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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

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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% CO_2 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 (Supplementary Data are available online at www.liebertpub.com/scd). 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 5×10^6 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.

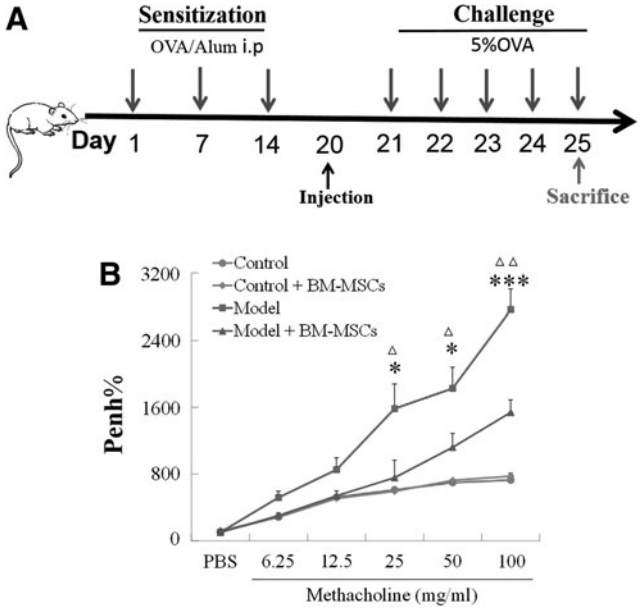

FIG. 1. The experimental protocol for the mouse asthma and airway hyperresponsiveness model. **(A)** BALB/c mice were sensitized on days 1, 7, and 14 by intraperitoneal injection of OVA with aluminum hydroxide and challenged with aerosolized 5% OVA from days 21 to 25. Purified human BM-MSCs (1×10^6) 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 ($n=6$). $^*P<0.05$; $^{***}P<0.001$, compare with control group. $^{\triangle}P<0.05$; $^{\triangle\triangle}P<0.01$, 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 histology 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 RT^2 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 involved in allergy, including the following: toll-like receptors, NFκB signaling pathways, mitogen-activated protein kinase (MAPK) pathways, Janus kinase activity pathways, signal transducer and activator of transcription (STAT) families, TGFβ superfamily receptors, 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 (ANOVAs). 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 $2^{\Delta\Delta Ct}$ method. Primer sequences for mRNA quantification were as follows: Forward-β-actin: TGAGACCTTCA ACACCCCAGCCATG, Reverse-β-actin: CGTAGATGG GCACAGTGTGGGTG. Forward-hAcvr2a: GATGGAA GTCACACAGCCCA, Reverse-hAcvr2a: GGTCCTGGG TCTTGAGTTGG. Forward-mAcvr2a: TCCTGTTACA CCGAAGCCAC, 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, 6 h, 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 24 h. 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 mg/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 perivessel 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 mice. 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 differently 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 progressions, 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).

