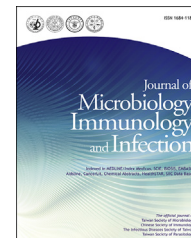


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Original Article

Characterization of microRNA profile in IgE-mediated mouse BMMCs degranulation

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KEYWORDS

Mast cell;
IgE;
miRNA;
Microarray;
Degranulation

Abstract *Background:* Mast cells play a central role in innate and adaptive immunity by releasing pre-formed and *de novo* synthesized mediators, which include microRNAs. Although miRNAs have been confirmed to function in cell proliferation, differentiation, apoptosis, and the immune response, their functions are still limited in mast cells degranulation.

Methods: Here, we survey miRNA expression profiles in activated mouse bone marrow-derived mast cells (BMMCs) with a miRNA microarray and compare the profiles to those from resting BMMCs. Partial miRNAs were selected for confirmation by qPCR, and let-7i was selected for function discover in mast cell degranulation process. TargetScan Mouse database were used for target genes prediction, gene ontology (GO) were used for gene molecular function classifications, and Cytoscape software were used to construct gene network of degranulation.

Results: We found 13 up-regulated miRNAs and 7 down-regulated miRNAs in DNP activated BMMCs by miRNA microarray; and let-7b, let-7c, let-7d, let-7f, let-7i, and miR-652 were up-regulated, and miR-296-3p was down-regulated in DNP-stimulated BMMCs by qPCR. In the function research, let-7i can inhibit mast cell degranulation by suppress Exco8 expression. Overall, the data indicate that miRNAs participate in mast cell activation, especial for mast cell degranulation process.

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Introduction

Mast cells are derived from hematopoietic progenitor cells; they differentiate and mature in various tissues and are especially prominent near surfaces that interact with the environment.¹ Mast cells are long-lived cells that can be stimulated to re-enter the cell cycle and proliferate,² and they are well-recognized by two kinds of highly expressed cell surface receptors: FcεRI, the IgE receptor, and c-kit (CD117), the stem cell factor (SCF) receptor. Mast cells are considered to play a key role in the immune system, both in innate and adaptive immunity. This includes immediate allergic reactions and chronic allergic inflammation.^{2,3} As effector cells, activated mast cells can release preformed inflammatory mediators like prostaglandins, histamines and cytokines by degranulation and can also release newly synthesized potent inflammatory mediators like leukotriene C4 (LTC4), prostaglandin D2 (PGD2), and cytokines.⁴ When released, these biologically potent mediators act on inflammatory reactions in the mucosa, resulting in asthma and allergic diseases.

MicroRNAs (miRNA) are single-stranded RNA molecules approximately 22 nucleotides in length that mediate the post-transcriptional gene silencing of target genes.⁵ They were first recognized as gene silencers in *Caenorhabditis elegans* in 1993.⁶ To date, hundreds of miRNAs have been identified in plants and animals, and more than 200 human and rodent miRNAs have been reported.⁷ MiRNAs are recognized as a distinct class of small regulatory RNAs in multiple species that regulate a wide variety of biological functions, such as cell proliferation, differentiation, apoptosis, stress responses, and immune responses.⁸ Recently, miRNAs have been reported to not only exist in cells but also in serum and plasma samples, where they remain stable in bodily fluids by forming complexes with lipid-protein carriers.⁹ This indicates that miRNAs may be ideal biomarkers for diagnosing specific diseases. MiRNAs are widely recognized as modulators of many parts of the immune response.¹⁰ For example, miR-146a is a NF-κB induced miRNA that serves as a feedback regulator for suppressing the activation of NF-κB in cancer cells and T regulatory cells.^{11–13} MiR-146a can also suppress the expression of Egr1, resulting in decreased B-cell oncogenesis.¹⁴

Despite the important roles that mast cells and miRNAs play in the immune response, little research has been done to investigate the regulation of miRNAs in mast cells. Recently, however, this area of research has been growing. Eighty-six miRNAs may be participated in mast cell development process,¹⁵ and 17 miRNA profiles are changed in IgE-DNP stimulated bone marrow-derived mast cells (BMMCs).¹⁶ Several miRNAs play important roles in the biological function of mast cells,¹⁷ and transcription of miR-146a is completely dependent on p50 for regulating mast cell homeostasis and cell survival.¹⁸ MiR-143 directly targets IL-13Rα1 and suppresses the expression of IL-13Rα1 in human mast cells, resulting in a down-regulation of allergic disease.¹⁹ Basal levels of miR-221 contribute to the regulation of the cell cycle in resting mast cells, while in activated mast cells, it regulates degranulation, cytokine production, and cell adherence.²⁰ Following KIT signaling,

miR-381 and miR-539 can suppress the expression and proper functioning of microphthalmia-associated transcription factor (MITF), resulting in an inhibition of mast cell proliferation.²¹ MiR-9 is highly transcribed in mast cell tumors and contributes to the invasive phenotype and spontaneous metastasis of malignant mast cells.²²

There is currently a limited amount of information regarding the potential role of miRNA dysregulation in mast cells degranulation process. The purpose of this study was to investigate the transcriptional differences between miRNAs from resting and activated mouse bone marrow-derived mast cells (BMMCs) using Microarrays, and discover miRNA participated in mast cell degranulation.

Materials and methods

Animals

Normal C57BL/6 mice were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). Animal protocols were approved by the Institution's Animal Care and Use Committees at Southwest University (Chongqing, China).

