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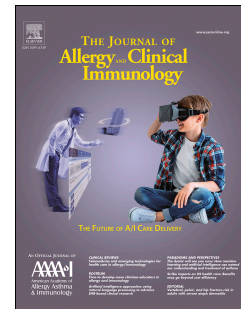
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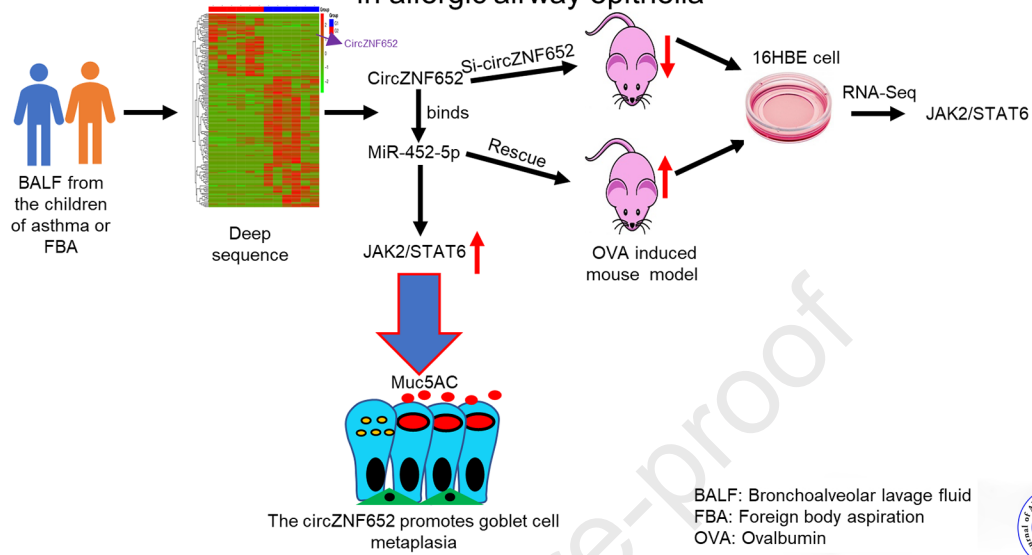
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CircZNF652/miR-452-5p/JAK2 promotes the goblet cell metaplasia in allergic airway epithelia



CircZNF652 promotes the goblet cell metaplasia by targeting the miR-452-5p/JAK2 signaling pathway in allergic airway epithelia

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Conflict of interest

The authors declared no conflict of interest.

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Abstract

Background: Circular RNAs (circRNAs) play potentially important roles in a variety of human diseases, however, their roles in the goblet cell metaplasia of asthma remain unknown.

Objective: To investigate the potential role and underlying mechanism of circZNF652 in the regulation of allergic airway epithelial remodeling.

Methods: The differential expression profiles of circRNAs were analyzed by transcriptome microarray, and the effects and mechanisms underlying circZNF652-mediated goblet cell metaplasia were investigated by RT-qPCR, RNA FISH, Western blot, RNA pull-down, and RNA immunoprecipitation analyses. Whereas the roles of circZNF652 and miR-452-5p in allergic airway epithelial remodeling were explored in both the mouse model with allergic airway inflammation and children with asthma.

Results: One hundred and sixty circRNAs were differentially expressed in bronchoalveolar lavage fluid (BALFs) of children with asthma versus children with foreign body aspiration, and 52 and 108 of them were significantly upregulated and downregulated, respectively. Among them, circZNF652 was predominantly expressed and robustly upregulated in airway epithelia of both the children with asthma and the mouse model with allergic airway inflammation. CircZNF652 promoted the goblet cell metaplasia by functioning as a sponge of miR-452-5p, which released the Janus kinase 2 (*JAK2*) expression and subsequently activated JAK2/STAT6 signaling in the allergic airway epithelia. In addition, epithelial splicing regulatory protein 1 (ESRP1), a splicing factor, accelerated the biogenesis of circZNF652 by binding to its flanking intron to promote the goblet cell metaplasia in allergic airway epithelial remodeling.

Conclusion: Upregulation of circZNF652 expression in allergic bronchial epithelia contributed to the goblet cell metaplasia by activating the miR-452-5p/JAK2/STAT6 signaling pathway, thus, blockage of circZNF652 or agonism of miR-452-5p provided an alternative approach for the therapeutic intervention of epithelial remodeling in allergic airway inflammation.

Key messages

CircZNF652 contributed to the goblet cell metaplasia by activating the miR-452-5p/JAK2/STAT6 signaling pathway in allergic airway remodeling, and splicing factor ESRP1 accelerated the biogenesis of circZNF652 to promote the goblet cell metaplasia.

Capsule summary

Upregulation of circZNF652 expression in allergic airway epithelia contributed to the goblet cell metaplasia by activating miR-452-5p/JAK2/STAT6 signaling pathway in asthma, thus, targeting circZNF652 may have promising therapeutic benefits for the treatment of asthmatic airway remodeling.

Keywords circZNF652; miR-452-5p; JAK2; goblet cell; asthma;

Abbreviations

circRNA: Circular RNA

miRNA: MicroRNA

BALF: Bronchoalveolar lavage fluid

FBA: Foreign body aspiration

OVA: Ovalbumin

CC10: Club cell 10 kDa protein

siRNA: Small interfering RNA

JAK2: Janus kinase 2

STAT6: Signal transducer and activator of transcription

RT-qPCR: Quantitative Real-time PCR

ICS: Intronic complementary sequence

- 85 RBP: RNA binding protein
- 86 RNA-FISH: RNA fluorescence in situ hybridisation
- 87 HBE: Human bronchial epithelial cell
- 88 Ago2: Argonaute 2
- 89 RIP: RNA Immunoprecipitation
- 90 NS: Normal saline

INTRODUCTION

Asthma is one of the most common chronic diseases. The World Health Organization predicts that asthma influences about 334 million people around the world¹. Airway wall remodeling is characterized by the structural alterations of the tissue composition, which includes mucous cell metaplasia and the airway epithelial cells remodeling, smooth muscle hypertrophy, hyperplasia, basement membrane thickening, and subepithelial fibrosis². Asthma is a result of complicated gene-environment interactions, there is an urgent need to identify the noninvasive biomarkers for diagnosing, characterizing, and understanding this disease. These biomarkers will enable the stratification of patients by phenotyping and predicting the patient's response to the therapy.

CircRNAs are a novel subgroup of endogenous noncoding RNAs and formed by back-splicing to be a covalently closed loop without a 5'-cap or a 3'-poly(A) tail. CircRNAs are characterized by the tissue specificity and structure stability³. They regulate gene expression by titrating microRNA (miRNA), interacting with RNA-binding protein, and interfering with pre-mRNA processing⁴. Recent reports have shown that aberrant circRNA expression is detected in cancers, immune responses, neurodegenerative diseases and cardiovascular diseases⁵⁻⁸. Several recent studies have emphasized the importance of circular RNAs in asthma⁹⁻¹², but the role of circRNAs in goblet cell metaplasia of asthma remains unknown.

Back-splicing is essential for circRNA biogenesis and modulated by the canonical spliceosomal machineries, which are regulated by the intronic complementary sequences (ICSs) flanking circle formation exons, core spliceosomal complexes, and RNA binding proteins (RBPs)³. Importantly, splicing factors, such as Muscleblind and Quaking, interact with the flanking introns of circRNAs to facilitate the back-splicing of circRNAs^{13, 14}.