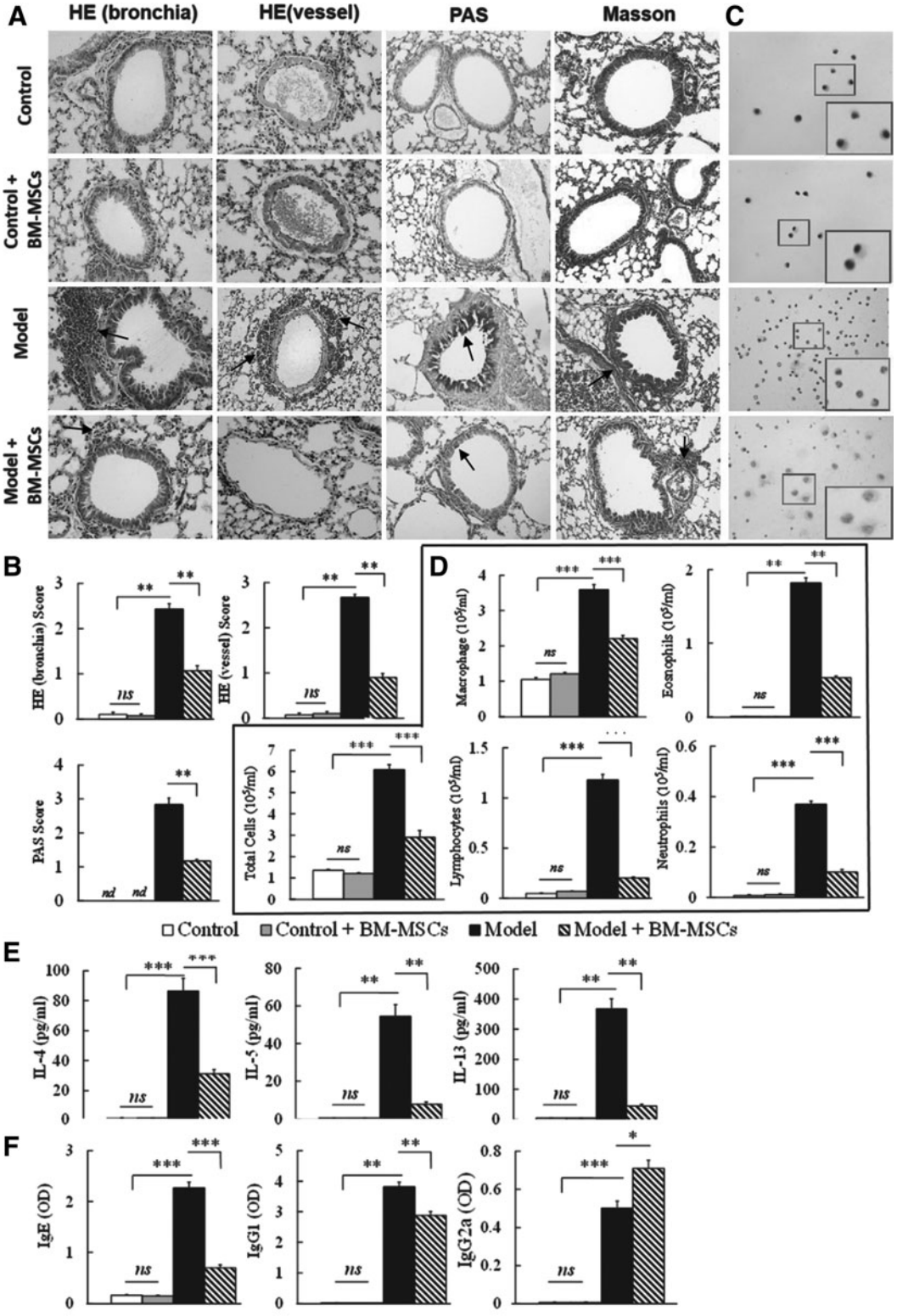

FIG. 2. Transplantation of human BM-MSCs attenuated airway inflammation in the lung and affected inflammatory cytokine production in the BALF and Ig proteins in the serum in asthmatic mice. **(A)** Representative photomicrographs of lung sections with H&E, PAS, and Masson staining around the bronchi 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.

