MicroRNAs Involved in Asthma After Mesenchymal Stem Cells Treatment

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Administration of human bone marrow-derived mesenchymal stem cells (BM-MSCs) significantly alleviates allergic airway inflammation. There are no studies that refer to the role of microRNAs (miRNAs) after the BM-MSCs treatment in airway allergic inflammation. We induced a mouse model of asthma and performed the transplantation of BM-MSCs. We analyzed aberrant miRNAs and key immune regulators using both miRNA and messenger RNA (mRNA) polymerase chain reaction (PCR) arrays. We identified that 296 miRNAs were differently expressed after the induction of asthma and/or the treatment of BM-MSCs, in which 14 miRNAs presented the reverse variation tendency between asthma induction and BM-MSCs transplantation. *Mmu-miR-21a-3p, Mmu-miR-449c-5p, and Mmu-miR-496a-3p were further confirmed to be differently expressed with additional samples and quantitative real-time PCR. With an mRNA PCR array, we identified 19 genes to be involved in the allergy induction and the administration of BM-MSCs. Further target genes analysis revealed that *mmu-miR-21a-3p was significantly correlated with the immune regulator activin A receptor, Type IIA (*Acvr2a). *Mmu-miR-21a-3p had opposite expression with *Acvr2a after asthma and BM-MSCs treatment. *Acvr2a had binding sites for *miR-21a for both mice and human, suggesting that *miR-21/Acvr2a axis is conserved between human and mice. Dual-luciferase reporter assay showed that *mmu-miR-21a-3p negatively regulated the transcript of *Acvr2a. In addition, has-miR-21a inhibitor significantly increased the expression of *Acvr2a mRNA in BEAS-2B cells under lipopolysaccharide stimulation. Our results suggest that there were different miRNA and mRNA profiles after asthma induction and BM-MSCs treatment, and the *miR-21/Acvr2a axis is an important mechanism for the induction of asthmatic inflammation.

Introduction

Allergic airway diseases have become a significant global public health concern and threatened patients’ quality of life while also causing substantial medical and financial burdens [1]. Airway hyperreactivity, mucus hypersecretion, and reversible airway obstructions are the hallmarks of allergic airway inflammation [2]. Allergic rhinitis and asthma share several common features, such as high serum IgE levels, increased cellular infiltration, and excessive T helper type 2 (Th2) activation [3,4]. Th2 cytokines, such as interleukin 4 (IL-4), IL-5, and IL-13, could affect asthma immunomodulatory properties [5]. Insufficient regulatory T-cell (Treg) suppression is responsible for the excessive Th2 response in allergic airway diseases [6].

Recently, microRNAs (miRNAs) were reported to contribute to the progress of allergic airway inflammation. miRNAs are endogenous short noncoding RNAs that predominantly silence target genes by binding to their 3’ untranslated regions (UTRs) to prevent translation or induce degradation [7]. A number of studies have confirmed the critical roles of miRNAs in a series of biological processes, including immune regulation [8]. Many miRNAs were reported to be differentially expressed and play important roles in allergic airway inflammation, and *miRNA-let-7, *miRNA-155, and *miRNA-126 have been studied most frequently. The *miRNA-let-7-inhibited IL-13 expression and knockdown of the *let-7 miRNA family inhibited both cytokine production and disease pathology in animal asthma models [9,10]. *miRNA-155-deficient mice were found to be immunodeficient and displayed increased airway remodeling [11]. Targeting miRNAs, such as *miR-126, in the airways may lead to anti-inflammatory treatments for allergic asthma [12]. Therefore, regulating the...
functions of miRNAs is a novel target for treating allergic diseases.

Mesenchymal stem cells (MSCs) are multipotent cells that are capable of differentiation into three mesenchymal cell types. Increasing evidence in animal studies and preliminary clinical trials has demonstrated that MSCs not only possess multipotent differentiation potential but also exhibit strong immunomodulation potential. Initial clinical trials have been completed or are underway with regard to inflammatory bowel disease, systemic lupus erythematosus (SLE), organ transplantation, acute respiratory distress syndrome, and acute kidney injury [13,14]. It has been conceived that bone marrow-derived mesenchymal stem cells (BM-MSCs) could suppress allergen-specific Th2 cell responses and, therefore, prevent allergic airway inflammation [15–17].

Human BM-MSCs have no or limited immunogenicity, and allogeneic human BM-MSCs are well tolerated and therapeutically active in rodent models without no rejection response [18,19]. Consequently, we and other groups reported that human-derived BM-MSCs have successfully activated the immunomodulation to alleviate the allergic inflammation in mice model [17,19]. However, the mechanisms underlying the immune regulation have still not been well documented, especially regarding the correlation between BM-MSCs and miRNAs. Currently, very few articles reported the complex roles of BM-MSCs and miRNAs in allergic responses. Only one report observed that human BM-MSCs suppress stretch-induced MiR-155 and cytokines in cultured human bronchial epithelial cells [20]. There are no studies that refer to the correlation between miRNA and the treatment of BM-MSCs in airway allergic inflammation.

In this study, we analyzed aberrant miRNAs and key immune regulators in a mouse asthma model with or without BM-MSCs treatment using both miRNA and messenger RNA (mRNA) PCR arrays. We identified a series of differently expressed miRNAs and protein-coding genes as key regulator candidates after the induction of allergic inflammation and BM-MSCs treatment. We further confirmed the key miRNA–target gene axis to mediate the asthma pathogenesis and BM-MSCs therapy.