Mast cell culture, activation, and degranulation

BMMCs were cultured as previously described.²³ Briefly, bone marrow cells were isolated from the femurs and tibias of C57BL/6 mice and cultured in complete medium (RPMI 1640 medium containing 10% fetal bovine serum, 5 ng/ml IL-3, 50 units/ml each of penicillin and streptomycin, 50 μM 2-mercaptoethanol, and 200 nM prostaglandin E2). After 4 weeks culture, the mast cell purity was greater than 98% as confirmed by toluidine blue staining and flow cytometry analysis for the expression of FcεRI and c-kit. To activate the mast cells, BMMCs were suspended in basic medium (RPMI 1640 medium containing 10% FBS) and passively sensitized with anti-DNP-BSA IgE (clone SPE-7, Sigma) for 18 h, after wash twice with RPMI 1640, then stimulated with 10 ng/ml of dinitrophenyl (DNP)-bovine serum albumin (BSA) (Biosearch Technologies, Novato, CA) for the indicated time period. Mast cell degranulation was determined by measuring β-hexosaminidase release.

To examine the effect of let-7i on its target genes, 100 nmol/L let-7i mimic, mimic negative control, let-7i inhibitor, or inhibitor negative control were mixed with Lipofectamine 3000 and BMMCs, and then electroporated using Gene Pulser Xcell system (Bio-Rad).²⁴ After 24 h, transfected cells were sensitized with anti-DNP-BSA IgE for 18 h and challenged with 10 ng/ml of DNP-BSA for indicated times.

MicroRNA microarray

Total RNA was extracted from untreated BMMCs or BMMCs treated with DNP for 1 h using TRIzol reagent (Life Technologies). Determination of the quantity and quality of extracted RNA and the miRNA microarray experiments were performed by Shanghai Oebiotech using a mouse & rat miRNA OneArray® (MRmiOA) V5 (Phalanx Biotech Company,

Table 1 microRNA sequences and qPCR primers.

Gene name	Primer sequence/forward primer	miR sequence/reverse primer	Product length
mmu-let-7a	GGTGAGGTAGTAGGTTGTATA	TGAGGTAGTAGGTTGTATAGTT	120bp
mmu-let-7b	GGTGAGGTAGTAGGTTGTGTG	TGAGGTAGTAGGTTGTGTGGTT	120bp
mmu-let-7c	GGTGAGGTAGTAGGTTGTATG	TGAGGTAGTAGGTTGTATGGTT	120bp
mmu-let-7d	GGAGAGGTAGTAGGTTGCATAG	AGAGGTAGTAGGTTGCATAGTT	120bp
mmu-let-7f	TGAGGTAGTAGATTGTATAGT	TGAGGTAGTAGATTGTATAGTT	120bp
mmu-let-7i	GGTGAGGTAGTAGTTTGTGCT	TGAGGTAGTAGTTTGTGCTGTT	120bp
mmu-miR652	AATGGCGCCACTAGGGTTGTG	AATGGCGCCACTAGGGTTGTG	120bp
mmu-miR125b-3p	ACGGGTTAGGCTCTTGGGAGCT	ACGGGTTAGGCTCTTGGGAGCT	120bp
mmu-miR296-3p	GAGGGTTGGGTGGAGGCTCT	GAGGGTTGGGTGGAGGCTCTCC	120bp
mmu-miR762	GGGGCTGGGGCCGGGACAGA	GGGGCTGGGGCCGGGACAGAGC	120bp
mmu-miR1195	TGAGTTCGAGGCCAGCCTGCT	TGAGTTCGAGGCCAGCCTGCTCA	120bp
U6 gene	TGGCCCTGCGCAAGGATG		150bp
Exoc8 (NM_198103)	CGCTACAACGCACTCTACCC	TCCAGCACTTCCAGCCACT	158bp
Lin7c (NM_011699)	GCGGTAGAGCTACTGAAAG	GGTCTGTTGTCTGCGTTTT	126bp
Mical3 (NM_00120475)	CACGCAGTTCTACGAGATGTT	AGATGGTCTGTCCGAGTTTG	137bp
miR reverse primer	CGAATTCTAGAGCTCGAGGCAGG		
miR RT primer	CGAATTCTAGAGCTCGAGGCAGGCACATGGCTGGCTAGTTAAGCTTG		
	GTACCGAGCTCGGATCCACTAGTCC(T)25VN (V is A, G or C; N is A, G, C or T)		

Hsinchu, Taiwan). The MRmiOA contains a total of 1265 unique, mature miRNA sequence probes from the mouse miRBase database release 19.0 and 48 experimental control probes. Each probe was provided in triplicate. miRNAs signals that were 5-fold above background with a signal intensity >120, a 1.5-fold difference between untreated and DNP-treated samples, and $p < 0.05$ were selected for further analysis.

Small RNA isolation and reverse transcription

Small RNAs were isolated from untreated BMMCs or BMMCs treated with DNP for 1 h using a mirVana miRNA Isolation Kit (Applied Biosystems) according to the manufacturer's instructions.²⁵ Briefly, 10^7 BMMCs were collected and washed with cold PBS and resuspended in 300 μ l of lysis/binding buffer. A 1/10 volume of miRNA Homogenate Additive was added, mixed, and incubated on ice for 10 min. Equal volumes of Acid-Phenol and Chloroform (1:1) were added to extract total RNA. The small RNA was isolated from the total RNA using a filter cartridge with 100 μ l of preheated (95 °C) nuclease-free water (Sigma). The isolated small RNAs (0.5 μ g) were polyadenylated with ATP using a Poly(A) Tailing Kit (Applied Biosystems) according to the manufacturer's instructions.²⁶ After phenol-chloroform extraction and isopropanol precipitation, the polyadenylated-RNAs were reverse-transcribed (RT) with SuperScript II Reverse Transcriptase (Invitrogen) and poly(T) adapter (5'-CGAATTCTAGAGCTCGAGGCGACATGGCTGGCTAGTTAA GCTTGGTACCGAGCTCGGATCCACTAGTCC(T)25VN-3'; V is A, G or C, and N is A, G, C or T).^{26,27}

miRNA target genes prediction and network construction

The target genes for miRNAs were predicted by public TargetScan Mouse database (Release 7.1, <http://www.targetscan.org>), and the selected target genes were evaluated for gene ontology (GO) classifications of molecular functions and cellular pathway. Cytoscape software were used to construct gene network of the exocytosis related genes and its regulatory miRNAs.

