Original Paper

Altered MicroRNA Expression Profiles in Activated Mast Cells Following IgE-FcεRI Cross-Linking with Antigen

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Key Words

MiRNA • Microarray • Mast cell • Immunoglobulin E • Fc receptor

Abstract

Background/Aims: MicroRNAs (miRNAs) are critical regulators of immune responses and immunologic disorders. However, little is known about miRNA expression and function during mast cell differentiation, proliferation and activation. This study aimed to determine the miRNA expression profiles in mast cells stimulated by immunoglobulin E (IgE) and antigen and to analyze the potential functions of specific miRNAs. **Methods:** Bone marrow-derived mast cells (BMMCs) generated from differentiated mouse bone marrow cells were untreated (Unstimu) or stimulated with IgE-antigen complexes for 1 h or 6 h (Stimu). The miRNA profiles were evaluated by miRNA microarray. MiRNA target gene prediction and enrichment analyses were performed using bioinformatics. **Results:** Seven significantly up-regulated and 10 down-regulated miRNAs were identified in the 1 h Stimu group relative to the Unstimu group (fold change > 2; $P < 0.05$). Of 8 miRNAs randomly selected from the 17 identified, the expression levels of 6 were confirmed by quantitative real-time PCR (qRT-PCR). The potential target genes of several candidate miRNAs were enriched in FcεRI signaling, response to stimulus and cellular exocytosis. **Conclusion:** The expression of many miRNAs changes following IgE-FcεRI cross-linking in activated mast cells, and these miRNAs probably play key regulatory roles in core signaling pathways and biological behaviors. Evaluating the functions of these characteristic miRNAs will further our understanding of IgE-associated allergic disease pathogenesis and the development of therapeutic strategies.

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Introduction

Type I hypersensitivity responses are immediate-type allergic reactions initiated by immunoglobulin E (IgE) antibodies and antigens, which are involved in the critical pathogenesis of IgE-associated allergic diseases, such as allergic rhinitis, asthma and atopic dermatitis. These reactions are triggered in IgE-sensitized individuals upon re-exposure to an innocuous antigen (allergen). Mast cells in the exposed tissue, which act as effector cells, can be immediately activated within seconds of allergen exposure. The activation of mast cells subsequently induces the secretion of inflammatory mediators, which leads to a series of allergic responses and disorders. For example, allergic rhinitis symptoms, including sneezing, nasal obstruction, nasal itching and rhinorrhea, occur when allergenic proteins come into contact with the mucous membranes of the nose. Current drug treatments for IgE-associated allergic diseases can effectively relieve allergic symptoms; however, such treatments are largely palliative rather than curative. Therefore, it is essential to elucidate the potential mechanisms of allergic diseases and to identify more effective treatments.

Mast cells are derived from hematopoietic precursors in the bone marrow but mature locally. These cells are widely distributed throughout vascularized tissues, particularly near surfaces that are exposed to pathogens and allergens [1]. In type I hypersensitivity responses, mast cells are typically activated by the antigen-induced cross-linking of specific IgE-bound high-affinity Fc receptors (FcεRI) on cell membranes [2-5]. Upon cell activation, a variety of biologically active pharmacological mediators that are pre-stored in cytoplasmic granules, including histamine, heparin, proteoglycans, and proteases, are rapidly released into the extracellular milieu. Additionally, multiple proinflammatory cytokines and lipid mediators are de novo synthesized and secreted [1, 6, 7]. Mast-cell proliferation, migration and adherence are also immediately initiated. Additionally, accumulating evidence has demonstrated that IgE can also induce a wide variety of biological responses of mast cells even in the absence of antigen, such as up-regulation of the FcεRI, survival, cytokine production, histamine synthesis, and adhesion to fibronectin, which is referred to as “monomeric IgE” responses [8-12]. Several intercellular molecules, such as spleen tyrosine kinase (Sky) and phospholipase Cγ (PLCγ), are involved in the signaling cascade following IgE binding to FcεRI or FcεRI aggregation, and regulate a series of IgE-mediated biological processes in stimulated mast cells [9, 13-15]. However, in these processes, the regulatory mechanisms that are involved in the expression of these signal molecules have not been extensively studied.

