter, and thus increased hyperreactivity. It has also been proposed that persons with a smaller initial lung volume are more likely to develop overt manifestations during the accelerated decline of lung function in COPD. Therefore, the genetic factors that limit lung development can be viewed as risk factors for later appearance of clinically significant disease. Genomewide association studies examining differences in forced expiratory volume in 1 s (FEV1), forced vital capacity (FVC), and the ratio FEV1/FVC have identified linkage to loci on 13 different human chromosomes (hCh) with replicated linkage reported on hCh 2, 5, and 6 (21, 42, 51–53).

In inbred mice, considerable interstrain differences in lung function and lung morphometrics have been reported (43, 46). Previously, we (34, 35) measured lung function in C3H/HeJ (C3H), BALB/cByJ (BALB), C57BL/6J (C57), A/J, and JF1/Msf (JF1) mouse strains and found that C3H and JF1 differed the most. For example, the total lung capacity (TLC, male and female) is 1,640 ± 54 μl in C3H mice, whereas TLC is 890 ± 15 μl in JF1 mice. Accordingly, we completed a quantitative trait loci (QTL) analysis of multiple lung phenotypes in offspring derived from the C3H (Mus musculus domesticus) and JF1 (Mus musculus molossinus) strains (35). With female N2 backcross and F2 intercross progeny, genomewide linkage analysis was performed for TLC, dead space volume (VD), lung compliance (CL), and diffusing capacity for carbon.
monoxide (DCO). Significant linkage was detected for TLC on mouse chromosome (mCh) 15 and 17 [logarithm of the odds (LOD) ≥ 5.0], Vd on mCh 5 and 15 (LOD ≥ 4.0), Ct. on mCh 15 and 19 (LOD ≥ 5.0), and DCO on mCh 15 and 17 (LOD ≥ 4.0).

After evaluating the possible physiological roles of genes harbored in these regions, we proposed possible candidate genes including superoxide dismutase 3, extracellular (Sod3, aka extracellular SOD) on mCh 5. This region is syntenic to a region on hCh 4 with suggestive linkage to FEV1/FVC identified in the National Heart, Lung, and Blood Institute Family Heart Study (51). We also proposed Relaxin 1 (Rln1) as a candidate for the QTL on mCh 19. To further explore these linkages, we performed a microarray analysis contrasting C3H with JF1 strain lung transcript levels with confirmatory quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot analyses. This was followed by exon sequencing of the candidate genes to predict protein structural modifications.

Western blot analyses. This was followed by exon sequencing of the candidate genes to predict protein structural modifications among six mouse strains, the five listed above and PWD. Volume, airway opening pressure (Pao), esophageal pressure, and CL was derived from the transpulmonary pressure-volume curve. Series VD was obtained by the Fowler method from single-breath washin measurements with 1% He in air administered for 3 s from relaxed expiratory level to TLC to slightly below FRC over 7 s without a breath hold. DCO was determined with a single-breath-holding method using a gas (0.3% C14O-1% He-21% O2-71.7% N2) started from the relaxed expiratory level to TLC over 3 s followed by a breath-holding time of 3 s and an expiration slightly below FRC. Alveolar volume (VA) was determined according to the method of Cotes et al. (8). Values of lung function measurements were also normalized to body weight (BW) or lung size (e.g., TLC/BW, Vd/TLC). Further details of the technique including calibration and reproducibility are described elsewhere (31a, 39). Age (12 wk), BW-, and sex-matched Sod3+/− mice were used as controls for comparisons of lung function with Sod3−/− mice (n = 6/group).

RNA Isolation and Microarray Analysis

For this initial transcript analysis of the lung we selected mice at 4 wk of age because this is a late adolescence, early adult stage in mice. In humans, the lung develops relatively late in gestation (with premature infants often being at risk of lung failure due to incomplete surfactant synthesis), continues to grow throughout childhood, and peaks at early adulthood (typically at 20–30 yr of age). With age lung function begins to decline, with an increasing rate of decline noted in chronic obstructive lung disease. Mice are born with incomplete lung morphology and continue to develop alveoli shortly after birth. The period of rapid alveolarization is nearly completed within the first 2 wk after birth and followed by further septation and lung growth at a continuously declining pace until adolescence (43). Therefore the age of 4 wk is a late adolescence and early adulthood stage, and we considered it to be the optimum time point for the first analysis. This stage can be considered as the transition between completion of lung development and beginning of the growth-maturation phase.

At exactly age 28 days, female C3H and JF1 mice (n = 8 mice/strain) were anesthetized (5 mg pentobarbital sodium ip; Abbott, Chicago, IL), and the lungs were removed and immediately frozen in liquid nitrogen. Total RNA was isolated from homogenized lung with TRIzol reagent (Invitrogen, Carlsbad, CA), and the concentration of total RNA was determined by spectrometric analysis. The quality of RNA was confirmed by running an aliquot of each sample on a denaturing formaldehyde-agarose-ethidium bromide gel and quantified by analysis with an Agilent Bioanalyzer (Quantum Analytics, Foster City, CA).

