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ORIGINAL RESEARCH

Role and Therapeutic Potential of miR-301b-3p in Regulating the PI3K-AKT Pathway via PIK3CB in Eosinophilic Chronic Rhinosinusitis

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Purpose: Type 2 inflammation and epithelial-mesenchymal transition (EMT) are critical components in the pathogenesis of eosinophilic chronic rhinosinusitis (ECRS), yet their upstream regulatory mechanisms remain poorly understood. This study aimed to explore the regulatory role of miR-301b-3p in these mechanisms and evaluate its therapeutic potential.

Methods: High-throughput miRNA sequencing of human and mouse nasal mucosa tissues identified miR-301b-3p as a key candidate molecule. Mendelian randomization (MR) analysis confirmed its causal relationship with chronic rhinosinusitis (CRS). The role of miR-301b-3p and its target gene PIK3CB in ECRS was further investigated using bioinformatics analysis, dual-luciferase reporter assays, adeno-associated virus (AAV)-mediated modulation of miR-301b-3p expression, histological staining, and a range of molecular biology techniques to elucidate the underlying mechanisms. The role of PIK3CB was assessed using the PIK3CB inhibitor TGX-221.

Results: hsa-miR-301b-3p was significantly downregulated in ECRS patient nasal mucosa (log2FC = –1.636, P = 0.010), and its expression level negatively correlated with disease severity; simultaneously, mmu-miR-301b-3p was significantly downregulated in the ECRS mouse model (log2FC = –2.256, P = 0.041). MR analysis demonstrated a causal relationship between reduced miR-301b levels and increased CRS risk (OR = 0.956; 95% CI, 0.918–0.996; P = 0.033). In vivo experiments revealed that miR-301b-3p knockdown led to PIK3CB upregulation and activation of the PI3K-AKT pathway, triggering type 2 inflammation and EMT. Conversely, overexpression of miR-301b-3p suppressed PIK3CB expression and mitigated these pathological changes. Notably, the PIK3CB inhibitor TGX-221 reversed PI3K-AKT hyperactivation induced by miR-301b-3p knockdown, alleviating type 2 inflammation and EMT.

Conclusion: miR-301b-3p regulates type 2 inflammation and EMT in ECRS by targeting PIK3CB and modulating the PI3K-AKT pathway, suggesting both miR-301b-3p and PIK3CB as promising therapeutic targets. Given the limited sample size of this study, we plan to expand our cohort and implement a longitudinal follow-up design to monitor dynamic changes in miR-301b-3p expression and their relationship to disease progression.

Keywords: miR-301b-3p, PIK3CB, eosinophilic chronic rhinosinusitis, PI3K-AKT pathway, epithelial-mesenchymal transition

Introduction

Chronic rhinosinusitis (CRS) is a highly heterogeneous inflammatory disease of the nasal cavity that affects over 10% of adults globally, making it a significant public health concern.[1–3] Recent studies have shown that CRS can be classified into non-type 2 and type 2 endotypes, characterized predominantly by neutrophilic and eosinophilic inflammation, respectively.[4–6] Among CRS patients, eosinophilic CRS (ECRS) with type 2 inflammation has a more complex etiology,

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more severe clinical symptoms, greater resistance to standard treatments, and a poorer prognosis.[6,7] The treatment of ECRS is becoming increasingly challenging due to the rising prevalence of ECRS worldwide, including in China and Japan.[8,9] Studies have shown that ECRS is characterized by massive eosinophilic infiltration in the nasal mucosa, accompanied by high levels of expression of type 2 cytokines such as IL-4. Excessive infiltration by these inflammatory cells can impair nasal epithelial function and induce epithelial-mesenchymal transition (EMT), and the persistence of EMT will lead to irreversible tissue remodeling of the nasal mucosa, which ultimately results in persistent inflammation and increased difficulty in treating ECRS.[10–14] However, the specific mechanisms of eosinophilic inflammation and EMT in ECRS have not been fully elucidated, and currently available therapeutic options are also limited. Therefore, identifying potential molecular targets and signaling pathways involved in the development of ECRS is crucial for advancing safer and more effective therapeutic strategies.

MicroRNAs (miRNAs) are a class of small non-coding RNAs consisting of about 22 nucleotides, which regulate gene expression by binding to the 3′ untranslated region of target mRNAs.[15,16] Studies have shown that miRNAs are important regulators of gene expression, which play a key role in the immune system and are closely related to the pathogenesis of many inflammatory diseases.[17] Since miRNAs can regulate multiple genetic pathways simultaneously and may determine the intrinsic phenotype of CRS by targeting mRNAs associated with Th1 and Th2 cell differentiation, miRNA dysfunction or dysregulation of the miRNA expression network may play an important role in the pathogenesis of CRS.[18,19] Recent studies have shown that miRNAs aberrantly expressed in ECRS are involved in the occurrence and development of ECRS by regulating immune cell function, pro-inflammatory signaling pathways, and EMT. For example, miR-205-5p is upregulated and positively correlates with IL-5 levels, eosinophil infiltration, and SNOT-22 scores;[3] miR-21-5p exacerbates type-2 inflammation via the GLP-1R/IL-33 pathway;[20] and miR-200a-3p is down-regulated and suppresses EMT and inflammation through the ZEB1/ERK-p38 pathway.[21] Therefore, changes in the expression of specific miRNAs may be potential biomarkers or targets for the diagnosis or treatment of ECRS.[22] However, the specific role and regulatory mechanism of miRNAs in ECRS are not fully understood yet, and therefore, further in-depth studies are needed to explore their potential as therapeutic targets or biomarkers.

The PI3K–AKT pathway plays a critical role in various respiratory inflammatory and remodeling processes. Single-cell analyses have revealed that HIF-2α is markedly upregulated in Th2 cells from asthma and CRS patients and promotes pathogenic Th2 differentiation via PI3K–AKT activation, thereby exacerbating airway inflammation.[23] Cigarette smoke extract stimulates MMP-2 expression in nasal fibroblasts through ROS/PI3K–AKT and NF-κB pathways, contributing to CRS-associated extracellular matrix remodeling.[24] Moreover, HIF-1α enhances IL-25 and IL-17RB expression via the PI3K–AKT axis in nasal polyposis, and inhibition of PI3K or HIF-1α significantly attenuates airway inflammation and polyp formation.[25] Although these studies elucidate the central functions of PI3K–AKT in airway inflammation and tissue remodeling, there remains a lack of systematic investigation into the specific mechanisms by which miR-301b-3p modulates this pathway in the pathogenesis of ECRS.

In this study, miR-301b-3p, a miRNA that was consistently differentially expressed in ECRS patients and a mouse model of ECRS, was identified by high-throughput sequencing of human and mouse nasal mucosa tissues. Mendelian randomization (MR) analysis showed a causal relationship between miR-301b and CRS. Using an in vivo ECRS model, bioinformatics analysis, and adeno-associated virus (AAV)-mediated regulation of miR-301b-3p expression, we found that knockdown of miR-301b-3p upregulated its target gene PIK3CB, which activated the PI3K/AKT signaling pathway. This activation induced type 2 inflammation and EMT, ultimately contributing to ECRS. Conversely, upregulating miR-301b-3p reduced PIK3CB expression, inhibited the PI3K/AKT signaling pathway, and alleviated type 2 inflammation and EMT, suggesting a potential salvage therapy for ECRS. The above findings provide a new insight into the regulatory mechanism of miRNAs in ECRS and offer a potential new strategy for its treatment.

Materials and Methods

Clinical Sample Collection

Nasal mucosa samples were obtained from patients who visited Yongchuan Hospital of Traditional Chinese Medicine affiliated with Chongqing Medical University from May 2023 to May 2024. The study subjects consisted of 23 patients

diagnosed with ECRS according to EPOS 2020 criteria and an eosinophil count of more than 10 per high-power field, as well as 11 healthy controls undergoing surgery for nasal septum deviation.[6,21] Exclusion criteria included cystic fibrosis, acute infections, fungal sinusitis, and patients who had used glucocorticoids or antibiotics within the last month. Clinical data from the study subjects are detailed in Table S1.