In the present study, we identified circZNF652 (hsa_circ_0000782) was highly and specifically expressed in the airway epithelia of both the children with asthma and mouse models with allergic airway inflammation, and this high expression promoted the bronchial

116 goblet cell metaplasia and mucous hypersecretion by activating miR-452-5p/JAK2/STAT6
117 signaling pathway. In addition, we identified ESRP1 as a potential RBP that was involved in
118 the biogenesis of circZNF652. Thus, the present study shed light on the uncharacterized role of
119 circZNF652 in the airway epithelial remodeling and provided the potential approach of
120 therapeutic intervention for asthmatic airway remodeling.

METHODS

BALFs from children with foreign body aspiration or allergic asthma

BALFs from children with foreign body aspiration (FBA) or allergic asthma were collected as previously described¹⁵⁻¹⁷. The study was approved by the Ethics Committee of the Children's Hospital of Zhejiang University School of Medicine (2015-HP-037 and 2019-IRB-032). Written informed consent was obtained from the children's guardians with no financial incentive. The clinical characteristics of the patients are included in online repository Table E1.

Statistical analyses

Statistical analyses were performed by using SPSS v22.0 software. Data distribution was examined with Shapiro-Wilk test, and the equality of variances was examined with Levene test. Statistical analysis was performed by one-way analysis of variance (normal distribution) or Kruskal-Wallis (non-normal distribution) test followed by Bonferroni or Dunn post hoc test. For all analyses, $P < 0.05$ and $P < 0.01$ were considered as statistical difference.

Expanded Materials and Methods can be found in the Online Repository Materials.

RESULTS

CircZNF652 expression is upregulated in airway epithelia of children with asthma and of mouse models with allergic airway inflammation

We determined the circRNAs expression profiles in 6 pairs of BALFs to recognize and distinguish differentially expressed circRNAs in asthma versus in FBA. We set the filter criteria as a fold-change ≥ 1 and a p-value < 0.05 and found 160 differentially expressed circRNAs, of which, 52 and 108 were upregulated and downregulated in asthma, respectively (online repository Table E2). Heat maps were used to illustrate the differentially expressed pattern of circRNAs in asthma and FBA groups (Fig. 1A), and RT-qPCR analyses were performed to confirm the microarray result and showed the 10 upregulated circRNAs (online repository figure E1A and E1B). We then searched for the mouse homologous genes of the top 5 upregulated human circRNAs, and found that only hsa_circ_0000782 had the mouse homologous gene, mmu_circ_0003573. The sequence identity between hsa_circ_0000782 and mmu_circ_0003573 was about 93% (online repository figure E1C). The host gene of hsa_circ_0000782 was *ZNF652*, hsa_circ_0000782 was therefore named circZNF652 in the following experiments.

To determine the expression pattern of circZNF652 in airway epithelia, BALFs from children with allergic asthma or FBA were prepared for cytopins and subsequent RT-qPCR and RNA fluorescence in situ hybridization (RNA-FISH) analyses. RT-qPCR analyses demonstrated that circZNF652 expression in the BALFs was obviously increased in children with asthma versus with FBA (Fig. 1B). Moreover, RNA-FISH analyses demonstrated that Club cell 10 kDa protein (CC10)-derived immune signals were obviously detected in the BALF cells of children with asthma or with FBA, and were apparently overlapped with the circZNF652-derived fluorescence in the BALF cells of children with asthma but not with FBA (Fig. 1C). We next generated mouse models with allergen-induced allergic airway inflammation to determine the

expression patterns of circZNF652. OVA challenge significantly increased the circZNF652 mRNA levels in lungs, and this upregulation predominantly occurred in both the bronchial and alveolar epithelia (Fig. 1D, E). Likewise, lung single cell suspension analyses indicated expression of circZNF652 was significantly high and low in CC10⁺ airway epithelial cells and in CD11C⁺ dendritic cells and CD4⁺ lymphocytes, respectively, after OVA challenge (online repository figure E2A-E2C). However, circDOPEY2 level in allergic lung tissues was essentially the same as that in normal lung tissues (online repository figure E2D).

We next used Sanger sequencing to confirm whether the exon 3-exon 5 of ZNF652 gene was back-spliced to form the closed loop structure (Fig. 1F). Because head-to-tail splicing could be the consequence of either trans-splicing or genomic rearrangements, we designed special convergent primers for ZNF652 mRNA and divergent primers to amplify circZNF652 by using cDNA and genomic DNA (gDNA), respectively. CircZNF652 was readily amplified by the divergent primers in cDNA but not in gDNA (Fig. 1G). Although linear ZNF652 mRNA was readily degraded by RNase R, circZNF652 was resistant to this digestion (Fig. 1H). In addition, RNA-FISH analyses for the subcellular distribution of circZNF652 in human bronchial epithelial cell (16HBE) cells indicated that circZNF652 was predominantly localized in the cytoplasm (Fig. 1I). Thus, circZNF652 generated by the back-splicing is apparently upregulated in the airway epithelia of both the mouse models with allergic airway inflammation and the children with allergic asthma.

CircZNF652 regulates goblet cell metaplasia in allergic airway epithelia

To explore the likely role of IL-4, IL-5, and IL-13, the key proinflammatory cytokines of asthma, in inducing the expression of circZNF652, we performed RT-qPCR in 16HBE cells. Both the IL-4 and IL-13 at 50 ng/mL but not the IL-5 at 50ng/ml significantly increased the mRNA levels of circZNF652, though IL-4 exerted more robust effect than IL-13 (Fig. 2A). However, neither IL-1 nor IL-17 promoted the circZNF652 expression (online repository figure

E3A). The dose- and time-dependent effects of IL-4 on induction of circZNF652 mRNA expression were confirmed by the RT-qPCR analyses (Fig. 2B, C). To determine whether IL-4-induced circZNF652 expression took part in the goblet cell metaplasia, we performed the *Muc5AC* luciferase reporter analyses in 16HBE cells transfected with circZNF652 small interfering RNA (si-circZNF652) and treated with IL-4 at 50 ng/ml. CircZNF652 siRNA significantly decreased not only the basal but also the IL-4-induced *Muc5AC* luciferase activities (Fig. 2D). Thus, IL-4-induced circZNF652 expression participates in the goblet cell metaplasia in bronchial epithelial cells.

To investigate the potential implication of circZNF652 upregulation in allergic airway remodeling, we designed three different in vivo siRNAs to silence circZNF652 expression. We selected in vivo siRNA #3 for circZNF652 silence, because it had maximal silencing efficiency to circZNF652 but had no influence on ZNF652 mRNA (online repository figure E3B). We then generated OVA-induced mouse models with allergic airway inflammation to determine the in vivo role of circZNF652 in the goblet cell metaplasia (Fig. 2E). Although instillation of in vivo si-circZNF652-3 at 1 mg/ml did not show any apparent effect on the number of inflammatory cells, instillation of in vivo si-circZNF652-3 at 3 mg/ml obviously attenuated the numbers of these inflammatory cells in BALFs (Fig. 2F). Histological examination of lung sections consistently demonstrated that OVA challenge caused a significant infiltration of inflammatory cells into peribronchial and perivascular connective tissues of lungs, whereas instillation of in vivo si-circZNF652-3 at 1 or 3 mg/ml slightly or robustly inhibited OVA-induced infiltration of these inflammatory cells into the peribronchial and perivascular connective tissues, respectively (Fig. 2G and online repository figure E3C). In addition, OVA challenge robustly increased the staining of Periodic Acid-Schiff (PAS) as well as the expression of circZNF652, MUC5AC, and SAM pointed domain containing ETS transcription factor (SPDEF), and apparently decreased the expression of forkhead box A2 (FOXA2) in bronchial epithelia,

however, in vivo si-circZNF652-3 at 1 or 3 mg/ml moderately or robustly inhibited the OVA-induced PAS staining and expression of circZNF652, MUC5AC, SPDEF, and restored the OVA-negated expression of FOXA2 (Fig. 2G, 2H and online repository figure E3C and E3D). Likewise, OVA challenge robustly increased the PenH values in response to methacholine ranging from 0.05mg/kg to 0.4 mg/kg, whereas in vivo si-circZNF652-3 at 1 or 3 mg/ml significantly inhibited the OVA-induced PenH values in response to methacholine at 0.4 mg/kg or ranging from 0.1 to 0.4 mg/kg, respectively (Fig. 2I). Thus, knockdown of circZNF652 expression attenuates the goblet cell metaplasia in the allergic airway inflammation.