Materials and Methods

Cell culture

Human adult BM-MSCs were obtained from Cyagen Biosciences (Jiangsu, China; passage 2, Cat. No. HUXMA-01001) and cultured in human MSC growth medium (Cyagen Biosciences) supplied with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin, and 1% glutamine. Human bronchus epithelial cells BEAS-2B were purchased from ATCC (Rockville, MD) and cultured in DMEM/F12 with 10% FBS and 1% penicillin–streptomycin. Human embryonic kidney cells 293T (ATCC) were cultured in Dulbecco’s modified Eagle medium (DMEM) supplied with 10% FBS and 1% penicillin–streptomycin. All cells were cultured in a humidified chamber under 5% CO2 at 37°C.

Animals

Female BALB/c mice (4–6 weeks of age) were purchased from the Guangdong Medical Laboratory Animal Centre (Guangzhou, China). All procedures were performed according to protocols approved by the Sun Yat-sen University Institutional Animal Care and Use Committee. The number of animals is 19, 11, 17, and 19 for control, control+BM-MSCs, model, and model+BM-MSCs groups, respectively.

The mouse asthma model and the transplantation of BM-MSCs

Mice were induced to asthma by sensitization and challenge with ovalbumin (OVA, grade V; Sigma, St. Louis, MO) as described in our previous report with a minor modification [17] (Fig. 1A). The details are presented in Supplementary Data. The BM-MSCs were used at passage six or lower and they were maintained in culture at 70%–80% confluency. The cells were suspended in sterile phosphate-buffered saline (PBS) at a density of $5 \times 10^6$ cells per mL, and 0.2 mL of the cells was intravenously injected through the tail vein on day 20 before the challenge. Similar volume of PBS was injected as the controls.

Airway responsiveness measurements

The details are presented in Supplementary Data.

FIG. 1. The experimental protocol for the mouse asthma and airway hyperresponsiveness model. (A) BALB/c mice were sensitized on days 1, 7, and 14 by intraperitoneal injection of OVA with aluminum hydroxide and challenged with aerosolized 5% OVA from days 21 to 25. Purified human BM-MSCs ($1 \times 10^6$) or PBS was administered through tail vein injection on day 20. The samples were collected with an over dose anesthetized 4 h after the last challenge on day 25. (B) The mice were assessed for airway responsiveness to an increasing dose of Mch (6.25, 12.5, 25, 50, and 100 mg/mL) with a whole body plethysmography on day 25 after the challenges from days 21 to 24. The data are shown with Penh%, which represents the percentage changes of Penh from the corresponding baseline values. The data are shown as mean±SEM (n=6). *P<0.05; ***P<0.001, compared with control group. ▲P<0.05; ▲▲P<0.01, compared with control+BM-MSCs. BM-MSCs, bone marrow-derived mesenchymal stem cells; OVA, ovalbumin; PBS, phosphate-buffered saline; SEM, standard error of the mean.
Histology analysis and inflammation scoring

Lung tissues were collected for histologic and inflammation analyses, as presented in Supplementary Data.

Bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid (BALF) collection method and the IL-4, IL-5, IL-13, and interferon (IFN)-γ levels in BALF were measured as presented in Supplementary Data.

Serum collection and OVA-specific Ig analysis

The details are presented in Supplementary Data.

The miRNA and mRNA PCR arrays

Samples for the miRNA PCR and mRNA PCR arrays were collected from lung tissues in mice of control, model, and model+BM-MSCs groups. RNA was isolated with Trizol reagent (Invitrogen, Paisley, UK) and cleaned up with the RNeasy® MinElute Cleanup Kit (SABiosciences, Qiagen, MD). Then, complementary DNA (cDNA) was synthesized with a cDNA synthesis kit (Exiqon, Vedbaek, Denmark) for the next experiments. For the miRNA array, the Exiqon miRCURY LNA™ Universal RT miRNA PCR profiling kit with two 384-well plates (Ready-to-Use Mouse & Rat Panel I–II V3, Cat. No. 20379; Exiqon; www.exiqon.com), which covered 752 murine miRNAs, was used with quantitative real-time PCR (qRT-PCR) technology. Six miRNAs and small RNAs were used as internal controls. The amplification curves were analyzed with the GenEx qPCR software (www.exiqon.com/mirna-pcr-analysis), both for Ct and melting curve analysis determinations.

Next, we examined the possible genes that were involved in our study with a 96-well RT² Profiler™ PCR array (SABiosciences, Frederick, MD). To obtain as much information about as many genes as we could, we studied previous reports regarding allergic airway inflammation or asthma and designed a custom-made mRNA PCR array (SABiosciences, Frederick, MD) that covered 11 different pathways or catalogs in allergic inflammation, including the following: toll-like receptors, NFKB signaling pathways, mitogen-activated protein kinase (MAPK) pathways, Janus kinase activity pathways, signal transducer and activator of transcription (STAT) families, TGFβ superfamilies, Notch pathways, transcription factors, Th2 cytokines and related genes, mast cells, IgE and eosinophils, and some others known to be involved in allergy (Supplementary Table S1).

The above cDNA from each sample was evaluated through the PCR array according to the manufacturer’s instructions, as in previous studies [21,22]. The raw data were obtained from the standard real-time PCR procedures. For both the miRNA and mRNA PCR arrays, P values were calculated with the one-way analyses of variance (ANOVA). The threshold for upregulation and downregulation was set as fold change ≥1.5 for miRNA and the genes, with a P value ≤0.05. Hierarchical clustering was performed based on differentially expressed miRNAs and mRNAs with the Cluster Treeview software from Stanford University (Palo Alto, CA).

