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Long ncRNA Landscape in the Ileum of Treatment-Naive Early-Onset Crohn Disease

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Background: Long noncoding RNAs (lncRNA) are key regulators of gene transcription and many show tissue-specific expression. We previously defined a novel inflammatory and metabolic ileal gene signature in treatment-naive pediatric Crohn disease (CD). We now extend our analyses to include potential regulatory lncRNA.

Methods: Using RNAseq, we systematically profiled lncRNAs and protein-coding gene expression in 177 ileal biopsies. Co-expression analysis was used to identify functions and tissue-specific expression. RNA in situ hybridization was used to validate expression. Real-time polymerase chain reaction was used to test lncRNA regulation by IL-1β in Caco-2 enterocytes.

Results: We characterize widespread dysregulation of 459 lncRNAs in the ileum of CD patients. Using only the lncRNA in discovery and independent validation cohorts showed patient classification as accurate as the protein-coding genes, linking lncRNA to CD pathogenesis. Co-expression and functional annotation enrichment analyses across several tissues and cell types showed that the upregulated LINC01272 is associated with a myeloid pro-inflammatory signature, whereas the downregulated HNF4A-ASI exhibits association with an epithelial metabolic signature. We confirmed tissue-specific expression in biopsies using in situ hybridization, and validated regulation of prioritized lncRNA upon IL-1β exposure in differentiated Caco-2 cells. Finally, we identified significant correlations between LINC01272 and HNF4A-ASI expression and more severe mucosal injury.

Conclusions: We systematically define differentially expressed lncRNA in the ileum of newly diagnosed pediatric CD. We show lncRNA utility to correctly classify disease or healthy states and demonstrate their regulation in response to an inflammatory signal. These lncRNAs, after mechanistic exploration, may serve as potential new tissue-specific targets for RNA-based interventions.

Key Words: Crohn disease, long ncRNA, RNAseq, RNA expression

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INTRODUCTION

The inflammatory bowel diseases (IBDs), Crohn disease (CD) and ulcerative colitis (UC), are caused by a complex interaction between host genetic background, microbial shifts, and environmental cues, leading to chronic activation of the mucosal immune system.\(^1\)\(^-\)\(^3\) Meta-analysis identified 163 different IBD risk loci,\(^4\) and a more recent analysis added 38 new loci.\(^5\) The majority of those IBD susceptibility loci are intergenic or intronic. These data are in agreement with the ENCODE project,\(^6\) showing that a large fraction of risk for complex disease is driven by noncoding genetic variation. Among those, approximately 10% of disease-associated single nucleotide polymorphisms (SNPs) are mapped to genetic variation. Among those, approximately 10% of disease-associated single nucleotide polymorphisms (SNPs) are mapped to genetic variation. Among those, approximately 10% of disease-associated single nucleotide polymorphisms (SNPs) are mapped to genetic variation. Among those, approximately 10% of disease-associated single nucleotide polymorphisms (SNPs) are mapped to genetic variation.

LncRNAs play fundamental roles in gene transcription regulation\(^7\)\(^-\)\(^\text{10}\) and overall exhibit more tissue-specific expression patterns than protein-coding genes. They are a large (>15,000\(^11\)) and diverse class of non-protein-coding genes longer than 200 nucleotides. Emerging data have highlighted their regulation of immunity.\(^12\) LncRNA are differentially regulated in virus-infected cells\(^13\) and in monocytes after lipopolysaccharide stimulation.\(^14\) The lncRNA NeST controls susceptibility to Thielers virus and Salmonella infection in mice through epigenetic regulation of the interferon-\(\gamma\) (IFN-\(\gamma\)) locus,\(^15\) and long interspersed ncRNA (lincRNA)–\(\alpha\)\(^1\)–\(3\)CoX2 acts as a broad-acting regulatory component of a circuit that controls the inflammatory response.\(^16\) Hrdlickova et al.\(^17\) revealed lncRNA enrichment in several immune-related disorders including IBD. Specifically, microarray analysis from colon biopsies of adults with long-standing IBD showed widespread dysregulation of lncRNA in inflamed and noninflamed tissue.\(^18\) However, there are no studies characterizing lncRNA in the ileum of treatment-naïve (not influenced by treatment), early-onset pediatric CD patients.

The Crohn’s and Colitis Foundation of America (CCFA)–sponsored RISK study is a prospective inception cohort study that enrolled 1098 pediatric CD patients at diagnosis at 28 sites in North America between 2008 and 2012.\(^19\)\(^-\)\(^21\) All patients were treatment naïve, with ileal biopsies obtained during the initial diagnostic colonoscopy. Using RISK RNAseq data and UCSC annotation, we have previously characterized transcriptomic signatures associated with CD pathogenesis focusing on protein-coding genes.\(^22\) We defined a core ileal CD (iCD) signature enriched by treatment), early-onset pediatric CD patients.