Construction of a Mouse Model of ECRS

Six-week-old male BALB/c mice were purchased from Hunan SJA Laboratory Animal Co., Ltd. A mouse model of ECRS was constructed with reference to a method in the literature.[26] Briefly, a mixture of 2 units of AP (Sigma-Aldrich) and 75 μg of OVA (Sigma-Aldrich) was diluted in sterile PBS to a total volume of 50 μL and was intranasally instilled into each mouse three times per week for five weeks.

Small RNA and mRNA Sequencing and Data Analysis

Small RNA and mRNA sequencing were performed on nasal mucosa samples from five healthy controls and four ECRS patients. In the mouse experiment, small RNA sequencing was performed on nasal mucosa samples from three healthy control and three model mice with ECRS. Samples were tested for RNA concentrations and integrity after extraction of total RNA to ensure sample eligibility. miRNA and mRNA sequencing libraries were constructed according to the manufacturer's recommendation and sequenced using Illumina NovaSeq6000 from BioMarker Co., Ltd. Raw reads underwent stringent quality control: for miRNA, adapters were removed, reads shorter than 18 nt or longer than 30 nt were discarded, and reads containing ≥10% unknown bases (N) or >50% low-quality bases ($Q \leq 20$) were filtered out; for mRNA, adapters were trimmed, and reads with ≥10% unknown bases or >50% low-quality bases ($Q \leq 10$) were removed. The resulting clean reads were used for alignment and quantification. Differential expression analysis was completed using the R package DESeq2 with a threshold set at $P < 0.05$ and $|\log2FC| > 1$.[27] KEGG enrichment analysis was based on DAVID. GSEA analysis was performed using GSEA_4.3.3. Inflammation and EMT gene sets were derived from the Hallmark gene sets on the official GSEA website. GSEA analysis results were verified using GSE72713, a public dataset from GEO.[28]

Quantitative Real-Time PCR (qRT-PCR)

Total RNA in the nasal mucosa was extracted using Trizol reagent (Accurate Biology), then reverse-transcribed using the miRNA First Strand cDNA Synthesis Kit (Sangon Biotech) and FastKing gDNA Dispelling RT SuperMix Kit (TIANGEN), and subjected to qRT-PCR analysis using SYBR Green fluorescence quantification reagents (TIANGEN). The relative expression level of a gene was calculated using the $2^{-\Delta\Delta Ct}$ method with U6 or GAPDH as an internal reference. Specific primer sequences are detailed in Table S2.

Fluorescence in situ Hybridization (FISH)

The probe mmu-miR-301b-3p was designed and synthesized by Wuhan Servicebio Technology Co., Ltd. Mouse nasal tissues were embedded in paraffin, then sectioned into 4-μm-thick slices, deparaffinized to water, and digested with Proteinase K for 25 minutes. A hybridization solution was added dropwise onto the tissue slices. They were hybridized overnight at 37°C in a thermostatic incubator, and then incubated in the dark at room temperature for 10 minutes with drops of DAPI. The tissue slices were mounted onto slides, and the expression sites of mmu-miR-301b-3p were observed by microscope.

MR Analysis

MR analysis was performed using the "TwoSample MR" package for R. First, the data on expression quantitative trait loci (eQTL) in miR-301b from 710 healthy blood donors were used as an exposure, and the CRS data (ID: ebi-a-GCST90018823) with the broadest genotype coverage from the Integrative Epidemiology Unit Open GWAS project were used as an outcome.[29] Single nucleotide polymorphisms (SNPs) were independently associated with miR-301b (clumping r^2 cut-off = 0.5 and clumping distance cut-off = 10) at a significance threshold of $P < 1e\text{-}5$.[30] Second, reverse MR was performed with CRS as an exposure and miR-301b as an outcome. SNPs were independently associated with

CRS (clumping r^2 cut-off = 0.001 and clumping distance cut-off = 10,000) at a significance threshold of P < 5e-6.[31] Five MR approaches were used, with the IVW approach as the main method for assessing causal effects.[31,32] To ensure the robustness of the causal relationship, analyses of heterogeneity, horizontal pleiotropy, and sensitivity were conducted.[32,33]

Immunohistochemistry (IHC)

After antigen retrieval and endogenous enzyme blocking, human and mouse nasal tissue slices were incubated with a primary antibody overnight at 4°C and a secondary antibody for 1 hour, followed by DAB staining and light hematoxylin counterstaining. The expression level of the target protein was assessed based on the staining intensity. Specific antibody information is shown in Table S3.

Western Blot (WB)

Nasal mucosal proteins were extracted using RIPA Lysis Buffer, separated by SDS-PAGE, and then transferred onto a PVDF membrane. The proteins were blocked at room temperature for 1.5 hours, then incubated with the primary antibody at 4°C overnight followed by the secondary antibody for 2 hours, and developed with ECL. Relative quantification of the expression of the target protein was performed using GAPDH as an internal reference and Image J. Antibody information is detailed in Table S3.

Target Gene Prediction

The target genes of hsa-miR-301b-3p and mmu-miR-301b-3p were predicted using TargetScan, MiRDB, miRPathDB, and ENCORI, and intersections were taken.[34–37] KEGG analysis was then performed, and a Cytoscape PPI network was constructed based on the STRING results.

Dual Luciferase Reporter Gene Assay

The binding site of miR-301b-3p for PIK3CB was predicted using TargetScan. Wild-type and mutant reporter plasmids of the PIK3CB gene were constructed using the H306 pMIR-REPORT System (Obio Technology) and co-transfected into HEK293T cells together with miR-301b-3p mimics or controls (NCs), respectively. At 24 hours after transfection, luciferase activity was determined using a luciferase reporter gene kit (Beyotime).

AAV Vector Construction and Intervention

To knock down mmu-miR-301b-3p, we constructed an AAV9-U6-mmu-miR-301b-3p-CAG-EGFP sponge vector (Sponge-miR-301b-3p; sponge sequence: 5′-GCTTTGACAATACCATTGCACTG-3′) and a non-targeting control vector (Sponge-Vector; sequence: 5′-CGCTGAGTACTTCGAAATGTC-3′). For overexpression, precursor mmu-pri-miR-301b sequences were cloned into an AAV9-CMV-eGFP backbone (Over-miR-301b-3p), with an empty AAV9-CMV-eGFP-Scramble vector serving as the control (Over-Vector). Mice were anesthetized with isoflurane and secured by gently grasping the scruff at a 45° angle while lightly closing the mouth. A total volume of 40 μL viral suspension (1×10^{11} vg per mouse) was administered by dropwise intranasal instillation: first into one nostril until fully inhaled, then repeated in the opposite nostril.[38] Peak transgene expression for AAV9 typically occurs at 3–5 weeks post-injection; thus, infection efficiency was confirmed by Western blot analysis at 5 weeks post-administration.[39]

Animal Experimental Design

Mice were randomly divided into the following groups: (a) WT group (no intervention), (b) ECRS group, (c) WT/Sponge-Vector group, (d) WT/Sponge-301b-3p group, (e) ECRS/Over-Vector group, (f) ECRS/Over-301b-3p group, (g) TGX-221+WT/Sponge-301b-3p group, and (h) DMSO+WT/Sponge-301b-3p group. TGX-221 (Selleck) at 40 mg/kg was administered by intraperitoneal injection once daily for five weeks.[40]

Pathohistological Analysis

Mouse nasal tissues were sectioned into 4-μm-thick slices, then stained with H&E to assess subepithelial inflammation, Sirius red to assess eosinophil infiltration, and PAS to evaluate goblet cell hyperplasia. Subepithelial inflammation was

scored from 0 to 4.[41] The analysis was conducted independently by two pathologists who were blinded to the experimental grouping.

Measurement of Total Serum IgE

Serum was tested for total IgE levels in each group using an ELISA kit (Solarbio).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9. Normally distributed data were expressed as mean ± standard deviation and compared using the unpaired *t*-test. Non-normally distributed data were expressed as median (interquartile range) and compared using the Mann–Whitney *U*-test. Categorical data were compared using the Chi-square test or Fisher's exact test. The Kruskal–Wallis test was used for non-parametric comparisons among multiple groups, and for parametric data, one-way or two-way analysis of variance (ANOVA) was conducted, followed by Tukey's test for multiple post-hoc comparisons of means to identify significant differences between groups. Correlations between various indicators were assessed by Spearman correlation analysis. $P < 0.05$ was considered statistically significant.