RNA extraction and miRNA quantification

Fourteen aberrant miRNAs, which exhibited opposite trends between the induction of allergic inflammation and the BM-MSCs treatment (up and down or down and up), were finally selected from the miRNA PCR array results and were confirmed with additional samples (n = 14 for the control group, n = 12 for the model group, and n = 14 for the model+BM-MSCs group). Total RNA was extracted with Trizol reagent (Invitrogen). For miRNA quantification, cDNA was synthesized with the M-MLV Reverse Transcriptase Kit (Promega, Madison, WI). Mature miRNAs were quantified by stem-loop real-time PCR with SYBR Premix Ex Taq (TaKaRa Bio, Otsu, Japan). The primers for miRNAs used in the reverse transcription and quantitative PCR assays are shown in Supplementary Table S2. Moreover, the activin A receptor, Type IIA (Acvr2a) mRNA expression in the mouse model was also confirmed by qRT-PCR.

For mRNA quantification, total RNA was reverse transcribed with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Inc., Rockford, IL), the synthesized cDNA was quantified by SYBR. Ct values were normalized to β-actin and relative expression was calculated using the 2ΔΔCt method. Primer sequences for mRNA quantification were as follows: Forward-β-actin: TGAGACCTTCA ACACCCCCAGGATG. Reverse-β-actin: CGTAGATGG GCACAGTGGGTG. Forward-hAcvr2a: GATGGAA GTCACACAGCCA, Reverse-hAcvr2a: GGTCCTGGG TCTTGAAGTGG. Forward-mAcvr2a: TTCAGTTACA CGGAAGCCAC, Reverse-mAcvr2a: GGTCCTGGGTCT TGAGTAGGA.

 Luciferase reporter assay

The 3’ UTRs of Acvr2a mRNA containing two mmu-miR-21a-3p binding sites (436–442 and 502–508) predicted by Targetscan were subcloned into a pMIR-REPORT vector (Applied Biosystems, Foster City, CA) immediately downstream of the luciferase gene. The Acvr2a-3’ UTR vector, mmu-miR-21a-3p mimics, or negative control was cotransfected into 293T cells. Each sample was detected for firefly luciferase and renilla luciferase. The relative luciferase activity was normalized with renilla luciferase activity.

The culture of BEAS-2B cells and the transfection of human miRNA inhibitor

Human BEAS-2B cells were exposed to 500 ng/mL lipopolysaccharide (LPS) for 0, 3, 6h, respectively. In one experiment, BEAS-2B cells were transfected with or without 500 pmol has-miR-21a inhibitor and cultured in the condition of LPS stimulation for 3 or 24h. Human Acvr2a mRNA levels were analyzed by qRT-PCR. Lip 2000 group served as control.

Statistical analysis

The experimental data are expressed as the mean ± standard error of the mean. All of the statistical analyses were performed with the SPSS software (version 19.0). For the Gaussian distribution data, one-way ANOVA followed by post-hoc Tukey (for equal homogeneity) or Dunnett T3 (for unequal homogeneity) tests was used for multiple comparisons between the different groups. A Kruskal–Wallis rank sum test followed by a Mann–Whitney U test was performed for comparisons that used abnormal distribution data. P < 0.05 was considered statistically significant.
Results

**BM-MSCs reduced airway hyperresponsiveness and airway inflammation**

We developed a mouse model of OVA-induced asthma and evaluated the effects of the systemic administration of human BM-MSCs on allergic inflammation (Fig. 1A). We first evaluated the airway inflammation response in the model of asthma. The asthmatic mice exhibited higher airway hyperresponsiveness (AHR) levels at high Mch concentrations (25, 50, and 100 μg/mL) (Fig. 1B, *P* < 0.05 or 0.001) and increased lung inflammatory infiltrates than the control group mice (Fig. 2).

And systemic BM-MSCs administration significantly inhibited AHR (Fig. 1B, *P* < 0.05 or 0.001) and clearly decreased peribronchial and perivascular inflammation (hematoxylin-eosin staining), mucus secretion from hyperplastic goblet cells (periodic acid–Schiff (PAS) staining), and peribronchial collagen deposition (Masson staining) in the lungs (Fig. 2A). Pathological scoring (H&E and PAS) in the model+BM-MSCs group was decreased two to threefold compared with the model group (Fig. 2B, all *P* < 0.01). The BALF analysis illustrated that the BM-MSCs-treated mice had significantly reduced macrophage, eosinophil, lymphocyte, and neutrophil levels, in terms of both total numbers and percentages compared with the model group (Fig. 2C, D).

**BM-MSCs affected the BALF inflammatory cytokine and circulating OVA-specific Ig protein levels**

The BALF Th2 cytokine levels, including IL-4, IL-5, and IL-13, were significantly higher in the model group than in the control group (*P* < 0.01, or 0.001, Fig. 2E). Correspondingly, the BM-MSCs treatment dramatically reduced their levels. No IFN-γ production was detected in the BALFs in any of the groups. Moreover, we confirmed an elevation in the serum OVA-specific IgE, IgG1, and IgG2a levels in the model mice (Fig. 2F). The BM-MSCs treatment significantly decreased the IgE and IgG1 levels. Interestingly, the IgG2a levels, a protective immune factor, increased after the induction of asthma and continued to increase with the BM-MSCs treatment.

