Silencing IncRNA CDKN2B-AS1 Alleviates Childhood Asthma Progression Through Inhibiting ZFP36 Promoter Methylation and Promoting NR4A1 Expression

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Abstract—LncRNA cyclin-dependent kinase inhibitor 2B antisense RNA 1 (CDKN2B-AS1) was found to be upregulated in plasma of patients with bronchial asthma. This study aimed to explore the roles and mechanisms of CDKN2B-AS1 in childhood asthma. We found that CDKN2B-AS1 was upregulated and zinc finger protein 36 (ZFP36) mRNA was downregulated in blood samples of children with asthma compared with healthy controls as measured by RT-qPCR. Human bronchial epithelial cell line BEAS-2B was treated with LPS to induce inflammation model. Small interfering RNA against CDKN2B-AS1 (si-CDKN2B-AS1) was transfected into LPS-treated BEAS-2B cells, and we observed that CDKN2B-AS1 silencing increased cell viability and inhibited apoptosis and inflammation cytokine levels in LPS-treated BEAS-2B cells. Methylation-specific PCR, ChIP, and RIP assays indicated that CDKN2B-AS1 inhibited ZFP36 expression by recruiting DNMT1 to promote ZFP36 promoter methylation. Co-immunoprecipitation (Co-IP) assay verified the interaction between ZFP36 and nuclear receptor subfamily 4 group A member 1 (NR4A1) proteins. Then rescue experiments revealed that ZFP36 knockdown reversed the effects of CDKN2B-AS1 silencing on BEAS-2B cell functions. ZFP36 overexpression facilitated apoptosis, inflammation, and p-p65 expression in BEAS-2B cells, while NR4A1 knockdown reversed these effects. Additionally, CDKN2B-AS1 silencing alleviated airway hyperresponsiveness and inflammation in ovalbumin (OVA)-induced asthma mice. In conclusion, silencing IncRNA CDKN2B-AS1 enhances BEAS-2B cell viability, reduces apoptosis and inflammation in vitro, and alleviated asthma symptoms in OVA-induced asthma mice in vivo through inhibiting ZFP36 promoter methylation and NR4A1-mediated NF-κB signaling pathway.

KEY WORDS: LncRNA CDKN2B-AS1; childhood asthma; ZFP36; NR4A1; NF-κB signaling pathway.

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Abbreviations LncRNAs; Long non-coding RNAs; Inc-BAZ2B, LncRNA bromodomain adjacent to zinc finger domain 2B; RMRP, RNA component of mitochondrial RNAase P; CDKN2B-AS1, Cyclin-dependent kinase inhibitor 2B antisense RNA 1; ANRIL, Anti-sense non-coding RNA in the INK4 locus; ZFP36, Zinc finger protein 36; NR4A1, Nuclear receptor subfamily 4 group A member 1; ELISA, Enzyme-linked immunosorbent assay; TNF-α, Tumor necrosis factor-α; IL-1β, Interleukin-1β; IL-6, Interleukin-6; MSP, Methylation-specific PCR; ChIP, Chromatin immunoprecipitation; RIP, RNA immunoprecipitation; Co-IP, Co-immunoprecipitation; OVA, Ovalbumin; BALF, Bronchoalveolar lavage fluid; HNECs, Human nasal epithelial cells; ADAM10, A disintegrin and metalloprotease 10
INTRODUCTION

Asthma is a common chronic inflammatory airway disease, which is characterized by various typical clinical symptoms, such as coughing, wheezing, chest tightness, shortness of breath, and dyspnea [1]. The pathological features of asthma include persistent airway inflammation, inflammatory cell infiltration, and release of pro-inflammatory cytokines and mediators [2]. Asthma usually develops during childhood and severely affects the lung function and quality of life of children. Diagnosis and treatment for childhood asthma have always remained a great challenge because of its complex pathogenesis and multiple triggers, such as genetic, epigenetic, developmental, and environmental factors [3]. Although most children with asthma can relieve asthma symptoms and achieve adequate asthma control through avoidance of triggering factors and medication, there is no available complete cure for childhood asthma up to now. Thus, more efforts are urgently required to clarify the molecular mechanism of childhood asthma progression and develop effective therapeutic targets.

Long non-coding RNAs (lncRNAs) are non-coding RNAs with more than 200 nucleotides in length, which have been revealed to play crucial roles in the aberrant regulation and pathogenesis of airway diseases. In recent years, a large number of lncRNAs related to the occurrence and development of childhood asthma have been continuously discovered and identified. For instance, lncRNA bromodomain adjacent to zinc finger domain 2B (lnc-BAZ2B) was found to be significantly upregulated in peripheral blood mononuclear cells of children with asthma, and lnc-BAZ2B promoted M2 macrophage activation and inflammation in children with asthma in a cockroach allergen extract-induced asthma model through stabilizing the pre-mRNA of its cis target gene BAZ2B [4]. A study revealed the pro-inflammatory and pro-fibrotic role of lncRNA RMRP in pediatric asthma through targeting microRNA-206/CCL2 axis [5]. Moreover, current evidence showed that lncRNA CDKN2B-AS1, also known as lncRNA ANRIL, was upregulated in plasma samples of bronchial asthma patients compared with healthy controls, and lncRNA CDKN2B-AS1 is potentially indicative of disease exacerbation, severity, and inflammation for bronchial asthma [6]. More importantly, a recent review has described the role of lncRNA CDKN2B-AS1 and highlighted its potential as a biomarker in various non-cancerous lung diseases [7]. However, whether lncRNA CDKN2B-AS1 is involved in the development of childhood asthma is still poorly understood.