The Crohn’s and Colitis Foundation of America (CCFA)–sponsored RISK study is a prospective inception cohort study that enrolled 1098 pediatric CD patients at diagnosis at 28 sites in North America between 2008 and 2012.\(^19\)\(^-\)\(^21\) All patients were treatment naïve, with ileal biopsies obtained during the initial diagnostic colonoscopy. Using RISK RNAseq data and UCSC annotation, we have previously characterized transcriptomic signatures associated with CD pathogenesis focusing on protein-coding genes.\(^22\) We defined a core ileal CD (iCD) signature enriched for genes induced by bacterial products and pro-inflammatory cytokine signaling, including IFN\(\gamma\), whereas genes induced by several nuclear receptors, including HNF4\(\alpha\), were suppressed. Functional analyses identified enrichment of innate antimicrobial responses and a profound loss of nuclear receptor–dependent lipid metabolic functions. However, differentially expressed lncRNAs that may play a central role in regulating the transcriptional landscape in a tissue-specific manner have not yet been defined. Here, we extend our analyses using GENCODE/ENCODE and Ensembl (http://useast.ensembl.org) annotation to characterize differentially expressed protein-coding and lncRNAs, extending the CD signature to now include 459 differentially expressed lncRNA genes. Importantly, we show that those lncRNAs can be utilized to correctly classify disease or healthy states in patients undergoing diagnostic endoscopies. We put forward a way to prioritize differentially expressed lncRNAs for future analyses, elaborate on their potential functions and tissue-specific expression, and validate specific prioritized lncRNA expression and regulation upon IL-1\(\beta\) triggering. As lncRNAs show higher tissue-specific expression in comparison with protein-coding genes, these tissue-specific lncRNA, after mechanistic exploration, may serve as potential new targets for RNA-based interventions that will be associated with fewer off-target toxic effects.

MATERIAL AND METHODS

The RISK Cohort

Ileal biopsy RNAs and associated clinical information were obtained from the RISK study,\(^22\)\(^-\)\(^24\) an ongoing, prospective observational IBD inception cohort sponsored by the CCFA. Newly diagnosed patients were enrolled and required to undergo baseline colonoscopy and confirmation of characteristic chronic active colitis/ileitis by histology before diagnosis and treatment.

RNA-seq Expression and Gene Enrichment Analysis

Ileal RNA extraction and mRNA-seq were performed as previously described (GEO series accession number GSE57945)\(^25\) with some modification as noted. To include lncRNAs in the analyses, reads were quantified by kallisto,\(^26\) using GenCode v23 as the reference genome and transcripts per million (TPM) as an output. Only 20,326 transcripts with a TPM above 1 in 20% of the samples were included in our downstream differential expression analysis. Samples were stratified into specific clinical subgroups including control (Ctl) and CD with ileal inflammation (iCD). Those groups were age- and gender-matched and were randomly assigned (ratio 4:1) to discovery or independent validation groups (Table 1). Differentially expressed genes were determined by the moderated \(t\) test method in GeneSpring next-generation sequencing software using the Benjamini–Hochberg false discovery rate correction (FDR, 0.05), and analyzed for fold change differences (FC) \(\geq 1.5\). Log2-transformed and baselined-to-median-level Ctl values were used for unsupervised hierarchical clustering using the Euclidean distance metric and Ward’s linkage rule to test for groups of ileal biopsies with similar patterns of gene expression. Pearson correlation based on trend and rate of change was performed for selected lncRNAs as indicated for a correlation co-efficient of 0.75 \(< r < 1\). ToppGene\(^27\) and ToppCluster\(^28\) software were used to test for enrichment of groups of genes within biologically relevant pathways. Visualization of the network was obtained using Cytoscape v3.0.28 and the ReVIGO approach, which converts a list of Gene Ontology terms into a semantic, similarity-based scatterplot after removing redundant terms.\(^29\) LncRNA prioritization was based on at least an average of 1 TPM in Ctl or
iCD and showed the highest fold change differences between iCD and Ctl. Further prioritization of the top 15 differentially expressed lncRNAs was based on the highest number of genes that coexpressed with the lncRNAs in our cohort. FASTQ files from human-derived peripheral blood cells (GSE64655) and human-derived intestinal biopsies (E-MTAB-1733) were analyzed using the same pipeline. Similar RNA extraction, RNAseq, and analysis pipelines were applied on the differentiated Caco-2 cells samples. Those samples’ data were deposited in GSE94578.

Caco-2 Cells
Caco-2 human colon carcinoma cell line was purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in standard culture conditions in DMEM (GIBCO 41965-039, Scotland) containing 20% (v/v) heat-inactivated fetal bovine serum (GIBCO 12657-029, Scotland). For experiments, cells were seeded on 6-well ThinCerts—0.4-um plate (Greiner bio-one 657641, Austria) for 21 days to obtain fully differentiated cells, and transepithelial electrical resistance (TEER) values were measured (World Precision Instruments, FL). Differentiated Caco-2 cells were either left untreated or treated with 25 ng/mL of IL-1β (MerckMillipore Human Recombinant Animal Free GF331, Germany). Cell fractionation was done using the PARIS kit (Thermo Fisher Scientific, CA, USA) according to the manufacturer’s directions.

Expression (Quantitative Polymerase Chain Reaction) Studies
Total RNA was isolated using Tri Reagent-LS (Sigma, T9424, Saint Louis, MO, USA). RNA concentration and purity were assessed on a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). First-strand cDNA was synthesized using a high-capacity RNA-to-cDNA reverse transcription kit (Applied Biosystems, 4387406). Quantitative real-time polymerase chain reaction (qPCR) was performed using a Fast SYBR Green Master mix (Applied Biosystems, 4385612) and qPCR machine with standard qPCR parameters to analyze the expression of indicated genes compared with the control gene GAPDH (primer list is in Supplementary Table 10). Results were analyzed with the comparative C_T method, and log10 (relative quantification values [Rq] values) are shown.

