A Role for Wnt Signaling Genes in the Pathogenesis of Impaired Lung Function in Asthma

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Rationale: Animal models demonstrate that aberrant gene expression *in utero* can result in abnormal pulmonary phenotypes.

Objectives: We sought to identify genes that are differentially expressed during *in utero* airway development and test the hypothesis that variants in these genes influence lung function in patients with asthma.

Methods: Stage 1 (Gene Expression): Differential gene expression analysis across the pseudoglandular (n = 27) and canalicular (n = 9) stages of human lung development was performed using regularized *t* tests with multiple comparison adjustments. Stage 2 (Genetic Association): Genetic association analyses of lung function (FEV₁, FVC, and FEV₁/FVC) for variants in five differentially expressed genes were conducted in 403 parent-child trios from the Childhood Asthma Management Program (CAMP). Associations were replicated in 583 parent-child trios from the Genetics of Asthma in Costa Rica study.

Measurements and Main Results: Of the 1,776 differentially expressed genes between the pseudoglandular (gestational age: 7–16 wk) and the canalicular (gestational age: 17–26 wk) stages, we selected 5 genes in the Wnt pathway for association testing. Thirteen single nucleotide polymorphisms in three genes demonstrated association with lung function in CAMP ($P < 0.05$), and associations for two of these genes were replicated in the Costa Ricans: Wnt1-inducible signaling pathway protein 1 with FEV₁ (combined $P = 0.0005$) and FVC (combined $P = 0.0004$), and Wnt inhibitory factor 1 with FVC (combined $P = 0.003$) and FEV₁/FVC (combined $P = 0.003$).

Conclusions: Wnt signaling genes are associated with impaired lung function in two childhood asthma cohorts. Furthermore, gene expression profiling of human fetal lung development can be used to identify genes implicated in the pathogenesis of lung function impairment in individuals with asthma.

Keywords: asthma; lung development; lung function; genetic variation; gene expression

Asthma is a common chronic respiratory illness, affecting 22 million people in the United States alone (1). Despite advances in our understanding of asthma pathogenesis and

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The trajectory of lung function growth appears to be set early in life. Animal models demonstrate that abnormal *in utero* expression of genes implicated in normal lung development can result in abnormal pulmonary phenotypes after birth.

What This Study Adds to the Field

Gene expression profiling of early human fetal lung development can be used to identify novel genes and pathways that influence lung function in subjects with asthma.

treatment, it remains the most common cause of pediatric hospital admission and lost days of school and work each year in the United States (2). A substantial proportion of children with persistent asthma (including those with mild disease) has reduced lung function compared with age-matched healthy controls and often fail to attain their maximal predicted lung function (3). Pulmonary function is among the most important health indicators, being a strong predictor of long-term morbidity and mortality and a marker of disease severity in asthma (4). Although most young people with asthma have a normal or only mildly reduced FEV₁, a substantial proportion have reductions in growth of FEV₁ during childhood (5). Furthermore, sustained treatment of childhood asthma with inhaled corticosteroids at conventional doses does not appear to influence long-term lung function growth (6).

Heritability estimates of lung function in the general population suggest that genetic factors explain 25 to 37% of the between-subject variability in FEV₁, FVC, and the ratio of FEV₁/FVC (7–10). Twin studies in subjects with asthma suggest that within this susceptible population, genetic factors may explain an even greater proportion of altered lung function (77–91% for FEV₁), even after accounting for smoking and the shared genetic effects of body size (11, 12). Although lung function has a substantial heritable component indicating a strong genetic contribution to this phenotype, only a small proportion of the genetic determinants of lung function have been identified.

Epidemiologic data demonstrate that lung function growth follows a set percentile curve over time, and that this trajectory appears to be established before age 6 years in most individuals (13). Furthermore, infants born to mothers who smoked throughout their pregnancy had significantly lower levels of

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lung function shortly after birth than individuals born to non-smoking mothers (14). Previous work from the Tucson Childhood Respiratory Study has shown that infants with reduced lung function in early life were more likely to develop wheezing related to respiratory illness within the first year (15). Longitudinal follow-up of these children also showed that those with early transient wheezing or persistent wheeze were more likely to have diminished lung function at 6 years of age (16). Another study suggests that infants with reduced lung function within the first week of birth were more likely to have an asthma diagnosis at 10 years of age (17). These studies suggest that the genetic programming of lung development and *in utero* exposures have important implications for lung function later in life.