Zinc finger protein 36 (ZFP36), also known as tristetraprolin or TTP, is identified as a prominent inflammatory regulator related to autoimmunity, which may suppress inflammatory response by regulating the mRNA stability of several important inflammatory cytokines [8]. More notably, a previous study analyzed the gene expression profiles in the blood of moderate asthma patients and healthy controls, and identified ZFP36 as one of the optimal asthma biomarkers [9]. Recent evidence demonstrated that ZFP36 expression was downregulated in hepatocellular carcinoma cells by methylation of a specific single CpG site in ZFP36 promoter, indicating that ZFP36 expression is affected by epigenetics modification [10]. Nuclear receptor subfamily 4 group A member 1 (NR4A1), also known as Nur77, is a member of the NR4A subfamily of nuclear hormone receptors, which plays critical roles in inflammatory diseases. More importantly, gene set enrichment analyses and ingenuity pathway analyses identified that NR4A1 was associated with COPD and allergic airway inflammatory disease [11]. Additionally, previous evidence showed that NR4A1 relieved ovalbumin (OVA)-induced airway inflammation response in a mouse model of allergic airway disease by counteracting NF-κB signaling in lung epithelial cells [12]. These findings indicated NR4A1 might be an important regulator in asthma.

In this study, we found that lncRNA CDKN2B-AS1 was upregulated in blood samples of children with asthma compared with healthy controls. Then we further explored the role of lncRNA CDKN2B-AS1 in childhood asthma progression in vivo and in vitro. This study revealed a novel regulatory mechanism among lncRNA CDKN2B-AS1, ZFP36, and NR4A1 in childhood asthma progression.

METHODS

Clinical Samples

In this study, a total of 30 children with asthma (18 males and 12 females, with an average age of 7.1 ± 3.1 years) and 30 healthy children (20 males and 10 females, with an average age of 7.7 ± 2.8 years) were recruited from Nanyang Central Hospital. Exclusion criteria were children having heart, liver, and kidney diseases, malignant hematological diseases, tumors diseases,
or other lung diseases. Blood samples were collected from children with asthma before treatment and health control during health examination, and plasma were isolated by centrifugation at 1000 g for 15 min and then stored at −80 °C. This study was approved by the Ethics Committee of Nanyang Central Hospital, and informed consent was obtained from each patient involved in this study.

Animals

Female BALB/c mice (6–8 weeks old, 20 ± 2 g) were obtained from the Laboratory Animal Center of Zhengzhou University. Mice were maintained in sterile cages under standard conditions (12-h light/dark cycle; temperature 22–25 °C; humidity, 55–60%) and free access to standard pellet food and water. All animal experiments in this study were approved by the Animal Ethics Committee of Nanyang Central Hospital.

Cell Lines and Culture

The human bronchial epithelial cell line BEAS-2B was obtained from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA), 100 μg/mL penicillin, and 100 μg/mL streptomycin (Sigma, St. Louis, MO, USA), and maintained with 5% CO₂ at 37 °C. When cells were grown to over 80% confluence, 1 μg/mL LPS (Sigma, St. Louis, MO, USA) was added to the BEAS-2B cell medium and incubated for 24 h.

Cell Transfection

Overexpression plasmids of lncRNA CDKN2B-AS1 (pcDNA-CDKN2B-AS1), ZFP36 (pcDNA-ZFP36), small interfering RNAs targeting CDKN2B-AS1 (si-CDKN2B-AS1) and ZFP36 (si-ZFP36), and their corresponding negative controls (vector and scramble) were obtained from RiboBio (Guangzhou, China). Cells were seeded in 6-well plates at a density of 2 × 10⁵ cells/mL, and when reaching 70% confluence, cell transfection was performed by using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Cells were collected after transfection for 48 h for further experiments.

RNA Extraction and RT-qPCR

The total RNA in plasma was extracted by using QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany), and the total RNA of BEAS-2B cells or tissues was extracted by using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The cDNA synthesis was performed by using a Prime Script RT reagent Kit (Takara, Dalian, China). RT-qPCR was conducted with SYBR Premix Ex Taq II (Takara, Dalian, China) on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) under the following conditions: 95 °C for 1 min, 35 cycles of 95 °C for 20 s, 56 °C for 10 s, and 72 °C for 15 s. PCR system contained 12.5 μL of SYBR Premix Ex Taq II, 1.0 μL of RT primer, 1 μL of cDNA sample, and 10.5 μL of double distilled H₂O. Relative gene expression was calculated normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and calculated by the 2⁻ΔΔCT method. Primers were as follows: lncRNA CDKN2B-AS1 (forward: 5′-TGC TCT ATC CGC CAA TCA GG-3′, reverse: 5′-GGG CCT CAG TGG CAC ATA CC-3′), GAPDH (forward: 5′-CTG GGC TAC ACT GAG CAC C-3′, reverse: 5′-AAG TGG TCG TGG CAC ATA CC-3′).

CCK-8 Assay

Cell viability was measured by using the Cell Counting Kit-8 (CCK-8, Dojindo, Japan) assay. Briefly, after treatment, BEAS-2B cells (1 × 10⁴ cells/well) were seeded into 96-well plates and cultured for 48 h. Then 10 μL of CCK-8 solution was added to wells and incubated for 2 h at 37 °C. The absorbance of each well was measured at 450 nm with a microplate reader (Bio-Rad, Hercules, CA).