In Situ Hybridization
3’ end biotin-labeled probes (Integrated DNA Technologies, Leuven, Belgium), recognizing HNF4A-AS1 and LINC01272, and a scrambled control probe were used at 180 nM for in situ hybridization on formalin-fixed, paraffin-embedded ileal biopsies. The alkaline phosphatase conjugate streptavidin (Roche, 11093266910, Basel, Switzerland) with subsequent BCIP/NBT color development substrate (Roche, 11681451001, Basel, Switzerland) was used to obtain the staining. A positive probe and a negative probe were included in the assay for quality control.

Ethical Considerations
The institutional review board at each site reviewed and approved the protocol, and informed written consent or assent was obtained in all cases from parents or guardians. All patients provided appropriate assent. This study was approved by national regulatory authorities and by local ethics committees or institutional review boards.

RESULTS
Widespread Dysregulation of Protein-Coding and lncRNAs in the Ileum of Treatment-Naive Pediatric CD Patients Using RNASeq and GENCODE/Ensembl Annotation
The majority of lncRNAs are generated by similar transcriptional machinery as mRNA, with a 5’ methylguanosine

TABLE 1. Clinical and Demographic Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Ctl Discovery (n = 30)</th>
<th>CD Discovery (L1,L3, n = 111)</th>
<th>Ctl Validation (n = 8)</th>
<th>CD Validation (L1,L3, n = 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (SD), y</td>
<td>11.2 (3)</td>
<td>11.9 (3)</td>
<td>11 (5)</td>
<td>12.2 (3)</td>
</tr>
<tr>
<td>Male gender, %</td>
<td>60</td>
<td>61</td>
<td>62</td>
<td>61</td>
</tr>
<tr>
<td>Mixed European descent (MED) ethnicity (3 of 4 grandparents), %</td>
<td>87</td>
<td>81</td>
<td>89</td>
<td>79</td>
</tr>
<tr>
<td>PCDAI ≤ 10 (inactive), %</td>
<td>—</td>
<td>8</td>
<td>—</td>
<td>11</td>
</tr>
<tr>
<td>PCDAI 11 to 30 (mild), %</td>
<td>—</td>
<td>39</td>
<td>—</td>
<td>36</td>
</tr>
<tr>
<td>PCDAI ≥ 31 (moderate-severe), %</td>
<td>—</td>
<td>53</td>
<td>—</td>
<td>53</td>
</tr>
<tr>
<td>Ileal deep ulcers, %</td>
<td>—</td>
<td>53</td>
<td>—</td>
<td>46</td>
</tr>
</tbody>
</table>

Abbreviations: L1, ileal location; L2, colon-only location; L3, ileo-colonic location; PCDAI, Pediatric Crohn Disease Activity Index at diagnosis before treatment.
cap, and are often spliced and polyadenylated. We were therefore able to utilize our previously published ileal biopsy poly-A selected RNAseq data set (GEO series accession number GSE57945) of subjects with newly diagnosed CD and non-IBD controls to test for differences in lncRNA expression (Table 1 and Supplementary Table 1). This includes CD patients with clinically affected (ileal CD [iCD], n = 139) ilea and non-IBD Ctl (n = 38). CD patients and Ctl participants were randomly divided and age- and gender-matched to the discovery (80%) and independent validation (20%) iCD and Ctl cohorts (Table 1). However, for more comprehensive analyses of the lncRNA, instead of using the UCSC annotation as a reference for alignment and quantification, we used the GENCODE/ENCODE project implemented into Ensembl (V23, GRCh38, Ensembl 81) as our reference. One main advantage of using the GENCODE/Ensembl annotation is that it clearly classifies each annotated gene as a protein coding or noncoding gene. The GENCODE v23 (GRCh38, Ensembl 81) version includes 60,498 genes; 19,797 protein-coding genes, 15,931 long noncoding genes, 9882 small noncoding genes, 14,477 pseudogenes, and 411 immunoglobulin/T-cell receptor genes. For quantification, we used kallisto software.

Our analyses comparing the iCD and Ctl ileal discovery samples identified 3022 genes (Supplementary Table 2) that were differentially expressed (FDR < 0.05 and fold change ≥1.5) (Supplementary Table 2 and Supplementary Fig. 1A). Unsupervised hierarchical clustering identified groups of patients with similar ileal gene expression profiles; this analysis tested whether patients with CD cluster together, whereas the transcriptional profile of non-IBD Ctl patients would cluster together. Unsupervised hierarchical clustering of the discovery cohorts showed that all Ctl patients grouped in cluster 1 and most of the CD patients grouped in cluster 2 (Supplementary Fig. 1B). We also applied principal component analysis (PCoA) as another approach to view patients’ separation using specific gene expression profiles, an approach that is used to reduce dimensionality of the input data. Loading the 3022 differentially expressed genes and the top 3 dimensions showed that most Ctl patients were separated from most of the CD patients (Supplementary Fig. 1C). Of note, although we used Ensembl annotation instead of the UCSC annotation that we previously used, we were able to show that >85% of the previously reported genes are within the new 3022 genes list. However, although our previously reported list contained <50 lncRNAs, using our current approach we identified 10 times more lncRNAs.

