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Gene expression analysis uncovers novel Hedgehog interacting protein (HHIP) effects in human bronchial epithelial cells

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Abstract

Hedgehog Interacting Protein (*HHIP*) was implicated in chronic obstructive pulmonary disease (COPD) by genome-wide association studies (GWAS). However, it remains unclear how *HHIP* contributes to COPD pathogenesis. To identify genes regulated by *HHIP*, we performed gene

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expression microarray analysis in a human bronchial epithelial cell line (Beas-2B) stably infected with *HHIP* shRNAs. *HHIP* silencing led to differential expression of 296 genes; enrichment for variants nominally associated with COPD was found. Eighteen of the differentially expressed genes were validated by real-time PCR in Beas-2B cells. Seven of 11 validated genes tested in human COPD and control lung tissues demonstrated significant gene expression differences. Functional annotation indicated enrichment for extracellular matrix and cell growth genes. Network modeling demonstrated that the extracellular matrix and cell proliferation genes influenced by *HHIP* tended to be interconnected. Thus, we identified potential *HHIP* targets in human bronchial epithelial cells that may contribute to COPD pathogenesis.

Keywords

Hedgehog interacting protein (*HHIP*); Gene expression profiling; COPD (Chronic obstructive pulmonary disease); extracellular matrix (ECM); network modeling

1. Introduction

Chronic obstructive pulmonary disease (COPD), the third leading cause of death in the U.S. [1], is a complex disease strongly influenced by cigarette smoking and genetic predisposition [2, 3]. COPD is characterized by emphysematous destruction of the alveoli and thickening of the airway walls in response to chronic exposure to cigarette smoke (CS). While the emphysematous destruction of the lung parenchyma probably results from the cytotoxic and pro-inflammatory activities of CS, the pathogenic mechanisms of the disease remain to be fully defined [4]. It has been shown that smoke-induced cell apoptosis and autophagy are likely to be important causes for lung injury during COPD development; imbalance of proteinases and proteinase inhibitors as well as abnormal tissue repair are other mechanisms likely involved in the pathogenesis of COPD [5].

Genome-wide association studies (GWAS) of COPD have provided compelling evidence for a disease susceptibility locus on chromosome 4q31 [6, 7]. This locus has been significantly associated with lung function in the general population [8, 9] as well as with COPD [10, 11]. Our previous work has identified the gene encoding hedgehog interacting protein (*HHIP*) as the causative gene for this highly replicated genetic association with COPD susceptibility [12]. Specifically, we have shown that SNPs upstream of the *HHIP* gene modulate expression of *HHIP*, implicating reduced *HHIP* gene expression in the pathogenesis of COPD [12]. However, the biological mechanisms by which *HHIP* influences COPD susceptibility have not been determined.

Hhip is a cellular membrane protein that directly binds to Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh), which trigger the hedgehog signaling pathway by binding to their membrane receptor, Ptch1. By competing with Ptch1 for binding of hedgehog ligands, Hhip negatively regulates the hedgehog pathway, a crucial pathway for the development of the lungs and other organs [13–15].

To study the biological pathways connecting *HHIP* to COPD pathogenesis, we determined the effect of *HHIP* knock-down through lentivirus-based shRNAs on gene expression in the bronchial epithelial cell line Beas-2B, which has detectable expression of *HHIP* at a relatively higher level than primary human airway epithelial cells (data not shown). Gene expression differences detected by expression array were validated by RT-PCR in Beas-2B and normal human bronchial epithelial cells (NHBE). In these systems, we evaluated the activity of the canonical Hedgehog pathway as well as other novel biological pathways identified by gene expression array analysis.

2. Results

2.1. Expression profiling in *HHIP* depleted Beas-2B cells

To identify the full range of cellular targets of *HHIP* in human bronchial epithelial cells, we performed expression microarray analysis in Beas-2B cells with and without knock-down of *HHIP* by stable infection with lentivirus-based shRNAs targeting *HHIP*. To limit possible off-target effects of the shRNAs, four different *HHIP* shRNAs were used, with up to four replicates for each shRNA (Figure 1B). Efficiency of *HHIP* knockdown was shown by at least 70% reduction in *HHIP* mRNA in Beas-2B cells after infection with *HHIP* shRNAs compared to cells infected with non-targeting control shRNA (NT) (data not shown).

Since there is no consensus in the literature regarding the optimal analytical approach for batch effect analysis of gene expression array data, two methods were used. Surrogate variable analysis [16] and its modification, independent surrogate variable analysis (iSVA) [17] coupled with the linear regression models with an empirical Bayes shrinkage method (limma), found 317 gene probes (representing 300 unique genes) and 469 gene probes (442 genes) respectively, to be differentially expressed (p adjusted < 0.05) in *HHIP* shRNA-infected Beas-2B cells compared to non-targeted shRNA-infected control cells. Of note, 296 genes were identified as showing differential gene expression by both analysis methods (Figure 1A). Figure 1B depicts the differential gene expression pattern for different biological and technical replicates with and without *HHIP* silencing with four individual *HHIP*-targeted shRNAs, showing that the effect of *HHIP* knock-down on gene expression is consistently greater than technical batch effects. The top 20 differentially expressed genes identified by iSVA analysis are shown in Supplemental Table 3. As expected, *HHIP* ranks first on this list. Protein level reductions of Hhip caused by shRNAs were confirmed by Western Blot (Supplemental Figure 1A).