To examine differential gene expression of 31,775 70-mer oligonucleotides, microarrays were fabricated by the Genomic and Microarray Laboratory, Center for Environmental Genetics, University of Cincinnati (http://microarray.uc.edu), using a commercial library (Qiagen-Operon, Alameda, CA) consisting of clones amplified by PCR and printed onto glass slides (Omnigrid Microarrayer, GeneMachines, San Carlos, CA). Eight microarrays per strain were compared, using 20 μg of total RNA per array. Each sample of mRNA was reverse transcribed and reciprocal tagged randomly with fluorescent Cy3 and Cy5 dyes at 20 pmol (i.e., the Cy3 and Cy5 dye switched, e.g., Cy3 for C3H strain and Cy5 for JF1 strain). Cy3 and Cy5 samples were cohybridized with the printed 70-mers. A sample from an individual mouse of one strain was randomly paired with a sample from a mouse of another strain and hybridized on a single array. After hybridization, slides were washed and scanned at 635 (Cy5) and 532 (Cy3) nm (GenePix 4000B, Axon Instruments, Union City, CA). For inclusion in analysis, an oligo covered its grid location on a slide by a minimum of 50% and a maximum of 400% of the 100-μm feature. The area of the feature was set to one-third of the background area and had an intensity above background. Each array was visually inspected to exclude artifacts or fused or streaked features. Consistency of fluorescence measurements in different hybridizations was assessed by calculating pairwise correlation coefficients between measurements for the same mouse in different hybridizations. All such pairwise...
Pearson’s correlation coefficients were ≥0.9, indicating the reproducibility of measurements.

**Microarray Data Normalization and Pathway Analysis**

Data normalization was performed in three steps for each microarray as described previously (24). Statistical analysis of microarray data was performed by fitting the following mixed-effects linear model for each gene separately as previously described (50). To further analyze the microarray data set, we used the Microarray Pathway Profile Finder (MAPPFinder) program to dynamically link microarray data to the Gene Ontology (GO) hierarchy database as illustrated previously (11). The results, calculated with Fisher’s exact test, are expressed as a “z score” for a given pathway, and values of at least 2.0 were considered to be significant. Gene Microarray Pathway Profiler (GenMAPP) was used to view microarray data on biological pathways (10).

**Confirmational qRT-PCR**

For qRT-PCR, 100 ng of lung RNA from C3H and JF1 mice (n = 5 mice/strain) was reverse transcribed into first-strand cDNA with a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) in a 100-μl reaction volume. cDNA (10 μl) was used in a subsequent PCR reaction using 25 μl of TaqMan Universal PCR Master Mix (Applied Biosystems), 2.5 μl of each primer mixture, and 12.5 μl of RNAse-free water. Primer mixtures for Tff2 (catalog no. Mm00447491_m1), Enpp2 (catalog no. Mm00516572_m1), Sod3 (catalog no. Mm00448831_m1), and actin, β, cytoplasmic (Actb) (catalog no. Mm00067939_s1) were purchased from Applied Biosystems, and analysis was performed with an Applied Biosystems 7900HT System and the following conditions: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. For relative quantification of expression of each gene between the C3H and JF1 strains, the comparative cycle number threshold (Ct) method (∆∆Ct) was used: ∆Ct = Ct (gene of interest) − Ct (Actb), and this value was calculated for each sample. The comparative ∆∆Ct calculation involved finding the difference between each sample’s ∆Ct and the mean ∆Ct for the JF1 strain. These values were then transformed to absolute values with a formula in which comparative expression level = 2−ΔΔCt.

**Western Blot Analysis**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with NuPAGE 4–12% Bis-Tris Gel (catalog no. L06010BOX, Invitrogen). Total lung protein (20 μl) was loaded in each lane. After SDS-PAGE, proteins were electrophoretically transferred to an Invitronol polyvinylidene difluoride membrane (catalog no. LC2005, Invitrogen). The membrane was then blocked with 5% fat-free milk in PBS-T (in mM: 170 NaCl, 2.68 KCl, 3.21 Na2PO4, and 1.47 KH2PO4 with 0.1% Tween 20, pH 7.4) for 1 h at room temperature. The membrane was then incubated with rabbit anti-mouse SOD3 antibody (kindly provided by Dr. Tim D. Oury) in 5% fat-free milk in PBS-T, followed by incubation with horseradish peroxidase-conjugated goat anti rabbit IgG. The antibody was then detected with an ECL detection system (Amersham Life Science, Little Chalfont, UK). Two bands signifying the intact and cleaved SOD3 at 32 and 31 kDa, respectively, were detected in all cases, and results were normalized to ACTB used as a protein loading control.

**Genomic DNA, PCR Amplification, and Nucleotide Sequencing**

Genomic DNA from C3H, BALB, C57, A/J, PWD, and JF1 mouse strains (listed from larger to smaller lung volume) was purchased from the Jackson Laboratory (http://www.jax.org/dnares/index.html). PCR amplification of each candidate gene was performed in an exonwise manner from the genomic DNA with standard procedures. PCR-amplified products were then commercially sequenced in both forward and reverse directions to obtain safe sequences (Sequiverse, Vaterstetten, Germany). The sequences of candidate genes from the five strains (BALB, C57, A/J, JF1, and PWD) were compared with C3H to identify single nucleotide polymorphisms (SNPs) that could result in amino acid changes. Lists of oligonucleotides used for PCR amplification are provided in the supplemental material for this article (Supplemental Table S1). 1

**Protein Structure Predictions and Hydropathic Profiles**

SwissModel First Approach Mode (20) was used to generate the Protein Data Bank (.pdb) files. The secondary structure of proteins was developed with the DeepView Swiss-PDB viewer (20) and MDL Chimie programs or PSIPRED Protein Structure Prediction Server (29). The DeepView Swiss-PDB viewer was used to calculate the total molecular surface area and electrostatic potential of the proteins. PROSITE scanning was performed to detect the location of each polymorphism in relation to the known functional domains of a protein. Hydropathic profiles of each protein from each strain were calculated and compared by the Kyte and Doolittle (28) method (default window size: 9). World Wide Web addresses for all the programs applied and further details of protein prediction studies are provided in the supplemental material for this article.