Results

miRNA Profiling Converges on miR-301b-3p

To enhance clinical translation and strengthen the evidence linking candidate miRNAs to disease, we conducted dual miRNA sequencing of human and mouse nasal mucosa tissues (Figure 1A). Hierarchical clustering analysis demonstrated significant differences in miRNA expression between ECRS patients and normal controls. Volcano plots identified 36 up-regulated and 47 down-regulated miRNAs in the nasal mucosa of ECRS patients (Figure 1B and C), including miR-21-5p, which has been previously associated with ECRS,[20] along with several previously unreported miRNAs (Table S4). In the mouse model, miRNA analysis revealed 11 up-regulated and 17 down-regulated miRNAs in ECRS mice (Figure 1D and E; detailed data provided in Table S5). Notably, miR-301b-3p showed a consistent downregulation trend in both human and mouse sequencing data (Figure 1F), which was confirmed by qRT-PCR. Both hsa-miR-301b-3p and mmu-miR-301b-3p were significantly down-regulated in ECRS (Figure 1G). FISH results further showed a marked decrease in miR-301b-3p levels in the epithelium and lamina propria of ECRS mouse nasal mucosa, corroborating the qRT-PCR findings (Figure 1H). Additionally, referencing the sequencing analysis by Ke Li et al of nasal mucosa tissues from ECRS patients and healthy controls, hsa-miR-301b-3p was significantly downregulated in ECRS patients (log2FC = –4.491, P= 0.000), further corroborating the robustness of miR-301b-3p downregulation observed in our study.[23] Moreover, miR-301b-3p expression was negatively correlated with the SNOT-22 score, indicating a relationship with ECRS severity (Figure 1I). MR analysis also demonstrated that miR-301b downregulation was associated with an increased risk of CRS [OR: 0.956; 95% CI: 0.918–0.996; P = 0.033]. All MR approaches showed consistent causal direction without significant heterogeneity or pleiotropy (Figure 1J–L). Reverse MR analysis indicated no significant causal effect of CRS on miR-301b expression, further supporting the previous conclusion (Figure 1M–O). The specific instrumental variables used in both forward and reverse MR analyses are presented in Tables S6 and S7, respectively.

miR-301b-3p Correlates with Type 2 Inflammation and EMT in Nasal Mucosa

To investigate the role of miR-301b-3p in the pathogenesis of ECRS, we performed mRNA sequencing on clinical samples. Differential gene expression analysis identified 442 up-regulated and 447 down-regulated genes in ECRS patients compared with normal controls (Figure 2A and B). Gene Set Enrichment Analysis (GSEA) highlighted significant activation of inflammatory and EMT pathways in ECRS patients, which was validated using public dataset GSE72713 (Figure 2C–F). Further analysis showed that IL-4, IL-5, and IL-13 levels were significantly elevated in the nasal mucosa of ECRS patients and negatively correlated with hsa-miR-301b-3p expression (Figure 2G–K). EMT marker gene analysis revealed down-regulation of E-cadherin, and up-regulation of N-cadherin and Vimentin, all of which correlated with hsa-miR-301b-3p expression (Figure 2L–O). IHC analysis confirmed that protein levels in the nasal mucosa of ECRS patients were consistent with transcriptional changes

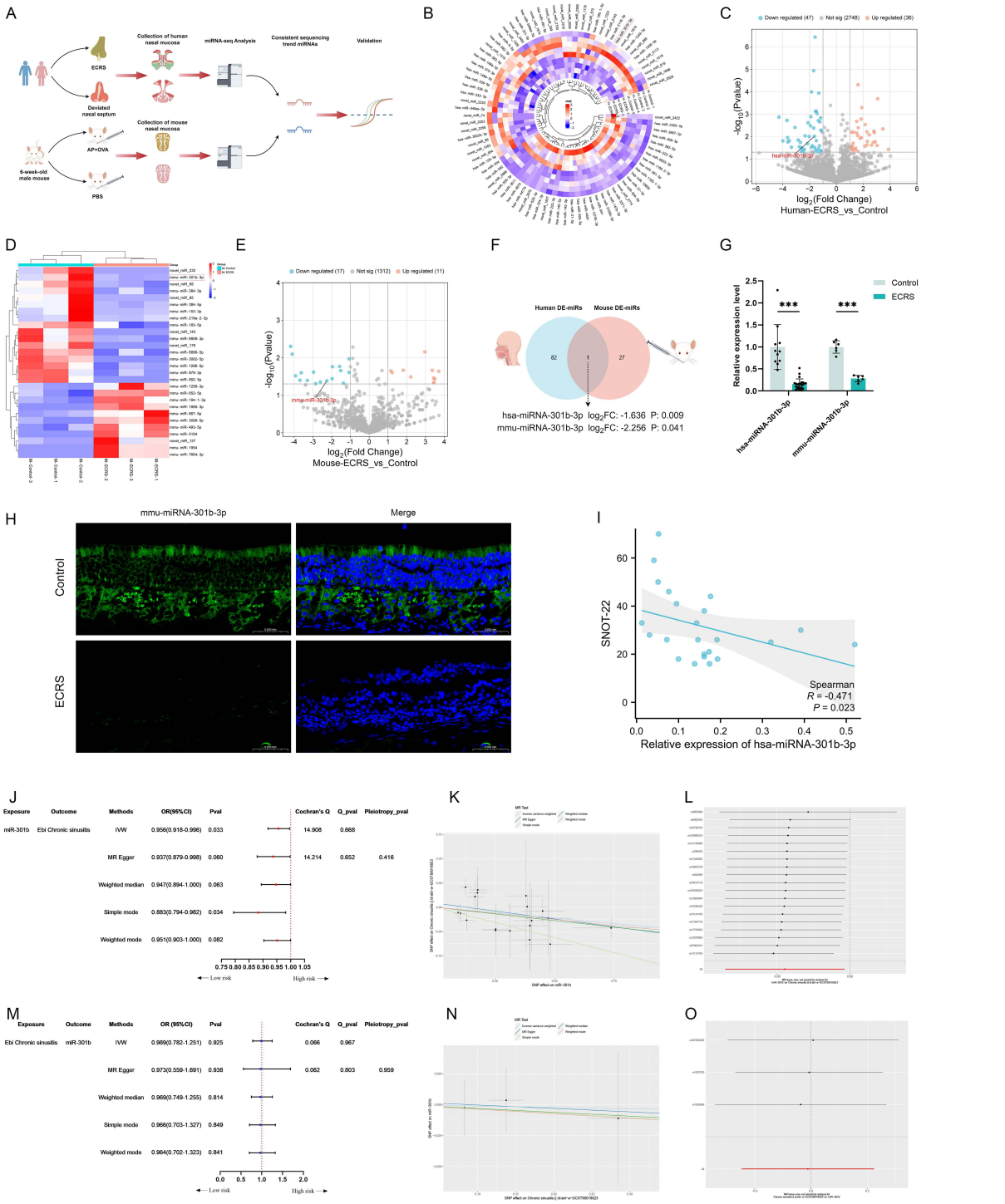

Figure 1 Expression of miR-301b-3p in ECRS Patients and Mouse Model, and Its Causal Relationship with Chronic Rhinosinusitis. (**A**) Study flowchart illustrating miRNA sequencing of human and mouse nasal tissues to identify differentially expressed miRNAs with consistent expression trends for further investigation. (**B** and **C**) Representative heatmap (**B**) and volcano plot (**C**) of miRNA expression in human nasal tissues showing differences between ECRS patients and normal controls. (**D** and **E**) Representative heatmap (**D**) and volcano plot (**E**) of miRNA expression in mouse nasal tissues showing differences between ECRS model mice and normal controls. (**F**) Venn diagram depicting commonly downregulated miRNAs in human and mouse nasal tissues, identifying miR-301b-3p as a shared candidate molecule. (**G**) qRT-PCR results further confirm the consistent downregulation of miR-301b-3p in the nasal tissues of both ECRS patients and the mouse model. (**H**) FISH analysis shows significantly reduced expression of miR-301b-3p in the epithelium and lamina propria of ECRS mouse nasal tissues. (**I**) Spearman correlation analysis indicates a negative correlation between miR-301b-3p expression levels and SNOT-22 scores in ECRS patients. (**J**–**L**) Forward MR analysis demonstrates a causal relationship between reduced miR-301b-3p expression and increased CRS risk, including the forward MR forest plot (**J**), scatter plot visualization (**K**), and leave-one-out sensitivity analysis (**L**). (**M**–**O**) Reverse MR analysis shows no significant causal effect of CRS on miR-301b expression, including the reverse MR forest plot (**M**), scatter plot visualization (**N**), and leave-one-out sensitivity analysis (**O**). ***P<0.001.