Previous work has demonstrated a link between developmentally regulated genes and disease susceptibility. For example, ADAM33, which was identified as a candidate gene in asthma by genome-wide linkage analysis of bronchial hyper-responsiveness (18), has been shown to surround developing bronchi and mesenchymal tissue in human fetal lung tissue from the early pseudoglandular stage of lung development (19). Although genetic association studies of ADAM33 with asthma susceptibility have been inconsistent, polymorphisms in the ADAM33 gene have been associated with reduced lung function between 3 and 5 years of age (20), suggesting the potential role for lung development genes in disease susceptibility. Furthermore, Demeo and colleagues previously demonstrated the ability to combine the gene expression signature of normal mouse lung development with existing human linkage studies to identify candidate genes for chronic obstructive pulmonary disease (COPD) (21). Previous genome-wide linkage analyses in extended pedigrees ascertained through subjects with severe, early-onset COPD participating in the Boston Early-Onset COPD Study demonstrated significant linkage on chromosome 2q to FEV₁/FVC ratio (22, 23). Three genes mapping to this linkage region were found to be highly expressed during normal mouse lung development (24). Of these, SERPINE2 was found to be the most differentially expressed gene during murine alveologenesis (postnatal days 4–10) with a 4.5-fold change in expression across the developmental time series. SERPINE2 expression was also found to be inversely correlated with post-bronchodilator FEV₁ in human lung microarray studies of patients with severe COPD (25), suggesting that increased expression of SERPINE2 was associated with reduced lung function in COPD. Sixteen single nucleotide polymorphisms (SNPs) in SERPINE2 demonstrated genetic association with FEV₁ and FEV₁/FVC in the Boston Early-Onset COPD cohort. Five of these SNPs demonstrated replicated association with lung function in a second, independent cohort of patients with COPD participating in the National Emphysema Treatment Trial (NETT) compared with control subjects from the Normative Aging Study (NAS) (21). These results demonstrate that combining genetic and genomic methodologies can facilitate the identification of candidate genes implicated in the pathogenesis of complex diseases.

Fetal development of the respiratory tract begins at approximately 4 weeks of gestation as the laryngotracheal groove emerges from the floor of the primitive foregut. Although airway growth continues into the postnatal period, the pattern of airway branching is established *in utero*. Branching morphogenesis, the programmed process of airway development, extends from the early pseudoglandular stage (6th wk) into the early canalicular period (17th wk), and is characterized by a distinct proximal-distal pattern of genetically programmed epithelial and mesenchymal differentiation (26). Murine models of lung development demonstrate that disruption of critical gene expression during both the pseudoglandular and canalic-

ular stages of airway development can lead to abnormal pulmonary phenotypes after birth (27–30). Evidence suggests that the study of the molecular pathways that regulate lung development may provide important information regarding the pathogenesis of pulmonary disease. It is clear that the early stages of lung development are dependent on complex molecular pathways, and evidence suggests that these molecular pathways can be recapitulated in postnatal life resulting in the pathologic changes of complex respiratory diseases (31, 32).

Based on these observations, we hypothesized that genes integral to airway development *in utero* harbor common genetic variants that are implicated in the pathogenesis of impaired lung function in asthma. Combining genomic and genetic studies in human populations, we demonstrate evidence of genetic association between measures of lung function and polymorphisms for several differentially expressed genes during *in utero* airway development in two ethnically distinct populations of children with asthma, supporting an important role for lung development genes in the pathogenesis of abnormal pulmonary function in later life. Some of the results of these studies have been previously reported in the form of an abstract (33).

METHODS

Stage 1: Gene Expression Profiling of Human Lung Development

Human Fetal Lung Tissue Samples. Thirty-six de-identified, unpaired fetal lung tissue samples were acquired through the tissue retrieval program sponsored by the National Institute of Child Health and Development, the University of Maryland Brain and Tissue Bank for Developmental Disorders (Baltimore, MD), and the Center for Birth Defects Research (University of Washington, Seattle, WA). Phenotypic information for the fetal lung tissue samples is limited to their estimated gestational age. Therefore, underlying pathologic conditions in the fetus are unknown.

Human Fetal Lung Tissue mRNA Extraction. Total mRNA from 36 human fetal lung tissue samples was isolated using the Illustra RNeasy mini RNA isolation kit (GE Healthcare, Piscataway, NJ) according to protocol. Sample quality was assessed by visualization on a 1% agarose gel and using the Agilent 2100 Bioanalyzer (Santa Clara, CA).