MiRNAs are a class of short, non-coding RNAs that have emerged as key post-transcriptional regulators of the expression of protein-coding genes. MiRNAs can specifically bind to target sites in the 3' untranslated region (3' UTR) of messenger RNAs (mRNAs), thereby triggering mRNA degradation or inhibiting mRNA translation [16-18]. The importance of miRNAs in many physiological and pathological processes has been well established. Interestingly, several miRNAs have also been found to be involved in rapid biological responses associated with acute stimulation, inflammation or injury [19-24]. For example, Srivastava et al. [20] found that 11 miRNAs had significantly altered expression in roots of *Brassica juncea* exposed to arsenate for 1 h. Mai et al. [21] reported that expression levels of miR-20a, -21, -19b, -34a, -34c, -140, and -200b were significantly down-regulated in MC3T3-E1 cells subjected to 1 h of fluid shear stress (FSS) at 12 dyn/cm². Additionally, an increasing number of studies have identified altered miRNA expression in diverse immune responses and immunologic disorders [25-33]. In contrast, the expression and function of specific miRNAs during the differentiation, proliferation and activation of mast cells has received little attention [34-41]. Mayoral et al. [37] reported that miR-221-222 were detectably up-regulated in BMMC stimulated for 1 h or 24 h with PMA and ionomycin, and regulated cell cycle checkpoints in mast cells in response to acute stimulation. Only individual miRNAs have been implicated in mast cell degranulation and cytokine production because of their role in the post-transcriptional fine-tuning mechanisms of gene expression, which are associated with the FcεRI signaling pathway [35, 36, 38, 42]. Furthermore, little is

to known about a global signature of miRNA expression in response to IgE-mediated mast cell activation or the regulatory network between miRNAs and their target genes during this biological event.

Considering that the mRNA expression levels of inflammatory mediators and signal molecules usually begin to be significantly changed 1 h after mast cell activation following IgE-FcεRI cross-linking with antigen [43, 44], we presumed that miRNA expression may be initiated at this time point to regulate the stabilities and expression levels of these mRNAs by post-transcriptional mechanism. Therefore, in this study, we characterized the miRNA expression profiles in BMMCs stimulated with IgE-Ag complexes for 1 h using a miRNA microarray and observed a dynamic regulation of the expression levels of selected miRNAs following different time points (1 h and 6 h) after BMMC activation. In addition, we analyzed the potential roles of selected miRNAs using bioinformatics methods to build a unique miRNA regulatory network that was closely linked to mast cell activation. This study aimed to lay a foundation for further investigations of the underlying mechanisms of IgE-associated allergic diseases that are caused by mast cell activation.

Materials and Methods

Preparation and culture of BMMCs

Male BALB/c mice (8-10 weeks old) were purchased from the Medicine Laboratory Animal Center of Fudan University, Shanghai, China. The animal experimental protocols were approved by the Animal Care and Use Committee of Fudan University. Mouse BMMCs were prepared as previously described [43, 45]. Briefly, BMMCs were generated by the *in vitro* differentiation of bone marrow cells that were isolated from the femurs and tibias of the BALB/c mice. Bone marrow cells were cultured at a concentration of $0.5\text{--}1.0 \times 10^6$ cells/ml in RPMI 1640 medium that was supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1 mM nonessential amino acids (Gibco, Invitrogen, Grand Island, NY, USA), 10 ng/ml recombinant murine interleukin (IL)-3 (Peprotech, Rocky Hill, NJ, USA) and 10 ng/ml recombinant murine stem cell factor (SCF; Peprotech). Non-adherent cells were transferred to fresh medium at least once a week.

