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A Long Noncoding RNA links TGF-β Signaling in Lung Fibrosis

Pulmonary fibrosis is an increasing cause of morbidity and mortality worldwide with limited therapeutic options. Idiopathic pulmonary fibrosis (IPF) is a particularly severe form of lung fibrosis, with no known etiology and a median survival of 2.5–3.5 years after diagnosis (1). The pathogenesis of IPF is complex and involves loss of epithelial integrity and excessive fibroblast activation (1, 2).

The TGF-β (transforming growth factor β) signaling pathway plays a central role in the initiation and progression of tissue fibrosis (3). Strategies to target the TGF-β signaling pathway have been extensively investigated in preclinical settings (4) and in clinical trials for patients with IPF. Owing to the pleiotropic nature of TGF-β, directly blocking TGF-β signaling may have adverse effects. Alternative strategies, such as partial inhibition of TGF-β using αvβ6 integrin antibodies, have been investigated (5).

In addition to protein-coding RNAs, many noncoding RNAs (ncRNAs), including microRNAs (miRNAs) and long ncRNAs (lncRNAs), have been recently described. miRNAs are short (∼22 nt in length), single-stranded ncRNAs that inhibit the production of target proteins or induce the degradation of mRNAs, thereby suppressing target gene expression. Dysregulation of miRNAs has been shown in the lungs of patients with IPF (6), as well as in animal models of lung fibrosis (7). The roles of miRNAs in lung fibrosis have been studied in humans and in mice (8, 9). lncRNAs are RNA transcripts that are more than 200 nt long and may play a role in gene transcriptional regulation, post-transcriptional regulation, and epigenetic regulation in development and diseases (10).

In this issue of the *Journal*, Savary and colleagues (pp. 184–198) report that the lncRNA *DNM3OS* (DNM3 opposite strand/antisense RNA) serves as an miRNA reservoir in TGF-β signaling (11). Using RNA sequencing and small RNA sequencing in a human lung fibroblast cell line (MRC-5) stimulated with TGF-β1, the authors found that the lncRNA *DNM3OS* was one of the most strongly induced lncRNAs. Fluorescent *in situ* hybridization

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targeting RNA molecules showed that the primary expression of *DNM3OS* was restricted to the fibrotic area of bleomycin-injured mouse lungs, specifically in *Acta2*$^{+}$ myofibroblasts.

DNM3OS and the miR-199a/miR-214 cluster are located on chromosome 1 at 1q24.3 on the opposite strand of the *DNM3* gene (encoding dynamin 3). However, *DNM3* mRNA (and its encoded miR-3120) was not modulated by TGF-β, suggesting that *DNM3OS* may not function as a *cis*-acting RNA mediating its host gene, but rather as a *trans*-acting lncRNA mediating TGF-β signaling components at distinct locations. lncRNAs can regulate gene expression and biological processes by giving rise to miRNAs. In this study, the lncRNA *DNM3OS* was shown to be a precursor that gives rise to three mature miRNAs (miR-199a-5p, miR-199a-3p, and miR-214-3p) in lung fibroblasts. With relevance to human disease, the study showed that *DNM3OS* and these three mature miRNAs were all upregulated in IPF fibroblasts and regulated by TGF-β.

The study further showed mechanistically that these three mature miRNAs regulate distinct TGF-β signaling activities. miR-199a-5p promoted lung fibrosis through a CAV1-dependent mechanism in lung fibroblasts *in vitro* and in a mouse model *in vivo*. miR-199-3p targets FGF7 and HGF, possibly in concert with TGF-β–induced suppression of HGF/FGF7 production. On the other hand, miR-214-3p targets GSK-3β and COX-2 in the β-catenin pathway, suggesting that *DNM3OS* may mediate the crosstalk between the TGF-β and Wnt profibrotic pathways.

This study further demonstrates the translational possibility of targeting lncRNAs as a therapeutic strategy for IPF. Loss-of-function experiments with gapmers (antisense oligonucleotides) showed that *DNM3OS* was a critical downstream effector of TGF-β signaling in lung fibroblasts *in vitro*, as well as in bleomycin-induced lung fibrosis in mice *in vivo*. The miRNAs miR-199a2 and miR-214 showed activity in response to TGF-β1 in mouse and human fibroblast lines *in vitro* and in mouse models *in vivo*. Two antisense oligonucleotides (antimiRs) against miR-199a-3p, miR-199a-5p and miR-214-3p, impacted fibrogenesis in cell lines and in bleomycin models in mice, respectively. These *in vivo* experiments have profound significance in that they suggest the ability of RNAse-H activating gapmers to silence the miRNA cluster in an *in vivo* setting. Thus, antisense oligonucleotide–based therapeutic strategies targeting either mature miRNAs or polycistronic miRNA precursor transcripts may represent an effective approach for the treatment of IPF.