CircZNF652 regulates goblet cell metaplasia in allergic airway epithelia by functioning as a miRNA sponge

CircRNAs can serve as miRNA sponges to regulate the cell functions¹⁸⁻²⁰. Given the fact that circZNF652 is predominantly localized in the cytoplasm and exhibits obvious stability, we explored whether circZNF652 exerted the biological functions by sponging the miRNAs. AS Argonaute 2 (Ago2) protein is a core complex of RNA-induced silencing complex (RISC) that combines miRNA complexes to target mRNAs²¹. RNA immunoprecipitation assays were performed and indicated that endogenous circZNF652 was specifically enriched in the immunocomplex of Ago2 (Fig. 3A). MicroRNA microarray analyses in BALFs of children with asthma or FBA indicated that a total of 65 miRNAs, including 43 downregulated and 22 upregulated miRNAs, was differentially expressed in the BALF cells (Fig. 3B and online repository table E4). Three databases were then used to forecast the possible target miRNAs, and 8 miRNAs were chosen from the overlap among these databases (Fig. 3C). Importantly, pull-down assays with a biotinylated circZNF652 probe showed that miR-452-5p, miR-23b-3p, miR-34b-5p, and miR-182-5p were robustly enriched in the RNA pools pulled down by the circZNF652 probe (Fig. 3D). Interestingly, among these miRNAs, the miR-452-5p was robustly down-regulated in the BALFs of children with asthma versus with FBA (online repository

figure E3E). To confirm whether these miRNAs participate in the goblet cell metaplasia, we performed the *Muc5AC* luciferase reporter assays and RT-qPCR analyses for *Muc5AC* mRNA in 16HBE cells treated with miRNA negative control (NC) or mimic. MiR-452-5p mimic significantly decreased the *Muc5AC* luciferase activities as well as the *Muc5AC* mRNA levels (Fig. 3E and 3F).

To determine whether IL-4 negates the miR-452-5p expression to participate in the goblet cell metaplasia, we performed the *Muc5AC* luciferase reporter analyses and immunostaining of MUC5AC in 16HBE cells treated with miR-452-5p mimic in combination with IL-4. IL-4 at 50 ng/ml increased both the *Muc5AC* luciferase activities and MUC5AC expression, whereas miR-452-5p mimic robustly attenuated the IL-4 induced either *Muc5AC* luciferase activities or MUC5AC expression (Fig. 3G and 3H). Moreover, circZNF652-driven luciferase reporter assays indicated that miR-452-5p mimic transfection significantly reduced the activities of luc-circZNF652-WT but not luc-circZNF652-MUT (Fig. 3I and 3J). The specific binding between miR-452-5p and circZNF652 was confirmed by the RNA-FISH assays, which indicated an apparent overlap between circZNF652- and miR-452-5p-derived fluorescent signals in 16HBE cells (Fig. 3K). Furthermore, RNA-FISH assays indicated miR-452-5p was robustly expressed in the CC10⁺ airway epithelia of BALFs from children and of mouse models, and down-regulated in the airway epithelia from children with allergic asthma and from mouse models with allergic airway inflammation (Fig. 3L and 3M). Thus, miR-452-5p directly interacts with circZNF452 to regulate the allergic goblet cell metaplasia.

JAK2 functions as the downstream target of circZNF652/miR-452-5p signaling

We predicted the potential target genes of miR-452-5p by overlapping the circZNF652-regulated genes, miRDB, and RNA-seq data (Fig. 4A and 4B), and selected six strikingly and differentially expressed genes, including *ELF5*, *RhoA*, *CDK6*, *JAK2*, *PPP1R10*, and *SRSF3*. RT-qPCR analyses revealed that four of them were regulated by circZNF652 and *JAK2* RNA

exhibited the most robust alteration (Fig. 4C). To confirm whether these genes participate in the goblet cell metaplasia, we performed the *Muc5AC* luciferase reporter assays in 16HBE cells and indicated that only silence of *JAK2* expression significantly decreased the *Muc5AC* luciferase activities (Fig. 4D). We next constructed the *JAK2* wild type and mutant sequence at 3'-UTR and performed luciferase reporter assays in 16HBE cells to confirm the molecular binding of miR-452-5p to *JAK2* (Fig. 4E and 4F). Transfection of miR-452-5p mimics and its inhibitor into the 16HBE demonstrated that miR-452-5p mimics and miR-452-5p inhibitor robustly decreased and increased the *JAK2* mRNA, respectively (Fig. 4G). Likewise, immunostaining revealed that CC10-derived immune signals were obviously detected in the BALF cells of children with FBA or asthma, whereas JAK2 were significantly activated and highly expressed in the BALF cells of children with asthma but not with FBA (Fig. 4H-4I). The p-JAK2 and JAK2 protein levels were further investigated in 16HBE cells transfected with miR-452-5p inhibitor or si-circZNF652 and treated with IL-4. MiR-452-5p inhibitor or si-circZNF652 apparently increased or decreased both the basal and IL-4-induced p-JAK2, p-STAT6, and JAK2 levels, respectively (Fig. 4J-4M). Thus, JAK2/STAT6 signaling pathway is the downstream of circZNF652/miR-452-5p in the regulation of goblet cell metaplasia.

CircZNF652-miR-452-5p-JAK2 signaling pathway regulates goblet cell metaplasia in allergic airway epithelia in vivo

To investigate the in vivo role of circZNF652-miR-452-5p-JAK2 signaling pathway in goblet cell metaplasia, we generated OVA-induced mouse models with allergic airway inflammation (Fig. 5A). The inflammatory cells in BALFs were significantly increased after OVA challenge, among them, eosinophils were most strikingly increased. MiR-452-5p antagomir significantly induced the number of inflammatory cells, and abolished the negating effect of si-circZNF652 on the number of inflammatory cells in OVA-challenged mice (Fig. 5B and online repository figure E4A). In addition, the expression of mmu-miR-452-5p, JAK2, p-

JAK2, and p-STAT6 were apparently localized in the bronchial epithelia, and OVA challenge robustly induced the expression of JAK2, p-JAK2, and p-STAT6 but significantly decreased the expression of mmu-miR-452-5p (Fig. 5C and online repository figure E4C, E5B and E5D). MiR-452-5p antagomir apparently induced the expression of JAK2, p-JAK2, and p-STAT6, and robustly abolished the negating effects of si-circZNF652 on the expression of them in response to the OVA challenge (Fig. 5D and online repository figure E4D). Consistent with these findings, miRNA-452-5p antagomir significantly increased the PAS staining and PenH values, and robustly abolished the negating effects of si-circZNF652 on the PAS staining and PenH values in response to the OVA challenge (Fig. 5C, 5E and online repository figure E4B, E4E, E5A, E5C). Thus, silence of circZNF652 releases miR-452-5p to inhibit the *JAK2* expression and in turn inactivates the JAK2/STAT6 signaling in the regulation of allergic airway goblet cell metaplasia.