After 3-4 weeks of culture, more than 95% of the cells were BMMCs as determined by toluidine blue staining and flow cytometric analysis using a combination of fluorescein isothiocyanate (FITC)-conjugated anti-mouse FcεRIα Armenian hamster IgG (clone MAR-1; eBioscience, San Diego, CA, USA) and phycoerythrin (PE)-conjugated anti-mouse CD117(c-Kit) Rat IgG (clone 2B8; eBioscience). Cell viability was determined by a trypan blue dye exclusion assay and was more than 98%.

BMMC stimulation

BMMCs were either left resting (Unstimu group) or were stimulated with IgE-Ag complexes for 1 h or 6 h (1 h or 6 h Stimu group). According to a previously described method [43, 46], the cells were suspended at a density of 1×10^6 cells/ml in fresh IL-3- and SCF-supplemented medium. To stimulate cells via FcεRI, the cells were sensitized with 2 µg/ml mouse anti-dinitrophenyl (DNP) monoclonal IgE (clone SPE-7; Sigma-Aldrich) overnight at 37°C. After washed twice with phosphate-buffered saline (PBS), the cells were suspended in Tyrode's buffer (10 mM HEPES, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 0.3 mM NaH₂PO₄ and 0.1% bovine serum albumin) at 2×10^6 cells/ml. The cells were challenged by the addition of 40 ng/ml DNP-Human Serum Albumin (DNP-HAS; Biosearch Technologies, Novato, CA, USA) and incubation for 1 h or 6 h at 37°C. The samples were collected by centrifugation at 120 g for 5 min at 4°C.

RNA extraction

Total RNA, including miRNA, was extracted from all of the cell samples and purified using the mirVana™ miRNA Isolation Kit (Cat# AM1560, Ambion, Austin, TX, USA) following the manufacturer's instructions. The RNA concentrations were quantified using a NanoDrop spectrophotometer (Thermo Fisher, Waltham, MA, USA). The integrity and quality of RNA was confirmed using an Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, USA), and only the RNA samples with an RNA integrity number (RIN) ≥ 6.0 and 28S/18S > 0.7 were used for the miRNA array analysis and reverse transcription (RT).

miRNA microarray assay

The RNA samples were further analyzed at Shanghai Biotechnology Corporation (Shanghai, China) using the Agilent Mouse miRNA Microarray (8×60K; version 19.0), which contains capture probes for a total of 1,247 mouse miRNAs based on the Sanger miRBase database (release 14.0). First, the miRNA molecules in total RNA were labeled using the miRNA Complete Labeling and Hyb Kit (Cat# 5190-0456, Agilent technologies, Santa Clara, CA, USA) according to the manufacturer's protocols. Each microarray slide was hybridized using 100 ng Cy3-labeled RNA in a hybridization oven (Cat# G2545A, Agilent technologies) under conditions of 55°C and 20 rpm for 20 hours. After hybridization, the slides were washed twice using the Gene Expression Wash Buffer Kit (Cat# 5188-5327, Agilent Technologies) in staining dishes (Cat# 121, Thermo Shandon, Waltham, MA, USA). The slides were scanned using the Agilent Microarray Scanner (Cat#G2565BA, Agilent Technologies), and the intensity of each hybridization signal was extracted using the default settings in the Feature Extraction software program (version 10.7, Agilent Technologies). Normalized data were obtained using the Quantile algorithm in the Gene Spring software program (version 11.0, Agilent Technologies), and these data were transformed to base 2. A relative fold change >2 in the differential expression of miRNAs and a *P* value <0.05 were considered significant. The Gene Cluster (version 3.0) and Java TreeView software programs were used to perform the hierarchical cluster analysis of differentially expressed miRNAs and to visualize the miRNAs.

qRT-PCR

Total RNA was reverse transcribed into cDNA using the miScript II RT Kit (Qiagen, USA). To validate the quantity of miRNA, qRT-PCR was performed using the 7900 HT Sequence Detection System (ABI, USA) with the miRcute miRNA qPCR Detection Kit (SYBR Green; Tiangen, China) according to the manufacturer's instructions. For mRNA analysis, RNA extracts were reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen). The synthesized cDNA was then quantified by SYBR green assay. All primers were synthesized by Invitrogen (Shanghai, China) and their sequences were presented in Table 1. RNU6 and β -actin were used as endogenous references for miRNAs and mRNAs, respectively, and the expression levels were calculated using the formula $2^{-\Delta\text{Ct}}$ ($\Delta\text{Ct} = \text{Ct}_{\text{miRNA/mRNA}} - \text{Ct}_{\text{RNU6}/\beta\text{-actin}}$). The experiments were independently performed three times in triplicate.