Quantitative PCR

Quantitative PCR was performed in an ABI 7500 Real-Time PCR system (Applied Biosystem) using SYBR Green (Invitrogen) to detect double-strand cDNA synthesis.^{27,28} Reactions were performed in a volume of 20 μ l containing 5 μ l of diluted cDNA (5 ng/ μ l), 1 $\times$ SYBR Green PCR Master Mix (Invitrogen), and 200 nM of each reverse and forward primer (Table 1). Dissociation curves for each amplicon were carefully examined to confirm the specificity of the primer pairs used. Relative transcript levels for each sample were obtained using the comparative Ct method. The threshold cycle (Ct) value obtained after each reaction was normalized to the Ct value of snU6, whose transcription was consistent across all conditions. The relative transcription levels were obtained by calibrating the $\Delta\Delta C_t$ values.

Statistics

Data are presented as the mean $\pm$ the S.E. for the indicated number of experiments. Statistical significance was determined by assessing the means with a paired *t*-test. Differences were considered significant at $*p < 0.05$, $**p < 0.01$.

Results

BMMC differentiation and maturation

BMMCs were incubated with IgE and stained with a FITC-conjugated rat anti-mouse IgE antibody (IgG1, Fig. 1A), or a FITC-conjugated rat anti-mouse CD117 antibody (c-kit, Fig. 1B), more than 98% of cells had high expression of Fc ϵ RI and c-kit. And when cells were stained by toluidine blue, they all contained granules (Fig. 1C), suggesting that mouse bone marrow cells were differentiating into mast cells. Mast cell degranulation by release of β -hexosaminidase (Fig. 1D) was further investigated through IgE-Fc ϵ RI

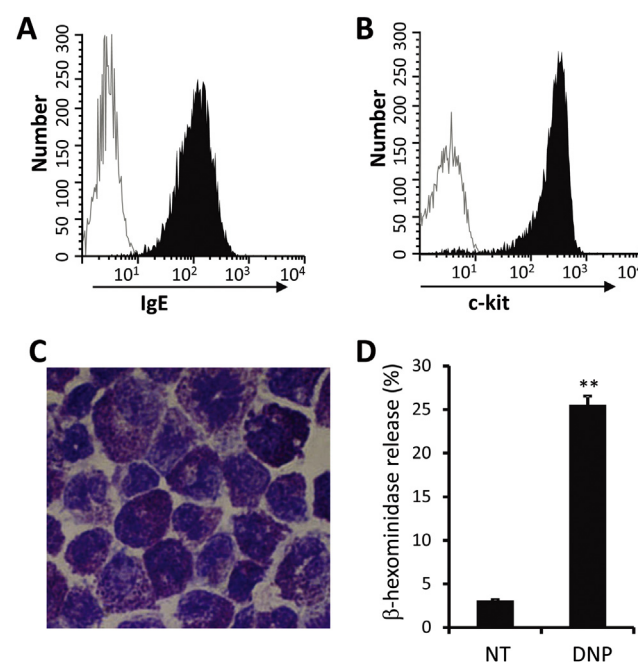


Figure 1. Bone marrow-derived mast cell maturation. A–B. Mast cell maturation was examined using flow cytometry for expression of the IgE receptor (A) and c-Kit (B). C. Cells were stained with toluidine blue to determine mast cell morphology. A representative photomicrograph is shown (original magnification = $\times 100$). Specimens were viewed on a Nikon Eclipse E600 microscope equipped with a DMX1200 camera with a CFI Plan-Fluor DDL 100 $\times$ /1.30 oil-immersion objective lens. Acquisition was performed with Nikon ACT-1 software version 2.20. Images were processed using Adobe Photoshop 5.0. D. Mast cell degranulation was determined by measuring β -hexosaminidase release after sensitization with anti-DNP IgE and stimulation with DNP-BSA for 20 min. Data are the mean $\pm$ SEM. $n = 4$, $**p < 0.01$.

stimulation. The release of β -hexosaminidase in four batches of BMMCs (each from an individual mouse) was over 20% ($25.53\% \pm 0.99\%$, mean $\pm$ S.E., $n = 4$). These results indicate that BMMCs are ready for further experiments.

MicroRNA OneArray analysis

To survey the different expression patterns of miRNAs in untreated and DNP-treated BMMCs, we performed a miRNA OneArray analysis. In this study, 616 miRNAs were detected in mouse BMMCs out of the 1265 currently known mouse miRNAs. Among these 616 miRNAs, 88 of them had an intensity over 120 in both untreated and DNP-treated BMMCs. Significant differences in intensity distribution were observed between untreated and DNP-treated mouse BMMCs. When the data were selected using our criteria (intensity > 120 and p value < 0.05), 25 miRNAs were up-regulated (Table 2), and 20 miRNAs were down-regulated (Table 3) in DNP-stimulated BMMCs when compared to untreated BMMCs. When an additional index (folds > 1.5) was added to the selection criteria, 20 miRNAs showed significant differences between untreated and DNP-treated BMMCs (Table 4). These included 13 miRNAs that were up-regulated and 7 miRNAs that were down-regulated. All of these 20 miRNAs have attracted the attention of our group and were selected for further research.