**RESULTS**

**Lung Function of PWD Mice**

In our previous survey of lung function in various mouse strains (34, 35), we initially assessed lung function in PWD mice and found TLC in this strain to be similar to that of the JF1 strain, which is much smaller than the TLC of the C3H strain. In this study, we further examined additional lung functions in PWD mice to determine whether this strain shares other lung function measurements with JF1 mice (Table 1). In PWD mice, TLC was 6% smaller in females (955 μl) than in males (1,015 μl) and was slightly larger than that of JF1 mice (females = 874 μl; males = 904 μl) but was significantly less than that in C3H mice (females = 1,443 μl; males = 1,837 μl). At full inflation Vd was not different between sexes, and the mean Vd (200 μl) in PWD mice was similar to the mean Vd (197 μl) of JF1 mice, but significantly lower than that of C3H mice (234 μl). The value of mean Vd/TLC (20.4%) in PWD mice was also similar to the mean Vd/TLC (22.2%) in JF1 mice, and both strains had higher mean Vd/TLC than C3H mice (14.7%) (Table 1).

PWD mice also exhibited the least compliant lung (Ct or Cl/TLC) among all strains studied (34, 35). In PWD mice, Dco was also less than that of C3H mice, but when normalized to Va, Dco/Va in PWD was greater than that in C3H. Similar to the relationship between JF1 and C3H mice, these findings are consistent with higher gas exchange efficiency per unit of lung volume. On the basis of this analysis, PWD phenotypes were deemed to resemble those of the JF1 strain, and exon sequences of candidate genes of JF1 and PWD strains were compared with those of the C3H strain.

**Lung Transcript Levels Differing Between C3H and JF1 Strains**

For microarray analysis, we considered expression values significantly different between C3H and JF1 mice when the
false discovery ratio (FDR) was <0.05 and an average intensity was ≥150. Of the 31,775 genes that were present on the microarray, 18,399 were detected in the lung RNA on each array, and of those 933 transcripts significantly differed between strains (FDR < 0.05) (submitted to ArrayExpress; accession no. E-MEXP-855). The assigned chromosomal location of the 933 genes was obtained from the Ensembl Mouse database with the NCBI m36 mouse assembly (C57 reference strain).

We focused the genomewide search in transcripts located within a previously identified chromosomal region with significant linkage (35) and that contained a 5′-untranslated region (UTR) SNP differing between C3H and JF1 as determined in silico. Using these criteria, we identified Sod3 (C3H vs. JF1 = 2.0 ± 0.2-fold; P < 0.0000001), previously identified as a candidate gene on mCh 5, and two novel candidate genes, Tff2 (C3H vs. JF1 = 2.4 ± 0.5-fold; P < 0.004) located on mCh 17 and Empn2 (C3H vs. JF1 = 1.6 ± 0.3-fold, P < 0.000002) located on mCh 15. Confirmation of differences in lung expression of these genes between the C3H and JF1 strains was performed by qRT-PCR (Fig. 1A). Of these three candidates, the strain difference in SOD3 transcript levels was the greatest, and therefore a confirmational Western blot was performed (Fig. 1, B and C).

We also evaluated the transcript levels of other candidate genes previously identified based on possible physiological significance and chromosomal location within QTLs associated with lung function (35). Transcript levels of Rxrb and Tgfbr3 were not significantly different between the two strains, and transcripts for Rln1 and Pdgfb were not detected in lung RNA from 4-wk-old JF1 or C3H mice.

Although transcript expression profiling has been supportive of QTL detection in the past (12, 23, 24), it is salutary to consider the limitation of this approach (15). Variants at the level of protein structure may not influence gene expression, transcript stability, or steady-state transcript levels (yielding false negatives), or they may illicit compensatory mechanisms (yielding false positives). In addition, expression differences may be limited to distal tissues (e.g., hormonal or neural signaling) or to developmental stages not examined. Finally, the physical proximity of a gene that is expressed in a linkage region does not prove causality. Thus it is important to sequence suspected candidate genes and to explore the strain differences in structure.

**Pathway Analysis**

Using MAPMFinder, we further evaluated the functional significance of the lung expression differences between C3H and JF1 mice as assessed by microarray. In comparing classification terms in the GO Consortium categories of cellular components, biological processes, and molecular function, several molecular pathways were identified from the 933 significant transcript expression differences that contained an overrepresented number of altered transcripts (i.e., z score ≥2.0 with ≥7 transcripts altered). Several of the affected pathways were likely to play a role in lung development, function, and remodeling and included extracellular matrix (z = 3.0), metalloendopeptidase activity (z = 3.0), growth factor activity (z = 2.8), and vasculature development (z = 2.4).