Prediction and enrichment analysis of miRNA target genes

The target genes for all of the significantly differentially expressed miRNAs were predicted using multiple public bioinformatics databases, including TargetScan Mouse Release 6.2 (<http://www.targetscan.org/>), miRanda (<http://www.microrna.org/>) and miRWalk (<http://mirwalk.uni-hd.de/>), which were comparatively analyzed using miRWalk online tool. Predicted targets were included in at least two databases. miRDB (<http://mirdb.org/miRDB/>) was a supplement only when some miRNAs were not in three miRNA databases above. The gene ontology (GO) and cellular pathway analyses of the predicted target genes were conducted using an online functional annotation tool (DAVID, <http://david.abcc.ncifcrf.gov/>). GO terms and pathways with a *P* value <0.01 and a false discovery rate (FDR) <0.01 were retained.

miRNA target gene network construction

Based on the functions of the target genes in the enrichment analysis, several interesting genes were selected, and a regulatory miRNA target gene network was constructed and visualized using the Cytoscape software. The correlation between the miRNAs and their target genes is presented in an image drawn using Adobe Illustrator CS6 (Adobe Systems, Inc.).

Table 1. Primer sequences used for qRT-PCR

Symbol	Primer (5' to 3')
mmu-miR-92b-3p	TATTGCACTCGTCCCGG
mmu-miR-874-3p	CCTGACCCTAGGGACCGA
mmu-miR-5117-5p	GCGATTAGTGATGATCAATAAAGTTA
mmu-miR-3113-5p	GTCCTAGCCCTTGTCCGG
mmu-miR-212-5p	ACCTTGCTCTAGACTGCTTACT
mmu-miR-21a-3p	CAACAGCAGTCGATGGAAA
mmu-miR-125a-3p	ACAGGTGAGGTTCTTGGGAG
mmu-miR-721	CAGTGAATTAAAAGGGGGA
RNU6	GCTCGCTTCGGCAGCACAT
Lyn	F: AACTTCGGCTGCTTCACT R: GTTGGTTCTCCCTGGGTA
Scamp1	F: ATGTCGGATTTTGACAGCAACC R: GTCCCGTGGCACAATTTCT
Vav3	F: ATGCAGACTCCAATTTCCATGAT R: ATGGCCCTTGTTCAACGGAAT
Csf2	F: GGCCTTGAAGCATGTAGAGG R: GGAGAACTCGTTAGAGACGACTT
β -actin	F: AGAGGGAAATCGTGCCTGAC R: CCATACCAAGAAGGAAGGCT

Fig. 1. Differentially expressed miRNAs involved in IgE-mediated BMMC activation. (A) A hierarchical cluster analysis of 17 miRNAs whose expression differed significantly between the 1 h Stimu group and the Unstimu group (fold change > 2; $P < 0.05$). Each row represents a miRNA, and each column represents a sample. The miRNA expression levels are illustrated using a color key and histogram. The red and green colors denote high and low expression, respectively. (B) The qRT-PCR validation of expression-alerted miRNAs that were screened by the microarray assay. Up-regulated and down-regulated miRNAs are indicated by bars above and below the horizontal axis, respectively. The qRT-PCR results were representative of three independent experiments.