**Aberrant miRNAs in the model and BM-MSCs-treated mice**

Because many miRNAs were reported to be involved in allergic airway inflammation, here we performed an miRNA PCR array with real-time PCR technology to evaluate the possible miRNAs involved in asthma after the treatment of BM-MSCs. The miRNA array used covers the short transcripts of 752 miRNAs in mice. Hierarchical clustering of aberrant miRNAs showed that systemic miRNA variations existed between the control, model, and model+BM-MSCs groups (Fig. 3A). Total of 296 aberrant miRNAs were observed to be differentially expressed after the induction of asthma model and/or BM-MSCs transplantation (Fig. 3B). The curves for the miRNAs changes between the model and control groups (the first lines in the boxes of Fig. 3B,a) and, subsequently, between the model+BM-MSCs and the model groups (the second lines) exhibited eight different patterns with a total of 296 miRNAs (Fig. 3B).

The key miRNA regulators that presented the reverse variation tendency between asthma induction and BM-MSCs transplantation should have more significance for our exploration of the possible mechanisms of MSC-mediated immunomodulation. Therefore, we next selected two patterns with opposite directions (down then up or up then down) after the model induction and after BM-MSCs treatment for further study, including 10 miRNAs with the pattern of down then up and 4 miRNAs with the pattern of up then down miRNAs, as shown as the 2 orange boxes in Fig. 3B,a. The expression of the 10 miRNAs, such as mmu-miR-496a-3p, declined after asthma induction and then increased with the BM-MSCs treatment; whereas the 4 miRNAs, such as mmu-miR-21a-3p, increased first and later declined (Fig. 3B).

To confirm that total of 14 miRNAs selected previously were specific for the model induction and BM-MSCs treatment progresses, further hierarchical clustering analyses were performed for them. We found that clustering of 14 selected miRNAs was significantly different between the model and control groups and between the model+BM-MSCs and model groups (Fig. 3C and Table 1). More importantly, the miRNA clusters in the BM-MSCs-treated mice were more similar to those in the control mice. Our results revealed that the induction of asthma and the treatment of BM-MSCs affected the miRNAs profiles in mice.

**miRNAs in the asthma model with the BM-MSCs treatment**

The abundance of the 14 significant miRNAs selected was further estimated based on the average Ct value in the control group in our miRNA PCR array. Clear significant expression differences were found for the 14 miRNAs both after allergic induction and BM-MSCs treatment (Fig. 4A). We further confirmed the expression of the 14 miRNAs using qRT-PCR for the current samples (n = 6 for each group) together with more control (n = 8), model (n = 6), and model+BM-MSC (n = 8) group mice, and we confirmed 3 out of the 14 miRNAs that were differentially expressed between the 3 groups (Fig. 4B–D and Supplementary Table S3). Mmu-miR-21a-3p and mmu-miR-449c-5p increased after allergic induction and then decreased as a result of BM-MSCs treatment (*P* < 0.01 or 0.001). The allergic inflammation induction downregulated the mmu-miR-496a-3p expression levels, whereas transplanting the human BM-MSCs rescued the miRNA transcriptional levels (*P* < 0.01 or 0.001). Then, these three miRNAs were selected as key regulatory candidates for our further study.

**Target genes of the selected miRNAs in the OVA/BM-MSC mouse model**

Next, we determined the possible targets of mmu-miR-21a-3p, mmu-miR-449c-5p, and mmu-miR-496a-3p. To screen the series of potential functional target genes, we performed a protein-coding gene PCR array with 88 genes that covers most of the immunomodulatory factors and important proteins that are involved in allergic airway inflammation. We found that 17 genes were significantly different between the model and control groups. Interestingly, 70 genes were significantly different between the model+BM-MSCs and the model groups (Table 2).
FIG. 2. Transplantation of human BM-MSCs attenuated airway inflammation in the lung and affected inflammatory cytokine production in the BALF and Ig proteins in the serum in asthmatic mice. (A) Representative photomicrographs of lung sections with H&E, PAS, and Masson staining around the bronchi and vessels from each group. The arrows show the significant increases in inflammatory infiltrates (H&E), mucus accumulation at the bronchi luminal surface (PAS staining) and blue collagen staining (Masson staining). Original magnification ×200. (B) Representative photomicrographs of Diff-Quick staining for the inflammatory cells in the BALF. (C) Statistical analyses for the inflammation score were quantified with H&E staining and the mucus hypersecretion levels were quantified with PAS scores. (D) Inflammatory cells (total cells, macrophage, eosinophils, lymphocytes, and neutrophils) were counted in the BALF. The data are shown as the mean ± SEM (n = 6). The BALF cytokine levels (E) and the OVA-specific serum IgE, IgG1, and IgG2a levels (F) were measured with enzyme-linked immunosorbent assays. The data are expressed as the mean ± SEM (n = 6). *P < 0.05; **P < 0.01; ***P < 0.001. BALF, bronchoalveolar lavage fluid; H&E, hematoxylin-eosin; PAS, periodic acid–Schiff.
Most of the differently expressed genes included toll-like receptor (TLR), MAPK signaling pathway, Th2 cytokine, and related genes. In detail, a total of six different patterns for the genes were found to be expressed differently in the model versus control and the model+BM-MSCs versus the model groups. For example, the TLR2, IL-10, IL-33, chemokine (C-C Motif) ligand 17 (CCL17), colony-stimulating factor 2 (granulocyte-macrophage) (CSF2), and IL-4 mRNA levels were upregulated in the model group compared with the control groups. The second lines in the boxes represent increased or decreased miRNA expression after the BM-MSCs treatment compared with the model group. The numbers in the boxes represent the number of miRNAs that obey the development pattern of that box. Two patterns with a total 14 aberrant miRNAs were selected and marked with orange for next experiments. (b) A detailed pattern of 10 miRNAs in the upper orange box and 4 miRNAs in the lower orange box in (B, a) for the three groups. (C) Hierarchical clustering analysis of the said 14 miRNAs marked with orange in (B, a). Red, expression above the median; green, expression below the median. miRNAs, microRNAs.
Acvr2a was the target gene of mmu-miR-21a-3p mRNA PCR microarray. To confirm the relationship between Acvr2a after the induction of asthma and an increase after BM-MSCs, miR-21a-3p negatively regulated the transcript of Acvr2a via the 3′ UTR of Acvr2a, suggesting that mmu-miR-21a-3p negatively regulated the transcript of Acvr2a and Acvr2a was the target gene of mmu-miR-21a-3p (Fig. 5F).