Abbreviations: ECRS, eosinophilic chronic rhinosinusitis; MR, Mendelian Randomization; DE-miRs, differentially expressed miRNAs.

(Figure 2P–R). In ECRS mice, WB also showed elevated levels of type 2 inflammatory proteins (IL-4, IL-5, IL-13) and decreased E-cadherin, along with increased Vimentin and Slug expression (Figure 2S–V).

miR-301b-3p Mediates PI3K-AKT Pathway Activation via PIK3CB

To elucidate the downstream mechanism of miR-301b-3p, we predicted 188 common target genes using multiple databases (Figure 3A). KEGG enrichment analysis of these targets revealed significant enrichment of the PI3K-AKT pathway among the top ten pathways (Figure 3B). Given the correlation between miR-301b-3p and type 2 inflammation and EMT phenotypes, we cross-referenced differentially expressed genes with inflammation and EMT gene sets, identifying 38 relevant genes (Figure 3C). KEGG enrichment of these 38 genes again highlighted the PI3K-AKT pathway (Figure 3D). GSEA confirmed significant PI3K-AKT pathway activation in ECRS patients, which was validated by external datasets (Figure 3E and F). PPI network analysis of the 188 target genes identified PIK3CB as a key hub gene (Figure 3G). In the ECRS mouse model, WB analysis showed increased expression of PIK3CB and activation of the PI3K-AKT pathway (Figure 3H and I). Similarly, human samples showed significant upregulation of PIK3CB, which was negatively correlated with miR-301b-3p expression (Figure 3J and K). The dual-luciferase reporter assay indicated that miR-301b-3p directly inhibited PIK3CB by targeting its 3'UTR (Figure 3L and M).

Knockdown of miR-301b-3p Induces Type 2 Inflammation and EMT via PI3K-AKT Activation, Leading to ECRS

To confirm the role of miR-301b-3p in ECRS, we used an AAV vector to knock down miR-301b-3p, with an empty vector as the control (Figure 4A). WB analysis at five weeks post-knockdown confirmed efficient viral infection with well-expressed GFP (Figure S1). qRT-PCR confirmed significant down-regulation of miR-301b-3p in mice in the WT/Sponge-301b-3p group (Figure 4B). Histological staining (H&E, Sirius red, PAS) and serum IgE analysis showed increased inflammatory infiltrates, elevated eosinophil and goblet cell numbers, and higher serum IgE levels in miR-301b-3p knockdown mice (Figure 4C–G), consistent with observations in ECRS mice. qRT-PCR and WB showed significant upregulation of type 2 cytokines, down-regulation of E-cadherin, and up-regulation of N-cadherin, Slug, and Vimentin in knockdown mice (Figure 4H–M). Furthermore, PIK3CB and P85 expression, as well as phosphorylated AKT levels, were significantly increased (Figure 4N and O). IHC results were consistent with qRT-PCR and WB findings (Figure 4P–T).

miR-301b-3p Overexpression Ameliorates ECRS by Inhibiting the PI3K-AKT Pathway

To evaluate the therapeutic potential of miR-301b-3p, we overexpressed miR-301b-3p in ECRS mice (Figure 5A). WB confirmed effective overexpression (Figure S2), with significantly increased miR-301b-3p expression in the nasal mucosa (Figure 5B). Overexpression significantly reduced nasal inflammation, eosinophil and goblet cell numbers, and serum IgE levels compared with the empty vector control (Figure 5C–G). miR-301b-3p overexpression also significantly ameliorated type 2 inflammation and EMT at both the transcriptional and protein levels (Figure 5H–M). WB results indicated that the levels of PIK3CB, P85, and phosphorylated AKT were significantly reduced after miR-301b-3p overexpression (Figure 5N and O). The IHC results for the expression of EMT markers and PIK3CB were also consistent with the qRT-PCR and WB results (Figure 5P–T).

PIK3CB Mediates Type 2 Inflammation and EMT Induced by miR-301b-3p Deficiency

To determine whether the lack of miR-301b-3p induces type 2 inflammation and EMT via the PIK3CB-mediated PI3K-AKT signaling pathway, we treated miR-301b-3p knockdown mice with the PIK3CB inhibitor TGX-221 (Figure 6A). WB analysis showed that TGX-221 significantly down-regulated PIK3CB, P85, and phosphorylated AKT levels that were elevated due to miR-301b-3p deficiency (Figure 6B and C). qRT-PCR and WB assays further showed that TGX-221 treatment significantly reduced type 2 inflammation caused by miR-301b-3p deficiency (Figure 6D–F). TGX-221 also increased E-cadherin expression while decreasing mesenchymal markers such as N-cadherin (Figure 6G–I). These results suggest that inhibition of PIK3CB can mitigate type 2 inflammation and EMT resulting from miR-301b-3p deficiency.