Statistical analysis

Statistical analyses were performed using the SPSS software for Windows (version 16.0; SPSS, Inc., Chicago, IL, USA). The differences between the two groups were determined using a two-tailed Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Significantly altered miRNA expression in activated BMMCs following IgE-FcεRI cross-linking

To identify the miRNAs that were potentially involved in IgE-mediated mast cell activation, we examined the miRNA expression profiles of BMMCs in the 1 h Stimu group and the Unstimu group using the miRNA microarray platform. The cluster analyses revealed that a total of 17 miRNAs were significantly differentially expressed in the BMMCs from the 1 h Stimu group relative to those from the Unstimu group (fold change > 2; $P < 0.05$; Fig. 1A). Among these altered miRNAs, 7 were up-regulated and 10 were down-regulated. Notably, miR-21a-3p and miR-3113-5p were the most remarkably up-regulated and down-regulated miRNAs in activated BMMCs according to the bioinformatics algorithm (Table 2).

Validation of the candidate miRNAs by qRT-PCR

To validate our microarray data, qRT-PCR was performed to quantify the expression levels of 8 miRNAs that were randomly selected from 17 differentially expressed miRNAs in the microarray experiment. The qRT-PCR results for 6 miRNAs (miR-21a-3p, miR-212-5p, miR-125a-3p, miR-5117-5p, miR-874-3p and miR-3113-5p) were consistent with the microarray results, and the results for miR-92b-3p and miR-721 were inconsistent, resulting

Table 2. List of differentially expressed miRNAs between the 1 h Stimu group and the Unstimu group detected by miRNA microarray assay

miRNA	Accession No.	log ₂ Fold change (vs. Unstimu group)	P value
mmu-miR-21a-3p	MIMAT0004628	4.959	5.418E-03
mmu-miR-23a-5p	MIMAT0017019	4.464	5.341E-03
mmu-miR-222-5p	MIMAT0017061	4.085	7.571E-03
mmu-miR-212-5p	MIMAT0017053	1.830	1.642E-02
mmu-miR-212-3p	MIMAT0000659	1.645	8.647E-05
mmu-miR-132-3p	MIMAT0000144	1.458	2.243E-06
mmu-miR-92b-3p	MIMAT0004899	1.103	4.902E-02
mmu-miR-3113-5p	MIMAT0014959	-6.684	4.224E-05
mmu-miR-874-3p	MIMAT0004853	-5.734	1.460E-06
mmu-miR-5117-5p	MIMAT0020625	-5.057	1.616E-05
mmu-miR-3058-3p	MIMAT0014814	-4.796	3.055E-03
mmu-miR-6392-3p	MIMAT0025142	-3.962	8.709E-03
mmu-miR-3472	MIMAT0015643	-3.441	2.380E-02
mmu-miR-149-3p	MIMAT0016990	-1.251	7.942E-04
mmu-miR-5109	MIMAT0020617	-1.118	9.454E-03
mmu-miR-721	MIMAT0003515	-1.078	3.531E-04
mmu-miR-125a-3p	MIMAT0004528	-1.014	1.576E-03

Fig. 2. Measurement of miRNA expression levels using qRT-PCR at different time points (1 h and 6 h) after BMMC activation. The results were representative of three independent experiments. Data are represented as mean±SD (standard deviation). * P<0.05.

in a concordance rate of 75.0% (6/8). Furthermore, of these 6 miRNAs, miR-21a-3p and miR-212-5p were up-regulated in the 1 h Stimu group, whereas miR-125a-3p, miR-5117-5p, miR-874-3p and miR-3113-5p were down-regulated 1 h after IgE-dependent BMMC activation (Fig. 1B). To observe a dynamic regulation of these 6 miRNAs following different time points after BMMC activation, we found that the expression levels of these miRNAs except

Fig. 3. An enrichment analysis of the predicted target genes. (A-C) GO functional enrichment of miRNA potential targets: (A) Biological process, (B) Cellular component, (C) Molecular function. (D) Pathway annotation of the target genes of the miRNAs. Ordinate is the significant GO/pathway term, and the abscissa is the P value of the negative logarithm ($P < 0.01$).

miR-5117-5p and miR-3113-5p remained to be significantly altered in BMBCs stimulated with IgE-Ag complexes for 6 h (Fig. 2).