2.2. Genetic Association Analysis of Differentially Expressed Genes

To assess the biological importance of these differential gene expression signals, we evaluated the affected genes for common variant associations with COPD based on our previously published genome-wide association study of four cohorts: The National Emphysema Treatment Trial/Normative Aging Study, GenKOLS, ECLIPSE, and COPDGene (first 1000 subjects) [18].

Among the 296 differentially expressed genes after *HHIP* silencing (including the *HHIP* genic region but not the COPD GWAS region upstream from the *HHIP* gene), 271 were located on autosomes and harbored high-quality imputed or genotyped SNPs within a window of 5kb upstream and 1kb downstream from the transcribed gene boundaries. One of the 271 genes, *RAB4B* is located within the chromosome 19 GWAS region shown to be strongly associated with COPD. While no other SNPs located within the 271 genes showed an association with COPD that reached genome-wide significance after a conservative Bonferroni correction within this subset, 115 genes contained at least one SNP showing association with COPD with a nominal P value of < 0.05 . Based on the method by Lipman and colleagues [19], these results are consistent with a significant enrichment in low P -values ($P = 0.02$). A histogram plot of LD-pruned p -values used for the Lipman analysis demonstrated the enrichment of lower p values (Supplemental Figure 2), supporting a role of these differentially expressed genes in COPD pathogenesis.

2.3. Extracellular matrix genes are differentially expressed by *HHIP* silencing

Next, we evaluated enrichment of the 296 genes differentially expressed after *HHIP* silencing in Gene Ontology (GO) biological processes, using DAVID [20] functional annotation analysis (<http://david.abcc.ncifcrf.gov/home.jsp>) and GeneMANIA (<http://>

www.genemania.org/) [21]. Both found significant enrichment ($P < 0.01$, using DAVID or GeneMANIA) of genes involved in extracellular matrix remodeling (ECM, Table 1). Based on these pathway analysis results, we used SYBR-green based RT-PCR analysis to attempt to validate differential expression of 18 ECM-related genes in Beas-2B cells infected with three *HHIP* shRNAs or one control shRNA (Table 2).

Using a linear mixed effects (LME) model (see details in Supplemental Materials and Methods section), we found that SYBR-green RT-PCR expression analysis confirmed the microarray results for seven genes, demonstrating significantly ($P < 0.01$) increased (*FBLN7*, *SERPING1*, *RECK*, *TULP3*) or decreased (*SERPINB7*, *WNT5B*, *SDC1*) mRNA levels following *HHIP* silencing in RT-PCR (Table 2). For one gene, *NTN4* (Netrin 4), SYBR-green RT-PCR results contradicted the microarray results. For the remaining ten genes, expression changes measured by SYBR-green RT-PCR did not reach statistical significance, although a trend was noted for *PI3* ($P = 0.077$). Furthermore, TaqMan-based RT-PCR confirmed the significant or near-significant SYBR-green based RT-PCR results, except for *NTN4*, which showed no statistically significant expression changes (Figure 2). Similar results were obtained when TBP was used as an additional reference gene (data not shown).

2.4. Hedgehog pathway activity lacks significant changes in *HHIP* silenced Beas-2B cells

Hh has been shown to inhibit the Hedgehog pathway by competitively binding to three Hedgehog morphogens (Shh, Ihh and Dhh) during development [13]. Surprisingly, we did not observe a significant enrichment of canonical Hedgehog pathway members other than *HHIP* among genes that were found to be differentially expressed by microarray analysis after *HHIP* knockdown. To further explore the Hedgehog pathway activity in Beas-2B cells with *HHIP* silencing, we used TaqMan based qPCR to measure the expression of five genes coding for critical components of the canonical Hedgehog pathway (*SHH*, *PTCH1*, *GLI1*, *GLI2*, *GLI3*, *IHH*, and *DHH* [22]) in Beas-2B cells stably infected with two of the *HHIP* shRNAs that were used in the microarray analysis.

No statistically significant expression changes were detected for *PTCH1* or *GLI3*, and *SHH* expression was under the detection limit in Beas-2B cells (Figure 1B); this low expression level of SHH in airway cells was confirmed in primary small airway epithelial cells (data not shown). *GLI1* demonstrated increased expression with *HHIP* shRNAs but was still expressed at a relatively low level. In addition, *GLI2*, previously reported to regulate cell proliferation during lung development [23], demonstrated a statistically significant reduction of 20–30% in expression after *HHIP* knock-down with any of the three *HHIP* shRNAs (Supplemental Figure 1B). Two other Hedgehog pathway ligands, *IHH* and *DHH*, were also barely detected in Beas-2B cells by qPCR at baseline and after *HHIP* silencing (data not shown).

These qPCR data indicate that, *HHIP* silencing by shRNAs had a limited effect on canonical Hedgehog pathway members in our current experimental settings. Hence, these minimal changes of the canonical Hedgehog pathway may not explain all of the other gene expression changes that we observed. These results raised the possibility that other biological pathways may be influenced by silencing *HHIP*.

2.5. Cell proliferation-related genes have reduced gene expression upon *HHIP* silencing

Ten differentially expressed genes, including two additional genes identified by the iSVA analysis method [BDNF (brain-derived neurotrophic factor) and BTG2 (B-cell translocation gene 2)], associated with cell growth were chosen for further validation by TaqMan based RT-PCR in Beas-2B cells (Figure 3A). Two cell-cycle related genes, *CCND1*, encoding

Cyclin D1, and *G0S2* (G0/G1 switch regulatory protein 2) demonstrated more than 50% reduced expression by *HHIP* shRNAs in Beas-2B cells.