Splicing factor ESRP1 accelerates the biogenesis of circZNF652 in the regulation of JAK2/STAT6 signaling

Although it is well established that circRNAs are produced from a distinct back-splicing process, the details of circRNA biogenesis remain largely unknown²². Some studies point out that back-splicing of exons occurs when RBPs that bind to the flanking introns of the back-spliced exons, dimerize, bringing exons closer to each other¹³. To identify the underlying mechanism of circZNF652 production, we investigated the biogenesis of circZNF652 in 16HBE cells. Splicing factors (SFs) have been shown to regulate cell-type specific circRNA expression^{13, 14, 23}, we therefore supposed that SF may be involved in the generation of circZNF652. Since ESRP1, a member of RBP subgroup, has been showed to regulate the circularization of circRNAs^{23, 24}, we investigated the potential role of ESRP1 in the biogenesis of circZNF652. Immunostaining showed that ESRP1 was robustly upregulated in the BALF cells of children with asthma or in the OVA-challenged mouse lungs, as compared to in the

BALF cells of children with FBA or in the normal saline (NS)-challenged mouse lungs, and the expression pattern of ESRP1 was essentially the same as that of circZNF652 in the BALF cells of children with asthma or in the OVA-challenged mouse lungs (Fig. 6A and 6B). To examine whether ESRP1-binding sequences were vital for circZNF652 formation, we searched for ESRP1-binding sequences (GGT-rich)^{23, 25, 26} of flanking introns in circZNF652 and constructed the wild type minigenes and their mutant that harbored the mutation in the potential ESRP1-binding sites (Fig. 6C). RNA-binding protein immunoprecipitation (RIP) analyses showed that ESRP1 apparently bound to the wild type minigenes but not the mutants (Fig. 6D). Likewise, RT-qPCR analyses revealed that ESRP1 knockdown significantly decreased the RNA levels of circZNF652 as well as the protein levels of JAK2, p-JAK2, and p-STAT6 (Fig. 6E and 6F). Thus, splicing factor ESRP1 is required for the biogenesis of circZNF652 by binding to its flanking intron.

DISCUSSION

To the best of our knowledge, the present study is the first work that identifies the ESRP1 /circZNF652/miR-452-5p/JAK2/STAT6 signaling pathway as a hitherto uncharacterized mechanism controlling the goblet cell metaplasia, airway hypersecretion, and airway hyperresponsiveness in allergic airway inflammation. In this molecular event, splicing factor ESRP1 promotes the biogenesis of circZNF652, which sponges miR-452-5p to induce the expression of *JAK2* and in turn to activate JAK2/STAT6 signaling, resulting in the goblet cell metaplasia, airway hypersecretion, and hyperresponsiveness in mouse models with allergic airway inflammation and possibly in children with allergic asthma (Fig. 6G).

The high-speed development of next-generation sequencing and bioinformatics technology allowed to find the vital roles of circRNAs in different biological processes, especially in carcinogenesis^{27, 28}, neurogenesis^{29, 30}, and innate immune response^{31, 32}. Little is known about the functions of circRNAs in the goblet cell metaplasia of asthma, assessment of the molecular mechanism of circRNAs in the regulation of goblet cell metaplasia therefore offers a new opportunity for the asthma therapy. Though ZNF proteins are the important transcription factors associated with cell growth, proliferation, differentiation and apoptosis^{33, 34}, up to date, no study has showed the role of circZNF652 in asthma. In the present study, we identified circZNF652 derived from exons 3, 4 and 5 of *ZNF652* gene as an important circRNA involved in the pathogenesis of allergic airway remodeling.

CircZNF652 serves as an endogenous sponge to repress miR-452-5p activity, resulting in the induction of *JAK2* expression. Thus, the present study confirms that competing endogenous RNAs (ceRNAs) sponge for miRNAs through their binding sites, and that alteration in ceRNA abundance affects miRNAs to transcriptionally activate or inactivate their target genes³⁵. Abundant circRNAs serve as the miRNA sponges, and we have identified 8 likely miRNAs with multiple binding sites for circZNF652 by using computational algorithm. The accurate and

authentic interplay between circZNF652 and miR-452-5p is confirmed by Pull-down, RIP, luciferase reporter and FISH analyses, and reveals that miR-452-5p not only has a strong binding capacity with circZNF652 but also exhibits the vital functions in allergic airway remodeling. CircZNF652 serves as a miR-452-5p sponge and restrains miR-452-5p activity, and circZNF652 overexpression in airway epithelia becomes a sink for miR-452-5p, and releases the suppressive effect of miR-452-5p on JAK2/STAT6 signaling pathway.

Accumulating evidence has showed that several factors can modulate the biogenesis of circRNA from the precursor-mRNA, such as alternative splicing factors QKI¹³, muscleblind¹⁴, eukaryotic translation initiation factor 4A3³⁶, transcription factor Twist1³⁷, and YY1 super-enhancer³⁸. The present study indicates ESRP1 is involved in the biogenesis of circRNAs by acting as an important splicing factor. Consistent with this finding, previous studies have demonstrated that ESRP1 facilitates the circBIRC6 biogenesis in the human embryonic stem cell²³, and that ESRP1 facilitates the biogenesis of circUHRF1 in the oral squamous cell carcinoma³⁹. In the present study, we find that ESRP1 shares the GGT-rich region with the flanking intron of 3, 4 and 5 exons of ZNF652, whereas mutation of the ESRP1-binding motifs in the introns flanking of circZNF652 leads to an apparent reduction of circZNF652 biogenesis. These results imply that ESRP1 may directly modulate back-splicing to facilitate circZNF652 biogenesis by interacting with the potential binding motifs in flanking introns of circZNF652. However, the exact mechanism governing the upregulation of ESRP1 expression in allergic airway epithelia remains unknown.

Though we have found that EIF5, RhoA, JAK2, and SRSF3 are regulated by circZNF652, only JAK2 is participated in the goblet cell metaplasia. EIF5 is related with the proliferation and invasion of lung cancer cells⁴⁰. Our team have found that protein tyrosine phosphatase 2 (SHP2) and RhoA were robustly activated in the airway eosinophils of children with allergic asthma and of mouse models with allergic airway inflammation, and SHP2 regulates eosinophil

recruitment into lungs through RhoA/ROCK signaling in allergic asthma⁴¹. RhoA play an important role in regulating allergic airway inflammation and remodeling. In our present study, we had found that instillation of in vivo si-circZNF652-3 attenuated the number of inflammatory cells in BALFs, which may be resulted from the regulation of RhoA expression. SRSF3 is a multi-functional protein. It has been shown to be involved in multiple cellular functions, such as alternative splicing⁴², DNA repair⁴³, RNA export⁴⁴, alternative RNA polyadenylation⁴⁵, and protein translation⁴⁶. The relationship with asthma is not clear.

In conclusion, we demonstrated that upregulation of ESRP1-mediated circZNF652 biogenesis in the airway epithelia of children with asthma and of mouse model with allergic airway inflammation contributed to the goblet cell metaplasia by sponging the miR-452-5p to enhance the transcription of *JAK2* and the subsequent activation of JAK2/STAT6 signaling. Thus, the present study proposes a noncoding RNA based approach for the therapeutic intervention of chronic airway inflammation.

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Figure Legends

Fig. 1. CircRNA expression profiling recognizes circZNF652 as a potential regulator of asthma. (A) Hierarchical cluster analysis was conducted to obtain the overview of circRNA expression profiles between FBA and asthma. (B) The expression level of circZNF652 in 10 pairs of asthma and FBA control tissues. (C) Expression levels of circZNF652 was detected by RNA FISH in BALFs from children with FBA and asthma (n=10). (D) The expression level of mmu-circZNF652 in BALF cells of OVA- or normal saline (NS)-challenged mouse model (n=10). (E) Expression levels of circZNF652 was detected by RNA FISH in OVA- or normal saline (NS)-challenged mouse model (n=10). (F) Schematic illustration showing ZNF652 exons 3–5 circularisation to form circZNF652. The presence of circZNF652 was confirmed by Sanger sequencing. Arrow represents head-to-tail circZNF652 splicing sites. (G) The presence of circZNF652 was confirmed in 16HBE cells by RT-PCR. GAPDH was used as a negative control. (H) The expression of circZNF652 and ZNF652 mRNA in 16HBE cells treated with or without Rnase R were quantified by RT-qPCR. (I) RNA FISH showed the predominant localization of circZNF652 in the cytoplasm. CircZNF652 probes were labelled with Cy-3. Nuclei were stained with DAPI. **p<0.01 versus FBA or NS.