GO analysis of miRNA target genes

To investigate the biological functions of the differentially expressed miRNAs, we used public online databases to obtain miRNA-regulated putative target genes. We performed a GO function analysis of miRNA targets to obtain the significantly over-represented GO terms ($P < 0.01$; FDR < 0.01). The results indicated that several important GO terms were possibly related to cell activation, such as intracellular signal transduction, protein phosphorylation, metabolic process, positive regulation of gene

Table 3. List of miRNAs and their potential target genes related to the biological process of cellular exocytosis

Target Gene	Accession No.	miRNA
Trim72	NM_001079932	miR-3113-5p
Lgl1	NM_001159404	miR-874-3p
Myo5a	NM_010864	miR-222-5p
Stx3	NM_152220	miR-149-3p
Stxbp1	NM_001113569	miR-212-5p
Unc13b	NM_021468	miR-21a-3p
Lin7c	NM_011699	miR-21a-3p
Rala	NM_019491	miR-21a-3p; miR-5117-5p
Rapgef4	NM_019688	miR-21a-3p
Scamp5	NM_020270	miR-149-3p
Exoc8	NM_198103	miR-3058-3p
Exoc5	NM_207214	miR-212-3p
Scamp1	NM_029153	miR-125a-3p
Mical3	NM_153396	miR-3058-3p
Stxbp51	NM_172440	miR-23a-5p
Exoc3	NM_177333	miR-3113-5p

Fig. 4. The potential influence of miRNAs on the FcεRI signaling pathway in activated mast cells following IgE-FcεRI cross-linking.

expression, response to stimulus, and exocytosis. (Fig. 3A-3C). Furthermore, in the biological process of exocytosis, several miRNAs (miR-3113-5p, miR-874-3p, miR-21a-3p, miR-3058-3p, and miR-125a-3p) may regulate the expression of a distinct set of target genes that are associated with cellular exocytosis (Table 3).

Specific miRNAs involved in the FcεRI signaling pathway in mast cells through targeting signal molecule genes

To determine whether the differentially expressed miRNAs regulated the expression levels of signal molecule genes in response to the IgE-mediated activation of mast cells, we conducted a pathway analysis. We discovered 38 significant pathways of the putative targets of these miRNAs, including the chemokine signaling pathway, the phosphatidylinositol signaling system, the FcεRI signaling pathway, the calcium signaling pathway, and the MAPK signaling pathway (Fig. 3D). As illustrated in Fig. 4, various miRNAs (miR-5117-5p, miR-212-5p, miR-874-3p, and miR-149-3p) potentially regulated the expression of critical signal molecules in the FcεRI signaling pathway upon IgE-mediated mast cell activation.

The miRNA target gene network reflecting the potential mechanism of IgE-mediated BMMC activation

To visualize the complex potential relationship between miRNAs and putative target genes and to provide some valuable clues for further exploring the potential function and regulatory mechanism of specific miRNAs following IgE-mediated BMMC activation, we constructed an interaction network of miRNAs and target genes. As shown in Fig. 5A, more than ten target genes were predicted for each miRNA. Several genes were considered the potential targets of two or more miRNAs. Two genes (Akt3 and Pip4k2c) were the predicted targets of miR-149-3p and miR-212-5p. Plnxa2 was the predicted target of miR-3113-5p, miR-149-3p and miR-212-5p. In addition, to determine whether the mRNA expression levels of predicted target genes were altered accordingly during IgE-mediated BMMC activation, we selectively analyzed their expression levels by qRT-PCR and found that the mRNA expression levels of Lyn, Scamp1, Vav3 and Csf2 were significantly up-regulated following down-regulation of corresponding miRNAs, which indicated that bioinformatics data on target gene prediction should be considered to be reliable (Fig. 5B).