Transcription profiles of miRNAs

Based on the miRNA OneArray data, 11 miRNAs were selected to confirm their differential transcription levels by qPCR. Six of the 11 miRNAs (let-7b, let-7c, let-7d, let-7f, let-7i, and miR-652) were up-regulated, and 2 (miR-125b-3p and miR-296-3p) were down-regulated in DNP-stimulated BMMCs. The remaining 3 miRNAs (let-7a, miR-762, and miR-1195) had high levels of transcription, but no significant differences existed between the untreated and DNP-stimulated BMMCs. To test the specificity of the primers (Table 1) and to optimize the PCR conditions, particularly the annealing temperature, a regular PCR was performed, and the PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. As shown in Fig. 2, all of the primers amplified the expected product size, and no template PCR (NTC) was set as a control. We then performed qPCR using 25 ng cDNA samples to quantify the transcription levels of the 11 selected miRNAs. Among them, miR-1195 was the most abundantly expressed in both untreated and DNP-treated samples (average Ct 18.82 for NT and 18.95 for DNP) (Table 5), whereas miR-125b-3p was the least expressed (average Ct 26.86 for NT and 27.50 for DNP). For the selected internal control, U6 was high and equally expressed in both untreated and DNP-treated samples (average Ct 19.65 for NT and 19.615 for DNP).

Table 2 Up-regulation of miRNAs in DNP-treated mouse BMMCs. Selected by intensity > 120 and $p < 0.05$.

Selection criteria: Intensity > 120 and p value < 0.05		Normalized Intensity		SE ($n = 3$)		folds	p value * $p < 0.05$ ** $p < 0.01$
ID	Name	NT average	DNP average	NT	DNP	DNP/NT	DNP vs NT
PH_mr_0000150	mmu-let-7i	1409.5	3415.6	117.7	127.8	2.42	0.0003**
PH_mr_0000206	mmu-let-7f	1318.8	2600.1	72.7	79.9	1.97	0.0003**
PH_mr_0002259	mmu-miR-320	199.4	373.6	16.9	10.7	1.87	0.0010**
PH_mr_0000477	mmu-miR-324-5p	132.6	245.5	7.8	9.6	1.85	0.0008**
PH_mr_0000169	mmu-let-7c	2058.2	3774.5	104.3	80.2	1.83	0.0002**
PH_mr_0001125	mmu-miR-652	1459.2	2597.0	106.9	171.7	1.78	0.0049**
PH_mr_0003510	mmu-miR-210	429.1	755.4	28.8	51.2	1.76	0.0051**
PH_mr_0002762	mmu-miR-674	835.9	1460.2	56.6	75.9	1.75	0.0027**
PH_mr_0001696	mmu-miR-20a	227.7	372.9	7.5	19.0	1.64	0.0021**
PH_mr_0000163	mmu-let-7b	1446.2	2313.6	67.5	126.7	1.60	0.0038**
PH_mr_0000994	mmu-miR-130b	169.3	268.1	6.7	14.3	1.58	0.0033**
PH_mr_0000207	mmu-let-7d	1047.2	1632.7	47.7	113.2	1.56	0.0089**
PH_mr_0000204	mmu-let-7a	2421.7	3726.3	121.2	241.3	1.54	0.0085**
PH_mr_0000505	mmu-miR-155	135.5	189.9	5.7	10.7	1.40	0.0110*
PH_mr_0001629	mmu-miR-484	162.1	226.1	1.7	8.5	1.39	0.0018**
PH_mr_0001893	mmu-miR-30d	454.8	629.7	6.0	19.2	1.38	0.0010**
PH_mr_0001321	mmu-miR-466f-5p	464.1	608.9	23.4	24.9	1.31	0.0133*
PH_mr_0001873	mmu-miR-744	274.2	353.1	15.5	2.8	1.29	0.0075**
PH_mr_0002382	mmu-miR-328	164.7	209.0	6.5	3.6	1.27	0.0040**
PH_mr_0000458	mmu-miR-669b	121.6	152.3	6.7	5.4	1.25	0.0231*
PH_mr_0000352	mmu-miR-34a	186.8	233.2	2.8	8.2	1.25	0.0057**
PH_mr_0001874	mmu-miR-24	829.0	1003.0	22.2	29.9	1.21	0.0095**
PH_mr_0002874	mmu-miR-21	1142.2	1352.0	30.2	53.4	1.18	0.0268*
PH_mr_0003011	mmu-miR-709	56750.4	67070.0	1.3	0.9	1.18	0.0000**
PH_mr_0001929	mmu-miR-221	302.2	348.7	11.4	8.0	1.15	0.0292*

Table 3 Down-regulation of miRNAs in DNP-treated mouse BMMCs. Selected by intensity >120 and $p < 0.05$.