Other significantly decreased genes include *FGF2* (fibroblast growth factor 2), *BIRC5* (baculoviral IAP repeat containing 5), *TGM2* (transglutaminase 2), *CREB3L2* (cAMP responsive element binding protein 3-like 2), and *ATF5* (activating transcription factor 5), which are all known to promote cell growth and/or survival ([24], [25], [26], [27]), as well as *LETM1* (leucine zipper-EF-hand containing transmembrane protein 1) and *BDNF*.

In contrast, *BTG2*, the only anti-proliferation gene included here [28], demonstrated significantly increased expression up to ~5 fold in *HHIP* shRNA-infected cells. For *BIRC5*, *BDNF*, *FGF2* and *BTG2*, the effect of *HHIP* knock-down on expression levels was validated in primary human bronchial epithelial cell lines (NHBE, Lonza) (Figure 3B). We attempted to validate protein level changes for three of our differentially expressed genes, *CCND1*, *TGM2* and *BIRC5*; we detected protein level reductions in Beas-2B cells infected with *HHIP* shRNAs for all three of these genes (Figure 3C).

2.6. HHIP target genes are also differentially expressed in human COPD lung tissues

We have previously shown that *HHIP* expression is reduced in human COPD compared to control lung tissues [12]. To provide additional evidence that *HHIP* target genes associated with cell proliferation or ECM are relevant to COPD pathogenesis, we examined expression of 11 selected *HHIP* target genes in human COPD and control lung tissues from the Lung Tissue Research Consortium (LTRC) by RT-PCR to determine whether COPD is associated with expression patterns similar to those seen following knock-down of *HHIP* in vitro.

Among-ECM related genes, *PI3* showed a trend of increased expression in human COPD lung tissues compared to control tissues from ex-smokers ($p=0.08$); while *WNT5B* and *SDC1* demonstrated significantly decreased expression in human COPD lung tissues (Figure 4A, $p<0.01$, unpaired *t* test), recapitulating the pattern seen after *HHIP* knock-down in cell culture. Among genes associated with cell growth, *CREB3L2*, *CCND1*, *ATF5* and *LETM1* showed significantly decreased expression ($p<0.05$) and *TGM2* showed a trend for decreased expression ($p=0.052$) in human COPD lung tissues compared with controls, again recapitulating the effect of *HHIP* knock-down in cell culture.

However, for *RECK* (Figure 4A, which was statistically significant in the direction opposite from our expectation based on RNAi studies), *G0S2* and *BTG2* (data not shown), the expression pattern in our set of COPD and control lung tissues did not confirm the statistically significant changes observed after *HHIP* knock-down. Overall, these data suggest that most of the novel *Hhip* targets identified in this study may participate in human COPD pathogenesis.

Furthermore, we measured gene expression of Sonic Hedgehog pathway members including *SHH*, *PTCH1*, *GLI1*, *GLI2* and *GLI3* in human lung tissue samples that contain mixed cell populations. There are relatively low expression levels of *GLI1* and *SHH* in human lung tissues, consistent with previous reports [29]. Compared to samples from control smokers, we detected significantly decreased expression of *SHH*, *PTCH1*, *GLI1*, *GLI2*, and *GLI3* (Supplemental Figure 3) in lung tissue samples from COPD subjects. Hence, the canonical Hedgehog pathway might participate in COPD pathogenesis in other cell types (or in epithelial cells after cellular injury) [29].

2.7. Network building based on Beas-2B cell microarray analysis

To understand the relationships between the genes found to be regulated by *HHIP*, we performed data mining and curation using the Predictive Networks web application [30]

with default parameters. Top differentially expressed genes (n=442) with p-adjusted <0.05 found using iSVA after *HHIP* silencing were used as seeds for network analysis. The resulting network includes 7489 connections among 1958 genes, including 87 genes that were differentially expressed by *HHIP* shRNAs (highlighted in red circles in Supplemental Figure 4). In this network, gene clusters centered on some validated target genes such as *CCND1*, *SERPINE1*, and *RECK* were observed (supplemental Figure 4).

Next, we asked whether the network showed interconnections between *HHIP* target genes associated with cell growth and extracellular matrix. Within this network analysis model, we found several primary and secondary connections between the 18 validated cell proliferation and extracellular matrix genes (Figure 5); in addition, 26 additional differentially expressed genes showed direct interactions with the 18 validated genes, and 220 differentially expressed genes are within two degrees of separation from the validated genes. The large number of connections observed between differentially expressed genes suggests that the genes which have differential expression after *HHIP* knock-down may have shared biological functions.

To estimate the statistical significance of the number of connections between ECM and proliferation genes seen in our network, we generated 10,000 gene sets of the same size as in our validated gene set, drawing randomly from the genome; for each, we generated literature-based networks using the network reconstruction protocol described previously. While the number of connections between ECM genes in our validated gene set is not significant (p=0.4, Supplemental Figure 5A left panel), the number of connections between cell proliferation genes is significantly greater than expected by chance (p<0.01, Supplemental Figure 5A middle panel).