Fig. 5. (A) A miRNA target gene network. The rounded rectangle nodes represent miRNAs, and the cycle nodes represent target genes. The lines on the edge represent the inhibition of mRNAs by miRNAs. The quantitative regulatory relationship between miRNAs and target genes is indicated by different colors and the areas of these nodes. (B) Relative mRNA expression of predicted target genes. The mRNA expression levels of Lyn, Scamp1, Vav3 and Csf2 following 1 h after BMMC activation were measured by qRT-PCR. The results were representative of three independent experiments. Data are represented as mean $\pm$ SD (standard deviation). * $P < 0.05$.

Discussion

Mast cells are bone marrow-derived cells and are well known as key effector cells in IgE-associated immune responses and allergic diseases [47, 48]. The cells are activated mainly via the IgE- and antigen-mediated crosslinking of Fc ϵ RI. Besides rapid degranulation of mast cells, the cell proliferation, migration and adherence are gradually initiated, and proinflammatory cytokines and lipid metabolites are de novo synthesized after IgE and antigen stimulation [1, 6, 49]. In this study, we found that the mRNA expression levels of some key signaling molecule genes (Lyn, Scamp1, Vav3 and Csf2) were significantly altered in activated mast cells following IgE-Fc ϵ RI cross-linking with antigen. However, it is vital to adequately clarify whether and how the expression of intracellular signaling molecule genes is fine-tuned at the post-transcriptional level in these biological processes. Such information will contribute to the discovery of potential therapeutic targets for the control of mast cell activation in IgE-associated allergic disorders.

As an epigenetic regulator of gene expression, miRNAs play important roles in the regulation of various cell processes, including cell differentiation, proliferation, apoptosis, function and responses to environmental stimulation [31, 50-52]. Nevertheless, the miRNA expression profiles and regulatory functions of mast cells following their physiological and pathophysiological responses have not been extensively documented. A miRNA microarray analysis is typically performed to select differentially expressed miRNAs before beginning

miRNA function experiments. Recently, Yang et al. [40] analyzed the miRNA expression profiles of cell cultures at the initiation of BMMC differentiation using Agilent microRNA array slides, and the authors identified 86 miRNAs that were characterized by a fold change >5, including 45 up-regulated miRNAs and 41 down-regulated miRNAs. Furthermore, using stringent statistical and function enrichment analyses, the authors predicted that 7 specific miRNAs may be directly associated with the expression of c-Kit and FcεRIα and that 18 miRNAs may enhance the expression levels of three critical transcription factors (Mittf, GATA1 and c/EBPα) that directed the differentiation of mast cells. To provide a substantial basis for elucidating the roles of miRNAs during IgE-mediated mast cell activation, we detected the miRNA profiles in BMMCs that were treated with or without IgE and antigen for 1 h using a microarray-based screening method. We found that 17 candidate miRNAs had a 2-fold or greater change in expression and that these miRNAs may participate in IgE- and FcεRI-dependent mast cell activation. Subsequently, 8 of these miRNAs were analyzed by qRT-PCR to validate the reliability of the microarray data, which indicated a high concordance rate of 75.0%. Furthermore, these miRNA expression levels were dynamically regulated following different time points (1 h and 6 h) after mast cell activation.