Fig. 2. CircZNF652 regulates airway epithelial remodeling in bronchial epithelial cells and mouse models with allergic airway inflammation. (A) 16HBE cells were treated with medium or 100 ng/ml IL-4, IL-5, and IL-13 for 24 h and then subjected to RT-qPCR assays of circZNF652 mRNA levels. (B and C) 16HBE cells were treated with the indicated concentrations of IL-4 for 24 h or 100 ng/ml IL-4 for the indicated times and then subjected to RT-qPCR analyses. (D) 16HBE cells were transfected with *Muc5AC* luciferase and Renilla luciferase reporter constructs. After transfection, cells were treated with 100 ng/ml IL-4 in the presence of si-circZNF652 or its control (si-NC) for 48 h and then subjected to dual-luciferase

assays. (E) Schematic representation of the OVA-induced mouse model with allergic airway inflammation. Intratracheal instillation of 50 μ l/mouse of si-circZNF652-3 or si-circRNA-NC at the concentration of 1 and 3 mg/ml was performed on day 22. (F) BALFs were prepared for cell counting and classification. (G) Lungs were subjected to paraffin-embedded sectioning for H&E and PAS staining. (H) Lungs were subjected for immunofluorescent staining. (I) Mice were subjected to determination of airway hyperresponsiveness in response to the stimulation of methacholine at the indicated concentrations. * p <0.05, ** p <0.01 versus NS/si-circRNA-NC; + p <0.05, ++ p <0.01 versus OVA/si-circRNA-NC. each n =10.

Fig. 3. CircZNF652 serves as a sponge for miR-452-5p in vitro. (A) RIP assay for circZNF652 levels in 16HBE cells transfected with Ago2. (B) High-throughput sequencing for differentially expressed miRNAs between FBA and asthma. (C) Schematic illustration to show the overlapping target miRNAs of circZNF652 predicted by miRNA Profile, Targetscan and MiRanda. (D) Lysates prepared from 16HBE cells transfected with circZNF652 or vector were subjected to RNA pull-down assays. (E and F) MiR-452-5p mimic, miR-23b-3p mimic, miR-34b-5p mimic, miR-182-5p mimic, or NC were co-transfected with a luciferase reporter construct of *Muc5AC* into 16HBE cells, or the expression of *Muc5AC* mRNA was detected by RT-qPCR. (G) MiR-452-5p mimic or NC was co-transfected with a luciferase reporter construct of *Muc5AC* and treated with 100 ng/ml IL-4 for 48 h. (H) 16HBE were transfected with miR-NC or miR-452-5p mimic and stimulated with or without 100ng/ml IL-4. (I) Schematic illustration demonstrates complementary to the miR-452-5p seed sequence with circZNF652. (J) MiR-452-5p mimic or NC were co-transfected with a luciferase reporter construct containing circZNF652 WT or circZNF652 MUT into 16HBE cells. (K) RNA FISH showed that circZNF652 was co-localized with miR-452-5p in 16HBE cells. (L and M) MiR-452-5p expression was detected by RNA FISH in airway epithelia of BALFs from children with

asthma or FBA, or in airway epithelia of mouse model with allergic airway inflammation (n=10). *p<0.05, **p<0.01 versus GAPDH, control probe and luc-circZNF652-WT+NC.

Fig. 4. JAK2 serves as a direct target of miR-452-5p. (A) Schematic illustration to show the overlapping of the target mRNAs of miR-452-5p predicted by miRDB, and circZNF652 regulated genes. (B) The differentially dysregulated mRNAs by circZNF652 knockdown was detected by RNA-seq in 16HBE cells (n=3). (C) Expression of 6 miRNAs were detected by RT-qPCR when circZNF652 was knocked down in 16HBE cells. (D) Si-NC, si-JAK2, si-EIF5, si-RhoA or si-SRSF3 were co-transfected with a luciferase reporter construct of *Muc5AC* into 16HBE cells. The luciferase reporter activities are shown. (E) Schematic illustration demonstrates complementary to the miR-452-5p seed sequence with JAK2. Green letters indicate mutated nucleotides. (F) MiR-452-5p mimic or NC were co-transfected with a luciferase reporter construct containing JAK2 WT or JAK2 MUT into 16HBE cells, and the luciferase reporter activities were assayed. (G) RT-qPCR analysis of JAK2 in 16HBE cells when treated with si-circZNF652 or miR-452-5p inhibitor. (H-I) Phospho-JAK2 and JAK2 expression were detected by immunofluorescence in CC10⁺ airway epithelia of BALFs from children with asthma or FBA (n=10). (J-M) Western blot analyses of JAK2 and STAT6 in 16HBE cells treated with si-circZNF652 or miR-452-5p inhibitor. *p<0.05, **p<0.01 versus si-NC and luc-JAK2-WT+NC.

Fig. 5. CircZNF652-miR-452-5p-JAK2 signaling pathway regulates goblet cell metaplasia in a mouse model with OVA-induced allergic airway inflammation. (A) OVA-sensitized mice were aerosolized with 1% OVA or an equal volume of normal saline (NS) for 30 min once daily for 7 days. After each OVA challenge (2 h), mice were intratracheally (i.t.) instilled with either 50 μ l/mouse of in vivo si-circZNF652-3 and/or mmu-miR-452-5p antagomir at the

concentration of 3 mg/ml or si-circRNA-NC and/or mmu-miRNA antagomir-NC. On day 29, mice were subjected to a variety of analyses and euthanized for sampling. (B) BALFs were prepared for cell counting and classification by a flow cytometer. (C and D) Lungs were subjected to paraffin-embedded sectioning for H&E and Periodic acid-Schiff (PAS) staining, immunofluorescence staining or western blotting analyses. (E) Mice were subjected to determination of airway hyperresponsiveness in response to the stimulation of methacholine (Mch) at the indicated concentrations. PenH, enhanced pause. * $p < 0.05$, ** $p < 0.01$ versus NS/Vehicle group; + $p < 0.05$, ++ $p < 0.01$ versus OVA/Vehicle group. each $n = 10$.

Fig. 6. Splicing factor ESRP1 facilitates the biogenesis of circZNF652. (A) ESRP1 expression was detected by immunofluorescence in airway epithelia of BALFs from children with asthma or FBA ($n = 10$). (B) ESRP1 expression was detected by immunofluorescence in airway epithelia of mouse models with allergic airway inflammation ($n = 10$). (C) Several ESRP1 motifs (GGT-rich) were found in the flanking of circZNF652. The minigenes of these motifs were constructed, including the wild type and mutants. (D) RNA-binding protein immunoprecipitation (RIP) presented the binding of ESRP1 with the motifs minigenes, including wild type and mutants. (E) RT-qPCR showed the circZNF652 expression in 16HBE cells transfected with ESRP1 silencing si-RNA or its control. (F) Western blotting analyses showed the ESRP1, p-JAK2, JAK2 and STAT6 expression in 16HBE cells transfected with ESRP1 silencing si-RNA or its control. (G) A working model of the present study. Splicing factor ESRP1 promotes the biogenesis of circZNF652, which sponges miR-452-5p to induce the expression of *JAK2* and in turn to activate JAK2/STAT6 signaling, resulting in the goblet cell metaplasia in allergic airway remodeling.