**Significant Enrichment of Known IBD-Associated SNPs Within Differentially Expressed and Annotated lncRNAs**

Similar to previously reported data, we tested for the presence of known IBD-associated SNPs (total of 233 SNPs) within annotated lncRNAs. We identified IBD loci-associated lncRNAs genes by intersecting the IBD susceptibility loci, which was defined as a 500-kb long genomic region with the IBD risk variant in the middle. In total, 1051 IBD loci-associated lncRNAs were identified, out of which 41 unique lncRNAs were found to be differentially expressed (Supplementary Table 3). These differentially expressed 41 lncRNAs co-localized with 47 unique IBD risk variants, and were found to be enriched within IBD loci (P < 0.0001, chi-square test).

**lncRNAs Can Be Utilized to Correctly Classify Disease or Healthy States in Patients Undergoing Diagnostic Endoscopies**

Classification of the 3022 genes into gene biotypes based on GENCODE annotation is shown in Table 2 and includes 2160 protein coding genes (71%) and 459 lncRNAs (15%). Table 2 further subdivided the lncRNAs into specific types. Unsupervised hierarchical clustering using only the 459 lncRNAs demonstrated that all discovery Ctl samples grouped in cluster 1 and most of the discovery CD patients grouped in cluster 2 (Fig. 1A), similar to clustering using the entire 3022 genes (Supplementary Fig. 1). PCoA to view patients’ separation using the 459 differentially expressed lncRNAs and the top 3 dimensions showed that most Ctl patients are separated from most of the CD patients (Fig. 1B). Importantly, unsupervised hierarchical clustering using only the 459 lncRNAs on an independent validation cohort also demonstrated that all Ctl samples grouped in cluster 1 and most of the validation CD patients grouped in cluster 2 (Fig. 1C), and PCoA showed that most Ctl patients are separated from most of the CD patients (Fig. 1D). Interestingly, we noted that a higher fraction of the lncRNAs (83%, 380/459, of the lncRNAs) was downregulated

**TABLE 2. Differentially Expressed Ileal Gene Types**

<table>
<thead>
<tr>
<th>Gene Type</th>
<th>Number of Genes (n = 3022)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein coding genes</td>
<td>2160</td>
</tr>
<tr>
<td>lncRNA</td>
<td>459</td>
</tr>
<tr>
<td>3’ UTR overlapping</td>
<td>1</td>
</tr>
<tr>
<td>Antisense</td>
<td>184</td>
</tr>
<tr>
<td>LincRNA</td>
<td>219</td>
</tr>
<tr>
<td>Processed transcript</td>
<td>30</td>
</tr>
<tr>
<td>Sense intronic</td>
<td>19</td>
</tr>
<tr>
<td>Sense overlapping</td>
<td>6</td>
</tr>
<tr>
<td>IG/TR</td>
<td>234</td>
</tr>
<tr>
<td>Pseudogenes</td>
<td>150</td>
</tr>
<tr>
<td>TEC</td>
<td>19</td>
</tr>
</tbody>
</table>

Abbreviations: IG/TR, immunoglobulin/T cell receptors; TEC, transcripts to be experimentally confirmed; UTR, untranslated region.
in iCD vs Ctl (Fig. 1E) in comparison with the fraction of the downregulated protein-coding genes (51%, 1099/2160).

Supervised classification algorithms may be used to develop models to classify unknown patient groups based on gene expression data that differ within a training set of known patient groups. The Support Vector Machine (SVM) algorithm is a machine learning approach, which is commonly used to classify 2 patient classes based on differential expression of biologic data. We conducted a supervised classification analysis using the 2160 protein-coding genes and 459 lncRNA genes differentially expressed between iCD and Ctl on a discovery cohort comprising 111 iCD and 30 Ctl patients to develop a classification model using the SVM algorithm (80% of the samples) (Table 1). We then tested the accuracy of the model on an independent validation cohort of 28 iCD and 8 Ctl (Table 3). Applying the model to the discovery and independent validation cohorts resulted in comparable accuracy using the 459 lncRNA and 2160 protein-coding genes (Table 3). Importantly, those unsupervised (hierarchical clustering and PCoA) and supervised (SVM algorithm) analyses of discovery and validation cohorts further link altered expression of lncRNA to CD pathogenesis.

Prioritizing the Differentially Expressed lncRNAs Based on Highest Fold Change and Co-expression Analyses

Our initial prioritization of differentially expressed lncRNA was based on the highest fold change differences between iCD and Ctl (Tables 4 and 5) and focused on the top 15 down- and 15 upregulated ileal lncRNA. Within our pediatric treatment-naïve cohort, we identified CDKN2B-AS1 (ANRIL) lncRNA, previously shown to regulate gene transcription in cis (in vicinity) and in trans (at distant loci) in carcinogenesis and cardiovascular disease, as one of the top 15 downregulated genes. Interestingly, CDKN2B-AS1 was within the top 10 downregulated transcripts in the colon of treated adult patients with IBD, whereas downregulation of HNF4A-AS1 lncRNA was unique to our ileal cohort. We also identified LINC01272 and RP11-44K6.2 within our top 10 upregulated lncRNAs that were also within the top 10 upregulated lncRNA transcripts in the colon of adult IBD patients.16

Co-expression analyses (Table 3) of lncRNAs with well-characterized protein-coding mRNA is usually used to gain insights into lncRNA potential functions. As we were interested in prioritizing potential functional lncRNAs, we used co-expression as another factor for prioritization. We further prioritized the top 15 differentially expressed lncRNAs to those that showed co-expression with the larger set of genes, as a read out for their potential functionality. We used Pearson correlation analysis (r > 0.75) to identify co-expression of lncRNAs within our Ctl and iCD samples. Of the top 15 downregulated genes, both HNF4A-AS1 and CDKN2B-AS1 showed the highest number of co-expressed genes (Table 4). The 314 HNF4A-AS1 downregulated co-expressed genes remarkably overlapped with the 411 CDKN2B-AS1 co-expressed gene list (70%) and included 3 other lncRNAs (CDKN2B-AS1, RP11-116D2.1, and RP11-132E11.2) also within the top 15 downregulated lncRNAs. Of the top 15 upregulated lncRNAs, LINC01272 showed the highest number of 187 upregulated co-expressed genes (Table 5).