**Polymorphisms and Predicted Protein Structure of Candidate Genes**

On the basis of the microarray analysis and previous linkage analysis (35), Sod3, Tff2, Rln1, Tgfbr3, Pdgfb, and Rxrb were selected for further exon sequence analysis to assess amino acid polymorphisms among the mouse strains (including JF1, PWD, C3H, BALB, C57, and A/J) (Table 2). Several polymorphisms were detected in the nucleotide sequences of five genes (Sod3, Tff2, Rln1, Tgfbr3, Pdgfb). Of these, no obvious differences in predicted protein resulted from four polymorphisms detected in Tgfbr3 (Supplemental Fig. S6) or one polymorphism detected in Pdgfb (Supplemental Fig. S7). Thus the three remaining candidates, Sod3, Tff2, and Rln1, were further analyzed.

**Sod3.** Four missense polymorphisms were detected in Sod3 that included 1) A → G in Sod3<sup>JF1,PWD</sup>, Asn-21-Asp, uncharged polar → acidic; 2) G → C in Sod3<sup>JF1,PWD</sup>, Glu-57-Gln, acidic → uncharged polar; 3) C → T in Sod3<sup>JF1</sup>, Pro-152-Leu, nonpolar → nonpolar; and 4) G → T in Sod3<sup>BALB,JF1,PWD</sup>, Ala-186-Ser, nonpolar → uncharged polar (Supplemental Fig. S1). The predicted amino acid sequence of SOD3 is identical among C3H, C57, and A/J strains. Under identical coordinates of rotational angle by either Swissprot or MDL Chimie anal-

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### Table 1. Body weight and lung function values of PWD/PhJ mice in comparison with C3H/HeJ and JF1/Msf mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PWD/PhJ Female</th>
<th>PWD/PhJ Male</th>
<th>JF1/Msf Female</th>
<th>JF1/Msf Male</th>
<th>C3H/HeJ Female</th>
<th>C3H/HeJ Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>15.8 ± 0.2</td>
<td>19.1 ± 0.4‡</td>
<td>16.5 ± 0.4</td>
<td>22.7 ± 0.7</td>
<td>22.4 ± 0.6</td>
<td>29.0 ± 0.7</td>
</tr>
<tr>
<td>Lung volumes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLC, µl</td>
<td>955 ± 17†</td>
<td>1,015 ± 20†</td>
<td>874 ± 17†</td>
<td>904 ± 11†</td>
<td>1,443 ± 29</td>
<td>1,837 ± 79</td>
</tr>
<tr>
<td>TLC/BW, µl/g</td>
<td>60.7 ± 1.6*</td>
<td>53.2 ± 0.9†</td>
<td>53.6 ± 1.7†</td>
<td>40.2 ± 1.5†</td>
<td>64.9 ± 2.1</td>
<td>65.3 ± 2.8</td>
</tr>
<tr>
<td>Vt, µl</td>
<td>202 ± 5†</td>
<td>198 ± 4†</td>
<td>193 ± 3†</td>
<td>201 ± 4†</td>
<td>227 ± 2</td>
<td>245 ± 4</td>
</tr>
<tr>
<td>Nu/TLC, %</td>
<td>21.3 ± 0.7†</td>
<td>19.5 ± 0.6†</td>
<td>22.1 ± 0.5†</td>
<td>22.3 ± 0.3†</td>
<td>15.8 ± 0.3</td>
<td>13.6 ± 0.5</td>
</tr>
</tbody>
</table>

Values are means ± SE. For comparison, data from C3H/HeJ and JF1/Msf mice are provided (34, 35). BW, body weight; TLC, total lung capacity; Vt, dead space volume; Cl, static lung compliance; DCO, diffusing capacity for carbon monoxide; VA, alveolar volume. *Significant differences from JF1/Msf (P < 0.01); ‡significant differences from C3H/HeJ (P < 0.05); †significant difference between sex in PWD/PhJ (P < 0.05); ‡significant difference between sex in PWD/PhJ (P < 0.05).
Analysis of lung homogenates of 4-wk-old C3H/HeJ (C3H) and JF1/Msf (JF1) strains showing significantly low immunoreactive SOD3 protein level (both intact and cleaved forms) in the lungs of JF1 mice (Fig. 2A). In addition to altered protein structure, the predicted SOD3JF1 protein backbone for JF1 mice (SOD3JF1) was markedly different from that of other strains including BALB and PWD. Presumably this is due to the combined or singular effect of the polymorphisms at positions 152 and 186 (Fig. 2A and B). In Table 2, polymorphisms in candidate genes linked to lung function in mice and their predicted effect on the respective protein.