Cell Apoptosis Analysis

BEAS-2B cells were collected and stained with the Annexin V-FITC/PI apoptosis detection kit (BD Biosciences, San Jose, CA, USA). Briefly, cells were resuspended in 1 × binding buffer (1 × 10⁶ cells/mL). Then 5 μL Annexin V-FITC and 5 μL PI were added into cell suspension and incubated for 15 min in the dark at room temperature. Cell apoptosis was detected by using a flow cytometer (BD Biosciences, San Jose, CA, USA) according to the manufacturer’s instructions.
Enzyme-Linked Immunosorbent Assay (ELISA)

Following the indicated treatments, the supernatant of BEAS-2B cells or BALF was collected, and then the concentrations of inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) were determined with the commercial ELISA kits purchased from R&D systems according to the manufacturer’s instructions.

Western Blot Analysis

Proteins were extracted from BEAS-2B cells and quantified using the BCA method (Millipore, Billerica, MA, USA). Then equal amount of protein was subjected to 10% SDS-PAGE at 70 V for 30 min, then 120 V for 90 min. And the protein bands were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) at 300 mA for 2 h. The membranes were blocked with 5% nonfat milk for 1 h at room temperature and then incubated overnight at 4 °C with the following primary antibodies obtained from Abcam (Cambridge, UK): rabbit polyclonal anti-ZFP36 antibody (1:1000, ab83579), rabbit polyclonal anti-NR4A1 antibody (1:500, ab13851), rabbit monoclonal anti-p65 antibody (1:1000, ab32536), and rabbit polyclonal anti-GAPDH antibody (1:2500, ab9485), followed by incubation with horseradish peroxidase (HRP)–conjugated goat anti-rabbit IgG (1:2000, ab6721) for 1 h. Subsequently, the protein bands were visualized with ECL detection reagents and analyzed with ImageJ software (National Institutes of Health, Bethesda, MA, USA).

Methylation-Specific PCR (MSP)

MS-PCR was used to detect the methylation status of the ZFP36 promoter. Cell DNA extraction was conducted with the genomic DNA extraction kit (Tiangen Biochemistry Technology Co., Ltd., Beijing, China) according to the manufacturer’s instructions. The DNA concentration was determined using a UV spectrophotometer. The extracted DNA (10 μg) was added with 5.5 μL of 3 mol/L NaOH to denature at 37 °C for 10 min. Next, DNA was added with 20 μL of 10 mM hydroquinone and 520 μL of 40.5% sodium hydroxide sulfite, and then covered by 200 μL mineral oil. Then the modified DNA was purified by wizard DNA column. The purified solution was added with 5.5 μL of 3 mol/L NaOH to denature for 10 min, and then 5.5 μL of 3 M sodium acetate and 120 μL cold absolute ethanol were added to precipitate and recycle DNA. MS-PCR was conducted using an ABI7500 quantitative PCR instrument (ABI Company, Oyster Bay, NY). The reaction products were then analyzed by agarose gel electrophoresis and imaged with a gel electrophoresis imaging analysis system.

Chromatin Immunoprecipitation (ChIP)

EZ-Magna ChiP TMA kit (Millipore, Billerica, MA) was employed for chromatin immunoprecipitation (ChIP) analysis following the manufacturer’s guideline. Briefly, BEAS-2B cells were cross-linked with 1% formaldehyde for 10 min, and then 125 mM glycine was added to terminate the crosslinking. Next, cells were lysed in lysis buffer, and chromatin fragments at 200–1000 bp were obtained by cracking the cells through ultrasound. The supernatant was centrifuged and the fragments were collected in three tubes, which were supplemented with the target protein specific antibody (DNMT1, ab13537, Abcam) or the negative control antibody (IgG, ab10948, Abcam) for incubation at 4 °C overnight. The DNA–protein complex was precipitated with Protein Agarose/Sepharose. The precipitated DNA fragments were purified and subjected to RT-qPCR analysis.

RNA Immunoprecipitation (RIP) Assay

The binding of lncRNA CDKN2B-AS1 with DNMT1 was determined by using the Magna RIP RNA-Binding Protein Immunoprecipitation kits (Merck Millipore, Billerica, MA). Briefly, cells were lysed by using a RIPA lysate buffer (Beyotime Biotechnology Co., Shanghai, China) for 5 min, and the supernatant was collected by centrifugation at 4 °C. Subsequently, 50 μL of protein A/G-beads was resuspended with 100 μL of RIP wash buffer, and 5 μg of anti-DNMT1 antibody (ab13537, 1:100, Abcam) or NC antibody (IgG, ab10948) was added and incubated for 30 min at the room temperature. After washed, the protein A/G-bead-antibody complexes were resuspended with 900 μL of RIP wash buffer and incubated with 100 μL of supernatant overnight at 4 °C. After immunoprecipitation, the protein A/G-beads were collected and washed with RIP wash buffer and deposited 5 times. Finally, the protein A/G-bead-protein complexes were blended with proteinase K to extract RNA. Relative RNA expression was analyzed with RT-qPCR.
Co-Immunoprecipitation (Co-IP) Assay

BEAS-2B cells were lysed with RIPA buffer (Beyotime, Shanghai, China), and the supernatant was collected and incubated with ZFP36 antibody at 4 °C overnight. Then the mixture was incubated with 100 μL of protein A/G agarose beads (Takara Biotechnology, Dalian, China) overnight at 4 °C. Subsequently, the agarose beads-antigen–antibody complex was collected by instantaneous centrifugation and washed with PBS for three times. Next, the complex was boiled with protein loading buffer for 5 min. The supernatant was collected by centrifugation and analyzed by using Western blot to detect the expression of interaction proteins.