Table 1. Selected MicroRNAs and Target Genes in Bone Marrow-Derived Mesenchymal Stem Cells-Treated Asthma Model.

| miRNAs       | Average Ct in control | Fold change (model vs. control) | P (model vs. control) | Fold change (model+BM-MSCs vs. BM-MSCs) | P (model+ BM-MSCs vs. model) | Target gene |
|--------------|-----------------------|--------------------------------|-----------------------|-----------------------------------------|--------------------------------|-------------|
| mmu-miR-496a-3p | 32.88                 | 0.05                           | 0.0063                | 7.58                                    | 0.0258                        | Stat5b      |
| mmu-miR-20b-3p  | 34.37                 | 0.14                           | 0.0178                | 4.36                                    | 0.0463                        | Postn, Il33 |
| mmu-miR-27a-5p  | 30.07                 | 0.21                           | 0.0020                | 1.87                                    | 0.0214                        | Smad1, Iga4 |
| mmu-miR-3107-5p | 25.61                 | 0.28                           | 0.0020                | 1.83                                    | 0.0444                        | Stat5b, Nfe212, Elk1, Gsk3b, Ira2 |
| mmu-miR-150-5p | 22.90                 | 0.32                           | 0.0003                | 1.57                                    | 0.0268                        | Notch1, Stat6 |
| mmu-miR-709    | 23.59                 | 0.32                           | 0.0079                | 1.96                                    | 0.0478                        | Akt1, Gsk3b |
| mmu-miR-434-3p | 29.95                 | 0.35                           | 0.0004                | 1.77                                    | 0.0108                        | Tslp, Mapk14 |
| mmu-miR-764-5p | 32.40                 | 0.42                           | 0.0069                | 1.99                                    | 0.0105                        | Stat1, Rela |
| mmu-miR-449c-5p| 29.65                 | 0.48                           | 0.0063                | 1.50                                    | 0.0215                        | Notch1, Stat6 |
| mmu-miR-184-3p | 28.79                 | 0.69                           | 0.0376                | 1.80                                    | 0.0078                        | Acvr2a      |
| mmu-miR-21a-3p | 31.12                 | 1.77                           | 0.0079                | 0.24                                    | 0.00003                       | Gata3, Elk1, Gsk3b, Mapk10, Jak2, Stat6, Tnfsf4, Tlr4 |
| mmu-miR-135b-5p| 34.66                 | 8.27                           | 0.0004                | 0.63                                    | 0.0153                        |  |

aAbundance of miRNAs was represented by average Ct value in control mouse.
bFor analysis of different expression levels between two groups, student’s t-tests were used. Results were considered statistically significant at P < 0.05.

Target genes of miRNAs, which should be differently expressed in our polymerase chain reaction array, were predicted in TargetScan. Acvr2a, activin A receptor, Type IIA; BM-MSCs, bone marrow-derived mesenchymal stem cells; Gata3, GATA binding protein 3; Gsk3b, glycogen synthase kinase 3 beta; Ila3, interleukin 33; Ira2, interleukin-1 receptor-associated kinase 2; Iga4, integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor); Jak2, Janus kinase 2; Mapk10, mitogen-activated protein kinase 10; Mapk14, mitogen-activated protein kinase 14; miRNAs, microRNAs; Nfe2l2, nuclear factor, erythroid 2-like 2; POSTN, periostin, osteoblast-specific factor; Rela, V-Rel Avian reticuloendotheliosis viral oncogene homolog A; Smad1, SMAD family member 1; Stat1, signal transducer and activator of transcription 1; Stat5b, signal transducer and activator of transcription 5B; Stat6, signal transducer, and activator of transcription 6; Tlr4, toll-like receptor 4; Tnfsf4, tumor necrosis factor (ligand) superfamily, member 4; Tslp, thymic stromal lymphopoietin.

the STAT family and the MAPK signaling pathways, were the targets of the 14 miRNAs. Certain genes, such as STAT5b and Gsk3b, are regulated by more than one miRNA.