### Table 2. Polymorphisms in candidate genes linked to lung function in mice and their predicted effect on the respective protein

<table>
<thead>
<tr>
<th>Gene</th>
<th>Associated Lung Function</th>
<th>Polymorphisms Detected and Predicted Amino Acid Changes</th>
<th>Secondary Protein Structure</th>
<th>Total Molecular Surface Area</th>
<th>Electrostatic Potential</th>
<th>Hydropathic Profile</th>
<th>Functional Domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sod3</td>
<td>Cl. and Vd</td>
<td>Total: 4 (JF1, PWD, BALB)</td>
<td>+ (JF1), - (PWD)</td>
<td>++ (JF1)</td>
<td>++ (JF1)</td>
<td>+ (JF1)</td>
<td>(between amino acid positions 140 and 200)</td>
</tr>
<tr>
<td>Tff2</td>
<td>TLC and DcO</td>
<td>Total: 3 (PWD)</td>
<td>- (JF1), - (PWD)</td>
<td>+ (JF1)</td>
<td>- (JF1)</td>
<td>(between amino acid positions 70 and 90)</td>
<td></td>
</tr>
<tr>
<td>Rbl1</td>
<td>Cl.</td>
<td>Total: 3 (JF1, PWD)</td>
<td>+ (JF1), - (PWD)</td>
<td>NA</td>
<td>NA</td>
<td>+ (JF1)</td>
<td>(between amino acid positions 90 and 120)</td>
</tr>
<tr>
<td>Pdgfb</td>
<td>TLC and DcO</td>
<td>Total: 1 (C57, JF1, PWD)</td>
<td>- (JF1), - (PWD)</td>
<td>- (JF1), - (PWD)</td>
<td>- (JF1), - (PWD)</td>
<td>- (JF1), - (PWD)</td>
<td>- (JF1), - (PWD)</td>
</tr>
<tr>
<td>Tgfbr3</td>
<td>Cl. and Vd</td>
<td>Total: 4 (JF1, PWD)</td>
<td>+ (JF1), - (PWD)</td>
<td>NA</td>
<td>NA</td>
<td>+ (JF1, PWD)</td>
<td>(between amino acid positions 90 and 120)</td>
</tr>
</tbody>
</table>

All polymorphisms among the mouse strains including JF1/Msf (JF1), PWD/Pjl (PWD), BALBcByJ (BALB), C57BL/6J (C57), and A/J are determined in comparison to C3H/HeJ (C3H). Strains analyzed but with no polymorphism listed are identical to C3H for the respective protein. mCh, mouse chromosome; Sod3, superoxide dismutase 3; extracellular; Tff2, trefoil factor 2; Rbl1, relaxin 1; Pdgfb, platelet-derived growth factor, β polypeptide; Tgfbr3, transforming growth factor, β receptor III; Rsrb, retinoid X receptor β; +, alteration observed; ++, disruptive alteration observed; NA, not applicable because .pdb file could not be generated; --, no change observed. Lung functions are associated to the respective chromosome region (35).
protein had different electrostatic potential (Fig. 2C), molecular surface area (Fig. 2D), and hydropathic profile (Fig. 2E) in the amino acid region 140–200 (which contains 2 predicted amino acid conversions). The PSIPRED prediction of SOD3 protein structure also varied in JF1 mice compared with that of other strains (Supplemental Fig. S2). The Asn-21-Asp is within the propeptide sequence of SOD3JF1 and could lead to difference in peptide processing. The amino acids in positions 57 and 152 are conserved among mouse, rat, and human.

Tff2. Three missense polymorphisms were detected in Tff2 that included 1) G → A Tff2PWD, Arg-4-Gln, basic → nonpolar; 2) A → C Tff2PWD, Glu-77-Ala, acidic → nonpolar; and 3) A → C Tff2PWD, Lys-89-Arg basic → basic (Supplemental Fig. S3). The influence of the nucleotide changes on amino acids at positions 77 and 89 is illustrated in the predicted protein structures (Fig. 3A) and electrostatic potential (Fig. 3B). The observed Tff2PWD SNPs could also alter the hydropathic profile (Fig. 3C) between amino acids 70 and 90 (containing 2 polymorphisms). No significant alteration was detected in the predicted total molecular surface area of TFF2PWD. The PSIPRED prediction of TFF2PWD protein structure also varied compared with other mouse strains (Supplemental Fig. S4). The predicted Lys-89-Arg conversion is located within the P-type trefoil domain signature and is proximal to a protein kinase C phosphorylation site (PROSITE scanning). Moreover, the Arg-4-Gln conversion is within the signal sequence of the protein. The amino acid position 4 in TFF2 is conserved among mouse, rat, and human, whereas position 77 is conserved among only mouse and human.

Rln1. Compared with the C3H mouse strain, JF1 and PWD mice had three missense polymorphisms in Rln1 that included 1) T → C conversion, Leu-98-Pro, nonpolar → nonpolar; 2) C → T conversion, Leu-109-Phe, nonpolar → aromatic; and 3) G → A conversion, Val-173-Ile, nonpolar → nonpolar (Supplemental Fig. S5). The PSIPRED RLN1JF1,PWD protein prediction revealed an altered helical structure (amino acid region 98–109).
acid position 80–120) and coiling of the protein (amino acid position 161–175) (Fig. 4A). The amino acid 90–120 region of predicted RLN1JF1,PWD also had an altered hydropathic profile compared with other strains (Fig. 4B). The polymorphism Leu-109-Phe is located within a casein kinase II phosphorylation site (PROSITE scanning). This nonpolar to aromatic amino acid shift could have severe functional consequences in that the biochemical properties of the related protein region would be significantly affected. However, none of the amino acids associated with these polymorphisms is conserved among mouse, human, and rat.