Given the representation of ECM and proliferation genes in the validated gene set, the enrichment of connections between these genes may not be surprising. To test the statistical significance of the observed associations, we constructed 10,000 random networks consisting of only ECM genes (N=7) and proliferation genes (N=11). Based on this analysis, the connections within the ECM gene subset, and within the cell proliferation gene subset, compared to the random networks are not significant (p=0.67 and 0.09, respectively). However, the number of observed connections between ECM genes and cell proliferation genes is significantly greater than we would expect by chance (p=0.0035, Supplemental Figure 5B), suggesting that we may have discovered important new connections between the ECM and proliferative processes in the cell. Using GO annotations, 12 of the 18 validated genes are connected to cell proliferation genes, and 9 are connected to at least one extracellular matrix modification gene. For example *LETM1* (a cell proliferation gene) has connections with *MMP2* and *MMP9*, which are inhibited by *RECK* (extracellular matrix genes).

3. Discussion

Genetic variants near *HHIP* have been consistently associated with COPD susceptibility, and a functional variant influencing *HHIP* expression has been previously reported by our group [12]. Based on these genetic association results, we hypothesized that *HHIP* would influence COPD susceptibility through the Hedgehog pathway. The Hedgehog (HH) signaling pathway is critical for organogenesis and carcinogenesis [22].

Vertebrate organisms have three types of Hedgehog ligands: Sonic Hedgehog (SHH), Indian Hedgehog (IHH), and Desert Hedgehog (DHH). Binding of the Hedgehog ligand to its membrane receptor, protein patched homolog 1 (PTCH1), triggers a series of cytoplasmic signaling cascades and eventually activates transcription factors GLI1, GLI2, and GLI3 [22].

HHIP, through its extracellular domain, binds to all three types of HH ligands and inhibits HH signaling [13, 31, 32].

In adults, detectable expression of *HHIP* was previously found in both whole lung [12] and primary bronchial epithelial cells (unpublished data). However, in our study, *SHH* was barely detectable in both Beas-2B and primary small airway epithelial cells. Moreover, induced expression of *PTCH1* and *GLI1* are usually regarded as hallmarks for the activation of the canonical Hedgehog pathway. Nonetheless, no significant expression changes of *PTCH1* or *GLI3* were observed after *HHIP* knockdown, and *GLI1* demonstrated increased but still relatively low expression in Beas-2B cells with *HHIP* silencing.

Only a subset of the *HHIP* targets identified here, such as *TULP3* [33] and *CCND1* [23, 33], are known targets of the Hedgehog pathway. Based on these observations, it is unlikely that the canonical Hedgehog pathway can explain all of the gene expression changes that we observed in the Beas-2B cells treated with *HHIP* shRNAs. However, given the complexity of the Hedgehog pathway, which includes both ligand-dependent and ligand-independent activation [34, 35] mechanisms, more comprehensive evaluations are needed to address whether Hedgehog signaling regulates expression of these novel *HHIP* targets in human bronchial epithelial cells through non-canonical pathways. Interestingly, when we assessed the canonical Hedgehog pathway in human COPD lung tissues by qPCR targeting the major pathway components: *SHH*, *PTCH1*, *GLI1*, *GLI2* and *GLI3* (Supplemental Figure 3), we detected overall reduced pathway activity in COPD subjects compared to controls. This may indicate a plausible role of the Hedgehog pathway in human COPD. Based on the gene expression levels that we observed in lung epithelial cell lines, we speculate that the involvement of the Hedgehog pathway in COPD could be mediated in other cell types or in lung epithelial cells only after cellular injury.

We found 296 genes with significant alterations in their expression levels after *HHIP* silencing in Beas-2B cells. The occurrence of the *RAB4B* gene (within the chromosome 19 COPD GWAS region) and the observed enrichment of other COPD genetic association signals which do not reach conventional levels of genome-wide significance suggest that SNPs within other members of this set of differentially expressed genes could influence COPD susceptibility. Further work to clarify which of these genes are associated with COPD in larger sample sizes and other study designs (e.g., eQTL analysis) could lead to the identification of novel genes for COPD susceptibility that interact with *HHIP*.

Our study provides some helpful insights regarding the mechanisms through which *HHIP* may affect COPD susceptibility. In particular, the results shown here suggest that *HHIP* may influence an individual's susceptibility to smoke-induced COPD by regulating extracellular matrix (ECM) remodeling and cell growth pathways. The expression levels of multiple extracellular matrix-related genes were significantly changed by *HHIP* shRNAs based on gene enrichment analysis.

Additionally, a subset of these ECM-related genes also demonstrated significant differential expression in human COPD lung samples. For example, *SDC1* expression was significantly decreased in both Beas-2B cells with *HHIP* silencing and in human COPD lungs. *SDC1* encodes a heparan sulfate proteoglycans transmembrane protein that is an important component of ECM as well as regulating matrix assembly [36]. It also protects lung epithelial cells from injury by removing C-X-C chemokines during inflammation [37].

Another differentially expressed gene, *PI3*/Elafin, is an inhibitor for neutrophil elastase, while *RECK* (a membrane-bound protein) can exert inhibitory effects on the transcription, synthesis, activation, and activity of MMPs, including *MMP2*, *MMP7*, and *MMP9* [38–41]. It is not entirely surprising to detect significant expression changes of *RECK* in human

COPD lungs given its crucial roles in regulating the activities of MMPs, and the importance of imbalance between proteinases and anti-proteinases in human lungs contributing to the pathogenesis of COPD/emphysema.

However, how HHIP affects expression of ECM-related genes requires further mechanistic investigations. It is possible that HHIP, as a cellular membrane protein [13], directly cross-talks with other cellular transmembrane proteins such as Syndecan-1 to facilitate its anti-inflammatory and extracellular matrix functions.