Selection criteria: Intensity >120 and p value < 0.05		Normalized Intensity		SE ($n = 3$)		folds	p value * $p < 0.05$ ** $p < 0.01$
ID	Name	NT average	DNP average	NT	DNP	DNP/NT	DNP vs NT
PH_mr_0003251	mmu-miR-877	250.8	225.0	8.8	2.7	0.90	0.0481*
PH_mr_0000377	mmu-miR-669c	1560.1	1371.4	52.6	6.5	0.88	0.0236*
PH_mr_0003506	mmu-miR-1907	142.7	124.6	4.5	2.9	0.87	0.0281*
PH_mr_0003501	mmu-miR-1892	1083.6	901.5	20.1	21.1	0.83	0.0033**
PH_mr_0000820	mmu-miR-665	219.9	179.6	7.8	6.5	0.82	0.0168*
PH_mr_0000465	mmu-miR-297b-5p	352.5	279.0	8.1	15.0	0.79	0.0126*
PH_mr_0002901	mmu-miR-466k	159.5	121.2	3.5	4.0	0.76	0.0020**
PH_mr_0002563	mmu-miR-92a	383.7	291.6	13.5	3.0	0.76	0.0027**
PH_mr_0003497	mmu-miR-1895	396.7	299.8	5.6	12.9	0.76	0.0023**
PH_mr_0002856	mmu-miR-466c-5p	633.1	468.9	21.3	42.9	0.74	0.0265*
PH_mr_0002761	mmu-miR-466h	315.0	232.9	12.9	15.0	0.74	0.0142*
PH_mr_0000460	mmu-miR-297a	182.3	132.8	5.8	6.9	0.73	0.0055**
PH_mr_0000379	mmu-miR-574-5p	2230.1	1598.2	130.6	156.8	0.72	0.0363*
PH_mr_0003200	mmu-miR-705	1026.4	671.7	18.5	15.5	0.65	0.0001**
PH_mr_0001880	mmu-miR-711	553.1	353.8	17.3	3.9	0.64	0.0004**
PH_mr_0001774	mmu-miR-125b-3p	3613.1	2174.9	362.9	72.1	0.60	0.0177*
PH_mr_0003495	mmu-miR-1897-5p	428.5	255.4	2.3	9.3	0.60	0.0001**
PH_mr_0002420	mmu-miR-30c-1	668.1	390.0	6.1	8.4	0.58	0.0000**
PH_mr_0002410	mmu-miR-296-3p	3325.0	915.5	142.9	38.0	0.28	0.0001**
PH_mr_0001524	mmu-miR-872	781.3	196.7	31.5	10.9	0.25	0.0001**

Table 4 Significant transcription of miRNAs between untreated and DNP-treated mouse BMMCs. Selected by intensity >120, fold change >1.5, and $p < 0.05$.

Selection criteria: Intensity >120, Fold >1.5, p value < 0.05		Normalized Intensity		SE		folds	p value * $p < 0.05$ ** $p < 0.01$
ID	Name	NT average	DNP average	NT	DNP	DNP/NT	DNP vs NT
PH_mr_0000150	mmu-let-7i	1409.5	3415.6	117.7	127.8	2.42	0.0003**
PH_mr_0000206	mmu-let-7f	1318.8	2600.1	72.7	79.9	1.97	0.0003**
PH_mr_0002259	mmu-miR-320	199.4	373.6	16.9	10.7	1.87	0.0010**
PH_mr_0000477	mmu-miR-324-5p	132.6	245.5	7.8	9.6	1.85	0.0008**
PH_mr_0000169	mmu-let-7c	2058.2	3774.5	104.3	80.2	1.83	0.0002**
PH_mr_0001125	mmu-miR-652	1459.2	2597.0	106.9	171.7	1.78	0.0049**
PH_mr_0003510	mmu-miR-210	429.1	755.4	28.8	51.2	1.76	0.0051**
PH_mr_0002762	mmu-miR-674	835.9	1460.2	56.6	75.9	1.75	0.0027**
PH_mr_0001696	mmu-miR-20a	227.7	372.9	7.5	19.0	1.64	0.0021**
PH_mr_0000163	mmu-let-7b	1446.2	2313.6	67.5	126.7	1.60	0.0038**
PH_mr_0000994	mmu-miR-130b	169.3	268.1	6.7	14.3	1.58	0.0033**
PH_mr_0000207	mmu-let-7d	1047.2	1632.7	47.7	113.2	1.56	0.0089**
PH_mr_0000204	mmu-let-7a	2421.7	3726.3	121.2	241.3	1.54	0.0085**
PH_mr_0003200	mmu-miR-705	1026.4	671.7	18.5	15.5	0.65	0.0001**
PH_mr_0001880	mmu-miR-711	553.1	353.8	17.3	3.9	0.64	0.0004**
PH_mr_0001774	mmu-miR-125b-3p	3613.1	2174.9	362.9	72.1	0.60	0.0177*
PH_mr_0003495	mmu-miR-1897-5p	428.5	255.4	2.3	9.3	0.60	0.0001**
PH_mr_0002420	mmu-miR-30c-1	668.1	390.0	6.1	8.4	0.58	0.0000**
PH_mr_0002410	mmu-miR-296-3p	3325.0	915.5	142.9	38.0	0.28	0.0001**
PH_mr_0001524	mmu-miR-872	781.3	196.7	31.5	10.9	0.25	0.0001**

According to the qPCR data (Table 5 and Fig. 3), after DNP stimulation let-7i (2.70-fold) had the highest fold change in up-regulation, whereas miR-296-3p (0.25-fold) had the highest fold change in suppressed transcription. When

comparing the MicroRNA OneArray data, the only miRNA of the 11 that displayed no significant difference in transcription by DNP stimulation was miR-125b-3p. However, it did still show suppressed transcription in the DNP