Genome-wide Gene Expression Profiles for Human Lung Development. Genome-wide gene expression profiles of 22,177 probes representing more than 22,000 genes were generated using Illumina HumanRef8 v2 BeadChips (San Diego, CA) according to the manufacturer's protocol, as described here. Total RNA was used to generate cDNA by reverse transcription, followed by biotin-labeled cRNA synthesis using the MessageAmp kit (Ambion, Austin, TX) (34). Labeled cRNA was combined with formamide and hybridization buffer, followed by overnight hybridization to Human Ref8 v2 BeadChip arrays (eight samples per chip), with deliberate randomization of samples across chips and their chip positions to avoid batch effects (for example, correlation of gestational age and BeadChip). Following hybridization, chips were washed, blocked, stained with streptavidin-Cy3 dye, and scanned on the BeadArray scanner (Illumina) with images captured using the Illumina BeadStudio software with default setting and background correction.

Microarray Preprocessing. All expression microarray analyses were performed in R 2.7/Bioconductor (35). Quantile normalization and log₂ transformation were performed using the *lumi* package (36). Variance-based nonspecific filtering to remove uninformative probes was performed using the *genefilter* package, specifying a minimum interquartile range of 1 on the log₂ scale, leaving 13,306 of the 22,000 genes represented on the microarray.

Statistical Analysis of Genome-wide Gene Expression of Human Lung Development. Human fetal lung tissue samples were assigned to the pseudoglandular stage (gestational age <112 d, n = 29) or canalicular stage (gestational age >112 d, n = 9). Differential gene expression analysis was performed using the Significance Analysis of Microarray (SAM) test statistic as implemented in the *siggenes* package (37). SAM

incorporates regularized t tests, which add a constant that determines the relative contribution of gene-specific and global variances to the denominator. The effect of this parameter is to smooth out the effect of underestimated variance and provide a more reliable assessment of whether a gene is differentially expressed (38, 39). An estimate of the number of differentially expressed genes across the two stages of lung development was derived using a false discovery rate of 0.10 (40–42).

Gene Ontology Analysis. Gene ontology (GO) pathway analysis was performed using the list of differentially expressed genes in the *Safe* package (43). GO is a structured vocabulary that classifies genes and gene products using a consistent description across databases. Because a gene or gene product may be active in one or more biological processes, GO analysis allows one to determine whether gene sets are enriched for certain biologic processes (more genes than would be expected by chance are from a given biologic pathway). GO can be used to narrow gene selection, when the testing of specific genes or gene products must be limited.

Lung Development Candidate Gene Selection. Using the list of differentially expressed genes and the GO pathway enrichment results, we sought consensus between two unblinded experts in lung development to select five lung development candidate genes from a single pathway. Genes in the Wnt pathway that were differentially expressed during early lung development were selected based on the known role of the pathway in lung development as demonstrated in animal models, its biologic plausibility for association with lung function impairment, and a lack of evidence in the literature demonstrating a role for the Wnt pathway in asthma pathogenesis. The five genes from the Wnt signaling pathway that were selected for follow-up genetic association testing were Wnt inhibitory factor 1 (WIF1), Wnt1-inducible signaling pathway protein 1 (WISP1), secreted frizzled-related protein 2 (sFRP2), sFRP5, and Dickkopf 1 (DKK1). To our knowledge, none of the genes chosen for association testing have been previously associated with impaired lung function in human populations.

Stage 2: Genetic Association Testing of Lung Development Candidate Genes with Lung Function in Asthma

Childhood Asthma Cohorts. Lung development candidate gene association testing was performed in two populations: The Childhood Asthma Management Program (CAMP) and the Genetic Epidemiology of Asthma in Costa Rica cohort. Details regarding these cohorts have been previously described (6, 44, 45). CAMP participants had mild to moderate persistent asthma based on increased airway responsiveness (a methacholine provocative concentration causing a 20% decrease in FEV₁ $\leq$ 12.5 mg/ml) and at least one of the following: asthma symptoms at least twice weekly, use of inhaled bronchodilator at least twice weekly, or use of daily asthma medication for at least 6 months in the year before screening. Participating children were followed for a mean time of 4.3 years in the randomized trial; lung function measurements were obtained at the time of randomization in the clinical trial (baseline), at 2 months, 4 months, and every 4 months thereafter throughout the clinical trial. Spirometry performance was required to meet American Thoracic Society criteria for acceptability and reproducibility. At least three spirometric maneuvers were performed, with at least two reproducible maneuvers required for each test. Post-bronchodilator spirometric values were obtained at least 15 minutes after the administration of two puffs of albuterol (90 μ g/puff). DNA samples were obtained on 968 children, and 1,518 of their parents contributed DNA samples. Genome-wide SNP genotype data were generated using the Illumina Human Hap 550K SNP array by Illumina Inc. for 403 parent-child trios of self-reported non-Hispanic white ancestry with sufficient DNA for genome-wide studies (average genotyping completion rate was 99.75% per subject).