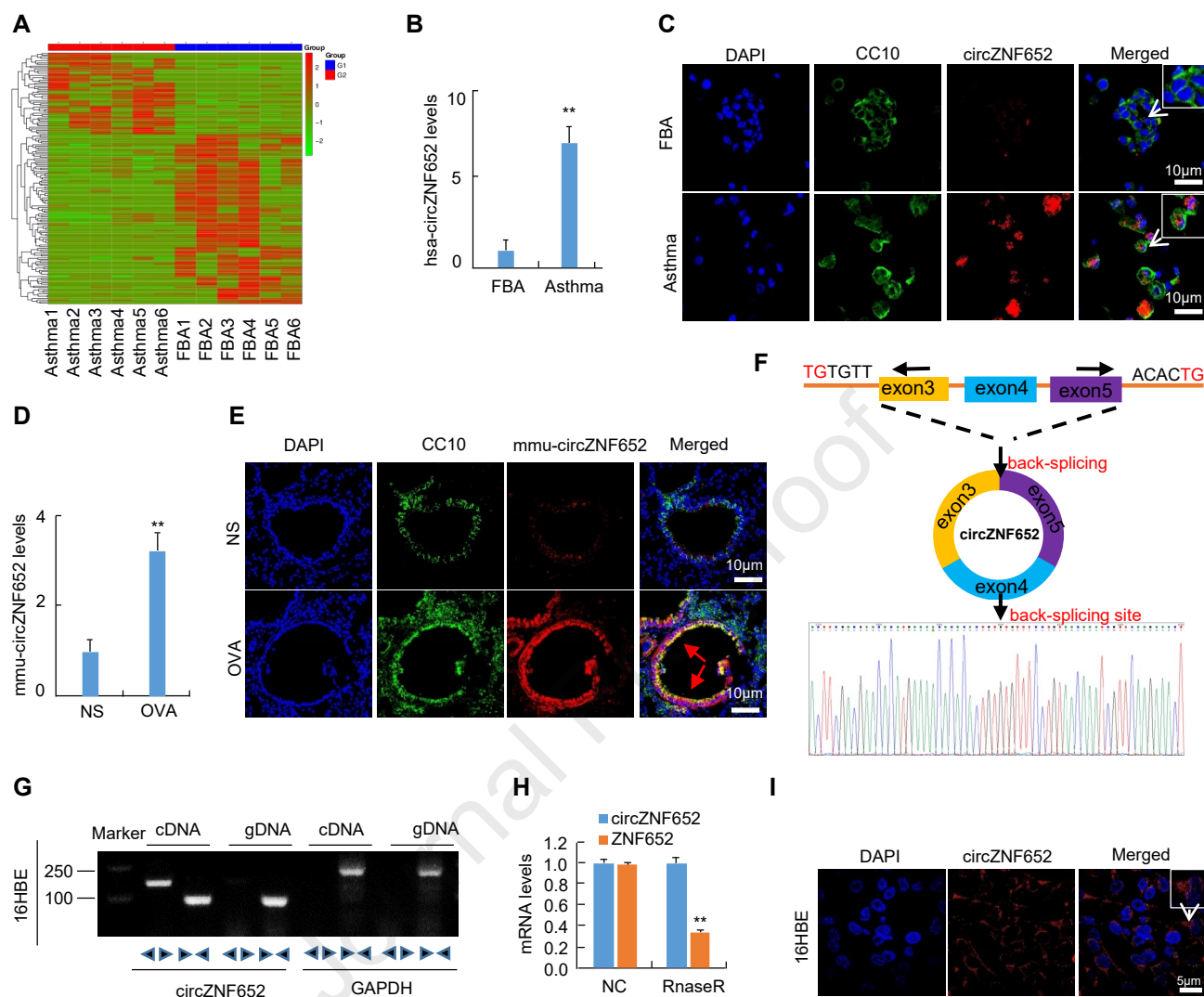


Fig. 1

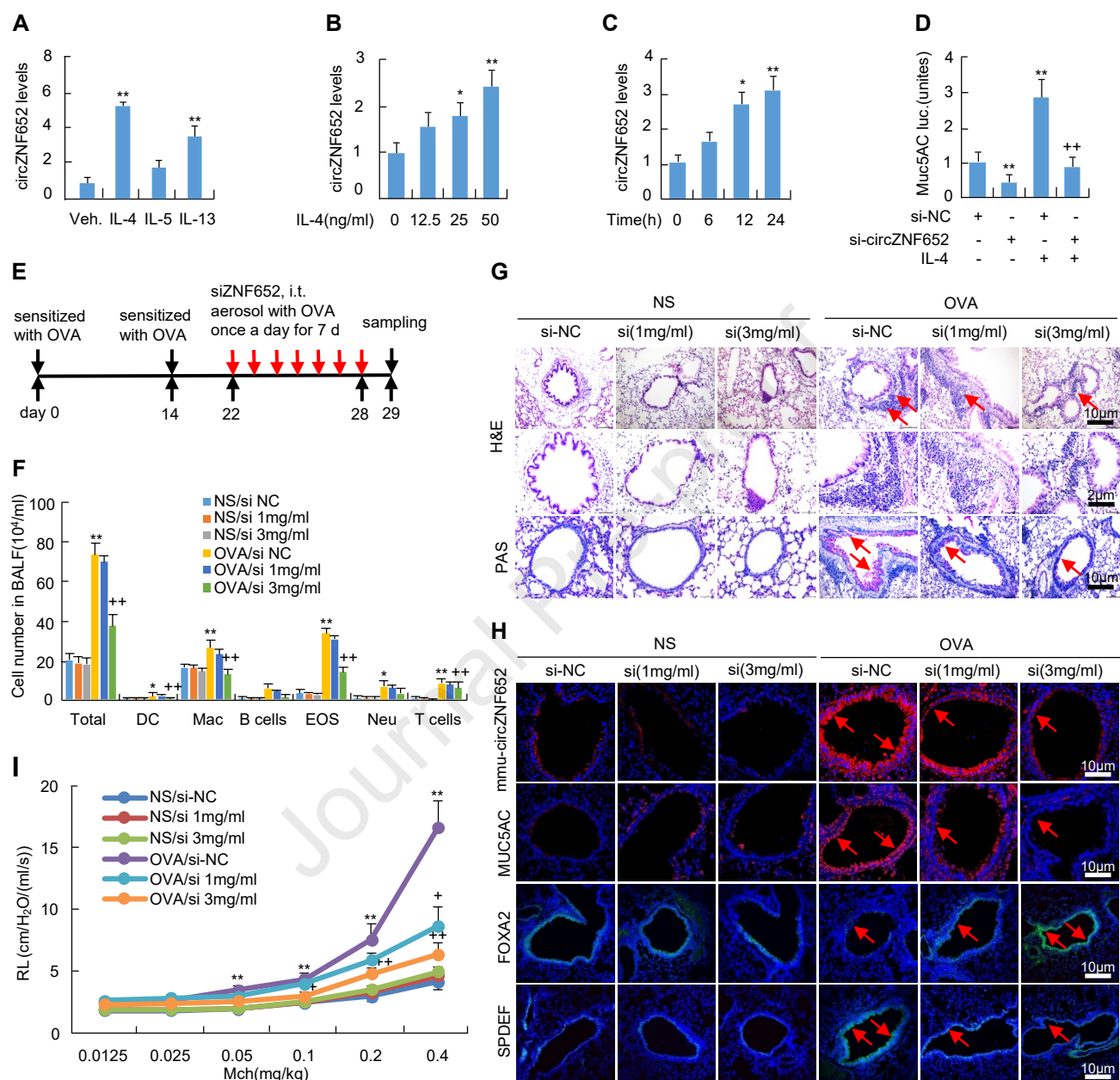


Fig. 2