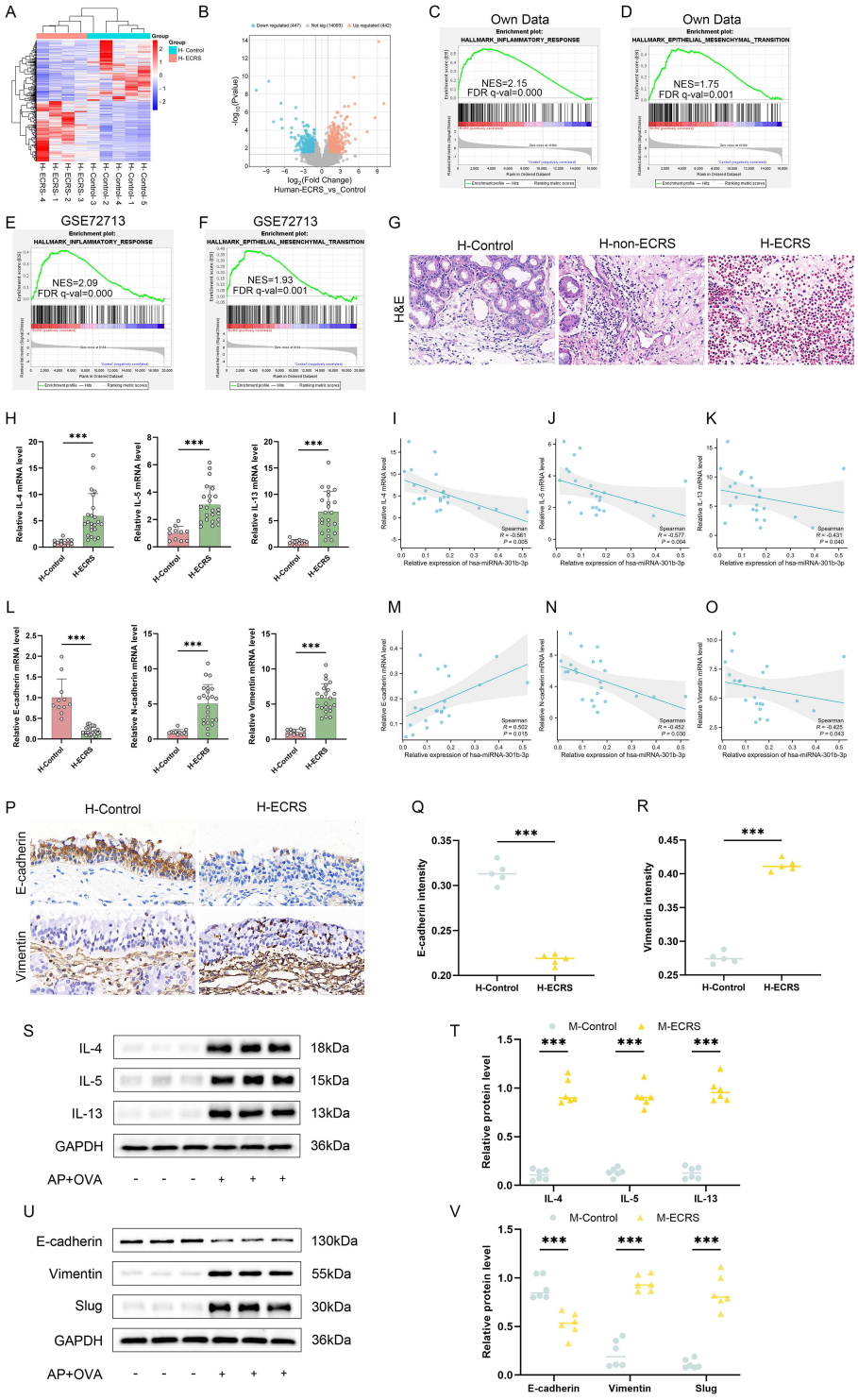

Figure 2 Correlation of miR-301b-3p with Type 2 Inflammation and EMT. (**A**) Clustered heatmap of differential mRNA expression levels in human nasal tissues obtained using the Illumina NovaSeq6000 platform. (**B**) Volcano plot showing differential mRNA expression between ECRS patients and normal controls. (**C** and **D**) GSEA analysis of proprietary mRNA sequencing data indicating significant activation of the Inflammatory Response (**C**) and Epithelial Mesenchymal Transition (**D**) pathways. (**E** and **F**) GSEA analysis of public dataset GSE72713 confirming significant activation of the Inflammatory Response (**E**) and Epithelial Mesenchymal Transition (**F**) pathways. (**G**) Representative HE-stained images of nasal tissues from normal controls, ECRS patients, and non-ECRS patients. (**H**) qRT-PCR results showing upregulated expression of IL-4, IL-5, and IL-13 in the nasal tissues of ECRS patients. (**I**–**K**) Negative Spearman correlation between miR-301b-3p expression and IL-4, IL-5, IL-13 levels. (**L**) qRT-PCR results showing decreased E-cadherin and increased N-cadherin and Vimentin expression in ECRS patients. (**M**–**O**) Positive correlation between miR-301b-3p expression and E-cadherin, and negative correlation with N-cadherin and Vimentin. (**P**–**R**) IHC showing decreased E-cadherin and increased Vimentin expression in the nasal tissues of ECRS patients. (**S** and **T**) WB results showing upregulated expression of IL-4, IL-5, and IL-13 in the ECRS mouse model. (**U** and **V**) WB results showing decreased E-cadherin and increased Vimentin and Slug expression in the ECRS mouse model. ***P<0.001.

Abbreviation: ECRS, eosinophilic chronic rhinosinusitis.

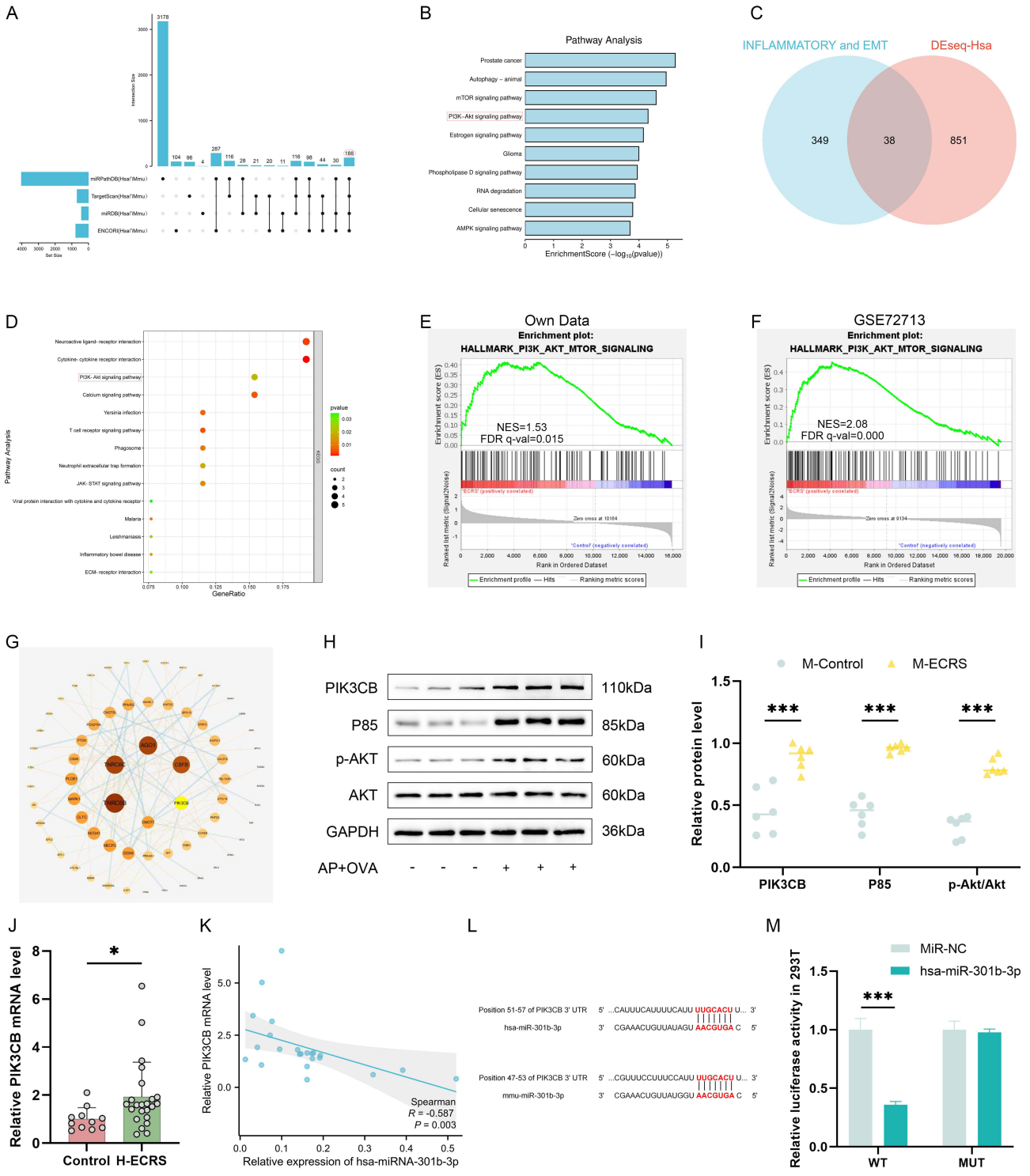

Figure 3 miR-301b-3p Potentially Mediates PI3K-AKT Pathway Activation via Target Gene PIK3CB. (**A**) Upset plot showing the intersection of 188 target genes for miR-301b-3p in humans and mice across four databases. (**B**) Top 10 enriched pathways in the KEGG analysis of 188 target genes. (**C**) Venn diagram showing the intersection of inflammatory and EMT gene sets with differentially expressed mRNAs from proprietary human nasal tissue sequencing, resulting in 38 intersecting genes. (**D**) Top 10 enriched pathways in the KEGG analysis of 38 intersecting genes. (**E**) GSEA analysis of proprietary mRNA sequencing data indicating significant activation of the PI3K-AKT signaling pathway. (**F**) GSEA analysis of public dataset GSE72713 confirming significant activation of the PI3K-AKT pathway. (**G**) PPI network constructed from 188 target genes showing PIK3CB as a key hub gene. (**H** and **I**) WB results showing upregulated expression of PIK3CB, P85, and phosphorylated AKT in the ECRS mouse model. (**J**) qRT-PCR results showing upregulated PIK3CB expression in the nasal tissues of ECRS patients. (**K**) Negative Spearman correlation between miR-301b-3p and PIK3CB expression in ECRS patient nasal tissues. (**L**) Binding sites of miR-301b-3p in human and mouse PIK3CB transcripts. (**M**) Dual-luciferase reporter assay confirming PIK3CB as a target gene of hsa-miR-301b-3p. *P<0.05, ***P<0.001.

Abbreviations: ECRS, eosinophilic chronic rhinosinusitis; DEseq-Hsa, differentially expressed mRNAs from proprietary human nasal tissue sequencing; WT, wildtype; MUT, mutant.