Animal Experimental Protocols

BALB/c mice were randomly divided into three groups (n = 8 per group): control group, OVA group, and OVA + si-CDKN2B-AS1 group. On days 1 and 14, the mice were sensitized with 20 μg of ovalbumin (OVA) with 2 mg aluminum hydroxide adsorbed in 200 μL of PBS by intraperitoneal injection. From days 21 to 23, mice were challenged by intranasal inhalations of 100 μg OVA adsorbed in 20 μL of PBS once a day. For si-CDKN2B-AS1 treatment, 100 μg of si-CDKN2B-AS1 was administered daily via intraperitoneal injection on days 21 to 23. For the NC group, mice were treated with an equal volume of PBS by using the same method. The mice were euthanized 24 h after the last challenge.

Measurement of Airway Hyperresponsiveness

Airway hyperresponsiveness (AHR) was detected by noninvasive whole-body plethysmography (Model PLY 3211; Buxco, Sharon, CT, USA). Within 24 h following the final OVA challenge, the mice in all groups were treated with 0, 5, 10, 25, and 50 mg/mL methacholine aerosol for 3 min, followed by 2 min of rest, and the enhanced pause (Penh) value of unrestrained mice within 5 min was recorded.

Collection of Bronchoalveolar Lavage Fluid (BALF) and Cell Counting

After mice were anesthetized, the tracheas were cannulated and lavaged with 0.8 mL aliquots of cold PBS twice to collect bronchoalveolar lavage fluid (BALF). The BALF samples were immediately centrifuged and kept at −80 °C. The cell pellets from BALF were resuspended in PBS (0.5 mL). Total cell counting was done with a hemocytometer. And the Kwik-Diff staining set (Thermo, USA) was used for counting of differential cell counts (eosinophils, macrophages, neutrophils, and lymphocytes) in BALF according to the manufacturer’s instructions.

Histopathological Analysis

Lung tissues were collected and fixed in 10% neutral buffered formalin, and then embedded in paraffin and sliced. Paraffin sections were stained with hematoxylin and eosin (H&E) using a standard protocol and analyzed by light microscopy.

Measurement of the Level of OVA-Specific IgE in Serum

On day 24 after the last OVA challenge, mice were euthanized and the blood samples were collected by puncturing the vena cava. The blood samples were centrifuged at 1000 g for 10 min to obtain serum samples. The level of OVA-specific immunoglobulin E (IgE) in serum was measured by ELISA kits (Abcam, Cambridge, UK) according to the manufacturer’s instructions.

Statistical Analysis

Data analysis was performed by SPSS version 22.0 software. Experimental results from three times independent experiments were presented as mean ± SD. Data distribution normality and homogeneity of variance were assessed using the Shapiro–Wilk normality test and Levene test, respectively. A one-way analysis of variance (ANOVA) or Kruskal–Wallis non-parametric test was applied for evaluating the significance among multiple groups, and parametric t-test and nonparametric t-test were performed to assess the significance between two groups, according to the data normal distribution and homogeneity of variances. ANOVA followed by Tukey HSD test, and nonparametric t-test followed by the post hoc analysis with Mann–Whitney U test. P < 0.05 was considered statistically significant.
RESULTS

LncRNA CDKN2B-AS1 Was Upregulated and ZFP36 Was Downregulated in Children with Asthma

To investigate whether CDKN2B-AS1 is involved in childhood asthma progression, we firstly detected the expression of CDKN2B-AS1 in children with asthma and healthy controls by using RT-qPCR. As shown in Fig. 1a, CDKN2B-AS1 was upregulated in blood samples of children with asthma compared with that in healthy controls ($P < 0.0001$). Moreover, we also found that the expression of ZFP36 mRNA was downregulated ($P < 0.0001$, Fig. 1b). Interestingly, we further found that CDKN2B-AS1 expression was negatively correlated with ZFP36 expression ($P < 0.0001$, Fig. 1c), which may imply a potential regulatory relationship between CDKN2B-AS1 and ZFP36 in the development of childhood asthma.

Silencing CDKN2B-AS1 Promoted BEAS-2B Cell Viability and Inhibited Apoptosis and Inflammation

To further explore the roles of CDKN2B-AS1 in childhood asthma progression, BEAS-2B cells were treated with LPS to induce inflammation model, and then si-CDKN2B-AS1 was transfected into BEAS-2B cells. RT-qPCR results showed that CDKN2B-AS1 expression was upregulated in LPS-treated BEAS-2B cells, while transfection of si-CDKN2B-AS1 decreased CDKN2B-AS1 expression ($P < 0.0001$, Fig. 2a). CCK-8 assay showed that BEAS-2B cell viability was inhibited by LPS treatment, but promoted by silencing CDKN2B-AS1 ($P < 0.0001$, Fig. 2b). Then we found that LPS treatment promoted BEAS-2B cell apoptosis, while silencing CDKN2B-AS1 inhibited LPS-induced cell apoptosis ($P < 0.0001$, Fig. 2c). Moreover, LPS treatment promoted the secretion of inflammatory cytokines including TNF-α, IL-1β, and IL-6 in BEAS-2B cells, while silencing CDKN2B-AS1 inhibited the secretion of inflammatory cytokines ($P < 0.0001$, Fig. 2d−f). All results indicated that silencing CDKN2B-AS1 promotes BEAS-2B cell viability and inhibits apoptosis and inflammation.