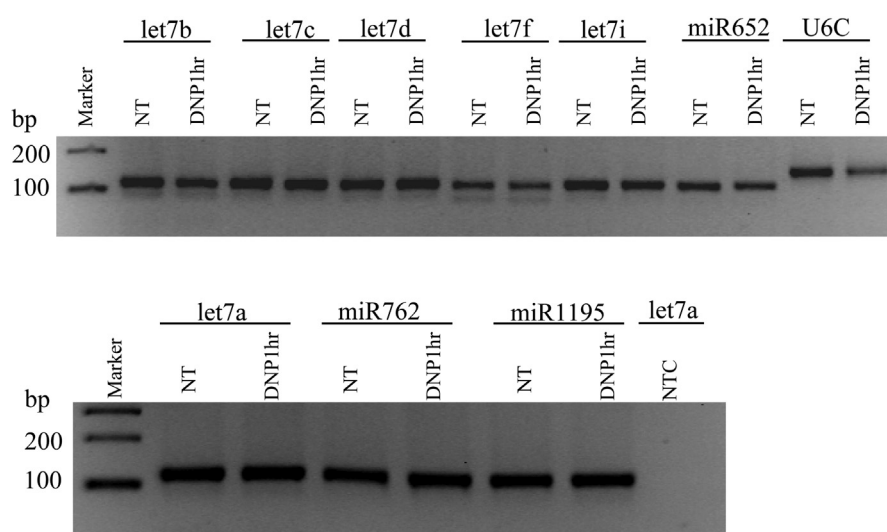


Figure 2. RT-PCR of selected microRNAs. Small RNAs from resting BMMCs and BMMCs stimulated with DNP for 1 h were isolated and reversed transcribed. PCR was performed by using designed miRNA primers, and PCR products were separated on agarose gels and stained with ethidium bromide. A representative gel is shown.

stimulated sample, but the p-value (0.1805) was larger than 0.05. These data suggested that miRNA participates in mast cell activation and may play a key role in the transcription of signaling molecules.

The miRNA regulate IgE-mediated BMMC degranulation

Degranulation is a main function of mast cell to participate in organism immunity. We surveyed the different expression miRNA target genes, and hundreds genes were selected, while after gene ontology classification of molecular functions, eleven exocytosis related genes were found. As shown in Fig. 4, each target genes were regulated

by several miRNA, and each miRNA regulates several target genes. Especially, let-7b, let-7c, let-7f and miR-705 regulates over 5 exocytosis related genes, and Exoc8, Exoc5, Lin7c, stxbp1, and Myo5a were targeted by 4 miRNAs. These results suggested that miRNA regulates IgE-mediated mast cell degranulation.

To confirm the microarray data for the observed miRNAs, we selected the let-7i, which expression is the highest fold change by TNP challenged, to performed degranulation and qPCR analysis. The results show that let-7i mimic do not affect mast cell degranulation, whereas let-7i inhibitor significant suppress mast cell degranulation (Fig. 5A). The results of real time PCR show that let-7i inhibitor suppress Exoc8 expression (Fig. 5B), whereas the expression of Mica13 (Fig. 5C) and Lin7c (Fig. 5D) has no changed. These data indicated that let-7i inhibit mast cell degranulation by suppress Exoc8 expression.

Discussion

Mast cells play an essential role in the development of allergic inflammatory reactions. The activation of mast cells through the high-affinity receptor for immunoglobulin E (FcεRI) results in the production and release of a number of preformed mediators and newly synthesized proinflammatory cytokines.²⁹ The key function of FcεRI is its role in allergic reactions.³⁰ Recent reports have demonstrated that miRNA is an effector molecule involved in the biological function of mast cells¹⁷; however, the reports involving microRNA transcription profiles in IgE-activated mast cells is very limited. The current study focused on the different transcription profiles of miRNAs between untreated and DNP-treated mouse BMMCs. Our data showed that 13 miRNAs are up-regulated and 7 miRNAs are down-regulated in DNP-stimulated BMMCs compared to resting BMMCs. This suggests that these 20 miRNAs may participate in IgE-mediated mast cell signaling. Interestingly, only one miRNA (mmu-miR-125b-3p) was detected in both our data

Table 5 Average threshold cycle (Ct) and standard error (SE) of the miRNAs used for qPCR ($n = 3$).

miRNA	Ct average		SE		$\Delta\Delta Ct$ fold	P value
	NT	DNP	NT	DNP	DNP/NT	
			(n = 3)			
miR-1195	18.82	18.95	0.11	0.15	0.94	0.8026
miR-762	19.16	19.31	0.40	0.26	0.88	0.3961
let-7a	19.58	19.44	0.36	0.26	1.11	0.6449
let-7b	20.16	19.34	0.32	0.18	1.75	0.0414*
let-7d	20.51	19.84	0.30	0.23	1.56	0.0029**
miR-296-3p	20.83	22.83	0.35	0.36	0.25	0.0005**
miR-652	21.02	20.24	0.13	0.18	1.69	0.0165*
let-7f	21.58	20.44	0.36	0.26	2.22	0.0319*
let-7i	21.78	20.32	0.36	0.17	2.70	0.0002**
let-7c	23.23	22.23	0.35	0.36	1.98	0.0088**
miR-125b-3p	26.86	27.50	0.40	0.44	0.66	0.1805
U6	19.65	19.61	0.24	0.22	—	—