Notably, in the predicted allergic inflammation and BM-MSC-mediated immune regulation targets, both mouse NOTCH1 and STAT6, respectively, contained one putative miRNA binding site for both mmu-miR-449c-5p and mmu-miR-21a-3p target site. Moreover, the 3′ UTR of mouse STAT5B and Acvr2a had a binding site for both mmu-miR-496a-3p and mmu-miR-21a-3p, respectively (Fig. 5C). Next, we performed a correlation analysis between the said three miRNAs and their predicted targets genes based on the miRNAs PCR array and miRNA PCR array data. The results showed that there was a significant correlation between the Acvr2a and mmu-miR-21a-3p expression levels (Pearson R = -0.824, P < 0.01, Fig. 5D). However, no strong correlation was found between NOTCH1, STAT6, and STAT5B with their corresponding miRNAs.

The qPCR analysis of Acvr2a in mice showed a decrease after the induction of asthma and an increase after BM-MSCs treatment (Fig. 5E), which was consistent with the results of mRNA PCR microarray. To confirm the relationship between mmu-miR-21a-3p with Acvr2a, we performed the dual-luciferase reporter assay by cotransfecting the firefly luciferase reporter vector harboring the 3′ UTR of Acvr2a with the miR-21a-3p mimics or miRNA negative control into 293T cells. The result showed that miR-21a-3p significantly reduced the luciferase activity of Acvr2a 3′ UTR, suggesting that mmu-miR-21a-3p negatively regulated the transcript of Acvr2a and Acvr2a was the target gene of mmu-miR-21a-3p (Fig. 5F).

Has-miR-21 regulates human Acvr2a expression

Next, we tried to understand the clinical significance of the miR-21a-3p/Acyr2a axis in human. We searched the online TargetScan database and found that the 3′ UTR of human Acvr2a has the same has-miR-21 binding site as the murine Acvr2a (Fig. 6A). This finding indicates that the miR-21/Acyr2a axis may be conservative from mouse to human, and it may have an important role in the physiological process of allergy. Moreover, we retrieved the Gene Expression Omnibus (GEO) database and found some data about the expression of hsa-miR-21 in asthma patients. The data from GEO database (GEO No. GSE25230) showed that compared with healthy donors, the asthmatic patients had a higher hsa-miR-21 level, but it was not significantly different (P = 0.0626, Fig. 6B). It may be because of the low number of samples with seven patients and seven controls. One study reported the up-regulation of has-miR-21 in human asthma [23].

To further identify the relationship between has-miR-21a and Acvr2a, we exposed the human bronchial epithelial cells, BEAS-2B, to LPS for different time points and also transfected the cells with has-miR-21a inhibitor. We found that Acvr2a mRNA expression showed a slight decline but with no significant difference at 3 h after the administration of LPS (Fig. 6C). Compared with control group lip2000, 1.3-fold of upregulation of Acvr2a mRNA expression was observed after transfection of has-miR-21a inhibitor with no stimulation of LPS (Fig. 6D). Moreover, the expression of Acvr2a mRNA in BEAS-2B significantly increased up to
eightfold after transfecting the cells with *has-miR-21a* inhibitor at 24 h with LPS stimulation (Fig. 6E). It suggests that *has-miR-21a* regulates the expression of *Acvr2a* especially under some inflammation stimulation.

**Discussion**

In this study, we used an miRNAs PCR array to identify 14 miRNAs that were differentially expressed after asthma induction and with BM-MSCs treatment. We further confirmed *mmu-miR-21a-3p*, *mmu-miR-449c-5p*, and *mmu-miR-496a-3p* with more samples. With an mRNA PCR array, we identified a total of 19 genes that were involved in allergy induction and the administration of BM-MSCs. Moreover, the analysis regarding the correlation between miRNAs and their target genes demonstrated that *mmu-miR-21a-3p* might modulate the immunomodulatory effects by negatively regulating the *Acvr2a* gene. Dual-luciferase reporter assay showed that *mmu-miR-21a-3p* negatively regulated the transcript of *Acvr2a*.

In addition, we found that *has-miR-21a* inhibitor significantly increased the expression of *Acvr2a* mRNA in BEAS-2B cells under LPS stimulation. We further confirmed that *miR-21a* regulates the expression of *Acvr2a* especially under some inflammation stimulation. Taken together, our results indicate that there were significantly different miRNA profiles after asthma induction and BM-MSCs treatment. Specifically, the *miR-21/Acvr2a* axis may play a critical role both in the asthma and BM-MSCs treatment mouse model and in clinical asthma patients. To the best of our knowledge, this study is the first to reveal the miRNA expression changes after BM-MSCs treatment in asthma.

Previous studies showed that human BM-MSCs effectively alleviated the inflammation in an OVA-induced asthma animal model, which included attenuating airway inflammation, OVA-specific IgE and Th2 inflammation cytokine levels [15–17]. We previously identified that BM-MSCs significantly decreased Th2 cytokine levels and promoted Treg cell immune responses after they were cocultured with peripheral blood mononuclear cells from allergic rhinitis patients [24]. Therefore, a better understanding of the underlying mechanisms of BM-MSCs with regard to their therapeutic role in asthma will help to achieve the clinical application for human BM-MSCs.

To date, several possible mechanisms regarding asthma have been reported. Th2 cytokines, such as IL-4, IL-5, IL-9,
and IL-13, are considered to be responsible for asthma immune dysregulation [5,25,26], and treatment with an anti-IL-5 mAb reduced the number of severe asthma exacerbations to some extent [27]. NFkB-deficient mice failed to mount an allergic inflammatory response because of a lack of Th2 differentiation capacity and the absence of Gata3 expression [28]. Recently, as an important negative regulator in a variety of biological processes, many types of miRNAs were reported to play a key role in allergic airway inflammation.