Among the most significant differentially expressed genes after *HHIP* silencing found by microarray analysis, we used RT-PCR to confirm reductions in expression of several cell cycle and proliferation-related genes, *GOS2*, *CCND1*, *FGF2*, *BIRC5* and *TGM2*, as well as increased expression of an anti-proliferation gene, *BTG2*. This apparent regulation by *HHIP* of cell growth genes in Beas-2B cells (Figure 3) is consistent with previous findings in *HHIP* conditional transgenic mice that over-expression of HHIP lacking the transmembrane domain led to hyper-proliferation of small intestine epithelium [42].

Moreover, *GLI2* has been reported to regulate lung epithelial growth by modulating Cyclin expression including Cyclin D1 [23], consistent with our findings of decreased expression of *CCND1* and *GLI2* (Supplemental Figure 1B) with *HHIP* shRNAs. Given that the *HHIP* gene locus was also associated with height in multiple populations [43], it is conceivable that HHIP may regulate cell growth and proliferation.

In addition, apoptosis has been implicated in COPD pathogenesis [44]. HHIP target genes *FGF-2* [45] and *ATF5* [27] positively regulate expression of Bcl-2 anti-apoptotic protein, which showed decreased expression accompanied by increased pro-apoptotic protein Bax expression in cigarette smoke (CS) induced cell death in vitro [46] and in vivo [47]. Additionally, *BIRC5*, a potent anti-apoptosis protein, interferes with cell death through caspase-dependent and independent mechanisms [48]. Hence, gene expression changes including decreased expression of *FGF2*, *ATF5* and *BIRC5* in HHIP-depleted cells may contribute to smoke-induced lung injury due to enhanced cell apoptosis.

When we used a recently developed network modeling approach to analyze our microarray results, we found not only that most of our validated hits are clustered in this “predictive network” (Supplemental Figure 4) but also that there are direct or indirect connections (0–1 connectors) between our validated cell proliferation and ECM-related genes (Figure 5), suggesting that the genes in these two pathways that are perturbed by *HHIP* knock-down may have shared biological functions. This network analysis included a variety of genes which have been previously implicated in COPD pathogenesis, but which were not directly identified by our gene expression analysis after *HHIP* silencing. For example, *EGR1* was identified as a key differentially expressed gene in a microarray gene expression analysis of human COPD and normal lung tissues [49]. Multiple members of the integrin family also appear in this *HHIP* network, including *ITGA V*, which has been implicated in TGF- β activation [50]. Thus, the network identified after *HHIP* depletion could relate directly to previously reported components of COPD pathobiology.

Nonetheless, there are some limitations related to our study. First, the gene expression changes induced by *HHIP* shRNAs may result from both primary effects of genetic perturbation of *HHIP* expression or the downstream signaling events related to these primary effects. Second, only a subset of the differentially expressed genes identified by microarray analysis were validated by RT-PCR; further work will be required to determine whether these inconsistencies represent false-positive microarray results or false-negative validation attempts. Finally, it is possible that the mixed cell population in whole lung homogenates from COPD subjects, phenotypic heterogeneity within COPD subjects, and the

moderate sample size of our COPD case and control lung tissue population accounts for our ability to confirm some, but not all, of the gene expression differences that we observed in cell-based gene expression analysis.

4. Conclusion

In summary, we have applied transcriptome profiling in human bronchial epithelial cell lines that were silenced with *HHIP* shRNAs to identify novel targets of the *HHIP* gene that may participate in the pathogenesis of human COPD. Our whole-transcriptome analysis suggests novel pathways by which *HHIP* may affect COPD pathogenesis. Further functional validation of the roles of these intriguing targets of *HHIP* in smoke-induced murine COPD models is warranted.

5. Materials and methods

5.1. Cell culture and human lung tissues

Human Beas-2B bronchial epithelial cells (#CRL-9609) were purchased from ATCC and cultured in complete DMEM medium supplemented with 10% fetal bovine serum, penicillin (50 units/ml), streptomycin (50 µg/ml) and gentamicin (10 µg/ml). Normal Human Bronchial Epithelial cells (NHBE) from healthy non-smoker donors and medium for culturing NHBE were purchased from Lifeline, Inc.

Human lung tissue samples from 15 COPD patients ($FEV_1 < 80\%$) and 18 control subjects with normal lung function were obtained from the NHLBI Lung Tissue Research Consortium (LTRC), a national biorepository of lung tissues.

5.2. Gene silencing of *HHIP* by RNA interference in Beas-2B cells

Four individual *HHIP*-targeting shRNAs and one non-targeting scrambled shRNA (Open Biosystems, Thermo Scientific) were transfected into 293T cells for packaging into lentivirus particles. Beas-2B cells were infected with lentivirus-based shRNAs using standard methods [49]. Approximately 24 hours post-infection, puromycin was added at 1.5 µg/ml for 3–4 days to select for successfully infected cells before RNA and protein extraction.

5.3. Detection of differential gene expression

Total RNA from Beas-2B cells was extracted using the RNeasy Mini Kit (Qiagen, Germantown, MD). Differential gene expression detected by gene expression microarrays was validated by real-time PCR with gene-specific primers on an ABI 7900 HT instrument (Applied Biosystems, Foster City, CA), using SYBR Green for detection. If statistically significant expression changes were confirmed, RT-PCR was repeated using TaqMan probes for detection. Relative gene expression was calculated based on the standard $2^{-\Delta\Delta CT}$ method, using *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) and control cells as references. *TBP* (TATA box binding protein) was also used as a reference gene in all TaqMan based RT-PCR validations. Primer and probe sequences are listed in the Supplemental Table 1 and 2.