**Lung Function of Gene-Targeted Sod3−−/− Mice**

To assess the loss of SOD3 on respiratory function, Sod3−−/− mice (age 12 wk; n = 6) were compared with strain-matched Sod3+/+ control mice. Baseline lung function values of the Sod3+/+ mice were not significantly different from those previously determined in C57BL/6J mice (34), the strain from which these mice were derived (5). The Sod3+/+ vs. C57BL/6J lung function values (means ± SE) were as follows: TLC = 1,203 ± 25 vs. 1,174 ± 34 μl for female and 1,337 ± 35 vs. 1,380 ± 33 μl for male; Vn/TLC = 18.8 ± 0.4% vs. 19.3 ± 0.3% for female and 17.2 ± 0.6% vs. 16.6 ± 0.3% for male; Cl = 56.7 ± 2.4 vs. 54.9 ± 1.9 μl/cmH2O for female and 61.2 ± 3.5 vs. 63.0 ± 2.0 μl/cmH2O for male; DCO = 11.4 ± 0.7 vs. 11.2 ± 0.2 μmol·min⁻¹·hPa⁻¹ for female and 13.1 ± 0.4 vs. 13.8 ± 0.3 μmol·min⁻¹·hPa⁻¹ for male.

The lung function values (means ± SE) of TLC (1,082 ± 33 μl), Cl (60.6 ± 3.0 μl/cmH2O), and DCO (10.4 ± 0.5 μmol·min⁻¹·hPa⁻¹) were compared with strain-matched Sod3+/+ control mice. Baseline lung function values of the Sod3+/+ mice were not significantly different from those previously determined in C57BL/6J mice (34), the strain from which these mice were derived (5). The Sod3+/+ vs. C57BL/6J lung function values (means ± SE) were as follows: TLC = 1,203 ± 25 vs. 1,174 ± 34 μl for female and 1,337 ± 35 vs. 1,380 ± 33 μl for male; Vn/TLC = 18.8 ± 0.4% vs. 19.3 ± 0.3% for female and 17.2 ± 0.6% vs. 16.6 ± 0.3% for male; Cl = 56.7 ± 2.4 vs. 54.9 ± 1.9 μl/cmH2O for female and 61.2 ± 3.5 vs. 63.0 ± 2.0 μl/cmH2O for male; DCO = 11.4 ± 0.7 vs. 11.2 ± 0.2 μmol·min⁻¹·hPa⁻¹ for female and 13.1 ± 0.4 vs. 13.8 ± 0.3 μmol·min⁻¹·hPa⁻¹ for male.

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\( \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{hPa}^{-1} \) in female \( Sod3^{-/-} \) mice were not significantly different from those of female \( Sod3^{+/+} \) mice. Similarly, \( C_l \) (60.9 ± 3.6 \( \mu \text{l/cmH}_{2}\text{O} \)) in male \( Sod3^{-/-} \) mice was not significantly different from that of male \( Sod3^{+/+} \) mice. A slight but significant \( (P < 0.05) \) decrease in TLC (1,167 ± 36 \( \mu \text{l} \)) and \( D_{CO} \) (12.2 ± 0.6 \( \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{hPa}^{-1} \)) was noted only in male \( Sod3^{-/-} \) mice compared with the values in male \( Sod3^{+/+} \) mice.
In contrast, Vt/TLC was consistently increased in both female (23.5 ± 0.4%) and male (22.1 ± 0.4%) Sod3−/− mice compared with the Vt/TLC in sex-matched Sod3+/+ mice (18.8 ± 0.4% in female and 17.2 ± 0.6% in male) (P < 0.001). Vt/TLC values in both female and male Sod3−/− mice were similar to those observed in PWD and JF1 strains, which are also significantly greater than C3H values (Table 1).

**DISCUSSION**

**Transcript Profiling**

To begin to uncover the genetic determinants of lung function and possible links to pulmonary disease, we previously performed (35) a genomewide linkage analysis study with inbred mice and identified multiple QTLs on chromosomes 5, 15, 17, and 19. While supporting the contention that lung function is a complex trait, the task of identifying candidate genes remains a considerable challenge, as noted with numerous other QTLs found in mice (19). Therefore, we employed a systematic approach utilizing microarray analysis to identify differences in transcript levels of genes harbored within the identified chromosomal regions followed by exon screening for SNPs and subsequent in silico analysis of the predicted structural alterations in candidate genes. We also performed functional analysis by measuring lung function in gene-targeted mice that lack one candidate gene.

The transcript profiling was limited to a single time during development and thus should be viewed with caution. Considering lung development, growth, and maturation in rodents (43), the age of 4 wk can be considered as late adolescence and early adulthood in mice. We considered this to be an optimum time for an initial microarray analysis because it can be considered as the transition between completion of lung development and beginning of the growth-maturation phase. Using stringent inclusion criteria (FDR < 0.05 and an average intensity ≥ 150), we detected 933 transcripts (of 18,399 detected) that differed between 4-wk-old C3H and JF1 mice. These results suggest that a relatively large portion (~5%) of the transcripts expressed in the lung is altered between these two strains. Functional assessment of these transcripts by MAPPFinder indicated that several of the affected pathways were likely to play a role in lung development, function, and remodeling and included pathways controlling the extracellular matrix (z = 3.0), metalloendopeptidase activity (z = 3.0), growth factor activity (z = 2.8), and vasculature development (z = 2.4).