CDKN2B-AS1 Reduced ZFP36 Expression by Promoting ZFP36 Promoter Methylation

To investigate the regulatory relationship and mechanism between CDKN2B-AS1 and ZFP36, we first transfected pcDNA-CDKN2B-AS1 or si-CDKN2B-AS1 into BEAS-2B cells; the transfection efficiency of pcDNA-CDKN2B-AS1 and si-CDKN2B-AS1 is shown in Fig. 3a ($P < 0.0001$). Moreover, transfection of pcDNA-CDKN2B-AS1 significantly decreased ZFP36 expression, while transfection of si-circ_0001756 increased ZFP36 mRNA and protein expression in BEAS-2B cells ($P < 0.0001$, Fig. 3b). Subsequently, CpG islands in the ZFP36 promoter region were analyzed using a 3000-bp fragment in the ZFP36 promoter region via the MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) website. The data indicated that the CpG islands existed in the ZFP36 promoter region (Fig. 3c), suggesting that

Fig. 1 LncRNA CDKN2B-AS1 is upregulated and ZFP36 is downregulated in children with asthma. A total of 30 children with asthma (18 males and 12 females, with an average age of 7.1 ± 3.1 years) and 30 healthy children (20 males and 10 females, with an average age of 7.7 ± 2.8 years) were recruited in this study. RT-qPCR was performed to detect the expression of CDKN2B-AS1 (a) and ZFP36 mRNA (b) in blood samples of children with asthma and healthy controls. (c) CDKN2B-AS1 expression was negatively correlated with ZFP36 expression in children with asthma. Data were presented as mean ± SD. a and b: The significance of differences was estimated by nonparametric t-test followed by the post hoc analysis with Mann–Whitney U test.
the expression of ZFP36 was influenced by promoter methylation. MS-PCR was further performed to investigate the methylation of ZFP36 promoter in BEAS-2B cells after transfection. As presented in Fig. 3d, ZFP36 methylation was enhanced following CDKN2B-AS1 overexpression and reduced following CDKN2B-AS1 silencing in BEAS-2B cells, and ZFP36 methylation was inhibited by addition of a DNA methyltransferase inhibitor (5-Aza-CdR), but rescued after CDKN2B-AS1 overexpression. Furthermore, ChIP results showed that DNA methyltransferase 1 (DNMT1) was enriched in the promoter region of ZFP36, and CDKN2B-AS1 overexpression promoted the
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Fig. 3 CDKN2B-AS1 promotes ZFP36 promoter methylation by recruiting DNMT1. BEAS-2B cells were transfected with pcDNA-CDKN2B-AS1, si-CDKN2B-AS1, and their corresponding negative controls, respectively. a The transfection efficiency of pcDNA-CDKN2B-AS1 and si-CDKN2B-AS1 was measured by RT-qPCR. b RT-qPCR and Western blotting were used to detect the expression of ZFP36. c The distribution of CpG islands within the ZFP36 promoter region was analyzed by the MethPrimer website. d MSP was adopted to detect the methylation level of the DPYD promoter region in BEAS-2B cells after CDKN2B-AS1 overexpression or silencing, and addition of DNA methyltransferase inhibitor 5-Aza-CdR (U, unmethylation; M, methylation; 5-Aza-CdR, 5-Aza-2′-deoxycytidine). e ChIP was employed to detect the enrichment of DNMT1 within the ZFP36 promoter region in BEAS-2B cells after CDKN2B-AS1 overexpression. f RIP verified the results of CDKN2B-AS1 binding to DNMT1. Data were presented as mean ± SD. a and b: The significance of differences was estimated by one-way ANOVA test; e and f: the significance of differences was estimated by parametric t-test.
enrichment of DNMT1 in ZFP36 promoter ($P < 0.0001$, Fig. 3e). RIP assay further illustrated that compared with IgG treatment, DNMT1 significantly increased the enrichment of CDKN2B-AS1 ($P < 0.0001$, Fig. 3f). Collectively, the obtained results demonstrated that CDKN2B-AS1 reduced ZFP36 expression by promoting ZFP36 methylation.

CDKN2B-AS1 Regulated BEAS-2B Cell Viability, Apoptosis, and Inflammatory Response by Inhibiting ZFP36 Expression

To explore whether CDKN2B-AS1 exerted its functions in childhood asthma progression by regulating ZFP36 expression, si-CDKN2B-AS1 was transfected into LPS-treated BEAS-2B cells alone or together with si-ZFP36. The results showed that LPS treatment reduced ZFP36 expression, and transfection of si-CDKN2B-AS1 increased ZFP36 mRNA and protein expression in LPS-treated BEAS-2B cells, while transfection of si-ZFP36 reversed this effect ($P < 0.0001$, Fig. 4a). Moreover, CCK-8 assay showed that BEAS-2B cell viability was suppressed by LPS treatment, silencing CDKN2B-AS1 increased LPS-treated BEAS-2B cell viability, which were then reversed by transfection of si-ZFP36 ($P < 0.0001$, Fig. 4b). In addition, flow cytometry and ELISA results showed that silencing CDKN2B-AS1 attenuated BEAS-2B cell apoptosis ($P < 0.0001$, Fig. 4c) and the secretion of TNF-α, IL-1β, and IL-6 ($P < 0.0001$, Fig. 4d–f) promoted by LPS treatment, while transfection of si-ZFP36 reversed these effects. These results
revealed that CDKN2B-AS1 regulates BEAS-2B cell viability, cell apoptosis, and inflammatory response by negatively regulating ZFP36 expression.