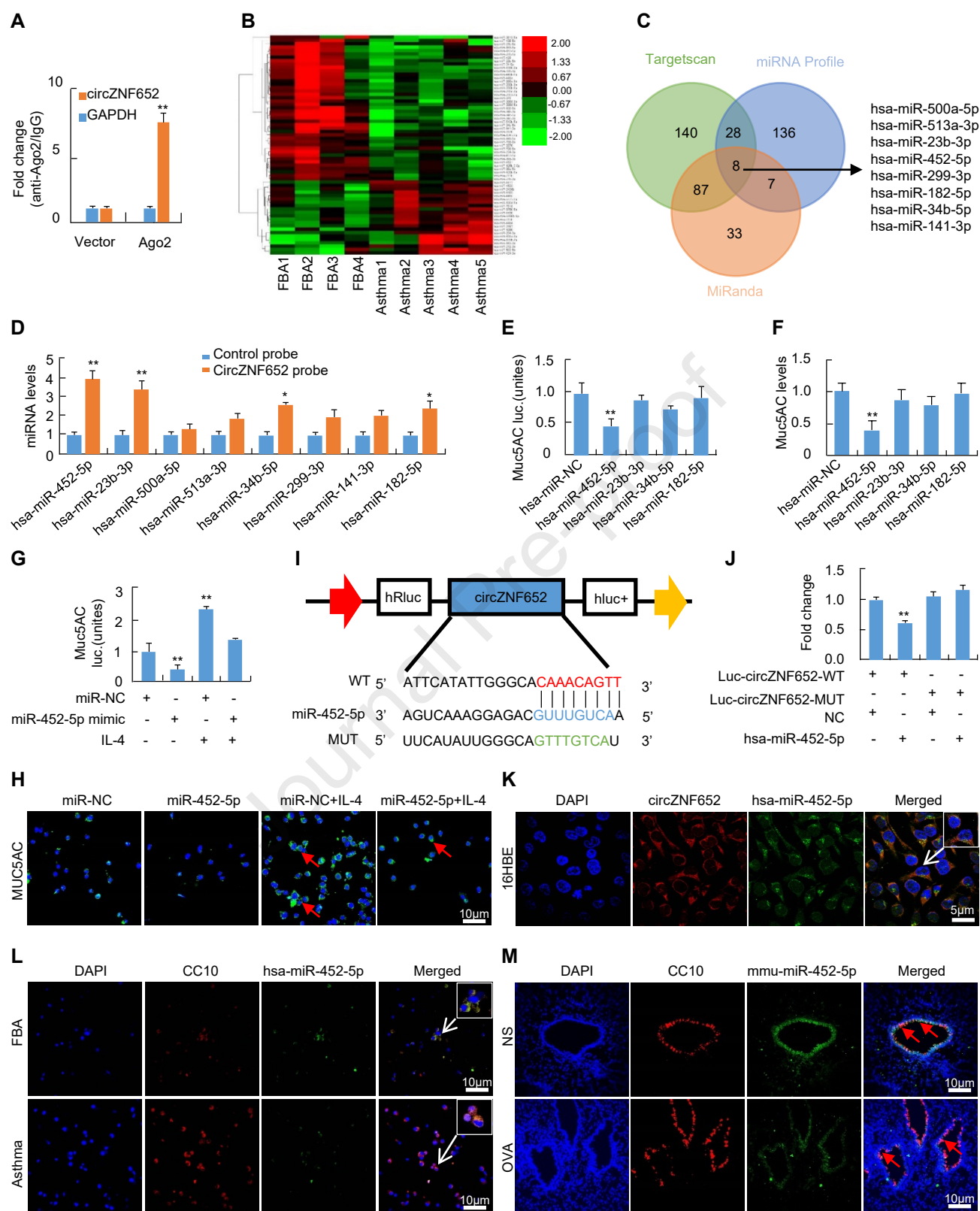


Fig. 3

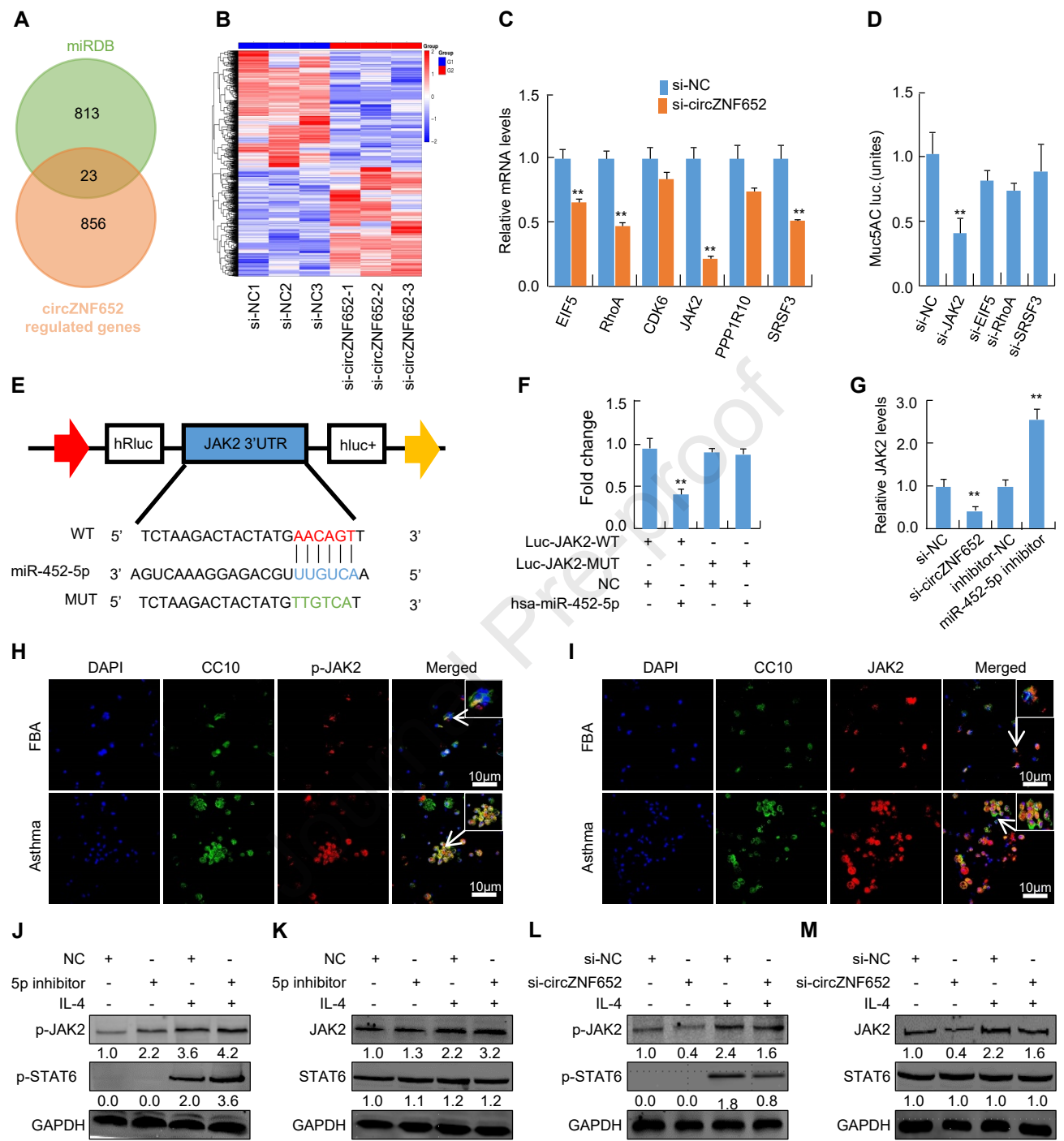


Fig. 4