Candidate genes identified from our microarray analysis that reside within the previously identified QTLs included Sod3 on mCh 5 [previously identified as a candidate (35)] and two novel candidates, Tff2 on mCh 17 and Enpp2 on mCh 15. The roles of these candidate genes are consistent with the biological pathways identified by MAPPFinder in eliciting downstream effects on key processes of lung development, such as extracellular matrix production and reduction, cell growth and differentiation, and vasculogenesis. In addition, preliminary histological studies comparing C3H and JF1 support the involvement of these pathways. JF1 mice have significantly lower mean alveolar air space chord length and higher elastin-to-collagen ratio in the interstitial compartment of alveolar septal walls compared with C3H mice (Heinz Fehrenbach, personal communication). These morphometric differences in the alveolus and pulmonary interstitial tissue are consistent with the observed differences in respiratory mechanics, particularly C1, inasmuch as compliance was determined in the linear region of the pressure-volume curve that is controlled by elastic fiber recoil (34, 35). The altered elastin-to-collagen ratio in JF1 mice supports the global changes in transcript expression that may be influenced by the candidate genes identified in this study in maintaining the elastin/collagen homeostasis.

**Exon Sequencing of Candidate Genes**

On the basis of the microarray results, we selected two differentially expressed candidate genes, Sod3 and Tff2, for sequencing. In addition, we sequenced four other previously identified candidates (Rhn1, Pdgfb, Tgfrb, and Rxrb) and predicted the consequence for protein properties. Exon sequencing identified polymorphisms in Sod3, Tff2, and Rhn1 nucleotide sequences that could influence predicted protein structure, property, and function. Effects of the polymorphisms detected in Tgfrb3 and Pdgfb were not immediately obvious. Additionally, no polymorphisms were detected within Rxrb that might result in an amino acid exchange. We have yet to sequence Enpp2 because of its complexity (i.e., 25 exons). The current mouse sequencing database (NCBI m36 mouse assembly) lists four SNPs in the 5′-UTR of Enpp2 that differed among the C3H, BALB, C57, and A/J strains and three SNPs in the 5′-UTR and one nonsynonymous SNP (T → C, Asp-743-Asn) that differed between the C3H and PWD strains.

**Sod3.** The combined microarray, RT-PCR, and Western blot results demonstrated a consistent two- to threefold difference in the level of SOD3 expression between strains. Although the fold difference of transcript and protein may be slightly different between strains, the two isoforms of the protein were not different within each strain. This suggests that the difference in expression between strains is probably due to differences at the transcript level. Altering the level of SOD3 protein is likely to have physiological significance for several reasons.

The protein encoded by Sod3 is highly expressed in the lung as an antioxidant enzyme associated with the extracellular matrix. Interestingly, Sod3 has been suggested as a candidate gene for susceptibility to lung function decline in COPD (56). In the mouse lung, Sod3 expression is highly localized to pulmonary epithelial cells (17), and augmentation of Sod3 expression protects against lung injury induced by hyperoxia (5, 16) or bleomycin (2). Similarly, gene-targeted Sod3−/− mice are sensitive to bleomycin or asbestos (14). Individuals vary greatly (as much as 10-fold) in serum SOD3 protein levels, a phenotype associated with Arg-213-Gly heterozygosity (38, 55). Moreover, this polymorphism is associated with development of tobacco smoke-induced COPD (22, 56) and is known to alter proteolytic SOD3 processing (33). In addition, JF1 mice had decreased lung SOD3 protein levels and missense exon polymorphisms that could lead to amino acid substitutions within conserved regions.

The predicted SOD3F1 protein with its altered structure, electrostatic potential, molecular surface area, and hydropathy, together with a lowering of steady-state expression levels, suggests susceptibility of JF1 mice to oxidative stress. Interestingly, C3H mice with the normal wild-type allele are resistant to bleomycin, and we would predict this phenotype on the
basis of the predicted protein structure. Significantly lowered expression of SOD3 protein in JF1 lung clearly indicates proportionally lowered enzymatic activity. The polymorphism Asn-21-Asp in the signal sequence of SOD3 is predicted to affect the cotranslational signal peptidase cleavage site of the protein. Therefore this polymorphism might affect the cleavage and secretion of SOD3 preprotein. Moreover, the two polymorphisms (Pro-152-Leu, Ala-186-Ser) in SOD3JF1 are located within the catalytic domain of the protein, and this region is conserved across species (17). However, it remains to be investigated whether novel polymorphisms uncovered in this study would lead to susceptibility of JF1 and PWD strains to oxidative stress.

Supportive evidence for a role of SOD3 in determining lung function was also obtained with gene-targeted Sod3−/− mice. Compared with strain-matched Sod3+/+ mice, gene-targeted Sod3−/− had increased Vd/TLC, a measure of the percentage of TLC that is occupied by the dead space of the conducting airways. Thus, with increasing Vd/TLC, ventilation efficiency is reduced. Compared with C3H mice, increased Vd/TLC was previously noted in JF1 mice, and we also found this to occur in PWD mice. The difference in Vd/TLC between Sod3−/− and Sod3+/+ mice accounted for ~67% of the phenotypic difference in Vd/TLC noted between JF1 and C3H mouse strains (Table 1). Previously, the QTL linked to difference in Vd between JF1 and C3H mice was localized to mCh 5 (LOD ≥ 4.0) and was mapped to 26 cM (50.6 Mbp) to 32 cM (54.5 Mbp) (delimited by D5Mit14 and D5Mit133, respectively). This led us to suggest Sod3, which is at 31 cM (52.6 Mbp), as a candidate gene for this trait. Because heritability of respiratory function is likely to be complex and not determined by a single gene, the observed difference suggests that other genes or gene-gene interactions contribute to this phenotype.