The Genetic Epidemiology of Asthma in Costa Rica cohort consists of 616 parent-child trios ascertained through children with asthma aged 6 to 14 years (a physician's diagnosis of asthma and two or more respiratory symptoms or asthma attacks in the previous year) and a high probability of having six or more great-grandparents born in the Central Valley of Costa Rica. All children included in the study completed a protocol including a questionnaire, pulmonary function testing, and methacholine challenge testing. Lung function-associated SNPs identified in CAMP (see below) were genotyped using the

MassARRAY iPLEX Gold platform (Sequenom, San Diego, CA) according to protocol.

Genetic Association Analysis. From the Illumina Human Hap 550K SNP set genotyped in CAMP, we selected polymorphisms that mapped to within 25 kb of five lung development candidate genes identified in Stage 1 that were in the Wnt signaling pathway. Family-based tests of association were conducted using the PBAT program (v 5.3) as implemented in GoldenHelix (<http://www.goldenhelix.com>) (46). We tested for SNP associations with three highly correlated lung function phenotypes: post-bronchodilator FEV₁, FVC, and FEV₁/FVC. To decrease the number of statistical tests performed, only additive genetic models adjusted for age, sex, and height were considered. All SNPs that attained a nominal level of significance ($P < 0.05$) for association with lung function in CAMP were carried forward for replication testing in the Costa Rican cohort using identical statistical procedures. To account for multiple comparisons, results were considered statistically significant when identical associations (i.e., same allele, same phenotype, same direction of genetic effect) were identified in both populations with a P value less than 0.05 and a Fisher combined P value less than or equal to 0.0005 (i.e., 0.05/100), which conservatively accounts for the 79 markers tested for association in CAMP, 13 of which underwent replication testing in the Costa Rican cohort.

Informed Consent

Fetal lung tissue samples were acquired through tissue retrieval programs sponsored by National Child Health and Development, the University of Maryland Brain and Tissue Bank for Developmental Disorders (Baltimore, MD), and the Center for Birth Defects Research (University of Washington, Seattle, WA). The collection of these tissues has been designated an institutional review board (IRB)-exempt protocol by the University of Missouri–Kansas City Pediatric IRB. Approval for the genetic portion of this analysis was obtained from the IRB of each CAMP institution before study enrollment and from the IRB of the Hospital Nacional de Niños (San José, Costa Rica) for the Genetics of Asthma in Costa Rica Study. Informed consent was obtained from parents of participating children, and assent was obtained from each child before study enrollment.

RESULTS

Stage 1: Differential Gene Expression Analysis of Early Human Fetal Lung Development

Illumina HumanRef8 expression array profiles were generated for 36 human fetal lung samples ranging in gestational age from 53 to 153 days (27 pseudoglandular, 9 canalicular, Table 1). There were no systematic differences between the human fetal lung samples across chips, and there was a high degree of correlation between the 10% of samples that were run as technical replicates ($r^2 \geq 0.9$). Nonspecific filtering of the approximately 22,000 probes represented on the Illumina platform identified 13,306 genes demonstrating variable expression levels across samples, which were then considered for further analysis.

Differential gene expression analysis across the pseudoglandular-to-canalicular transition was performed using the SAM statistic in Bioconductor. In total, 1,776 genes were identified as differentially expressed across the pseudoglandular and canalicular periods at a false discovery rate of 0.10. A SAM plot is shown in Figure 1, which illustrates the number of genes that deviate from the null expectation of no differential expression (*gray diagonal line*). The genes for which there is a difference in mean gene expression level across the pseudoglandular and canalicular stages of lung development are shown in gray. Genes differentially expressed across the pseudoglandular-to-canalicular transition of human lung development included the surfactant proteins, genes in the transforming growth factor- β pathway, and genes in the Wnt signaling

TABLE 1. HUMAN FETAL LUNG TISSUE SAMPLES

Developmental Stage	Gestational Age (d)	Count
Pseudoglandular (n = 27)	51–60	2
	61–70	2
	71–80	6
	81–90	5
	91–100	6
	101–112	6
Canalicular (n = 9)	113–125	3
	126–135	3
	136–145	2
	146–155	1

pathway (see Table E1 in the online supplement). For example, expression differences between the pseudoglandular and canalicular stages of lung development for the five Wnt genes selected for follow-up genetic association testing are shown in Figure 2.