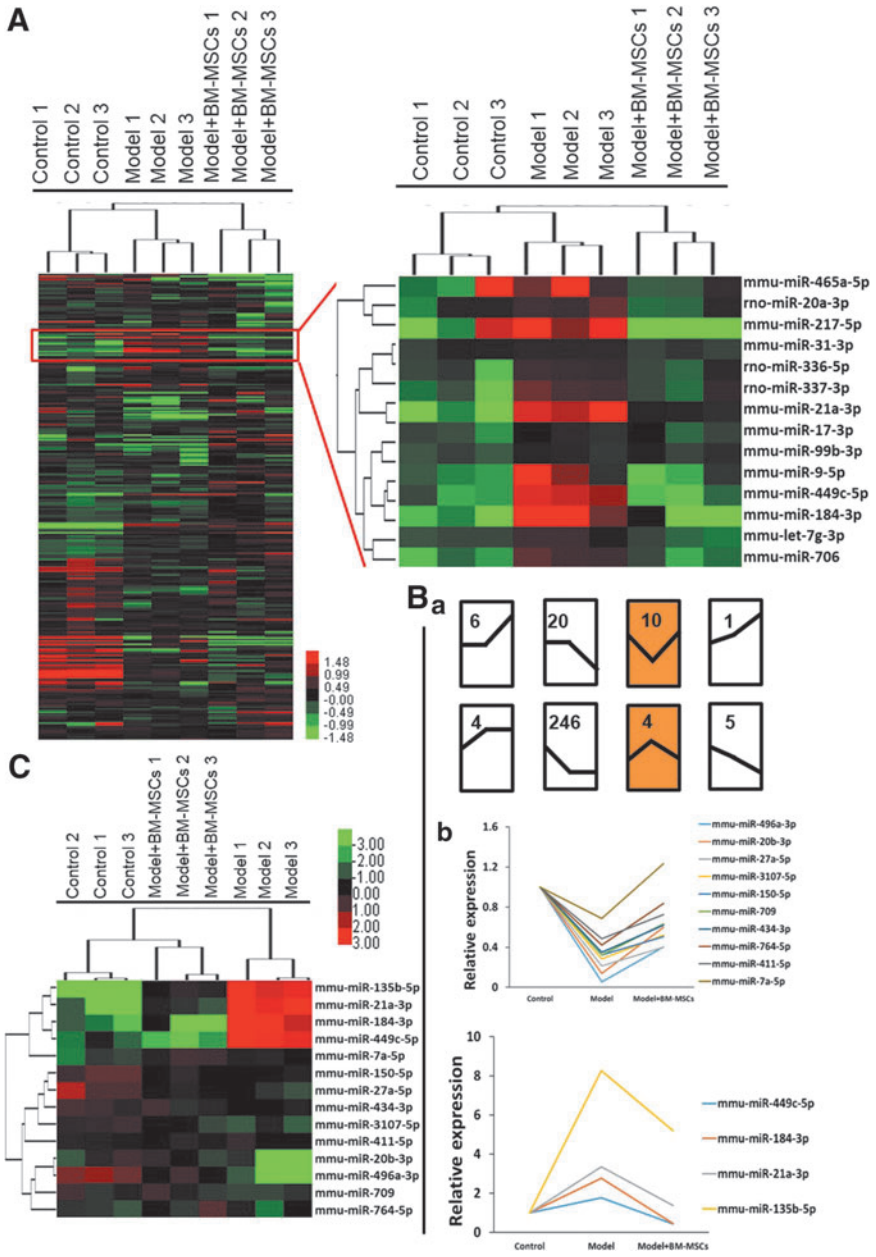

FIG. 3. miRNAs differently expressed during asthma and after BM-MSCs treatment. **(A)** Hierarchical clustering analysis of all the aberrant miRNAs that were differently expressed either between the model ($n=3$) and control groups ($n=3$) or between the model+BM-MSCs ($n=3$) and model groups (total $n=9$, greater than twofold; $P<0.05$). **(B)** The miRNA development patterns after asthma induction and then with the BM-MSCs. (a) The *first lines* in the *boxes* represent increased or decreased miRNA expression after asthma induction 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.

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 group. Next, the *TLR2*, *IL-10*, and *IL-33* levels continued to increase to higher levels, and the *CCL17*, *CSF2*, and *IL-4* levels decreased after the BM-MSCs treatment. The *TLR1*, *TLR4*, *TLR6*, *TLR7*, *TLR8*, *TLR9*, *CCL26*, *IL-21*, *IL-25*, programmed cell death 1 (*PDCD1*), tumor necrosis factor (ligand) superfamily, member 4 (*TNFSF4*), *Foxp3*, and *RORC* mRNA levels were not changed after the allergic inflammation induction. However, their levels were significantly increased after the BM-MSCs treatment. The *TLR3*, *IFNG* (*IFN-γ*), and *IL-12a* mRNA levels declined after allergic induction and increased with the BM-MSCs treatment.