TFF2. Initially, increased TFF2 protein was localized to mucosal epithelial restitution and wound healing in the gastrointestinal tract. However, additional studies have demonstrated a role for TFF2 in the lung. Transcript levels are increased after allergen challenge of sensitized mice (via a mechanism involving Th2 cytokines and STAT6 signaling (32)) and in airway epithelial cells in subjects with asthma (27). The altered amino acid sequence in the TFF2PROD protein predicted from the DNA sequence polymorphisms would slightly alter secondary structure. However, the predicted alteration in hydrophilic profile and electrostatic potential of TFF2PROD could be significant enough to influence its function. The polymorphism (Arg-4-Gln) in the signal peptide of the protein, and location of another polymorphism (Lys-89-Arg) in the P-type trefoil domain that is close to a protein kinase C phosphorylation site (PROSITE scanning), make it an interesting candidate for functional analysis. The decreased transcripts in JF1 lung suggest interference in injury, repair, and remodeling processes in the lung.

RLN1. Although RLN1 transcript levels did not differ between strains, RLN1 is the major circulating and stored form of relaxin in rodents and the detected sequence differences were noteworthy. RLN1 was originally thought mainly to be a hormone related to pregnancy, but recent studies support a role for RLN1 in the lung, heart, and brain (40) as an extracellular matrix remodeling hormone that reduces lung fibrosis and promotes wound healing (37). Progressive, age-related increases in lung weight, collagen content, and collagen concentration have been reported in gene-targeted Rln1−/− mice, resulting in the distortion of alveolar structure and altered lung function (36). Moreover, treatment of Rln1−/− mice with human relaxin 2 in early and developed stages of fibrosis resulted in the reversal of lung collagen deposition, confirming its ability to inhibit fibrosis by diminishing collagen biosynthesis and promoting collagen breakdown (37). Relaxins may also play critical roles in the regulation of collagen deposition in the airway/lung in animal models of allergic airway disease (31).

The identified Rln1 polymorphisms predicted to lead to Leu-98-Pro and Leu-109-Phe are contained within the connecting peptide domain, and the Val-173-Ile is within the A chain of the protein. Position 173 is thought to be determinative in the specificity of ligand binding to its receptor (3), and position 109 could influence the rate of phosphorylation of the protein. In addition, the predicted changes in hydrophilic profile within the connecting peptide (amino acids 58–156) and in protein folding might significantly influence the functionality of the Rln1JF1PWD protein. The predicted diminution of Rln1 function could contribute to the altered extracellular matrix (e.g., elastin-to-collagen ratio) and lung tissue architecture noted in JF1 mice.

ENPP2. Another possible candidate gene implicated by microarray was Enpp2. Initially found to increase cell motility in vitro (44), ENPP2 is released into the extracellular space (26), where it has shared function with purified extracellular lysophospholipase D (47, 49). The biological effects of ENPP2 are mediated by degradation of the abundant lung sphingosylphosphorylcholine and lysosphatidylcholine into sphingosine 1-phosphate and lysosphosphatidic acid (LPA), respectively (7, 18, 54). LPA can exert diverse biological effects pivotal to lung development and remodeling including mitogenesis (13) and collagen gel reduction by lung fibroblasts (30). LPA also mediates fibronectin release and epithelial cell spreading and migration (1), as well as smooth muscle cell proliferation (6) and contractility (48). Thus differences in lung transcript levels may be a genetic determinant of functions regulated through LPA-mediated signaling in multiple target lung cells. Cautionary consideration of Enpp2 as a suggestive candidate gene is limited because of the lack of additional exon sequence.

Summary

Global differential expression identified >900 transcripts that differed in C3H and JF1 lungs, many contributing to an enrichment in pathways related to lung development, function, and remodeling (e.g., extracellular matrix). This group was further narrowed down to three candidate genes, Sod3, Tff2, and Enpp2, based on localization to chromosome regions previously associated with differences in lung function. Lung function measurements of Sod3−/− mice suggest that a larger portion of TLC is occupied by dead space of the conducting airways, which is consistent with our previously identified QTL on mCh 5. In addition to differences in transcript levels, the predicted protein changes associated with polymorphisms in Sod3 and Tff2 could, in part, contribute to variation of lung function among commonly inbred laboratory strains of mice. Exon sequencing of another candidate gene, Rln1, identified several alterations of predicted protein structure. Because
gene-targeted Rln1−/− mice have altered lung structure (36) and Tff2−/− mice is altered in allergic airway sensitivity (32), it would be valuable to examine the lung function of Rln1−/− and Tff2−/− mice to further validate these candidates. These candidate genes also need to be functionally tested to influence the phenotype in a segregant cohort or haplotype across several other mouse strains that have been genotyped. Finally, the human variation in these genes clearly needs further study both in lung development and in the development of lung disease.

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