Therefore, targeting miRNA function is a novel strategy for asthma. The miRNA-let-7 inhibited IL-13 expression in vitro and alleviated the IL-13-dependent allergic airway inflammatory response in a mouse model [9]. miRNA-155 is required for B-cell, T-cell, and dendritic cell functions. miRNA-155-deficient mice were immunodeficient and displayed increased airway remodeling [11]. Inhibition of miRNA-126 function suppressed the Th2 cell response and allergic airway disease development in mice [12]. Inhibition of asthma by miRNA-145 antagonism is comparable with Gata3 expression [36]. In this study, we determined that mmu-miR-496a-3p and mmu-miR-449c-5p were consistently upregulated after asthma induction, and they were decreased as a result of BM-MSCs treatment. However, mmu-miR-496a-3p decreased after asthma induction, and human BM-MSC transplantation rescued its transcriptional level. MiRNA-449, which is a member of the miRNA-34/449 family, was recently identified as a critical regulator of airway epithelial cell differentiation into ciliated cells by directly repressing NOTCH1 transcript levels [30]. Moreover, miRNA-34/449 regulated motile ciliogenesis by a centriolar protein, CP110 [31]. The miRNA-34/449 family was differentially expressed specifically in epithelial cells from asthma subjects after IL-13 stimulation and these cells were resistant to corticosteroids [32]. In this study, we determined that mmu-miR-449c-5p expression increased after asthma induction and decreased with the BM-MSCs treatment in mouse lungs. Currently, there are no reports regarding miRNA-496 and allergic diseases.

More importantly, our study suggests that the miRNA-21-Acvr2a axis plays an important role in the induction of asthma. Murine miRNA-21 has been strongly conserved throughout evolution, and it has been reported to play a critical role in tissue injury and inflammation. miRNA-21 was functionally expressed in T cells and regulated T-cell apoptosis [33,34]. Furthermore, miRNA-21 was upregulated during allergic airway inflammation in mice and IL-13-induced miRNA-21 led to an anti-inflammatory effect by silencing IL-12 [35]. In addition, miRNA-21 was reported to inhibit lung inflammation by modulating TLR2 signaling [36]. In this study, we performed an miRNA PCR

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| Classify | M/C – MSCs/M ↑ | M/C – MSCs/M ↓ | M/C ↑ MSCs/M | M/C ↓ MSCs/M | M/C ↓ MSCs/M |
|----------|----------------|----------------|--------------|--------------|--------------|
| Toll-like receptors | TLR1, TLR4, TLR6, TLR7, TLR8, TLR9 | TLR2 | TLR3 | NFkB2 | MAPK10 |
| NFkB signaling | REL | MAPK1, MAPK14 | NFkB2 | MAPK10 |
| MAPK | JAK2, JAK3 | STAT4, STAT5b, STAT6 |
| Janus kinase activity | Notch pathway | Notch1 | Foxp3, RORC | IL-10, IL-33 | CCL17, CSF2, IL-4 |
| STAT family | Th2 cytokines and related genes | CC2L6, IL-21, IL-25, PDCD1, TTNFSF4 |
| TGFß superfamily receptors | Mast cells, IgE and eosinophils | CPA3 | IL-1r1 | CMA1 |
| Others | ACTB, GUSB | POSTN, TBX21, B2M |

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Table 2. Polymerase Chain Reaction Array Results and Fold Changes (>1.5) of Immune Response Genes Expression in Mice of Control, Asthma, and Bone Marrow-Derived Mesenchymal Stem Cells Treatment

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| Classify | M/C – MSCs/M ↑ | M/C – MSCs/M ↓ | M/C ↑ MSCs/M | M/C ↓ MSCs/M | M/C ↓ MSCs/M |
|----------|----------------|----------------|--------------|--------------|--------------|
| Toll-like receptors | TLR1, TLR4, TLR6, TLR7, TLR8, TLR9 | TLR2 | TLR3 | NFkB2 | MAPK10 |
| NFkB signaling | REL | MAPK1, MAPK14 | NFkB2 | MAPK10 |
| MAPK | JAK2, JAK3 | STAT4, STAT5b, STAT6 |
| Janus kinase activity | Notch pathway | Notch1 | Foxp3, RORC | IL-10, IL-33 | CCL17, CSF2, IL-4 |
| STAT family | Th2 cytokines and related genes | CC2L6, IL-21, IL-25, PDCD1, TTNFSF4 |
| TGFß superfamily receptors | Mast cells, IgE and eosinophils | CPA3 | IL-1r1 | CMA1 |
| Others | ACTB, GUSB | POSTN, TBX21, B2M |