Similarly, we performed a hierarchical clustering analysis for the differently expressed genes in the three groups. Systematic variations were found in the control, model, and model+BM-MSCs treatment groups (Fig. 5A). The results further suggested that allergic airway inflammation and the human BM-MSCs treatment caused significant changes to the immune states of the model animals. Furthermore, we selected 19 aberrant protein-coding genes based on the said variation patterns, such as *CCL17*, and conducted a hierarchical clustering analysis. The pattern-associated genes made a better clustering effect (Fig. 5B).

Moreover, we analyzed the target genes in the gene list of our PCR array of the 14 selected miRNAs with TargetScan (www.targetscan.org) (Table 1). A total of 22 genes, such as

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

miRNAs	*Average Ct in control*[a]	*Fold change (model vs. control)*	P *(model vs. control)*[b]	*Fold change (model+BM-MSCs vs. model)*	P *(model+ BM-MSCs vs. model)*	*Target gene*[c]
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	
mmu-miR-3107-5p	25.61	0.28	0.0020	1.83	0.0444	Smad1, Itga4
mmu-miR-150-5p	22.90	0.32	0.0003	1.57	0.0268	Stat5b, Nfe2l2, Elk1, Gsk3b, Irak2
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	
mmu-miR-764-5p	32.40	0.42	0.0069	1.99	0.0105	Tslp, Mapk14
mmu-miR-411-5p	29.65	0.48	0.0063	1.50	0.0215	
mmu-miR-7a-5p	28.79	0.69	0.0376	1.80	0.0078	Stat1, Rela
mmu-miR-449c-5p	31.12	1.77	0.0079	0.24	0.00003	Notch1, Stat6
mmu-miR-184-3p	35.76	2.76	0.0141	0.16	0.0336	
mmu-miR-21a-3p	31.68	3.36	0.0254	0.41	0.0285	Acvr2a
mmu-miR-135b-5p	34.66	8.27	0.0004	0.63	0.0153	Gata3, Elk1, Gsk3b, Mapk10, Jak2, Stat6, Tnfsf4, Tlr4

[a]Abundance of miRNAs was represented by average Ct value in control mouse.
[b]For analysis of different expression levels between two groups, student's *t*-tests were used. Results were considered statistically significant at $P < 0.05$.
[c]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; Il33, interleukin 33; Irak2, interleukin-1 receptor-associated kinase 2; Itga4, 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 *mmu-miR-449c-5p* 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 mRNA 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/Acvr2a* axis in human. We searched the online TargetScan database and found that the 3′ UTR of human *Acvr2a* has the same *hsa-miR-21* binding site as the murine *Acvr2a* (Fig. 6A). This finding indicates that the *miR-21/Acvr2a* 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