Although this was a comprehensive study involving extensive datasets, several points should be considered. First, the expression and function of *DNM3OS* in other cell types in the lung should be carefully examined. For example, a recent study using single-cell RNA sequencing identified that lncRNA *MEG3* was expressed in airway epithelial cells from healthy donors and IPF basal-like epithelial cells (12). Epithelial apoptosis pathways are activated in the lungs of patients with lung injury, in part by activation of the TGF-β signaling pathway. Deletion of TGFβR2 in epithelial cells protects mice from lung fibrosis (13). Impaired alveolar type 2 cell renewal may contribute to a dysregulated lung-injury response in IPF (14). Therefore, the role of these lncRNAs in epithelial cells should be further investigated. Second, the necessity of *DNM3OS* for TGF-β signaling can be established with the lncRNA knockout in mice. Moreover, it is hard to imagine that one lncRNA works alone in a complex disease such as IPF. An lncRNA circuit may exist to regulate the fibrogenic pathways. It was previously reported that embryonic stem cell pluripotency is mediated by several dozens of lncRNAs with diverse mechanisms (15). Third, the induction of cleavage of the target lncRNA *DNM3OS* with gapmers or antisense oligonucleotides should be examined biochemically, genetically, and pharmacologically. For example, gapmer-RNase H complex formation, RNase H1/H2 involvement, cleavage efficacy, and access to the injury area are worth careful investigation. Nevertheless, this study shows that gapmer-based therapy against the lncRNA *DNM3OS* may represent a new therapeutic approach to treat patients with IPF. ■

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Resequencing to Fine Map Known Idiopathic Pulmonary Fibrosis Risk Genes

Homing in on Causal Variants

Genetic determinants of familial interstitial pneumonia, the familial form of idiopathic pulmonary fibrosis (IPF), were initially recognized as far back as 2001 as rare variants in the genes encoding *SFTPC* (surfactant protein C) and *TERT* (telomerase reverse transcriptase) (1, 2). Subsequent targeted candidate gene and whole-exome sequencing studies found that rare variation in candidate genes for familial IPF in the telomere complex (*TERT*, *RTEL1* [regulator of telomere elongation helicase 1], and *PARN* [poly(A)-specific ribonuclease]) and surfactant protein pathways (*SFTPA2* [surfactant protein A2] and *SFTPC*) also determined risk for sporadic IPF in individuals without a family history of IPF (Figure 1) (3–6). A linkage study in 2011 was the first to identify a SNP on chromosome 11p15.5 within the promoter of the mucin 5 gene (rs35705950 in *MUC5B*), with large effects on both familial and sporadic IPF risk replicated in subsequent genome-wide association studies (GWASs) (7).

For nearly a decade, GWASs have discovered 17 common genetic variants (allele frequency >5%) scattered across the genome associated with risk for IPF (Figure 1) (8–12). Among the most successfully replicated of these IPF risk loci was rs35705950 on *MUC5B*, confirming previous linkage studies and the large effect of this locus on familial and sporadic IPF pathogenesis (7). This led to the hypothesis that IPF results from increased *MUC5B* expression, causing excess production of mucus in the airways and thus impairing lung defense (13). The risk allele frequency of rs35705950 mirrors the observed prevalence of IPF, with a risk allele frequency of 10% in European individuals, the group with the highest IPF prevalence, whereas the risk allele is nearly nonexistent in African descent populations, in which IPF is much less prevalent. Although the *MUC5B* promoter polymorphism explains approximately 30% of the observed genetic risk (14), IPF is determined by environmental interactions with variation in multiple genes related to different pathogenic pathways identified by GWAS discoveries, including genes related to host defense (*TOLLIP* [Toll-interacting protein]), telomere maintenance (*TERT*, *TERC* [telomerase RNA component]), signaling (*AKAP13* [A-kinase anchor protein 13]), and cell–cell adhesion (*DSP* [desmoplakin]) (8–12).

GWASs are based on chip genotyping data from subsets of SNPs to tag whole genomes and, more recently, additional imputed genotypes. Hence, GWASs are not usually sufficient to identify the causal variant and have the potential to miss uncovered or previously unknown rare variants. GWASs are also underpowered to detect rare variant associations owing to low frequency and the large number of rare variants throughout the genome. Because of these limitations, it can be difficult to conclusively determine the total number of causal variants within a genomic region. For example, there are contrasting reports of additional association signals within the chromosome 11p15.5 region independent of the *MUC5B* promoter polymorphism (9, 10). Hence, deep resequencing of known candidate genes for familial and sporadic IPF is required to fully characterize the genetic architecture for risk loci and map independent causative common and rare variants, both known and novel.

In this issue of the *Journal* (pp. 199–208), Moore and colleagues (15) describe targeted DNA resequencing of 16 genomic regions surrounding loci previously associated with risk for familial or sporadic IPF. Common variants (allele frequency ⩾3%) were investigated individually in 3,624 individuals with IPF and 4,442 control subjects, and rare variants were investigated using gene-level and region-based tests in a subgroup of 7,116 subjects with confirmed European ancestry. This is the largest resequencing study of known loci for this uncommon respiratory disease, which has been evaluated mostly in smaller IPF genetic studies. The size of this large IPF cohort in combination with the use of deep, targeted gene resequencing analyzed with conditional analyses and gene-level rare variant tests allowed for the most detailed estimates of the contribution of multiple common and rare variants to IPF risk currently possible.

This resequencing study demonstrated several important aspects of the genetic architecture of known IPF risk loci. First, this study confirmed reported associations for rare and common variations initially described for the genomic regions investigated. However, the top associations were not always at the initially reported sentinel variant (i.e., near *ZKSCAN1* [Zinc finger protein with KRAB and SCAN domains 1] and *IVD* [isovaleryl-coenzyme A dehydrogenase]), demonstrating the power of resequencing

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