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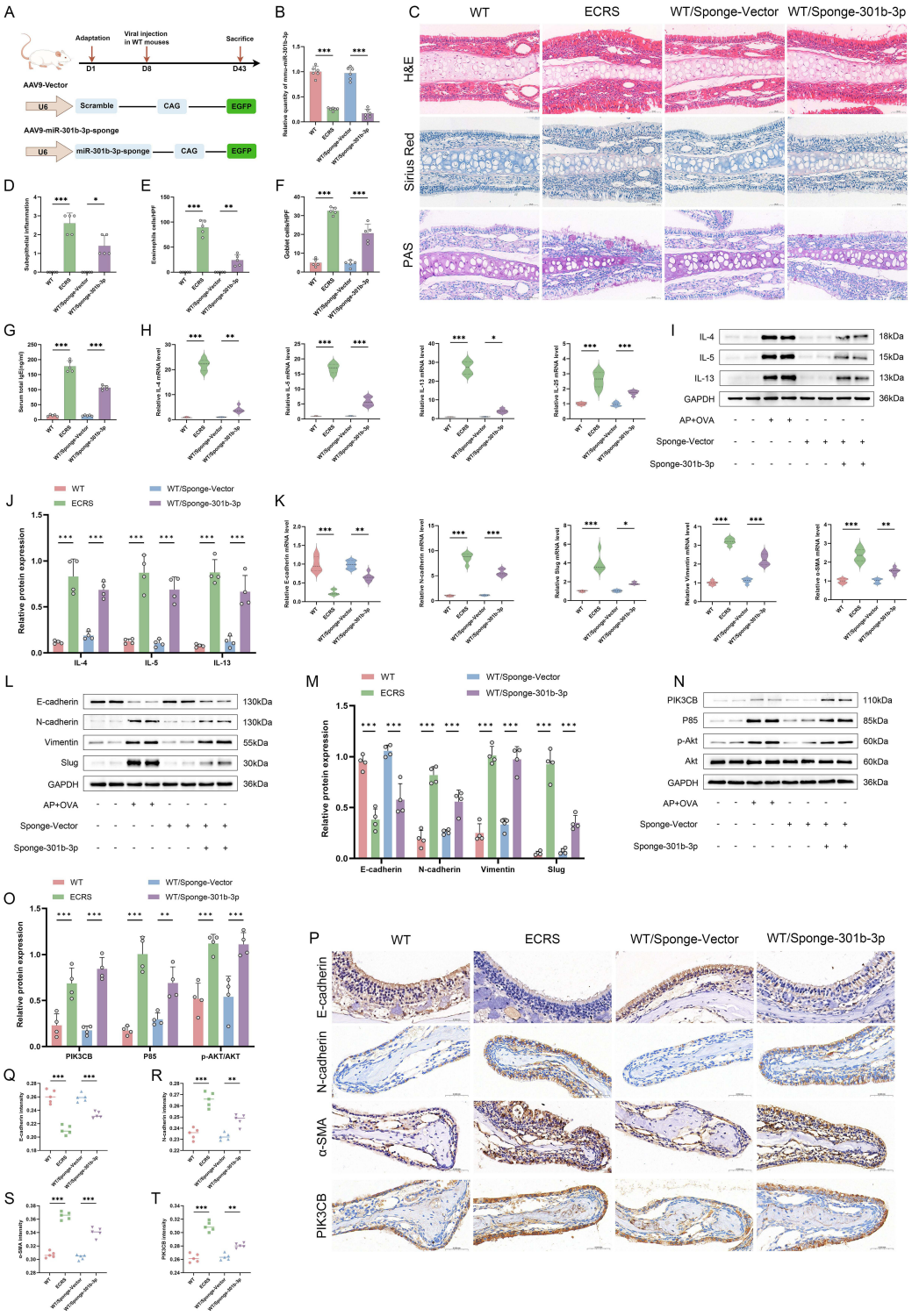

Figure 4 Knockdown of miR-301b-3p Induces ECRS-like Changes by Activating the PI3K-AKT Pathway, Triggering Type 2 Inflammation and EMT. (**A**) Schematics of AAV vectors engineered to knock down miR-301b-3p or a vector control construct and experimental paradigm for viral injection. (**B**) qRT-PCR was used to validate the efficiency of miR-301b-3p knockdown. (**C**–**F**) Histological staining (H&E, Sirius red, PAS) shows that knockdown of miR-301b-3p increases subepithelial inflammation, eosinophil count, and goblet cell numbers in mouse nasal tissues. (**G**) ELISA results indicate that knockdown of miR-301b-3p elevates serum IgE levels in mice. (**H**) qRT-PCR shows that knockdown of miR-301b-3p increases mRNA levels of type 2 inflammatory cytokines in mouse nasal tissues. (**I** and **J**) WB results show increased protein levels of type 2 inflammatory cytokines after miR-301b-3p knockdown. (**K**) qRT-PCR results indicate reduced mRNA levels of E-cadherin and increased levels of N-cadherin, Slug, Vimentin, and α-SMA in mouse nasal tissues after miR-301b-3p knockdown. (**L** and **M**) WB results show decreased protein levels of E-cadherin and increased levels of N-cadherin, Slug, and Vimentin after miR-301b-3p knockdown. (**N** and **O**) WB results show upregulated PIK3CB, P85, and phosphorylated AKT levels after miR-301b-3p knockdown. (**P**–**T**) IHC shows reduced protein levels of E-cadherin and increased levels of N-cadherin, α-SMA, and PIK3CB after miR-301b-3p knockdown. *P<0.05, **P<0.01, ***P<0.001.
Abbreviations: WT, wildtype; ECRS, eosinophilic chronic rhinosinusitis.