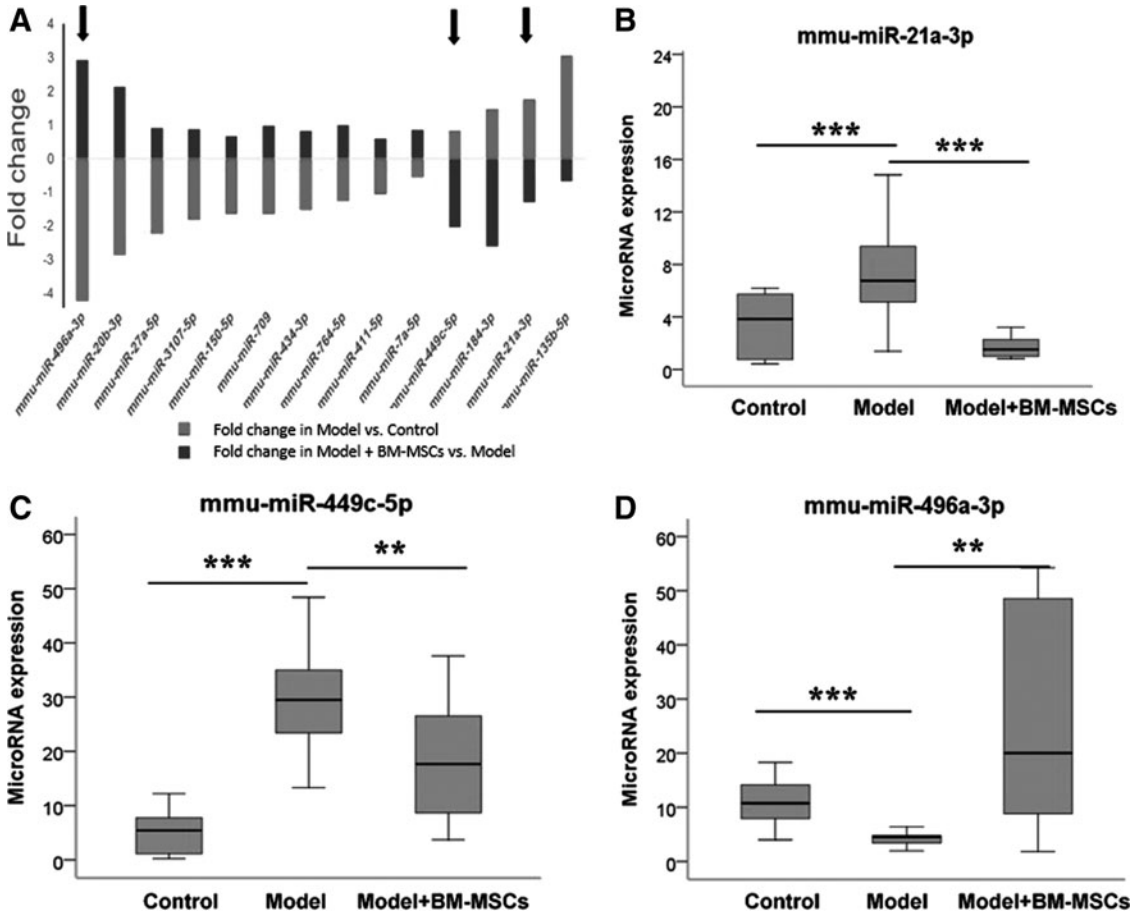

FIG. 4. *Mmu-miR-21a-3p*, *mmu-miR-449c-5p*, and *mmu-miR-496a-3p* were significantly differentially expressed in asthma and with BM-MSCs treatment. **(A)** The fold changes of the 14 selected miRNAs with the log ratio of the mean value are shown. The *arrows* indicate the miRNAs that were selected for the next experiments. The *mmu-miR-21a-3p* **(B)**, *mmu-miR-449c-5p* **(C),** and m*mu-miR-496a-3p* **(D)** expression levels were confirmed with real time PCR in subsequent lung tissue samples from the control ($n = 14$), model ($n = 12$), and model+BM-MSC ($n = 14$) groups. $^{**}P < 0.01$; $^{***}P < 0.001$. PCR, polymerase chain reaction.

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. Specially, 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,

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

Classify	*M/C – MSCs/M ↑*	*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	
NFκB signaling	REL	NFκB2				
MAPK	MAPK1, MAPK14					MAPK10
Janus kinase activity	JAK2, JAK3					
STAT family	STAT4, STAT5b, STAT6					
TGFß superfamily receptors					Acvr2a	
Notch pathway	Notch1					Hes1
Transcription factors	Foxp3, RORC					
Th2 cytokines and related genes	CCL26, IL-21, IL-25, PDCD1, TNFSF4		IL-10, IL-33	CCL17, CSF2, IL-4	IFNG, IL-12a	
Mast cells, IgE and eosinophils	CPA3				CMA1	
Others	ACTB, GUSB		IL-1r1		POSTN, TBX21, B2M	

–, no obvious change; ↑, raise, $P < 0.05$; ↓, decline, $P < 0.05$.