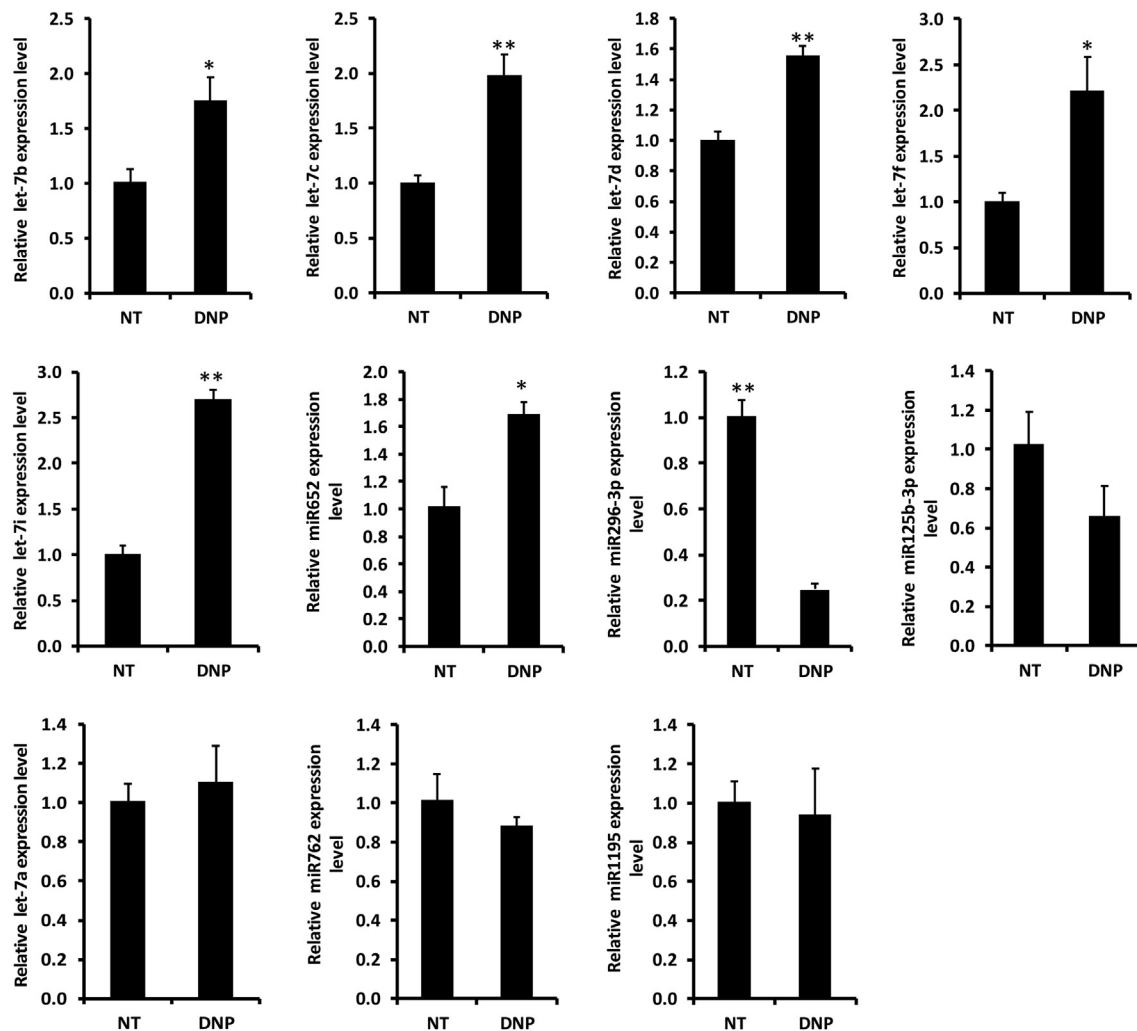


Figure 3. Quantitative PCR of microRNAs. Wild-type BMMCs were rested or stimulated with DNP-BSA for 1 h, and then, small RNA was isolated and analyzed for selected miRNAs by qPCR. Expression levels were normalized to the endogenous control snU6. The relative transcription levels were presented by calibrating the $\Delta\Delta C_t$ values. Data are the mean $\pm$ SEM. $n = 3$, * $p < 0.05$, ** $p < 0.01$.

and previously report,¹⁶ it would be caused by different mouse genetic background and different BMMC culture medium. In our current study, we used C57BL/6 mice and cell culture medium contain PGE₂, whereas previously study used BALB/c mice and cell culture medium contain SCF, it has been reported that SCF can promote immature cell proliferation to peritoneal mast cell type (PMC), while IL-3 alone divide immature cell to mucosal mast cell type (MMC).³¹ This result indicated that miRNA regulation network maybe difference in different cell types, whereas the comparative study should be perform under the same conditions.

MicroRNAs regulate various biological functions like growth and differentiation in normal cells. Let-7 directly interact with the mammalian lin41 3'UTR and regulate lin41 gene expression, which results in the control of mouse embryonic survival and neural tube closure.³² The let-7 family is the most abundantly expressed miRNA in mouse newborn ovary tissues,³³ let-7 activity is suppressed in mouse oocytes.³⁴ This suggests that let-7 is

important for cell differentiation and mouse development, but its function is still unclear. In T cells, let-7 is the direct target of IL-13, and when let-7 expression is inhibited, IL-13 is significantly up-regulated in in vitro models.³⁵ The opposite results were observed in an in vivo asthma model,³⁶ suggesting that the functions of miRNAs are still a mystery and require further investigation. In mast cell, miR-221 and miR-142-3p regulate mast cell degranulation,³⁷ miR-146a regulates mast cell apoptosis, and miR-126 regulates mast cell proliferation,⁸ but the mechanism is still undiscovered.

Although miRNAs are very important in cell differentiation, development, maturation, and function, they still shroud in mystery. For instance, most documents are discovered the phenomenon that miRNA can regulate mast cell degranulation or cytokine production, but the cellular signal pathway is still unknown.^{8,36} Further research should focus on the mechanism to discover miRNA functions, and miRNA knockout and knock-in cell line or animal should be employed. Anyway, our data suggest that some new miRNAs