**ZFP36 Interacts with NR4A1 and Positively Regulates NR4A1 Expression**

Previous evidence showed that NR4A1 overexpression alleviated ovalbumin (OVA)-induced airway inflammation response in a mouse model of allergic airway disease [12]. Therefore, we speculated that NR4A1 might be an important regulator in asthma. To confirm our speculation, the mRNA and protein levels of NR4A1 were detected in asthmatic children and healthy controls. As shown in Supplemental Fig. 1, the mRNA levels of NR4A1 have no differences in asthmatic children compared with healthy controls ($P = 0.3154$), but NR4A1 protein levels were markedly reduced ($P < 0.0001$). Next, we used the Genemania tool (http://genemania.org/) to predict the potential interacting proteins of ZFP36, which showed that there was a potential interaction between ZFP36 and NR4A1 (Fig. 5a). Subsequently, Co-IP assay verified that both ZFP36 and NR4A1 proteins could be detected by immunoprecipitation with ZFP36 antibody but not with IgG in BEAS-2B cells (Fig. 5b). Moreover, we found that ZFP36 overexpression significantly increased NR4A1 protein expression and ZFP36 silencing markedly inhibited NR4A1 protein expression ($P < 0.0001$), whereas ZFP36 overexpression ($P = 0.8123$) or knockdown ($P = 0.6356$) had no effect on the mRNA expression of NR4A1 in BEAS-2B cells (Fig. 5c and d). Next, we further examined the effect of ZFP36 on NR4A1 protein by using cycloheximide (CHX) to block translation. ZFP36 greatly extended the half-life of NR4A1 protein (Fig. 5e). Proteasomal inhibitor MG132 alleviated ZFP36 silencing-induced NR4A1 destabilization (Fig. 5f). Furthermore, ZFP36 dramatically decreased NR4A1 ubiquitination (Fig. 5g). Together, these results demonstrate that ZFP36 destabilizes NR4A1 protein through the ubiquitin–proteasome pathway. In addition, we observed that CDKN2B-AS1 overexpression decreased NR4A1 expression and CDKN2B-AS1 knockdown increased NR4A1 expression in BEAS-2B cells ($P < 0.0001$, Fig. 5h).

To further investigate whether the effects of ZFP36 and NR4A1 was involved in childhood asthma progression, pcDNA-ZFP36 were transfected into LPS-treated BEAS-2B cells alone or together with si-NR4A1. Western blotting results showed that LPS treatment repressed NR4A1 expression, and ZFP36 overexpression increased NR4A1 expression, while transfection of si-NR4A1 decreased NR4A1 expression in BEAS-2B cells ($P < 0.0001$, Fig. 6a). Moreover, CCK-8 assay showed that BEAS-2B cell viability was inhibited by LPS treatment, ZFP36 overexpression increased LPS-induced BEAS-2B cell viability, which were then reversed by transfection of si-NR4A1 ($P < 0.0001$, Fig. 6b). In addition, flow cytometry and ELISA results showed that ZFP36 overexpression suppressed BEAS-2B cell apoptosis ($P < 0.0001$, Fig. 6c) and the secretion of TNF-α ($P < 0.0001$), IL-1β ($P < 0.0001$), and IL-6 ($P < 0.0001$) (Fig. 6d–f) promoted by LPS treatment, while transfection of si-NR4A1 reversed these effects. Besides, we further found that LPS treatment increased p-p65 expression, and ZFP36
overexpression reduced p-p65 expression, while transfection of si-NR4A1 reversed the inhibited effect of ZFP36 overexpression on NF-κB p65 expression in BEAS-2B cells ($P < 0.0001$, Fig. 6g). These results demonstrated that ZFP36 regulates BEAS-2B cell viability, apoptosis, and inflammation by promoting NR4A1 expression and blocking the NF-κB signaling pathway.

To further confirm whether ZFP36 plays a role in LPS-induced BEAS-2B cells through regulating the NF-κB signaling pathway, LPS-induced BEAS-2B
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Fig. 6 ZFP36 regulates BEAS-2B cell viability, apoptosis, and inflammatory response by regulating NR4A1-mediated NF-κB signaling pathway. pcDNA-ZFP36 that were transfected into LPS-treated BEAS-2B cells alone or together with si-NR4A1. a Western blotting was conducted to measure the expression of NR4A1 protein. b CCK-8 assay was performed to evaluate BEAS-2B cell viability. c Flow cytometry was performed to evaluate BEAS-2B cell apoptosis. d–f The levels of TNF-α, IL-1β, and IL-6 in cell culture supernatant were measured by using ELISA. Data were presented as mean ± SD. a–g: The significance of differences was estimated by one-way ANOVA test.
cells were treated with si-CDKN2B-AS1 and/or si-NR4A1, before treatment with mangiferin, an inhibitor of the NF-κB signaling pathway. The results showed that silencing CDKN2B-AS1 increased protein expression of ZFP36 and NR4A1 \( (P < 0.0001) \), reduced expression of p-p65 and secretion of TNF-α, IL-1β, and IL-6, and promoted cell proliferation \( (P < 0.0001) \), whereas silencing NR4A1 promoted p-p65 expression, increased secretion of TNF-α, IL-1β, and IL-6, and inhibited cell proliferation \( (P < 0.0001) \), Fig. 7a–g). Mangiferin counteracted the effects of NR4A1 silencing (Fig. 7a–g). In summary, we determined that silencing CDKN2B-AS1 alleviates LPS-induced BEAS-2B cell injury through promoting ZFP36 and NR4A1 expression and blocking the NF-κB signaling pathway.