5.4. Network analysis

Unbiased, automated literature mining was performed on the differentially expressed genes (adjusted p-value < 0.05) using the Predictive Networks application [30]. We gathered gene pairs representing genetic interactions, regulatory functions, and pathways collected from PubMed and Medline abstracts and other published sources. Networks were visualized by Cytoscape (<http://www.cytoscape.org>). A sub-network centered around the RT-PCR

validated genes was produced to illustrate connections between the differentially expressed genes and cell proliferation/survival and extracellular matrix functions. First, only validated genes and their interactions were selected. Then, all non-differentially expressed genes with one interacting partner were removed, retaining only genes that connect to differentially expressed genes.

To estimate the statistical significance of the number of connections to ECM and proliferation genes seen in our network, we generated 10,000 random gene sets of the same size (18) as in our validated gene sets and used them to generate literature-based networks using the network reconstruction protocol described previously. We then counted the total number of connections in that network, the number of connections between ECM genes (defined by GO:0005576 and GO:0005615 classes), the number of connections between cell proliferation genes (defined by GO:0008283), and the number of connections between ECM and cell proliferation genes (excluding internal connections among ECM genes and connections among proliferation genes) for each of the random networks. The empirical p-values for enrichment are calculated by taking the fraction of random networks having a greater number of connections than the observed values. This was repeated with random gene sets generated by using the same numbers of ECM genes (7) and proliferation genes (11) as in the validated gene set.

5.5. Additional details are provided in the online supplemental material and methods

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Gene expression analysis was performed after silencing HHIP, a COPD GWAS gene.
- SNPs near differentially expressed genes were nominally associated with COPD.
- Extracellular matrix and cell growth genes were enriched and tend to be interconnected.
- Seven differentially expressed genes showed significant changes in human COPD lungs.
- Novel targets of HHIP that may contribute to COPD pathogenesis are identified.

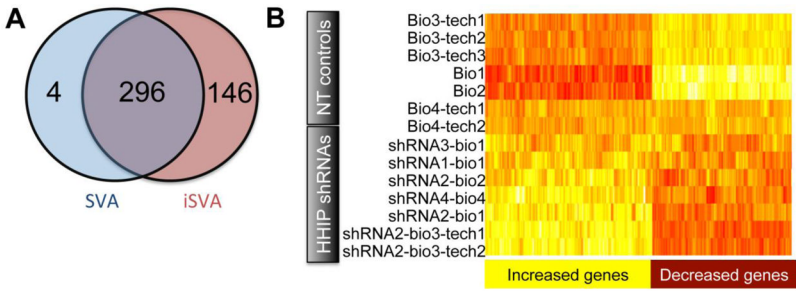


Figure 1. Gene expression profiling analysis in Beas-2B cells stably infected with *HHIP* shRNAs. A. Venn diagram showing the number of differentially expressed genes based on iSVA or SVA. B. Expression heat map of 296 differentially expressed genes (columns) (p adjusted <0.05). Each row represents a separate sample (array). Bio: biological replicate; tech: technical replicate; NT shRNA: non-targeting shRNA control; shRNA: *HHIP*-targeting shRNA

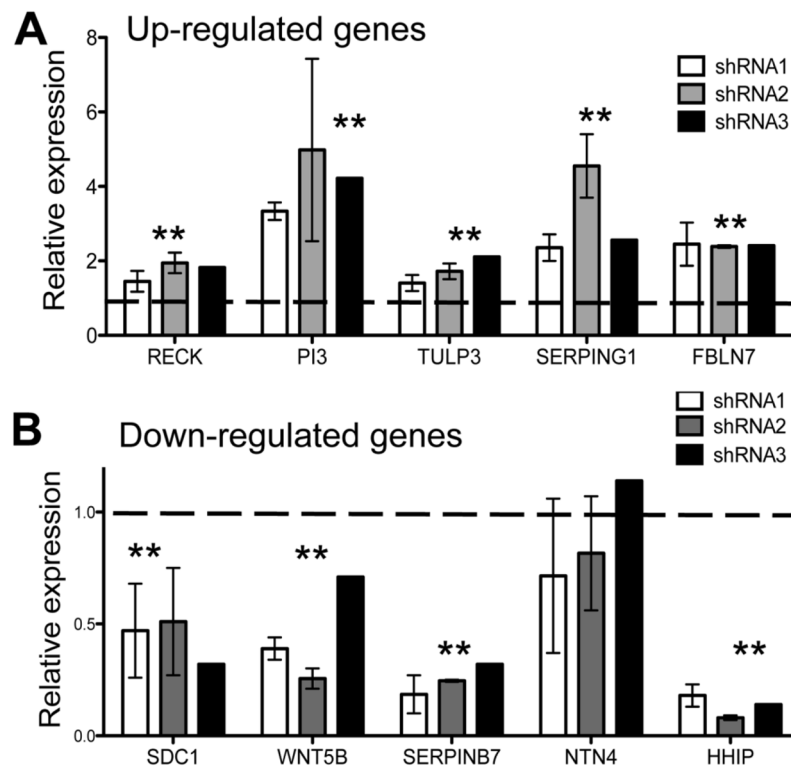


Figure 2. Significantly increased (A) and decreased (B) expression (**, $p < 0.01$) of HHIP target genes associated with extracellular matrix (ECM) after *HHIP* silencing in Beas-2B cells, as measured by TaqMan assay-based RT-PCR. Three different *HHIP*-targeted shRNAs were used for knock-down. Relative expression values were calculated as described in Materials and Methods and represent means \pm standard errors from two independent shRNA infections.