ACTB, actin, beta; B2M, beta-2-microglobulin; CCL, chemokine (C-C Motif) ligand; CMA1, chymase 1, mast cell; CPA3, carboxypeptidase A3 (mast cell); CSF2, colony-stimulating factor 2 (granulocyte-macrophage); Foxp3, Forkhead Box P3; GUSB, glucuronidase, beta; Hes1, Hes family BHLH transcription factor 1; IFNG, interferon, gamma; IL-1r1, interleukin 1 receptor, type I; M/C, model/control; MSCs/M, model+MSCs/model; NFκB2, nuclear factor of kappa light polypeptide gene enhancer in B-cells 2; PDCD1, programmed cell death 1; RORC, RAR-related orphan receptor C; TBX21, T-Box 21; Th2, T helper type 2.

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]. NFκB-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 glucocorticoid treatment [29]. Few articles studied the effects of MSCs on miRNAs for the diseases of respiratory system. One report revealed that human BM-MSCs suppressed stretch-induced *miR-155* expression and its relative cytokines in cultured human bronchial epithelial cells [20]. However, there were no reports regarding the role of miRNA in asthma after BM-MSCs treatment.

In this study, we identified that 296 out of total 752 miRNAs were differently expressed after the induction of asthma and/or the treatment of BM-MSCs. Fourteen out of 296 miRNAs that presented the reverse variation tendency between asthma induction and BM-MSCs transplantation were finally selected for further analysis. Among these miRNAs, *mmu-miR-21a-3p*, *mmu-miR-449c-5p*, and *mmu-miR-496a-3p* were further confirmed with more samples and qRT-PCR. We found that *mmu-miR-21a-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