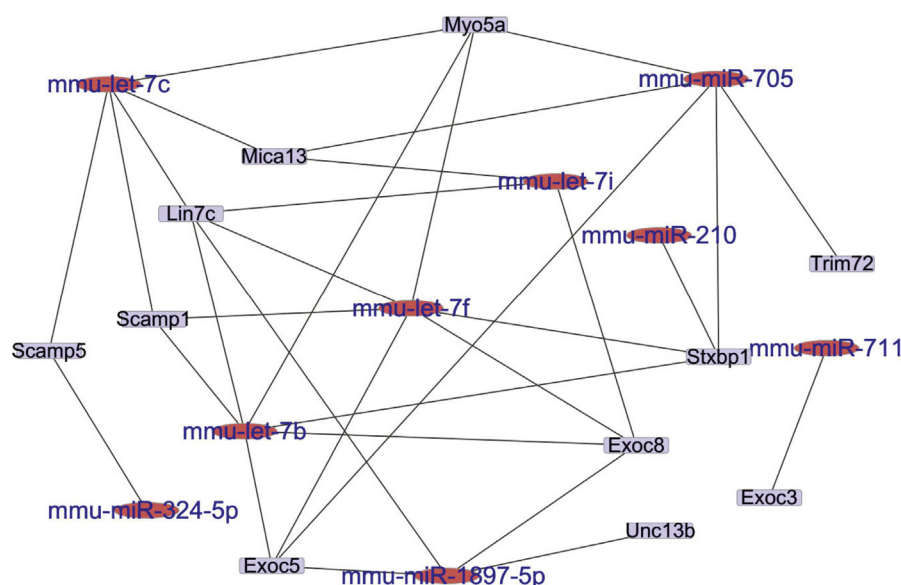


Figure 4. A miRNA target exocytosis gene network. Eleven exocytosis related genes were target by mentioned miRNA, and one miRNA can regulate several genes. The cycle nodes represent miRNAs, and the rounded rectangle nodes represent target genes.

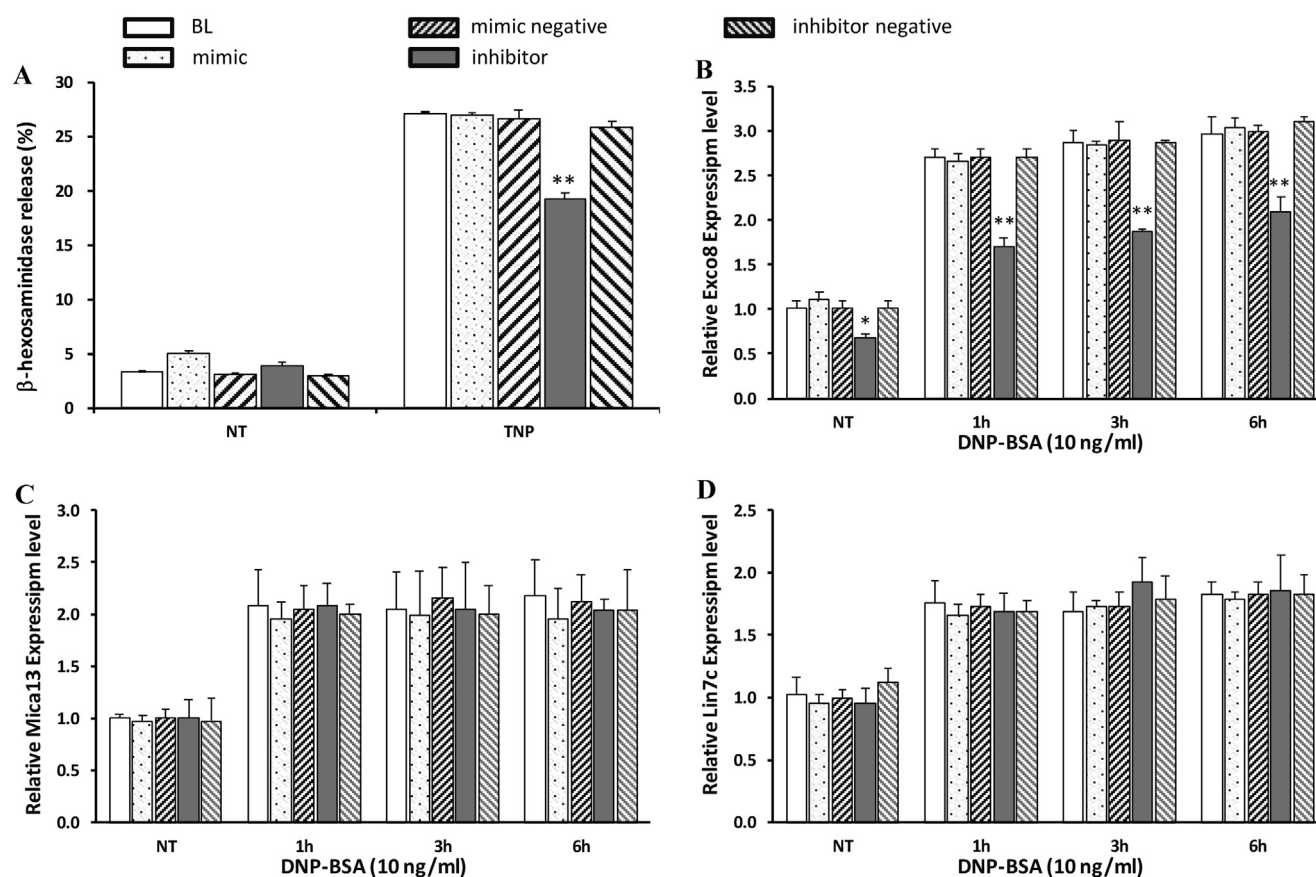


Figure 5. Let-7i regulate IgE-mediated mast cell degranulation by Exoc8 expression. let-7i mimic, mimic negative control, let-7i inhibitor, or inhibitor negative control were transfected into BMMCs, the transfected cells were culture 24 h, and then sensitized with anti-DNP-BSA IgE and challenged with 10 ng/ml DNP-BSA. mast cell degranulation by monitor β -hexosaminidase release (A), and relative genes expression level were detected by qPCR of Exoc8 (B), Mica13 (C), and Lin7c (D). Data are the mean $\pm$ SEM. $n = 3$, * $p < 0.05$, ** $p < 0.01$.

maybe participated in mast cell degranulation, these results will enlarge our knowledge of miRNA function in mast cell.

Conflicts of interest statement

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

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