GO analysis demonstrated enrichment of genes involved in several biologic processes, including neutral amino acid transport and spindle organization and biogenesis (both $P < 0.01$). Of note, biologic processes involving the Wnt signaling pathway were also significantly enriched in this analysis (Table 2).

Stage 2: Genetic Association of Lung Development Candidate Genes with Lung Function in Asthma

The baseline characteristics of the index cases in both the CAMP and Costa Rican trios genotyped for this study are presented in Table 3. Despite the differences in geographic and ancestral origin and methods of sample ascertainment, the baseline characteristics of the CAMP and Costa Rican probands were similar. There were more boys in both cohorts, in keeping with the known increased prevalence of childhood asthma among boys in this age group. Lung function measurements were similar between the CAMP cohort and the children with asthma in the Costa Rican cohort.

The initial genetic association screening was performed using high-quality genome-wide association data that were available on 403 parent-child trios in CAMP. Of 561,466 markers present on the BeadChip, 547,645 markers (97.54%) passed quality control measures. In total, 79 SNPs within 25 kb of the five Wnt pathway genes were tested for association with lung function in the CAMP cohort.

There was suggestive evidence of association of 13 SNPs from WIF1, WISP1, and SFRP5 with post-bronchodilator spirometric measurements of lung function in CAMP ($P < 0.05$), several of which were associated with two measures (Table E2). These 13 variants were therefore genotyped in the Costa Rican cohort. Of the 616 Costa Rican parent-child trios, 33 were excluded from this analysis because of Mendelian inconsistencies (n = 9) or inadequate genotypic data (n = 24), leaving 583 trios.

Variants in two genes demonstrated reproducible associations in both cohorts (Table 4). One variant (rs2929973), mapping approximately 1 kb downstream of the WISP1 gene, was strongly associated with FEV₁ in both cohorts ($P = 0.01$ in CAMP, $P = 0.005$ in Costa Rica), with the G allele conferring lower FEV₁ values (Fisher combined $P = 0.0005$). Furthermore, this variant was also associated with reduced FVC ($P = 0.008$ in CAMP, $P = 0.005$ in Costa Rica, Fisher combined $P = 0.0004$). Both of these associations were significant after correction for multiple comparisons.

One variant (rs6581612) approximately 25 kb upstream of the WIF1 gene was associated with FVC in both CAMP ($P = 0.01$) and Costa Rica ($P = 0.03$). For this variant, the C allele

Figure 1. Significance analysis of microarray plot demonstrating differential expression of 1,776 genes across the pseudoglandular-to-canalicular periods of human fetal lung development. The genes shown in gray (n = 1,776) are differentially expressed at a false discovery rate = 0.10.

was associated with a higher FVC in both populations (Fisher combined $P = 0.003$).

Another variant in the WIF1 gene (rs1596725), located approximately 22 kb upstream of the transcription start site, was associated with higher FEV₁/FVC values in both childhood asthma cohorts (Fisher combined $P = 0.003$). Although these associations did not meet strict correction for multiple comparisons, the evidence of differential expression of the WIF1 gene during early lung development and the consistency of genetic association replication (same SNP, same phenotype, and the same direction of association) in two populations suggests that this gene warrants further follow-up.

DISCUSSION

Using gene expression profiling across the pseudoglandular and canalicular transition of human fetal airway development, we demonstrate enrichment of differentially expressed genes regulating the Wnt signaling pathway during the critical stages of airway branching morphogenesis. Although murine models of lung development have demonstrated a complex role for the Wnt pathway in airway branching morphogenesis, the precise role of the Wnt genes in human lung development has yet to be defined. Using gene expression patterns of early human fetal lung development and genetic association testing in childhood asthma cohorts, we have identified two genes in the Wnt pathway that harbor polymorphisms associated with impaired lung function in two well-characterized cohorts of childhood asthma. To our knowledge, this is the first report demonstrating both differential expression of genes in the Wnt signaling pathway during *in utero* airway development using human fetal lung tissue and an association between variants within the WISP1 and WIF1 genes with lung function in two asthma populations. Based on these results, we demonstrate that gene expression profiling in early human fetal lung development may help elucidate the molecular processes underlying lung function impairment in patients with asthma.