**Silencing CDKN2B-AS1 Alleviated Asthma Symptoms in OVA-Induced Asthma Mice**

To further investigate the role of CDKN2B-AS1 in asthma progression *in vivo*, an OVA-induced asthma mice model was constructed and si-CDKN2B-AS1 was intraperitoneally injected for treatment. We confirmed the increased CDKN2B-AS1 expression and decreased ZFP36 and NR4A1 expression in lung...
tissues of the OVA group (\( P_{\text{OVA}} < 0.0001 \)), and this expression pattern was reversed by si-CDKN2B-AS1 treatment (Fig. 8a–c). In histological evaluation, lung samples in control group mice revealed normal lung structure without the obvious inflammatory infiltration. However, the mice in the OVA group displayed infiltration of inflammatory cells into the lung interstitium and alveolar spaces, thickening of alveolar walls, and intra-alveolar exudation, while si-CDKN2B-AS1 treatment inhibited OVA-induced inflammatory cell infiltration.
Fig. 8 Silencing CDKN2B-AS1 alleviated asthma symptoms in OVA-induced asthma mice. BALB/c mice were randomly divided into three groups (n=8 per group): control group, OVA group, and OVA + si-CDKN2B-AS1 group. The mice were sensitized and challenged with OVA by intraperitoneal injection to establish asthma model, and si-CDKN2B-AS1 was intraperitoneally injected for treatment. a The expression of CDKNAB-AS1 in lung tissues of mice was measured by using RT-qPCR. b The expression of ZFP36 and NR4A1 in lung tissues of mice was detected by using Western blotting. c The localization of ZFP36 and NR4A1 protein in the lung tissues of mice was detected with immunofluorescence. d The airway hyperresponsiveness of mice was detected through measuring the Penh value. e Lung tissue sections from each experimental group were processed for histological evaluation, and lung inflammation was determined by observation of lung tissue sections stained with H&E staining under light microscopy. f Total and differential inflammatory cell count in the BALF of mice in all groups. g The serum OVA-specific IgE levels of mice in all groups were detected by using ELISA. h The levels of TNF-α, IL-1β, and IL-6 in BALF were examined by using ELISA. i The levels of p-p65 in the nucleus and cytoplasm of mouse lung tissues were detected with Western blotting. Data were presented as mean ± SD. a, b, f, g, h, and i: The significance of differences was estimated by one-way ANOVA test; D: the significance of differences was estimated by two-way ANOVA test.

Fig. 9 A schematic model representing the mechanism by which lncRNA CDKN2B-AS1 promotes the progression of childhood asthma. LPS induces increased CDKN2B-AS1 expression in BEAS-2B cells, while CDKN2B-AS1 recruits DNMT1 to promote ZFP36 promoter methylation and then represses ZFP36 expression. ZFP36 inhibits the ubiquitinated degradation of NR4A1 by binding to NR4A1, thereby promoting the protein stability of NR4A1. In addition, phosphorylated p65 enters the nucleus to promote the transcriptional expression of downstream genes, whereas NR4A1 inhibits the phosphorylation of p65. Activation of the NF-κB/p65 signaling pathway promotes inflammatory responses, induces cell apoptosis, and contributes to the development of childhood asthma.
significantly inhibited OVA-induced serum IgE level and inflammatory cytokine levels \( (P < 0.0001) \). In addition, we also detected the expression of p-p65, a key protein in the NF-κB pathway, and found that expression of phosphorylated p65 was significantly increased in the nucleus and decreased in the cytoplasm of lung tissues from OVA-induced mice \( (P < 0.0001) \), while treatment with si-CDKN2B-AS1 significantly inhibited OVA-induced p-p65 incorporation into the nucleus \( (P < 0.0001, \text{Fig. 8i}) \).

In summary, we found that CDKN2B-AS1 is significantly upregulated in LPS-induced BEAS-2B cells and recruits DNMT1 to promote ZFP36 promoter methylation, which in turn represses ZFP36 expression. Furthermore, we observed that ZFP36 inhibits its ubiquitinated degradation by binding to NR4A1, thereby promoting the protein stability of NR4A1. In addition, NR4A1 was found to inhibit the phosphorylation of p65, and phosphorylated p65 would enter the nucleus and promote the transcriptional expression of downstream genes, thereby promoting inflammatory responses, inducing cell apoptosis, and contributing to the development of childhood asthma (Fig. 9).

**DISCUSSION**

In recent years, increasing evidence has revealed that the altered expression of lncRNAs affects inflammatory response, immune response, and lung function and may be used for the development of specific biomarkers for airway disease [13, 14]. It was previously reported that interleukin-13 treatment increased CDKN2B-AS1 expression in human nasal epithelial cells (HNECs), and knockdown of CDKN2B-AS1 may suppress the production of inflammatory cytokines and mucin in IL-13-treated HNECs via regulation of the miR-15a-5p/JAK2 axis [15]. Furthermore, CDKN2B-AS1 was found to be upregulated in plasma samples of bronchial asthma patients compared with healthy controls, and the expression of CDKN2B-AS1 was positively correlated with pro-inflammatory cytokine secretion exacerbation severity in bronchial asthma patients [6]. Given the role of CDKN2B-AS1 in bronchial asthma, we further explored its underlying role in childhood asthma progression. In the present study, we found that CDKN2B-AS1 was upregulated in blood samples of children with asthma compared with healthy controls. Moreover, silencing CDKN2B-AS1 significantly promotes BEAS-2B cell viability and inhibits apoptosis and inflammatory cytokine secretion in vitro, and alleviated airway inflammation and asthma symptoms of OVA-induced asthma mice in vivo. In addition, a recent study has implicated that CDKN2B-AS1 negatively regulated A disintegrin and metalloprotease 10 (ADAM10) expression via recruiting DNMT1 to promote ADAM10 DNA methylation, consequently preventing inflammatory response of atherosclerosis [16]. Therefore, CDKN2B-AS1 may regulate the expression of its downstream target genes by promoting DNMT1-mediated DNA methylation. Interestingly, our study found that CDKN2B-AS1 negatively regulated ZFP36 expression in BEAS-2B cells by recruiting DNMT1 to promote ZFP36 promoter methylation, thereby modulating cell viability, apoptosis, and inflammatory response in BEAS-2B cells.