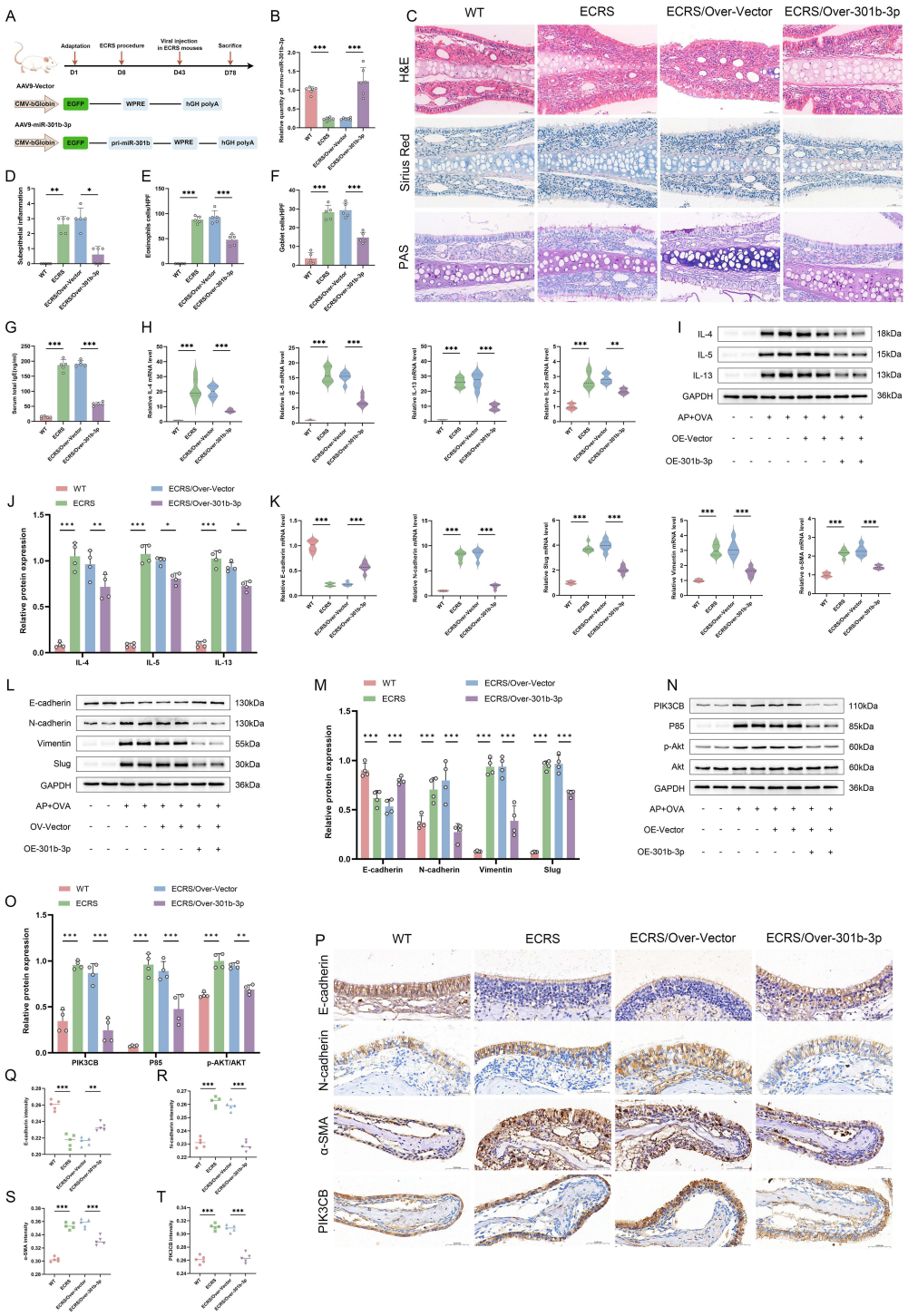

Figure 5 Overexpression of miR-301b-3p Suppresses Type 2 Inflammation and EMT via Inhibition of the PI3K-AKT Pathway, Providing Therapeutic Effects in ECRS. (**A**) Construct of AAV-miR-301b-3p for overexpression of miR-301b-3p and experimental paradigm involving ECRS, viral injection, and testing. (**B**) qRT-PCR showing the efficiency of miR-301b-3p overexpression in nasal tissues. (**C**–**F**) Histological staining (H&E, Sirius red, PAS) shows that overexpression of miR-301b-3p reduces subepithelial inflammation, eosinophil count, and goblet cell numbers in ECRS mouse nasal tissues. (**G**) ELISA results show that overexpression of miR-301b-3p lowers serum IgE levels in ECRS mice. (**H**) qRT-PCR shows that overexpression of miR-301b-3p reduces mRNA levels of type 2 inflammatory cytokines in ECRS mouse nasal tissues. (**I** and **J**) WB results indicate reduced protein levels of type 2 inflammatory cytokines after overexpression of miR-301b-3p. (**K**) qRT-PCR results show increased mRNA levels of E-cadherin and reduced levels of N-cadherin, Slug, Vimentin, and α-SMA in ECRS mouse nasal tissues after overexpression of miR-301b-3p. (**L** and **M**) WB results show increased protein levels of E-cadherin and reduced levels of N-cadherin, Slug, and Vimentin after overexpression of miR-301b-3p. (**N** and **O**) WB results show decreased levels of PIK3CB, P85, and phosphorylated AKT in ECRS mouse nasal tissues after overexpression of miR-301b-3p. (**P**–**T**) IHC shows increased protein levels of E-cadherin and reduced levels of N-cadherin, α-SMA, and PIK3CB after overexpression of miR-301b-3p. *P<0.05, **P<0.01, ***P<0.001.

Abbreviations: WT, wildtype; ECRS, eosinophilic chronic rhinosinusitis; OE-Vector, Overexpression Vector; OE-301b-3p, Overexpression miR-301b-3p.

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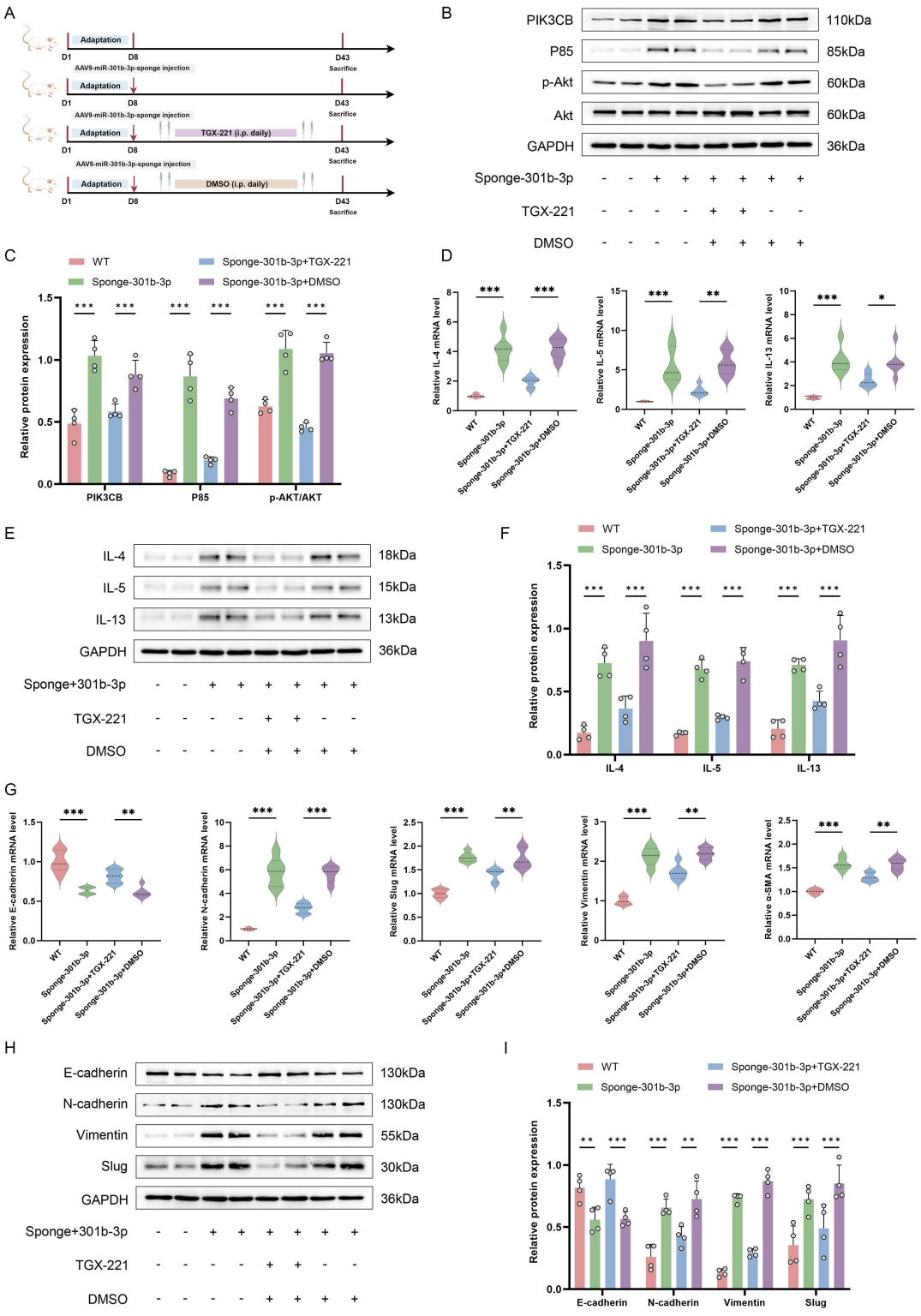

Figure 6 PIK3CB Inhibition Mitigates PI3K-AKT Pathway Activation and Type 2 Inflammation and EMT Induced by miR-301b-3p Deficiency. (**A**) Experimental paradigm showing the injection of AAV-miR-301b-3p sponge virus followed by administration of TGX-221, a specific inhibitor of PIK3CB. (**B** and **C**) WB results show that TGX-221 reduces the elevated levels of PIK3CB, P85, and phosphorylated AKT in mouse nasal tissues due to miR-301b-3p deficiency. (**D**) qRT-PCR shows that TGX-221 reduces the increased mRNA levels of type 2 inflammatory cytokines in mouse nasal tissues due to miR-301b-3p deficiency. (**E** and **F**) WB results show that TGX-221 decreases the elevated protein levels of type 2 inflammatory cytokines in mouse nasal tissues. (**G**) qRT-PCR results indicate that TGX-221 increases the mRNA levels of E-cadherin and decreases the mRNA levels of N-cadherin, Slug, Vimentin, and α-SMA elevated by miR-301b-3p deficiency. (**H** and **I**) WB results show that TGX-221 increases the protein levels of E-cadherin and decreases the protein levels of N-cadherin, Slug, and Vimentin elevated by miR-301b-3p deficiency. *P<0.05, **P<0.01, ***P<0.001.
Abbreviations: WT, wildtype; ECRS, eosinophilic chronic rhinosinusitis.