The Wnt signaling pathway constitutes a large family of highly conserved, secreted glycoproteins, which function as growth factors that are essential to organogenesis. The canonical Wnt

Figure 2. Evidence of differential expression of Wnt genes between the pseudoglandular period to the canalicular stage of human fetal lung development (Wnt inhibitory factor 1 [WIF1] *t* test, $P = 0.02$; Wnt1-inducible signaling pathway protein 1 [WISP1] *t* test, $P = 0.008$; secreted frizzled-related protein 2 [sFRP2] *t* test, $P = 0.007$; sFRP5 *t* test, $P = 0.002$; Dickkopf 1 [DKK1] *t* test, $P = 0.002$). The horizontal black bar in the box plot represents the mean expression value for the gene. The whiskers represent the standard deviation in expression value.

proteins bind to frizzled receptors, causing β -catenin inhibition and subsequent transcription of TCF/LEF target genes (47). Genes in the Wnt signaling pathway regulate cell fate and differentiation during embryogenesis, modulate cell proliferation, and are involved in homeostatic functions in adult tissues (48–50). To date, 19 Wnt proteins have been identified in humans with a vast array of biologic functions allowing for

redundancy in the pathway with compensation for the loss of certain Wnt ligands by other genes in the pathway (51). Furthermore, the Wnt signaling pathway is highly regulated by Wnt inhibitors, including the sFRPs and WIF. Branching morphogenesis in fetal lung development is mediated by a complex interaction between the epithelium and its surrounding mesenchyme during early lung development (49, 52). Genes

TABLE 2. BIOLOGIC PROCESSES GENE ONTOLOGY PATHWAY ANALYSIS OF GENES DIFFERENTIALLY EXPRESSED DURING AIRWAY DEVELOPMENT

GO ID	GO Term	P Value
GO:0007051	Spindle organization and biogenesis	0.001
GO:0015804	Neutral amino acid transport	0.001
GO:0001936	Regulation of endothelial cell proliferation	0.002
GO:0006752	Group transfer coenzyme metabolic process	0.002
GO:0048015	Phosphoinositide-mediated signaling	0.002
GO:0016055	Wnt receptor signaling pathway	0.003
GO:0030111	Regulation of Wnt receptor signaling pathway	0.003
GO:0007017	Microtubule-based movement	0.005
GO:0007059	Chromosome segregation	0.007
GO:0000226	Microtubule cytoskeleton organization and biogenesis	0.008

Definition of abbreviation: GO = gene ontology.
All GO pathways enriched at $P \leq 0.01$.

in the Wnt signaling pathway are expressed in the developing lung at sites of critical epithelial–mesenchymal interactions that involve cell–cell and cell–matrix interactions that are essential for normal lung development (28, 51–53). Murine models have shown that deletion of β -catenin signaling in the epithelium during early lung development results in profound perturbations of the normal epithelial and mesenchymal compartments resulting in decreased secondary and tertiary branching (54). Furthermore, studies have shown that Wnt signaling is critical to the proliferation and survival of airway submucosal gland progenitor cells. Alterations of Wnt signaling have been associated with pathologic alterations, including hypertrophy and hyperplasia of submucosal glands (55), which are common pathologic changes found in the airway wall of individuals with asthma. Thus, murine models suggest a plausible role for the Wnt genes in the pathogenesis of lung function impairment in the susceptible host.

Differential expression in human fetal lung tissue during the period of branching morphogenesis was a prerequisite for our considering a gene for genetic association testing. We were thus able to identify two genes (WISP1 and WIF1) that are associated with both intrauterine airway development and lung function impairment in susceptible populations. Neither WISP1 nor WIF1 has been previously implicated in the pathogenesis of obstructive airway diseases. However, recent work has demonstrated a role for the Wnt signaling pathway at sites of tissue injury and repair. Colston and colleagues demonstrated a role for WISP1 in postinfarction cardiac remodeling, by demonstrating the proliferative effect of WISP1 on fibroblasts (56). Aberrant Wnt signaling has also been implicated as a profibrotic mechanism underlying the pathogenesis idiopathic pulmonary fibrosis (IPF) (57–59). Specifically, WISP1 expression was increased in alveolar epithelial cells in both *in vitro* models and *in vivo* studies (60). In addition, WISP1 showed the greatest difference in expression between lung tissue of normal control subjects and in patients with IPF (60). Treatment with exogenous WISP1 resulted in increased type II cell proliferation, increased extracellular matrix deposition from lung fibroblasts, and increased fibrosis. Furthermore, depletion of WISP1 using neutralizing antibodies in a bleomycin-induced murine model of IPF resulted in a marked attenuation of lung fibrosis, decreased extracellular matrix deposition, partial restoration of pulmonary function assessed by lung compliance measures, and improved survival (60). Although WISP1 has not been specifically studied in asthma, the profibrotic effect of WISP1 may explain some of the characteristic changes of airway remodeling that occur in asthma, which includes subepithelial fibrosis, neovascularization, and increased smooth muscle deposition. These histopathologic changes may explain why variants in this gene are