MiRNAs are emerging as endogenous negative regulators of protein expression by combining with the 3' UTR of target mRNAs, thereby inhibiting protein production or destabilizing target transcripts [16-18]. In a study that aimed to understand how miRNAs regulate the various biological behaviors of mast cells, Mayoral et al. [37] discovered that miR-221-22, a family of miRNAs, was significantly up-regulated following mast cell stimulation. Under mast cell resting and stimulated conditions, the individual or combined overexpression of miR-221 and miR-222 resulted in an increase in the number of cells in the prominent G₁/G₀ peak with correspondently fewer cells in the G₂/M phase. Furthermore, their study further shed light on the miR-221-222 transcriptional regulation mechanism that enabled miR-221-222 to regulate the cell cycle through partially inhibiting p27^{Kip1} protein expression after acute stimulation. Additionally, in response to mast cell stimulation through IgE-antigen complexes, miR-221 had several mast cell-specific, activation-dependent functions that affected the extent of degranulation, cytokine production and cell adherence [36]. In contrast, another miRNA, miR-146a, promotes mast cell apoptosis but not cytokine synthesis [34]. During mast cell differentiation from bone marrow cells, the expression levels of miR-126 are down-regulated, and miR-126 positively regulates mast cell proliferation and FcεRI-mediated cytokine production via enhancing ERK activity and suppressing sprouty-related, EVH1 domain containing 1 (Spred1) expression [35]. In addition, miR-142-3p promotes FcεRI-mediated degranulation in mast cells [38]. With respect to the relationship between miRNAs and pathological conditions that are caused by malignant mast cell proliferation, Lee et al. [53] reported that the miR-539 and miR-381 cluster was significantly down-regulated by c-Kit signaling, and the expression of these two miRNAs decreased Mittf protein levels and the colony-forming ability of mastocytosis cell lines. To determine the impact of the global depletion of miRNAs on mast cells, Oh et al. [39] found that global miRNA expression was indispensable for murine mast cell development *in vivo*. MiRNAs have favorable roles in regulating mast cell differentiation, proliferation and activation; however, current data are preliminary, and future studies are needed to confirm the functions of miRNAs in mast cells.

The identification of miRNA target genes is widely considered to be an important step toward understanding miRNA regulatory mechanisms. Therefore, target prediction programs, as bioinformatics analysis tools, were run to detect the potential target genes of the candidate miRNAs from the microarray data in this study. To our knowledge, a large number of miRNA-target prediction databases have been established using different computational algorithms, most of which are publicly available. However, the single prediction database is quite limited to generate a list of target genes, which probably produces a huge amount of false positive and false negative results. Thus, it is very essential to carry out a comprehensive analysis of multiple databases. TargetScan and miRanda are well-known databases that are extensively used to predict biological targets of miRNAs on the basis of imperfect base-pairing between miRNA and target mRNA 3' UTR. However, emerging evidence shows a

novel mode of action of miRNAs by which they may directly regulate target gene expression by binding to promoter and amino acid coding regions. For this reason, Dweep et al. [54] have presented miRWALK database, which is used to predict all the possible miRNA binding sites by “walking” on the genes of three genomes (i.e. all protein coding genes, and their 10 kb upstream flanking regions and mitochondrial genes). In addition, it hosts predicted and validated miRNA binding sites information and integrates eight established databases for a comprehensive and comparative study. Therefore, in this study, we comprehensively analyzed the potential target genes of miRNAs using dry lab approaches mainly through three public databases above, and found ten to hundreds of target genes for each miRNA. A GO function enrichment analysis was subsequently conducted. Interestingly, a potential relationship was detected between several genes and cellular exocytosis. MiR-3113-5p may promote cellular exocytosis and intercellular mediator secretion via targeting Trim72 and Exoc3. The target gene of miR-125a-3p was Scamp1, which was closely linked with exocytosis. These findings suggest that these miRNAs may participate in the secretion processes of inflammatory mediators in activated mast cells. Our pathway analysis further confirmed that several putative targets were associated with pivotal signaling pathways that are involved in IgE-mediated mast cell activation, such as the FcεRI signaling pathway, the calcium signaling pathway, and the MAPK signaling pathway. The potential target of miR-5117-5p was Lyn, which was associated with the FcεRI signaling pathway. PI3K was the target of multiple miRNAs (miR-23a-5p, miR-21a-3p and miR-149-3p). PI3K is a core signaling molecule in the FcεRI signaling pathway. Collectively, the data from this study indicate that miRNAs may be a vital contributor to important biological behaviors and signaling pathways of activated mast cells.

This study provides informative data on the global miRNA profiles of activated mast cells following IgE-FcεRI cross-linking with antigen and the potential functions of miRNAs using bioinformatics methods, which will contribute to the identification of novel therapeutic targets for treating IgE-associated allergic diseases that are caused by mast cell activation.

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Disclosure Statement

All of the authors declare that they have no conflicts of interest.

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