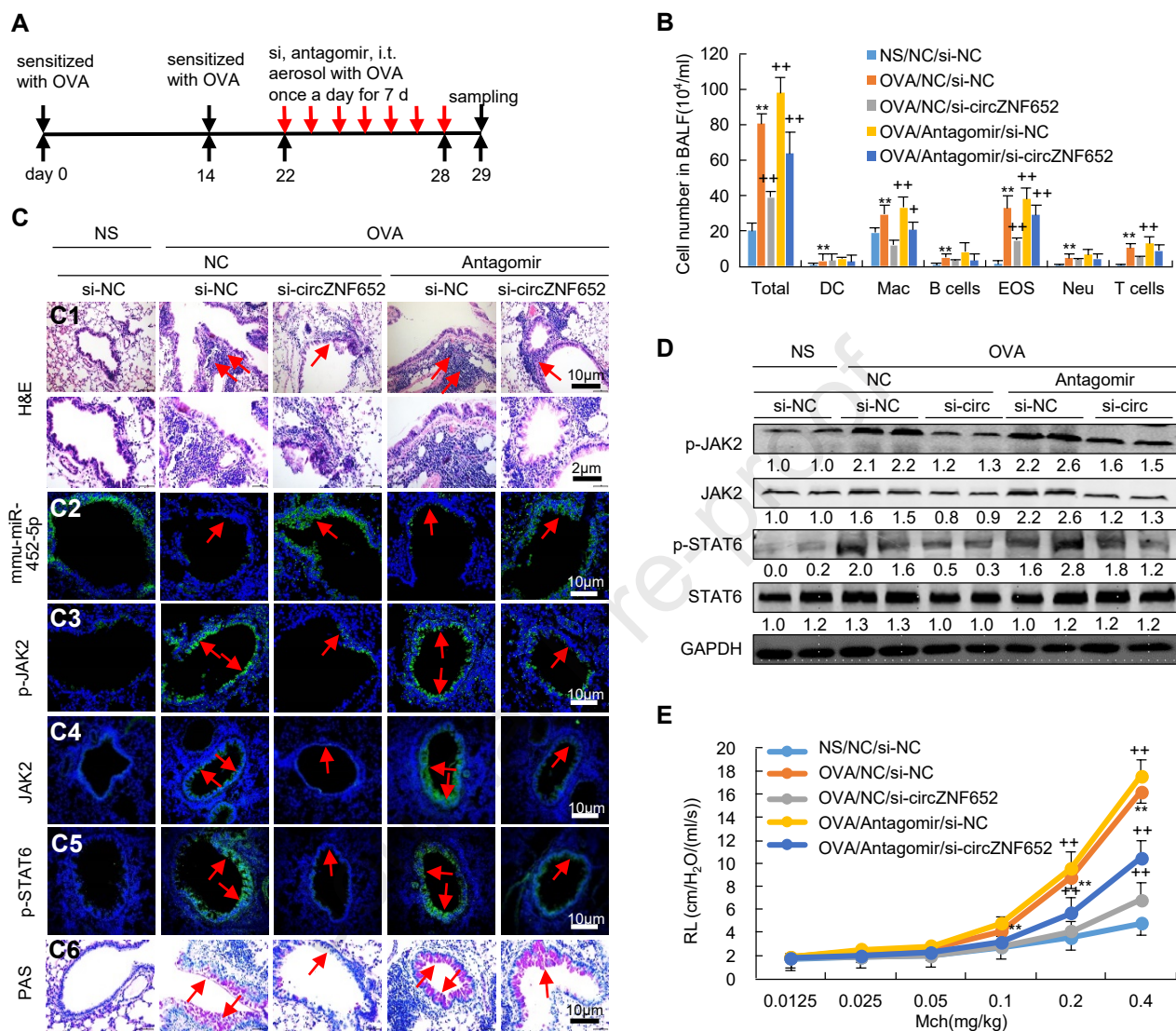


Fig. 5

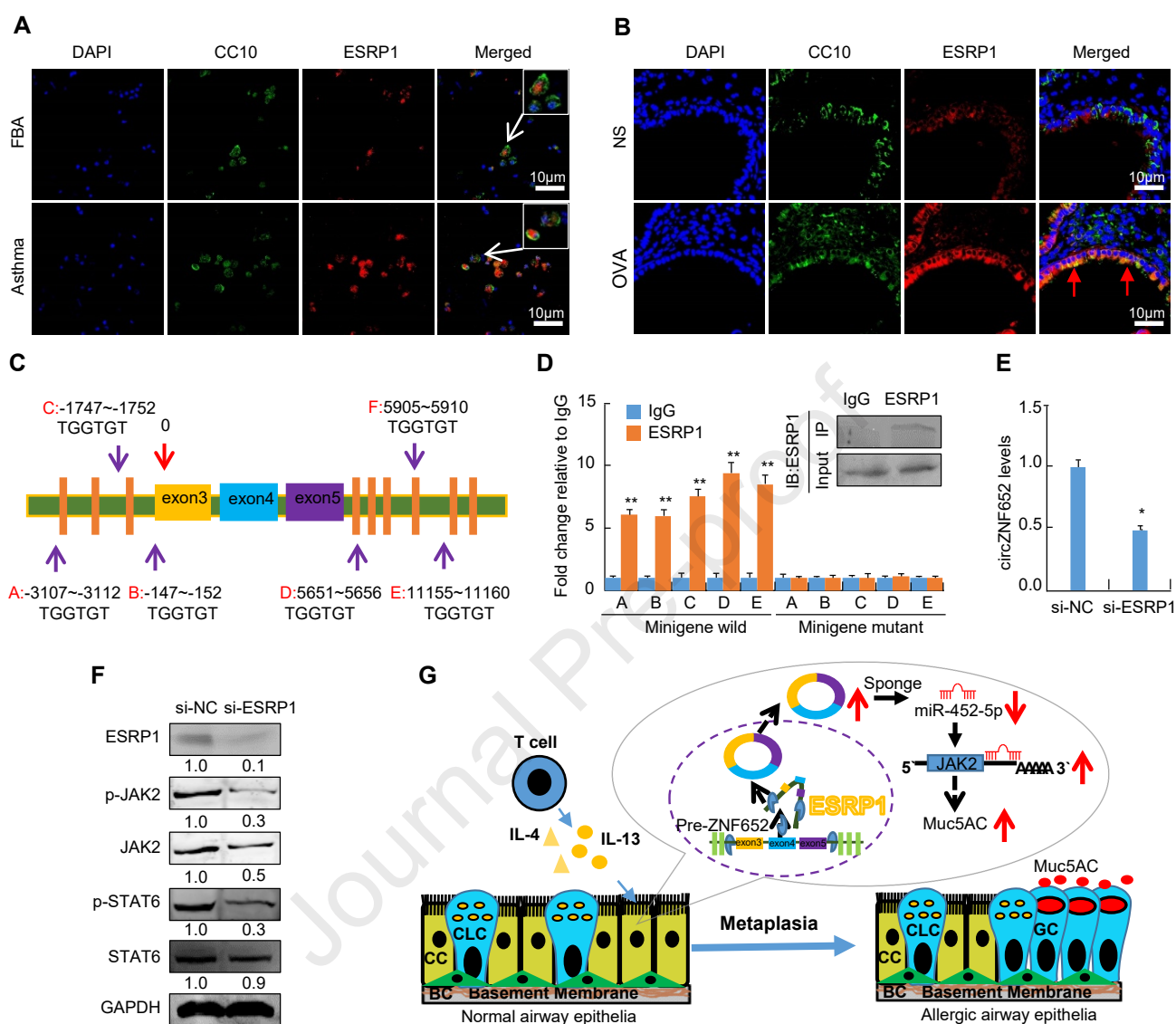


Fig. 6