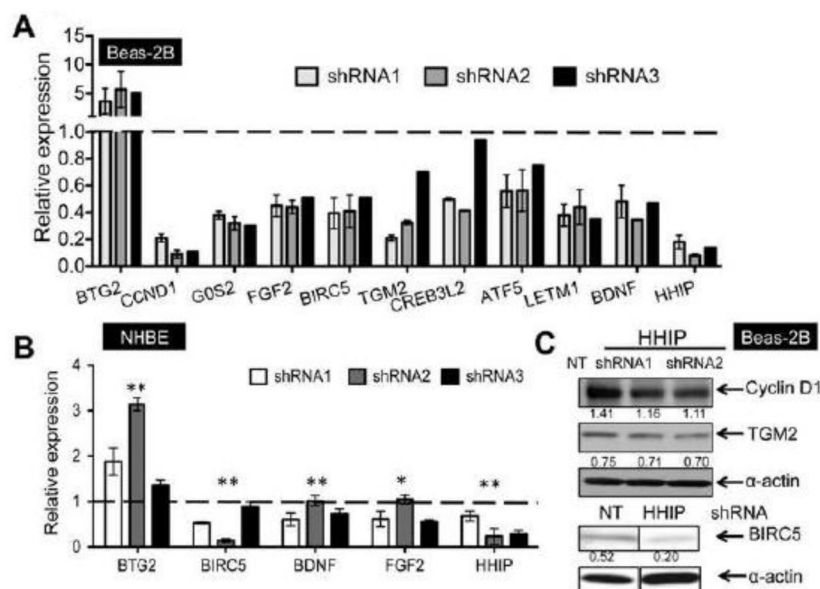


Figure 3.

Genes associated with cell growth that demonstrated significant differential expression ($p < 0.05$) after *HHIP*-knock-down in Beas-2B (A) or normal human bronchial epithelial cells (NHBE) (B), as measured by TaqMan assay-based RT-PCR. Three different *HHIP*-targeted shRNAs were used for knock-down. Relative expression values were calculated as described in Materials and Methods and represent means \pm standard errors from two (A) or three (B) replicates for each hairpin. P values in figure 3B are calculated based on two biological replicates and one is represented here. C. Expression of *CCND1*, *TGM2* and *BIRC5* in Beas-2B cells infected with *HHIP*-targeted or non-target (NT) control shRNA, as detected by western blot. α -actin was used as loading control. Band density was quantified using Image J and then normalized to α -actin control.

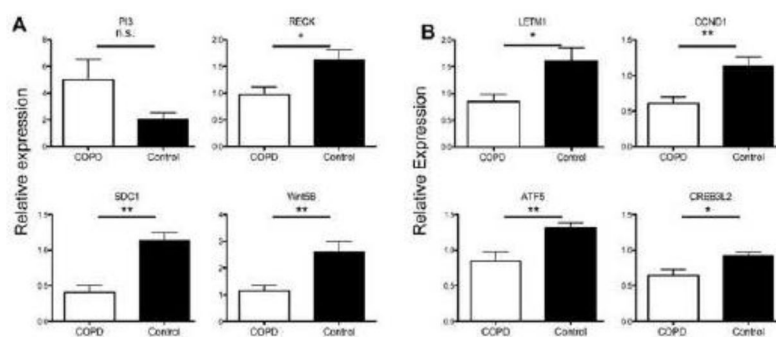


Figure 4.

Expression of HHIP target genes associated with ECM (A) or cell growth (B) were measured in human COPD and control lung tissues by RT-PCR. Relative expression values were calculated as described in Materials and Methods and represent means \pm standard errors for COPD (n=15) and Controls (n=18). **p<0.01 and *p<0.05. n.s., non-significant.