TABLE 3. BASELINE PHENOTYPIC CHARACTERISTICS OF INDEX CHILDREN IN CHILDHOOD ASTHMA MANAGEMENT PROGRAM AND COSTA RICA

Variable	CAMP (n = 403)	Costa Rica (n = 583)
Age, years	8.62 (6.97–10.44)	8.84 (7.79–10.48)
Female sex	148 (37%)	235 (40%)
Baseline post-bronchodilator FEV ₁ , L	1.79 (1.45–2.08)	1.74 (1.49–2.06)
Baseline post-bronchodilator FVC, L	2.11 (1.64–2.47)	2.05 (1.71–2.39)
Baseline post-bronchodilator FEV ₁ /FVC	86.0 (81.0–90.0)	86.41 (82.08–90.06)

Definition of abbreviation: CAMP = Childhood Asthma Management Program.
Median (interquartile range) or count (frequency) reported.

associated with lower levels of lung function in children with asthma. Further investigation of the functional role of this gene in asthma is warranted.

WIF1 acts as an inhibitor of Wnt signaling by directly binding to Wnt ligands, thus disrupting their ability to bind to the Wnt/frizzled receptor (48). Decreased WIF1 expression in human disease states, including lung cancer, has been related to hypermethylation of the promoter region of the gene (61). Sun and colleagues have previously shown that abrogation of Alk3-mediated bone morphogenetic protein (BMP) signaling in lung epithelial cells during early lung development (E16.5 in the murine model) disrupts cell differentiation and proliferation resulting in abnormal lung branching morphogenesis (62). Their murine model demonstrates that changes in BMP signaling subsequently cause respiratory distress syndrome in the early postnatal period. Analysis of the lung tissue of the Alk3 conditional knock-out mouse demonstrate that decreased BMP signaling is associated with increased canonical Wnt signaling activity as demonstrated by increased phosphorylation of the Wnt coreceptor LRP6 and activation of downstream β -catenin (62). Of note, WIF1 expression in the Alk-3 conditional knock-out mouse was reduced in the perinatal lung tissue, suggesting both a role for WIF1 in abnormal airway branching and the development of impaired lung function in later life. Although increased WIF1 expression may explain why variants in the WIF1 gene are associated with increased lung function in patients with childhood asthma, functional validation of these variants is necessary to further elucidate their mechanistic function. Furthermore, investigation of genotype-specific differences in the expression of Wnt signaling genes in lung tissue specimens from patients with asthma would help to further elucidate the role of both WISP1 and WIF1 in asthma pathogenesis.

We performed an extensive search of the medical literature for abstracts involving each of the 1,776 differentially expressed genes to identify those genes that were previously associated with abnormal lung pathology, as evidenced by (1) murine models demonstrating an association with abnormal pulmonary phenotypes, (2) implication of the gene in lung development, or (3) human data demonstrating gene associations with lung disease. Of the 1,776 differentially expressed genes, only 102 met at least one of these criteria, suggesting the usefulness of gene expression profiling in early human lung development to identify genes and molecular pathways that may have a novel biologic role in the pathogenesis of airflow obstruction in susceptible individuals. Furthermore, although the Wnt signaling pathway has been implicated in murine lung development, the role for the WISP1 and WIF1 genes in the pathobiology of asthma in human populations has not been identified to date. The current data suggest an association of genes within this pathway with lung function in human populations. However,

TABLE 4. REPLICATED ASSOCIATIONS WITH LUNG FUNCTION IN THE CHILDHOOD ASTHMA MANAGEMENT PROGRAM AND COSTA RICAN COHORTS UNDER AN ADDITIVE GENETIC MODEL

Phenotype	Gene	dbSNP rs#	CAMP				Costa Rica				Fisher Combined P Value
			Allele	Allele Frequency	No. Informative Families	P Value	Allele	Allele Frequency	No. Informative Families	P Value	
FEV ₁	WISP1	rs2929973	G	0.14	140	0.01 ↓	G	0.19	238	0.005 ↓	0.0005 ↓
FVC	WIF1	rs6581612	C	0.24	183	0.01	C	0.27	338	0.03	0.003
	WISP1	rs2929973	G	0.14	140	0.008 ↓	G	0.19	227	0.005 ↓	0.0004 ↓
FEV ₁ /FVC	WIF1	rs1596725	C	0.39	267	0.01	C	0.29	339	0.04	0.003

Definition of abbreviations: CAMP = Childhood Asthma Management Program; WIF = Wnt inhibitory factor; WISP = Wnt1-inducible signaling pathway protein.