Prioritized Differentially Expressed HNF4A-AS1 lncRNAs Show Epithelial-Specific Expression and Associations With Metabolic Functions

Many lncRNAs exhibit tissue-specific expression patterns.4 We therefore assessed the expression of prioritized LINC01272 and HNF4A-AS1 expression in independent noninflamed tissues and cells including publically available RNAseq of human blood–derived cell types (GSE64655)28 and human-derived intestinal tissues (E-MTAB-1733).39 We supplemented those analyses with RNAseq of our local differentiated
Caco-2 intestinal epithelial cell lines. Caco-2 cells, although derived from the colon, when differentiated and polarized, exhibit a phenotype, tissue marker expression, morphology, and cellular functions that resemble small intestine enterocytes. Indeed, in this independent data set, we show high expression of *HNF4-AS1* in small intestinal tissue (duodenum and ileum) and Caco-2 cells, remarkably low expression in neutrophils, and no expression (TPM level < 1 and read count < 10) in other cells and tissues (Fig. 2A).

To gain insights into those lncRNA potential functions, we employed co-expression analyses also in those new samples and tested for co-expression of lncRNA with well-characterized protein-coding mRNA. We used Pearson correlation analyses (*r* > 0.75) to identify co-expression and then

<table>
<thead>
<tr>
<th>Top 15 Downregulated</th>
<th>FC (iCD) vs Ctl</th>
<th>Ensembl Gene ID</th>
<th>lncRNA Subclass</th>
<th>Remark</th>
<th>Co-expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP11-347E10.1</td>
<td>−13.2</td>
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<td>FOXD1-AS1</td>
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<tr>
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<td>ENSG00000250271</td>
<td>lincRNA</td>
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<tr>
<td>RP11-91P17.1</td>
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<td>ENSG00000254001</td>
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<tr>
<td>CDKN2B-AS1</td>
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<td>antisense</td>
<td>also in adult colon</td>
<td>411</td>
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<td>lincRNA</td>
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<td>ENSG00000271952</td>
<td>lincRNA</td>
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<td>RP11-132E11.2</td>
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<td>ENSG00000237153</td>
<td>lincRNA</td>
<td></td>
<td>78</td>
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<tr>
<td>HNF4A-AS1</td>
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<td>ENSG00000229005</td>
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<td>314</td>
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<td>134</td>
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<td>RP11-689K5.3</td>
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<tr>
<td>RP11-122D19.1</td>
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<td>ENSG00000261760</td>
<td>lincRNA</td>
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<td>RP11-122K13.7</td>
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<td>ENSG00000226699</td>
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<td></td>
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<td>RP11-798K3.2</td>
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**TABLE 5. Top 15 Upregulated Differentially Expressed Ileal lncRNA Genes**

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<th>FC (iCD) vs Ctl</th>
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**TABLE 4. Top 15 Downregulated Differentially Expressed Ileal lncRNA Genes**

**TABLE 5. Top 15 Upregulated Differentially Expressed Ileal lncRNA Genes**

**Abbreviations:** Co-expression, number of additional differentially expressed ileal genes co-expressed with the lncRNA by Pearson correlation analysis; FC, fold change between iCD and control.
conducted functional annotation enrichment analyses to map groups of related genes to identify biological process, pathways, phenotypes, and molecular functions, where \( P \) values for the specific pathway, phenotype, and function were obtained as an output from ToppGene.\(^{24}\) Pearson correlation analyses on those tissues and cells identified 4102 genes that co-expressed with \( HNF4A-AS1 \) (Supplementary Table 4). Unsupervised hierarchical clustering for this gene set is shown in Fig. 2B, showing clear separation between small intestine and differentiated Caco-2 cells and neutrophils. Functional annotation enrichment analyses of those 4102 genes showed enrichment for Gene Ontology Biological Process terms involving epithelial development \( (P < 7.94E-5) \) and functions including the organic acid metabolic process \( (P < 4.15E-13) \), but also enrichment for
ncRNA processing \((P < 4.15E-13)\) and translation \((P < 8.42E-7)\) (Fig. 2C and Supplementary Table 4). Those results are visualized using the ReViGO approach,\(^\text{27}\) which converts a list of Gene Ontology terms into a semantic, similarity-based scatterplot after removing redundant terms. Bubble color indicates \(P\) value; size indicates the frequency of the GO term in the underlying GOA database (more general terms are larger). The full list of genes and the functional enrichment results and \(P\) values are in Supplementary Table 4. D, LINC01272 TPM levels in several human-derived peripheral blood cells and intestinal tissues are shown. E, Hierarchical clustering of the 521 genes that co-expressed with LINC01272 (expressed above 1 TPM and 10 read count) in the indicated cells and tissues is shown and visualized as a heat map, with genes higher and lower than the median levels of all samples in red and blue, respectively. F, Top 50 GO: Biological Process terms found for the LINC01272 co-expression network were visualized using the ReViGO approach, which converts a list of gene ontology terms into a semantic, similarity-based scatterplot after removing redundant terms. Bubble color indicates \(P\) value; size indicates the frequency of the GO term in the underlying GOA database (more general terms are larger). The full list of genes and the functional enrichment results and \(P\) values are in Supplementary Table 5. G, RNA in situ hybridization detection of HNF4A-AS1 in paraffin-embedded sections of ileal mucosal biopsies. Dark purple staining in those cells (Fig. 2C) is located predominantly in the nucleus of immune cells scattered in the intestinal lamina propria. Dark purple staining of LINC01272 is located predominantly in the nucleus of immune cells scattered in the intestinal lamina propria.