Discussion

The treatment of ECRS remains challenging. Current widely used glucocorticoids offer limited efficacy, while the high cost of dupilumab restricts its use despite its effectiveness, underscoring the need for new and improved treatment options for ECRS.[8,42] Molecularly targeted therapies that address ECRS pathogenesis hold promise as an alternative.[43] Recent studies highlight the significant roles of type 2 inflammation and EMT in ECRS pathogenesis, making it crucial to elucidate the upstream regulatory mechanisms of these processes for developing new therapeutic strategies.[10–13,20,21] In this study, we uncovered, for the first time, the role and downstream mechanisms of miR-301b-3p in ECRS pathogenesis, revealing that miR-301b-3p regulates type 2 inflammation and EMT by targeting PIK3CB, thereby modulating the PI3K-AKT signaling pathway. This provides new insight into ECRS pathogenesis and identifies a potential target for novel diagnostic and therapeutic strategies.

The regulatory role of miRNAs in airway inflammatory diseases, such as CRS, allergic rhinitis, and asthma, is well recognized; however, few miRNAs have been causally linked to ECRS and shown to regulate type 2 inflammation and EMT phenotypes.[20,21,44,45] By dual miRNA sequencing of human and mouse nasal mucosa tissues, we identified miR-301b-3p as a miRNA consistently downregulated in both species, and not previously reported in CRS. Interestingly, miR-301b-3p did not emerge as a major candidate in miRNA expression profiles of the ECRS animal model, consistent with Henshall et al's findings that emphasized the benefits of integrating heterogeneous miRNA expression data to better identify key disease targets.[46] We subsequently investigated the functional role and regulatory mechanism of miR-301b-3p, addressing a gap in the field of ECRS.

Our results showed that miR-301b-3p was significantly downregulated in the nasal mucosa of both ECRS patients and the mouse model. FISH results further confirmed the reduction of miR-301b-3p in the nasal mucosal epithelium and lamina propria of ECRS mice. Additionally, the expression of miR-301b-3p was negatively correlated with the severity of ECRS, suggesting its role in disease development. Bidirectional MR analysis indicated that decreased miR-301b expression increased the risk of CRS, while CRS had no causal effect on miR-301b expression. This suggests miR-301b acts as an upstream regulator in CRS pathogenesis and represents a potential therapeutic target. Similar MR approaches have previously demonstrated the causal role of specific miRNAs in disease, further supporting miR-301b-3p as a key regulator in ECRS.[47]

We further explored the regulatory mechanism of miR-301b-3p in ECRS. Through bioinformatics analysis and experimental validation, we identified PIK3CB as a target gene of miR-301b-3p. PIK3CB is an essential upstream component of the PI3K-AKT signaling pathway, which plays a critical role in AKT activation and has been implicated in cancer-related inflammation and EMT.[48–50] However, PIK3CB's role in ECRS had not been previously reported. We found that the PI3K-AKT pathway was significantly activated in ECRS patients and mice, consistent with studies linking aberrant activation of this pathway to inflammatory airway diseases, including asthma and allergic rhinitis.[25,51,52] Furthermore, the correlations of miR-301b-3p with inflammatory cytokines and EMT-related indicators in ECRS suggest that miR-301b-3p may affect the activation of the PI3K-AKT signaling pathway through the regulation of PIK3CB, which in turn regulates type 2 inflammation and EMT.

To test this hypothesis, we manipulated mmu-miR-301b-3p expression in mice using an AAV vector. Knockdown of miR-301b-3p significantly increased PIK3CB expression, activated the PI3K-AKT pathway, and promoted type 2 inflammation and EMT in the nasal tissues of mice. Conversely, overexpression of miR-301b-3p decreased PIK3CB expression, inhibited PI3K-AKT activity, and attenuated type 2 inflammation and EMT. Previous studies on miR-301b-3p have primarily focused on its regulatory roles in various pathological contexts, including cancer and inflammatory diseases. For example, miR-301b-3p has been reported to promote tumor proliferation, invasion, and metastasis in breast cancer and non-small cell lung cancer through lncRNA-mediated axes such as ADAMTS9-AS1/miR-301b-3p/TGFBR2/JAK-STAT and CASC19/miR-301b-3p/LDLR. In addition, miR-301b-3p participates in the regulation of inflammation and apoptosis in middle ear epithelial cells via the NEAT1/miR-301b-3p/TLR4 axis.[53–55] These findings underscore the context-dependent and multifaceted functions of miR-301b-3p, highlighting the need to further clarify its tissue- and disease-specific effects. Our study expands the understanding of miR-301b-3p by demonstrating its role in regulating type 2 inflammation and EMT in ECRS, suggesting it as a potential therapeutic target for future ECRS

treatments. Notably, our findings are consistent with miRNA-mediated regulatory mechanisms described in other airway inflammatory diseases.[56] For instance, miR-21-5p has been shown to exacerbate type 2 inflammation in CRSwNP via the GLP1R/IL-33 axis;[20] miR-1 attenuates eosinophil adhesion and migration in asthma and CRS models by modulating P-selectin and TSLP;[44] and miR-146a promotes regulatory T-cell differentiation and function in allergic rhinitis through targeting STAT5b.[45] This study not only enriches the miRNA regulatory network in airway inflammation but also lays the groundwork for developing therapeutic strategies based on individual miRNAs or their combinations.

To verify whether PIK3CB functions downstream of miR-301b-3p in regulating type 2 inflammation and EMT, we used the PIK3CB inhibitor TGX-221. TGX-221 effectively reversed the PI3K-AKT pathway hyperactivation caused by miR-301b-3p knockdown and ameliorated type 2 inflammation and EMT. This confirmed the critical role of PIK3CB in the miR-301b-3p regulatory network, consistent with previous studies on PIK3CB's role in inflammation and EMT, and suggests that targeting PIK3CB may be a viable therapeutic strategy for ECRS.[49,50,52] Moreover, signaling pathways such as JAK–STAT and AMPK also play critical roles in type 2 inflammation and EMT in airway diseases.[57,58] Future studies should investigate whether miR-301b-3p also engages with or cooperates through these pathways to regulate ECRS pathogenesis.

Despite these promising findings, our study has several limitations. First, the relatively small sample sizes for human and mouse sequencing may reduce the statistical power of gene expression analyses. Second, while we identified both upstream and downstream effects of PIK3CB, the detailed mechanisms underlying these effects warrant further exploration. Third, miRNAs often target multiple genes, and further studies are needed to fully understand the biological functions of miR-301b-3p. Fourth, our reliance on a mouse model, which does not fully replicate human immune responses, limits the translational applicability of our results. To address this limitation, future work will validate the regulatory effects of miR-301b-3p on PIK3CB and its downstream signaling pathways in primary human nasal epithelial cells. Finally, The clinical translation of miRNA-based interventions continues to face significant challenges. Although this study successfully employed an AAV vector to modulate miR-301b-3p expression in animal models—and AAV remains the first RNAi viral delivery platform used in human clinical settings—systemic administration often leads to substantial hepatic retention, hindering efficient targeting of other tissues. Consequently, delivery efficiency and tissue specificity must be further optimized.[59]

Conclusion

In summary, our study reveals for the first time the mechanism of miR-301b-3p in ECRS pathogenesis. By validating clinical samples and conducting functional experiments in a mouse model, we demonstrated that miR-301b-3p deficiency leads to upregulation of PIK3CB, activation of the PI3K-AKT pathway, and subsequent type 2 inflammation and EMT. Restoring miR-301b-3p expression or inhibiting PIK3CB effectively mitigated these pathological changes, suggesting that targeting miR-301b-3p and its downstream mediator, PIK3CB, may be a novel approach for managing inflammation and EMT in ECRS, providing a potential therapeutic strategy for this condition. Although this study is limited by sample size and the translatability of animal models, we are expanding our clinical cohort, performing in-depth validation in primary human cells, and initiating multicenter early preclinical studies to evaluate the safety and efficacy of this targeting strategy.

Data Sharing Statement

Data from this study can be obtained by making a reasonable request to the corresponding author.

Statement of Ethics

All procedures involving human participants were approved by the Ethics Committee of Yongchuan Chinese Medicine Hospital Affiliated to Chongqing Medical University (Approval No.: 2023-004-01) and were conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants. The animal experiments were approved by the Laboratory Animal Ethics Review Committee of Southwest University (IACUC-20230612-01). Furthermore, all animal procedures were carried out in strict accordance with the Chinese "Guidelines for the Ethical Review of Laboratory Animal Welfare" to rigorously uphold animal welfare ethics.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors have no conflicts of interest to declare.

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