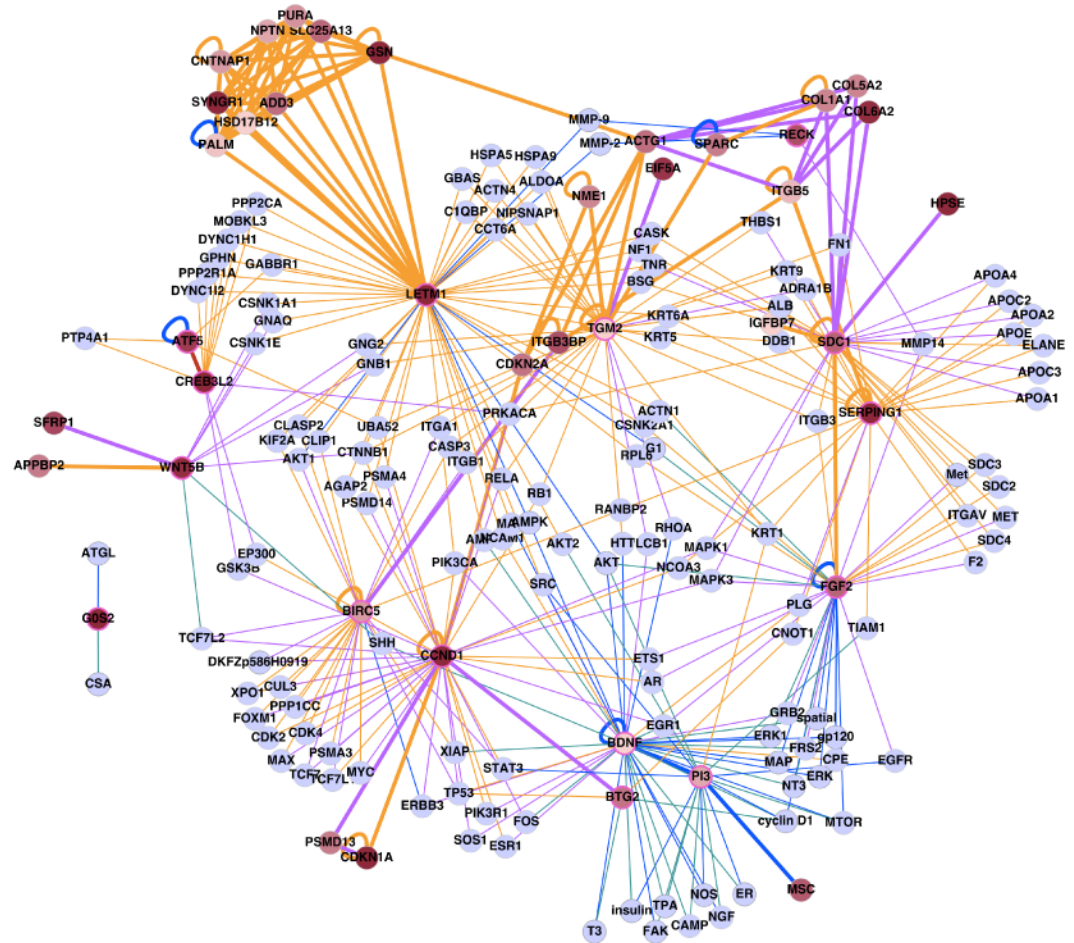


Figure 5.

Network analysis of validated HHIP targets related to ECM and cell proliferation (highlighted by pink circles). Edge colors represent sources of the interaction (brown: Pathway Commons, purple: functional interaction, blue: PubMed abstract, teal: Medline abstract, red: manual curation based on recently published literature) and node colors represent the level of differential expression (red: highly differential, gray: no expression change.) Thickened edges connect pairs of differentially expressed genes.

Table 1
DAVID and GeneMANIA functional annotation clusters for genes regulated by HHIP

DAVID					
Clusters	Enrichment score	Top GO term	Top term		Gene count
			Fold enrichment	P value	
1	2.12	GO:0031012~extracellular matrix	2.93	<0.001	14
2	1.75	GO:0047115~trans-1,2-dihydrobenzene-1,2-diol dehydrogenase activity	50.45	0.0013	4
3	1.74	GO:0042612~MHC class I protein complex	12.89	<0.001	5
GeneMANIA					
Clusters	GO annotation	FDR	coverage query genes		
1	extracellular matrix	1.82E-04	15/178		
2	proteinaceous extracellular matrix	1.82E-04	12/105		
3	DNA conformation change	4.86E-04	11/97		

Table 2

Effect of *HHIP* knock-down on expression of selected ECM genes, as measured by microarray and SYBR-green based RT-PCR.

Gene symbol	Gene name	Microarray		RT-PCR	
		Statistics*	adjusted p-value	Mean [§]	p-value [†]
OLFML2	Olfactomedin-like 2A	4.63	0.02	1.22	0.11
TIMP1	TIMP metalloproteinase inhibitor 1	4.21	0.04	1.01	0.05
EFEMP2	EGF-containing fibulin-like extracellular matrix protein 2	4.94	0.017	1.16	0.11
SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)	4.39	0.03	0.92	0.11
BGN	biglycan	4.63	0.02	1.31	0.24
NTN4	Netrin 4	4.01	0.049	0.58	0.08
COL6A2	Collagen type VI, alpha2	5.25	0.013	1.13	0.06
GSN	Gelsolin	5.95	0.006	1.37	0.14
ECM1	Extracellular matrix protein 1	4.68	0.02	1.39	0.14
FBLN7	Fibulin 7	5.78	0.007	2.07	0.21
SERPING1	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	5.60	0.009	4.35	1.88
PI3	peptidase inhibitor 3, skin-derived	4.19	0.039	3.50	1.18
RECK	reversion-inducing-cysteine-rich protein with kazal motifs	6.12	0.005	2.01	0.17
TULP3	Tubby like protein 3	6.56	0.004	1.47	0.07
SERPINB7	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 7	-5.33	0.012	0.20	0.03
WNT5B	Wingless-type MMTV integration site family, member 5B	-5.18	0.014	0.34	0.07
COL13A1	Collagen, typeXIII, alpha 1	-5.14	0.014	0.59	0.09
SDC1	syndecan 1	-4.56	0.026	0.50	0.11

* Moderated t-test statistic testing for the hypothesis that a gene probe is differentially expressed between HHIP hairpins arrays and control hairpin arrays adjusting for the 2 estimated independent surrogate variables (ISVA).

[§] mean and standard error of mean (sem) of log₂ fold change of hairpins versus controls.

[†] p-value for testing if a gene probe is differentially expressed between HHIP hairpins and NT controls based on linear mixed effects models. Genes with p<0.01 were regarded as significantly differentially expressed genes validated by RT-PCR, which were highlighted in bold.