**FIGURE 2.** Expression and functional annotation enrichment analyses of the HNF4A-AS1 and LINC01272 co-expression networks in diverse cellular and tissue samples. A, HNF4A-AS1 TPM levels in several human-derived noninflamed peripheral blood cells \((n = 2,\) PBMC, B cells, T cells, neutrophil, monocyte, NK cells, myeloid DC). Noninflamed intestinal tissues are shown \((3\) stomach, \(2\) duodenum \([\text{Duo}],\) \(4\) small intestine \([\text{SI}],\) \(3\) colon), and unstimulated differentiated Caco-2 cells. B, Hierarchical clustering of the 4102 genes that co-expressed with HNF4A-AS1 (expressed above 1 TPM and 10 read count) in the indicated cells and tissues is shown and visualized as a heat map, with genes higher and lower than the median levels of all samples in red and blue, respectively. C, The top 50 Gene Ontology (GO): Biological Process terms found for the HNF4A-AS1 co-expression network were visualized using the ReViGO approach, which converts a list of gene ontology terms into a semantic, similarity-based scatterplot after removing redundant terms. Bubble color indicates \(P\) value; size indicates the frequency of the GO term in the underlying GOA database (more general terms are larger). The full list of genes and the functional enrichment results and \(P\) values are in Supplementary Table 4. D, LINC01272 TPM levels in several human-derived peripheral blood cells and intestinal tissues are shown. E, Hierarchical clustering of the 521 genes that co-expressed with LINC01272 (expressed above 1 TPM and 10 read count) in the indicated cells and tissues is shown and visualized as a heat map, with genes higher and lower than the median levels of all samples in red and blue, respectively. F, Top 50 GO: Biological Process terms found for the LINC01272 co-expression network were visualized using ReViGO approach as in (C). The full list of genes and the functional enrichment results and \(P\) values are in Supplementary Table 5. G, RNA in situ hybridization detection of HNF4A-AS1 in paraffin-embedded sections of ileal mucosal biopsies. Dark purple staining of HNF4A-AS1 is located predominantly in the epithelia nucleus. H, RNA in situ hybridization detection of LINC01272 in paraffin-embedded sections of ileal mucosal biopsies. Dark purple staining of LINC01272 is located predominantly in the nucleus of immune cells scattered in the intestinal lamina propria.

In this independent data set, we show high expression of linc01272 to myeloid dendritic cells \((\text{Dcs}),\) monocytes \((\text{Mo}),\) peripheral blood mononuclear cells \((\text{PBMCs}),\) and neutrophils, and to a lesser degree to human-derived intestinal tissues and T cells, but no expression \((\text{TPM level < 1 and read count < 10})\) in B cells and Caco-2 cells (Fig. 2D). Performing Pearson correlation analyses \((r > 0.75)\) on those tissues and cells identified 521 genes that co-expressed with LINC01272. Unsupervised hierarchical clustering of this gene set is shown in Fig. 2E, sowing clear separation between myeloid cells and PBMCs from the intestinal tissues, NK cells, and T cells. Functional annotation enrichment analyses of those 521 genes (Supplementary Table 5) showed enrichment for Gene Ontology Biological Process terms involving inflammatory and immune response \((P < 1.96E-26)\) and more specifically myeloid functions \((P < 4.43E-13)\) and response to bacteria \((P < 1.68E-10)\) (Fig. 2F and Supplementary Table 5).

To validate HNF4A-AS1 and LINC01272 cell-specific expression and to test their subcellular localization, we employed RNA in situ hybridization on paraffin sections using HNF4A-AS1 antisense Biotin-labeled oligonucleotides probes. Consistent with the expression data, HNF4A-AS1 is specifically expressed in the epithelia, showing predominantly nuclear staining in those cells (Fig. 2G). In contrast, LINC01272 show no expression in epithelia, but are expressed in immune cells scattered in the intestinal lamina propria (Fig. 2H). Our observations regarding tissue-specific expression of LINC01272 to leukocytes and HNF4A-AS1 to epithelia are further supported by the publicly available GTEx RNAseq database, where HNF4A-AS1 showed the highest expression in small intestine and liver tissues and LINC01272 showed the highest expression in whole blood and spleen (Supplementary Fig. 2).