ZFP36 has been well-studied in inflammatory disorders and plays an important role in inducing the mRNA decay of inflammatory cytokines such as TNF-α, IL-3, and IL-8 [17]. It was found that carbon monoxide–induced ZFP36 mediates the protective effect of carbon monoxide against LPS-induced acute lung injury by enhancing the mRNA decay of proinflammatory cytokines [18]. Moreover, enhancing ZFP36 activity reduced the levels of cytokines and pulmonary inflammation and improved lung function in the cigarette smoke–induced experimental chronic obstructive pulmonary disease (COPD) mice model [19]. In this study, we demonstrated that ZFP36 served as an anti-inflammatory gene in LPS-treated BEAS-2B cells, and knockdown of ZFP36 repressed BEAS-2B cell viability and promoted apoptosis and inflammatory response. Besides, available studies have indicated that regulation of ZFP36 expression is affected by epigenetics modification of DNA methylation. For instance, resveratrol showed anticancer activity through suppressing the expression of DNMT1 and inducing demethylation of the ZFP36 promoter in non-small cell lung cancer cells [20]. Additionally, treatment with DNA methylation inhibitor (5-aza dC) increased ZFP36 expression in several HCC cell lines [21]. Similarly, our study implicated that ZFP36 expression was regulated by CDKN2B-AS1-mediated methylation in BEAS-2B cells.

NR4A1 is a key negative regulator of inflammatory responses. Accumulating evidence has suggested that NR4A1 is implicated in the regulation of inflammation and immunity disease [22, 23]. In particular, NR4A1 has been shown to have a protective function in
lung inflammation and diseases. For instance, the anti-inflammatory properties and protective effect of NR4A1 on acute respiratory distress syndrome were reported in a study, which indicated that NR4A1 decreased endothelin-1 expression by inhibiting NF-κB and p38 MAPK in LPS-stimulated A549 cells in vitro, and NR4A1 reduced endothelin-1 expression and lung injury in LPS-induced ARDS rats [24]. Moreover, emerging evidence has shown that NR4A1 decreased inflammatory cytokine secretion by inhibiting NF-κB signaling in lung epithelial cells, and in OVA-induced allergic airway inflammation mice model; NR4A1 knockout mice show significantly enhanced inflammatory infiltration and mucus secretion [12]. In the present study, we verified the interaction between ZFP36 and NR4A1 in BEAS-2B cells, and revealed that ZFP36 suppressed cell apoptosis and inflammatory cytokine secretion in LPS-treated BEAS-2B cells, while NR4A1 knockdown could reverse these effects. More notably, NR4A1 has been identified as a negative regulator of the NF-κB signaling. NR4A1 was found to regulate cerebral ischemia–induced brain injury through direct interaction with the p65 component of NF-κB and inhibiting the NF-κB signaling pathway [25]. Furthermore, it was reported that NR4A1 suppressed inflammatory response–related lung diseases by inhibiting NF-κB signaling both in vitro and in vivo [26]. Similarly, our findings showed that NR4A1 played an anti-inflammatory role in LPS-treated BEAS-2B cells through blocking the NF-κB signaling pathway.

Taken together, our findings suggested that CDKN2B-AS1 was upregulated in children with asthma compared with healthy controls. Moreover, silencing CDKN2B-AS1 significantly promotes BEAS-2B cell viability and inhibits apoptosis and inflammatory cytokine secretion in vitro, and alleviated airway inflammation and asthma symptoms of OVA-induced asthma mice in vivo. Mechanistically, CDKN2B-AS1 plays a role in childhood asthma via regulating ZFP36/NR4A1 axis and NF-κB signaling pathway by recruiting DNMT1 to promote ZFP36 promoter methylation. Our study may provide a novel regulatory mechanism and potential therapeutic target for the treatment of childhood asthma. However, the regulatory mechanism of CDKN2B-AS1 in childhood asthma may involve multiple pathways, only one of which was explored in our study, and more regulatory pathways need further exploration. In addition, whether silencing CDKN2B-AS1 has a role in the treatment of childhood asthma clinically is unknown and needs to be verified by future studies.

SUPPLEMENTARY INFORMATION

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AUTHOR CONTRIBUTION

Zhixin Chen designed the experiments. Zhixin Chen, Nuandong Fan, and Guangsheng Shen performed the experimental work. Jing Yang provided statistical analysis as well as figures for the manuscript. Zhixin Chen wrote the manuscript. All authors read and approved the final manuscript.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

DECLARATIONS

Consent for Publication The authors grant their consent to publish the material presented herein.

Ethical Approval This study was approved by the Ethics Committee of Nanyang Central Hospital, and all subjects had read and signed the informed consent. All animal care and experimental procedures in this study were approved by Animal Care and Use Committee of Nanyang Central Hospital.

Competing Interests The authors declare no competing interests.

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