Lung function phenotypes are post-bronchodilator values. The downward arrow represents the direction of effect based on the value of the z-score, which means the transmission of the minor allele is associated with lower values of the spirometric value of lung function.

further molecular characterization of the genetic variants within these genes is required to determine their precise role in the pathogenesis of impaired lung function.

Several limitations of our genomic analysis must be addressed. Our genome-wide gene expression analysis of lung development was limited to fetal lung tissue samples from the pseudoglandular and canalicular stages of development. Therefore, we are not able to determine the changes in gene expression profile that occur during the later stages of gestation. Although we acknowledge that investigation of gene expression patterns during the later stages of development during which the airway size increases may allow us to gain further insights into the pathogenesis of lung function impairment, the use of early developmental expression patterns is scientifically justified based on what we have already learned from genetic manipulation of mice wherein interval sacrifice throughout development is possible. It is quite well established that the same genes that are identified as having key functions in early embryonic lung development are reused during later fetal development, postnatal alveolarization, and response to injury (28, 59). In addition to its scientific merit, using the early fetal lung to do a genome-wide expression screen is also feasible because of the limits imposed by our ability to obtain fetal tissue during the later stages of gestation.

Like other intermediate asthma phenotypes, lung function is likely to be a complex process, resulting from the interplay of both genetic and environmental factors. We have previously shown that of early life exposures (including intrauterine tobacco smoke exposure) influence early postnatal lung function (14). Because our fetal lung tissue samples are de-identified samples obtained from a national tissue repository, we do not have access to maternal information. Therefore, we are unable to assess the effect that *in utero* exposures may have on gene expression patterns, which would result in a source of misclassification that would bias our results toward the null hypothesis. Despite this limitation, our results suggest that we can use gene expression profiling to investigate the genes and pathways that influence *in utero* lung development, which may help define the molecular processes involved in the pathogenesis of impaired lung function in the susceptible host.

We also recognize that the use of gene expression profiling of early lung development to identify genes associated with lung function may have broader implications in terms of lung function in both health and disease. Recent work has suggested a role for Wnt signaling genes in the pathogenesis of idiopathic pulmonary fibrosis and pulmonary arterial hypertension (59, 63); genetic association testing of variants in Wnt genes in these diseases should be considered.

Genetic association studies of lung function in obstructive airways diseases have yielded inconsistent findings. In addition

to differences in linkage disequilibrium (LD) patterns, gene-by-gene, or gene-by-environment interactions among study populations, potential explanations for these discrepant results include differences in statistical power, failure to control for multiple testing, and (for case-control studies) population stratification. Our study had adequate statistical power to detect associations of relatively large magnitude, and we have reduced the potential for false-positive results by replicating our findings in two family-based studies, which are not susceptible to population stratification. We recognize that some of our non-replicated results at the SNP level may be due to underlying differences between our study populations (e.g., ancestral history, LD, and environmental exposures). However, based on recent work from Rogers and colleagues, the Illumina genome-wide genotyping platform also provides variable coverage across genetic loci (64). Studies with sample sizes similar to ours can detect associations with relatively large effect size (odds ratios ≥ 1.5), but are underpowered to detect genes with small effect sizes, which may be expected for complex traits like lung function. Furthermore, important genetic variation (functional or regulatory) may not have been genotyped as part of the HumanHap550 platform. Therefore, sequencing and functional validation studies for the lung development candidate gene set identified in this study are still required.

In summary, genes in the Wnt signaling pathway are associated with impaired lung function in two ethnically distinct cohorts of childhood asthma. Furthermore, in concordance with previous experimental animal data, we have demonstrated that genes in critical lung development pathways influence lung function later in life. Our findings provide evidence that genome-wide gene expression profiles of lung development using human fetal lung tissue can be used to elucidate candidate genes involved in the pathogenesis of impaired lung function in children with asthma.

Conflict of Interest Statement: S.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. V.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.J.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.L.-S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.C.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.K. has received a U.S. patent 6,703,228 for methods and products related to genotyping and DNA from Massachusetts Institute of Technology. A.R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.S.Q. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. L.A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.G. does not have a financial relationship with a commercial

entity that has an interest in the subject of this manuscript. S.L. has received advisory board fees from GlaxoSmithKline (\$1,001–\$5,000) and NIH-sponsored grants for \$50,001–\$100,000. J.T. has received lecture fees from Sepracor for up to \$1,000 and has received an industry-sponsored grant from Philip Morris Foundation for \$100,001 or more (unrelated to this study), an NIH grant for \$100,001 or more, and from TRDRP for \$100,001 or more. D.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.T.W. has received less than \$10,000 for serving as a consultant for Genentech.

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