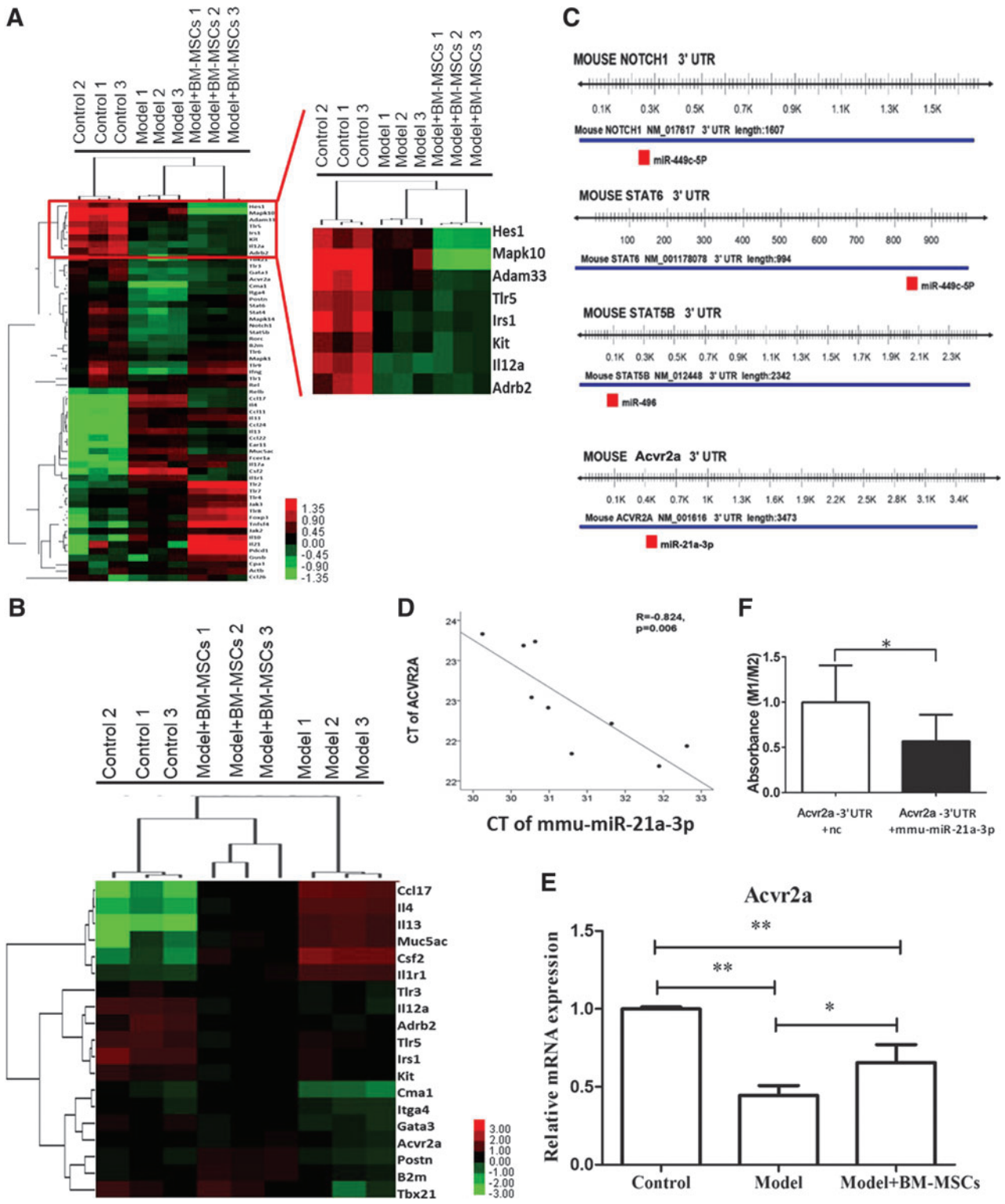

FIG. 5. The mRNA profile in asthma with the treatment of BM-MSCs and the relationship between *mmu-miR-21a-3p* and *Acvr2a*. **(A)** A hierarchical clustering analysis of all the aberrant protein-coding genes in the PCR array that were differentially expressed either between the model and control groups 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.