**Prioritized Differentially Expressed LINC01272 Show Specific Myeloid Expression and Association With Myeloid Immune Activation**

To support those IncRNA potential functions and validate the above co-expression analyses, we employed...
co-expression analyses and functional annotation enrichment analyses also within our Ctl and inflamed CD ileal samples. Pearson correlation (r > 0.75) analysis identified 314 downregulated genes that co-express with HNF4A-AS1 (Supplementary Table 6). Functional annotation of these 314 suppressed genes (Fig. 3A and Supplementary Table 7) showed enrichment for entities associated with the organic acid metabolic process (P < 1.5E-17), lipid metabolic process (P < 2.16E-14), oxidation-reduction process (P < 7.54E-12), vitamin digestion and absorption (P < 4.67E-7), and brush border cellular component (P < 6.13E-26). Interestingly, functional annotation to transcription factor binding sites showed top enrichment for HNF4 (P < 5.6E-4). To model the effect of the inflammatory signal on prioritized lncRNA in an epithelial model system, we stimulated Caco-2 cells with IL-1β (25 ng/mL), which were previously shown to be upregulated in the ileum of patients with CD. As expected, we noted an increased expression of IL8 after incubation with IL-1β. We also detected a significantly increased expression of DUOX2 and decreased expression of APOA1 (Fig. 4), similar to the increased and decreased expression detected in the ileum of CD patients.17 We further detected a significant decrease of CDKN2B-AS1, RP11-132E11.2, RP11-347E10.1, and RP11-798E3.2 in the Caco-2 system after IL-1β stimulation, as was observed in CD biopsies in comparison with Ctl. Importantly, both CDKN2B-AS1 and HNF4A-AS1 showed enrichment to the nuclear fraction in the Caco-2 model system like the established nuclear lncRNA MALAT1, which may suggest a transcriptional regulatory role in intestinal epithelia (Supplementary Fig. 3).

Pearson correlation analysis identified 187 upregulated genes that co-express with the upregulated LINC01272 (Supplementary Table 8). Functional annotation of these 187 genes (Fig. 3B and Supplementary Table 9) showed enrichment for entities associated with inflammatory response (P < 2.32E-29), response to the molecule of bacterial origin (P < 7.12E-26), CD11b activated granulocyte myeloid cells (P < 4.36E-69), cytokine activity (P < 5.36E-10), and immunoglobulin binding (P < 2.44E-6). Altogether, combining co-expression and functional annotation enrichment analyses indicated a predominant epithelial-related signature of those downregulated CDKN2B-AS1 and HNF4A-AS1 co-expressed lncRNAs, in contrast to the granulocyte-associated signature of the upregulated LINC01272. This was in agreement with our results across a diverse set of noninflamed tissues and cells (Fig. 2).

**HNF4A-AS1 and CDKN2B-AS1 Expression Significantly Correlates With More Severe Mucosal Injury**

We tested for correlation between LINC01272 and HNF4A-AS1 and clinical disease activity indices and the severity of mucosal injury. Using the Pediatric Crohn’s Disease Activity Index (PCDAI) as a continuous value or stratifying the iCD group to those inactive (PCDAI ≤ 10), with mild symptoms (PCDAI ≤ 30), and with moderate-severe symptoms (PCDAI > 30) at diagnosis showed no significant correlation or differences, respectively, for both LINC01272 expression and HNF4A-AS1 expression (Fig. 5). However, dividing the iCD group into 2 groups based on the presence of deep ulcers (DU, iCD-DU, and iCD-noDU), which are associated with endoscopic severity scaling and unfavorable clinical outcome,35 demonstrated a significant increase of LINC01272 in iCD-DU in comparison with iCD-noDU (Fig. 6). These results are further supported by the significant positive correlation noted between S100A8 (calprotectin), our best current clinical biomarker for tissue inflammation, and LINC01272 (r = 0.9,
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P < 0.001). In contrast, a significant negative correlation with S100A8 was noted with HNF4A-AS1, and a significant decrease of HNF4A-AS1 expression was observed in iCD-DU vs iCD-no DU (Fig. 6). These results emphasize that lncRNAs show positive correlation with tissue inflammation and lack of correlation with clinical disease activities (PCDAI). These significant correlations between lncRNA expression and mucosal tissue injury further highlight the need to elucidate their potential function in CD pathogenesis as a first step toward utilizing those lncRNAs as targets for potential future interventions.

DISCUSSION

We previously defined a core ileal CD (iCD) signature enriched for genes coding for innate antimicrobial responses and a profound loss of nuclear receptor–dependent lipid metabolic functions. However, differentially expressed lncRNAs that may play a central role in regulating the transcriptional landscape in a tissue-specific manner have not yet been defined. Growing evidence suggests that lncRNAs contribute to various aspects of gene regulation in the immune system. However, there are only a few studies focusing on lncRNAs in human gut pathogenesis, and specifically, there are no studies of the ileum of treatment-naive pediatric CD patients. Here, based on the largest prospective pediatric treatment-naive inception cohort, we extend the CD signature to now include 459 differentially expressed lncRNA genes. We show that lncRNA can be utilized to correctly classify disease or healthy states in patients undergoing diagnostic endoscopies. Prioritizing differentially expressed genes based on the highest fold change differences, and more uniquely to our study on higher numbers of co-expressed genes, directed us to epithelial-specific and myeloid-specific lncRNA expression and potential functions. We validated the lncRNA-specific expression using in situ hybridization in intestinal biopsies. Finally, we captured a significant correlation between HNF4A-AS1 and LINC01272 expression and a presence of more severe mucosal ulcers. Those results are illustrated in Fig. 7. The tissue specificity of lncRNA may offer a clear interventional advantage as a future pharmacologic target that will have a less off-target toxic effect.