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Table 2. Polymerase Chain Reaction Array Results and Fold Changes (>1.5) of Immune Response Genes Expression in Mice of Control, Asthma, and Bone Marrow-Derived Mesenchymal Stem Cells Treatment
FIG. 5. The mRNA profile in asthma with the treatment of BM-MSCs and the relationship between mmu-miR-21a-3p and Acvr2a. (A) A hierarchical clustering analysis of all the aberrant protein-coding genes in the PCR array that were differentially expressed either between the model and control groups or between the model+BM-MSCs and model groups (≥1.5-fold; \( P < 0.05 \)). (B) A hierarchical clustering analysis of 19 aberrant protein-coding genes in the PCR array. Red, expression above the median; green, expression below the median in (A). (C) Schematic diagrams showing the interaction sites between the mmu-miR-21a-3p, mmu-miR-449c-5p, and mmu-miR-496a-3p and the 3' UTR of their corresponding mRNAs. (D) Mmu-miR-21a-3p was negatively correlated with the Acvr2a gene in miRNAs array and mRNA PCR array. y, the mean Acvr2a Ct value in the three groups. x, the mean mmu-miR-21a-3p Ct value in the three groups. (E) qRT-PCR analysis of Acvr2a in lung tissue samples from the control, model, and model+BM-MSC groups. \( n = 6 \) in each group. (F) Dual luciferase assay of 293T cells cotransfected with firefly luciferase vector containing the Acvr2a 3' UTRs and the mmu-miR-21a-3p mimics or scrambled oligonucleotides as the miRNA negative control. *\( P < 0.05 \); **\( P < 0.01 \). Acvr2a, activin A receptor, Type IIA; mRNA, messenger RNA; UTR, untranslated region; qRT-PCR, quantitative real-time PCR.
array and a corresponding mRNA PCR array to detect miRNAs and their potential targets simultaneously. We found that a total of 19 genes were involved in the allergy induction and the treatment of BM-MSCs. And there was significant negative correlation between the Acvr2a gene and mmu-miR-21a-3p in mice. Both our PCR array results and further qRT-PCR confirmation showed that Acvr2a mRNA levels decreased after the induction of asthma and increased after the treatment of BM-MSCs. More importantly, we performed the dual-luciferase reporter assay and found that miR-21a-3p significantly reduced the luciferase activity of Acvr2a 3′ UTR.

Moreover, the miRNA-21-Acvr2a axis was not only functional in our asthma mouse model but also functioned in human. After analyzing the online TargetScan database, we found that human Acvr2a harbors a miRNA-21 binding site at the 3′ UTR, suggesting the conservation of the miRNA-21-Acvr2a axis from mouse to human. Furthermore, we searched the data from the GEO database (GEO No. GSE25230) and found that the asthmatic patients had a high hsa-miR-21 level but with no significant difference, partly because of the limited number of samples. Moreover, a previous study also described the upregulation of has-miR-21 in human asthma [23]. Using BEAS-2B cell line, we identified that has-miR-21a inhibitor significantly upregulated human Acvr2a mRNA levels with no stimulation. Surprisingly, has-miR-21a inhibitor dramatically increased Acvr2a mRNA levels with the LPS stimulation. Our data suggest that miRNA-21-Acvr2a axis plays an important role in the induction of asthma both for mice and human.

Numerous studies provide controversial evidence for anti-inflammatory effects and proinflammatory effects of activin A and also in asthma [37]. Similarly, the data about activin receptor suggest controversial findings. There was increased Acvr2a expression in atopic asthmatics after allergen challenge [38]. However, some studies reported that decreased frequency of Acvr2a expression was found in epithelial cells in asthma [39,40], which was similar with our results about the decrease of Acvr2a expression after the induction of asthma. It suggests that there are different expression levels of activin A and its receptor Acvr2a for the different asthma subtypes and asthma severity, and activin A binding with its receptors exerts distinct effects on immune responses dependent on the underlying signal pathway in the different states of asthma [37]. Our findings about the decreased levels of Acvr2a after the induction of asthma and increased with BM-MSCs administration indicate that Acvr2a may play anti-inflammatory roles in our
asthma model. Previous studies have shown that overexpression of activin A reduced the Th2 polarization and blocking activin A with antibodies could enhance the Th2 cytokine production [40].

In addition, some studies indicated the role of miR-21 in Th2 response activation [41]. It is very interesting to see whether the involvement of the miR-21a-3p-Acvr2a axis is crucial in the BM-MSC-induced beneficial effects in asthma. Inhibiting miR-21a-3p-Acvr2a axis through Acvr2a antagonism, or miRNA antagonism in the asthma therapeutic model, would be of great interest to demonstrate its essential role in the therapeutic effect of BM-MSCs. The potential mechanisms of miR-21a-3p-Acvr2a axis in BM-MSCs treatment may be toward a less Th2 environment. Of course, the detailed roles of miR-21-Acvr2a axis in MSCs therapeutic effects should be carefully addressed in the future.

To screen for potential functional target genes that expression was changed after the BM-MSCs treatment in asthma, we designed a custom-made protein-coding gene PCR array that covered 11 different pathways or catalogs with 88 genes that include most of the immunomodulatory factors and important proteins that are involved in allergic airway inflammation. Seventeen genes were found to be different after asthma induction. However, the BM-MSCs administration affected 70 genes that included TLR, MAPK signaling pathway, Th2 cytokines, and other related genes. A hierarchical clustering analysis for the genes exhibited different expression levels in three groups. Especially, the MSCs treatment increased the expression of genes transcripts expression and several miRNAs after the challenge, which will be closer to the clinical settings. In addition, the possible signaling pathways that are involved in the effects of activin A and Acvr2a should be further investigated.

Taken together, our results indicate that there were different expression levels for a number of functional protein genes transcripts expression and several miRNAs after the induction of asthma and BM-MSCs treatment. A study evaluating aberrant miRNAs both in asthma and with human BM-MSCs treatment will provide us with a better understanding regarding the mechanisms underlying allergic airway inflammation and help to promote human BM-MSCs therapeutic applications in the clinic.

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Author Disclosure Statement

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