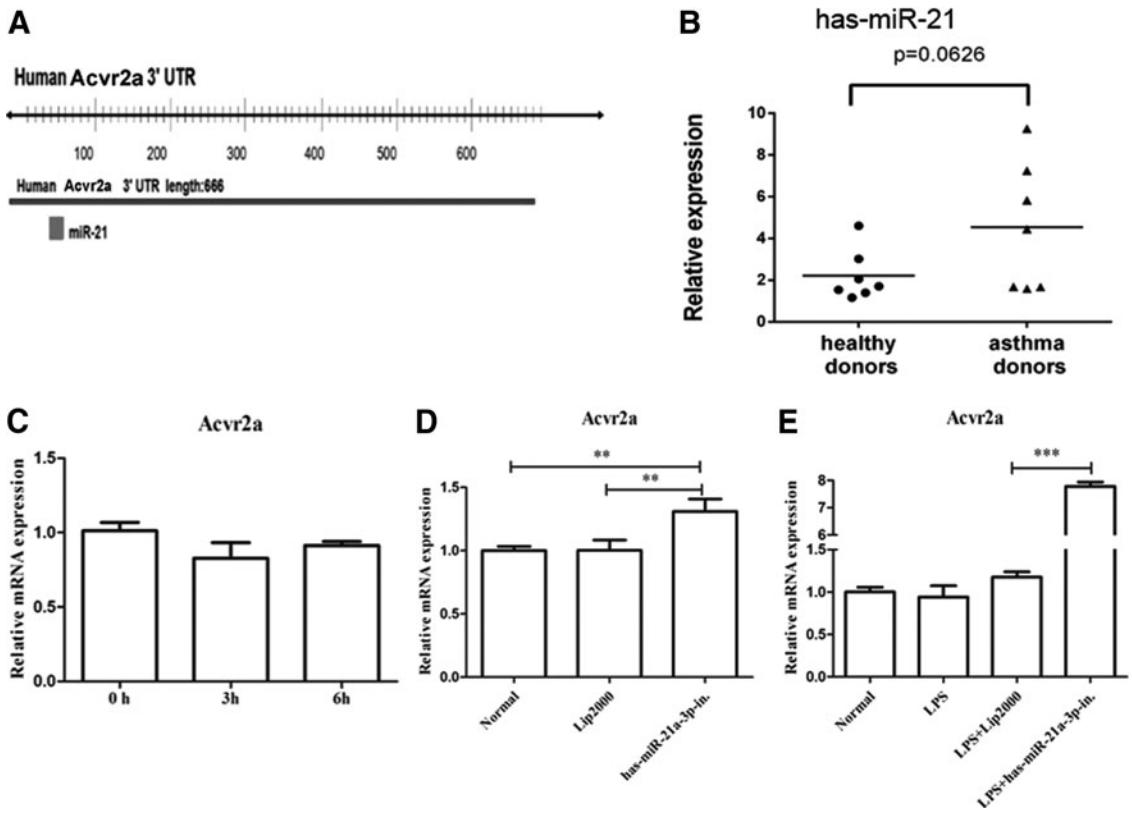

FIG. 6. *Has-miR-21* regulates human *Acvr2a* expression. **(A)** The schematics show the interaction site for *has-miR-21* that is harbored in the 3′ UTR of the human *Acvr2a* mRNA transcript. **(B)** The mRNA expression levels of human *hsa-miR-21* in asthma patients are shown. The *hsa-miR-21* expression levels increased; however, they were not significantly different between the human bronchial epithelial cells from the asthmatic donors compared with the healthy donors. The *hsa-miR-21* expression data were collected from NCBI Gene Expression Omnibus (GEO; GEO No. GSE25230). **(C)** BEAS-2B cells were cultured in indicated condition and exposed to 500 ng/mL LPS for 0, 3, and 6 h, respectively. **(D)** BEAS-2B cells were transfected with or without 500 pmol *has-miR-21a* inhibitor and cultured for 24 h. Lip 2000 group served as control. **(E)** BEAS-2B cells were transfected with or without 500 pmol *has-miR-21a* inhibitor for 24 h and then exposed to 500 ng/mL LPS for 3 h. *Acvr2a* mRNA levels were analyzed by qRT-PCR for **(C–E)**.$^{**}P < 0.01$; $^{***}P < 0.001$. LPS, lipopolysaccharide.

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 it 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 *Foxp3* and *IL-10*, which are associated with Treg function. This was consistent with a previous study in which adipose-derived MSCs ameliorated allergic airway inflammation by inducing Tregs in a mouse model of asthma [42]. *STAT4* was reportedly decreased in asthma [43], and in our study, its gene levels increased after MSCs administration. In addition, previous studies showed high gene levels of *CCL17*, *CSF2*, and *IL-4* in asthma [44–46].

Our data showed that MSCs treatment decreased the gene levels of these factors. The data that we got from the gene PCR array at least provided us with many avenues for further investigation of the effects of BM-MSCs in allergic airway inflammation. To the best of our knowledge, this study is the first to investigate the effects of BM-MSCs on the functional target genes comprehensively with an mRNA PCR array that covered most of the previously reported genes that are involved in asthma.

We acknowledge that this study contains some flaws and some questions should be further addressed. For example, we did not identify the roles of *miR-21a-3p-Acvr2a* axis in the therapeutic effect of BM-MSCs in our asthma model. Second, we did not observe the effects of BM-MSCs administrated 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

No competing financial interests exist.

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