The rapid growth of genome-wide transcriptome data sets has uncovered a fast-growing list of lncRNAs (>15,000).
However, most of those lncRNAs are uncharacterized, and their associations with human pathogenesis are only beginning to emerge. LncRNA differential expression in the colon of adult patients has only recently been associated with CD and UC using microarray analyses.\textsuperscript{16, 36} We have been using RNAseq rather than microarray and propose a pipeline for lncRNA identification using Ensembl/Gencode annotation and lncRNA prioritization. Using Ensembl/Gencode annotation, we were able to capture 10 times more lncRNAs than using the UCSC annotation.\textsuperscript{17} We show similar altered downregulation of CDKN2B-AS1 and upregulation of LINC01272 in the CD ileum, as was previously reported in the colon of UC and CD patients\textsuperscript{16, 36} within our top 15 down- and upregulated lncRNAs and the previously reported downregulation of DIO3OS (Supplementary Table 2), supporting the validity of our approach. However, we identified HNF4A-AS1, which shows remarkably high expression specific to the small intestine and Caco-2 cells in the enterocytes system, and we confirmed its expression to predominantly the epithelial nucleus. We also show that 41 differentially expressed lncRNA genes co-localized with 47 IBD risk variant loci and are enriched within IBD loci. Those 41 lncRNAs included our prioritized lncRNAs LINC01272 and HNF4A-AS1. Interestingly, and unlike previously described data in the colon of UC patients who show an increase of 329 lncRNAs and a decrease of 126 lncRNAs in active UC tissues compared with normal controls, we noticed a significantly higher fraction of the lncRNAs (83% of the total 459 differentially expressed lncRNA) to be decreased in active ileal CD (iCD) mucosa in comparison with controls. This observation was unique to lncRNAs in the ileum as an equal number of protein coding-genes (51%) were downregulated in iCD vs Ctl (Fig. 2) in the same samples. H19 and SPRY4-IT1 lncRNAs have both been recently associated with the regulation of

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**FIGURE 6.** HNF4A-AS1 and LINC01272 lncRNA show significant correlation with severity of mucosal injury and with ileal S100A8 gene expression. A, LINC01272 log10(TPM) of all patients (iCD \(n = 139\)) and Ctl \(n = 38\)) were plotted against their log10(TPM) of S100A8 as a continuous value (right) or after stratifying the iCD group to those with or without deep ulcers (iCD-DU \(n = 72\)) and iCD-noDU \(n = 67\), respectively). B, HNF4A-AS1 log10(TPM) of all patients plotted against their log10(TPM) of S100A8 as a continuous value (right) or after stratifying the iCD group, as in (A). Analysis of variance with Bonferroni’s multiple comparison test was calculated for the stratified groups, and Pearson correlation was calculated for the continuous variables. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\).
intestinal epithelial barrier function.\textsuperscript{37, 38} As co-expression analyses suggest that the downregulated gene signal is coming from the epithelia, it will be highly important to further understand the role of the remarkably decreased lncRNA expression on epithelial integrity and repair.

There is a clear necessity in the IBD field for novel drugs that target major pathogenic pathways with minimal toxicities. The 12-month remission rate with currently available treatments is below 65\textsuperscript{\%}\textsuperscript{39} and is associated with increased risk for cancer and infections. While anti–tumor necrosis factor (anti-TNF) and newer antibodies offer effective medications, these drugs substantially increase the economic cost of IBD treatment. Moreover, at least for anti-TNF treatment, only 50\% will maintain long-term remission,\textsuperscript{40} in part due to development of antidrug antibodies that reduce the effect of the drug. RNA-based therapies conjugated to cholesterol to facilitate cellular uptake are currently being evaluated in clinical trials for different disorders including IBD.\textsuperscript{41, 42} A specific inhibitor of miR-122 showed prolonged dose-dependent reductions in HCV RNA levels without evidence of viral resistance,\textsuperscript{43} and Mongersen, an oral \textit{SMAD7} antisense oligonucleotide, was shown in a double-blind phase II clinical trial to be efficacious in active CD.\textsuperscript{42} One advantage in harnessing lncRNA-directed therapy is its higher tissue specificity expression in comparison with protein-coding genes, which should be associated with fewer off-target effects. Along those lines, we have captured a specific expression of \textit{HNF4A-AS1} to epithelial cells, and \textit{LINC01272} to myeloid DC, monocytes, and neutrophils. Another advantage in targeting lncRNA is their potential key regulatory role on gene transcription. \textit{CDKN2B-AS1} (\textit{ANRIL}) lncRNA has previously been shown to regulate gene transcription in cis (in vicinity) and in trans (at distant loci) in carcinogenesis and cardiovascular disease, whereas the role of the other prioritized lncRNAs is not yet known and will be tested in future studies. An important future approach to better characterize lncRNA function will include employing specific modulations of \textit{HNF4A-AS1} and \textit{CDKN2B-AS1} levels. This approach will provide strong evidence of a transcriptional role in intestinal epithelia, which is more convincing than showing expression alone.

In conclusion, we define differentially expressed lncRNAs in the ileum of treatment-naive pediatric CD patients. We show lncRNA utility to correctly classify disease or healthy states in both discovery and independent validation cohorts. We prioritized lncRNA and confirmed their tissue-specific expression...
and demonstrate their regulation in response to an inflammatory signal in an enterocyte model system. Characterizing lncRNA expression is the first step toward elucidating lncRNAs’ molecular mechanisms, which will provide further more comprehensive insights into CD pathogenesis and ultimately lead to novel therapeutic strategies.

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REFERENCES