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hsa-miR-20a-5p attenuates allergic inflammation in HMC-1 cells by targeting HDAC4

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ABSTRACT

micro-RNAs (miRNAs) are non-coding RNAs which play important role in human diseases. Dysregulated miRNAs have been identified in asthma patient while their precise roles in asthma are not well elucidated. We compared the expression level of total 11 miRNAs between PMA/A23187-treated and control HMC-1 mast cells. We determined the effect of miR-20a on inflammation by overexpressing miR-20a mimic or its antagonist. We further predicted histone deacetylase 4 (HDAC4) as potential target of miR-20a and explored the effects of miR-20a on HDAC4 expression and histone modification. miR-20a was down-regulated in PMA/A23187-treated HMC-1 cells. miR-20a inhibited expression of pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β) and Interferon gamma (IFN-γ) while promoted Interleukin 10 (IL-10) production. miR-20a targeted HDAC4 and suppressed its expression, which contributed to epigenetically regulation of IL-10 expression by miR-20a. hsa-miR-20a-5p attenuates allergic inflammation in HMC-1 cells by targeting HDAC4.

1. Introduction

Asthma is a common chronic inflammatory disease of lung airways and affects more than 350 million people over the world (Martinez-Nunez et al., 2018). Largely reversible airflow obstruction, airway hyper-responsiveness and episodic respiratory symptoms including wheezing, productive cough, and the sensation of breathlessness and chest tightness have been characterized in asthma (Bousquet et al., 2000).

Although the underlying mechanisms of asthma remain incompletely understood, mast cells have been implicated in asthma (Reuter et al., 2010). In asthma, mast cells initiate and promote airway inflammation by secreting several mast cell-produced mediators. The activation of mast cells also leads to an increased chemotaxis of inflammatory cells as well as T cell activation (Reuter et al., 2010). It has also been implicated that cytokines regulate the airway inflammation underlying asthma (Kips, 2001). TNF-α has been shown to be an important element in determining asthma severity. Exogenous administration of TNF-α induces airway neutrophilia and hyperresposiveness (Kips et al., 1992). Severe asthma is characterized by high levels of IFN-γ and neutralization of IFN-γ results in inhibition of airway hyperactivity (Leavy, 2015). IL-1β has been reported to drive inflammatory response and contributes to airway smooth muscle responsiveness in asthma (Whelan et al., 2004). In contrast, IL-10 production is reduced in asthma patient and IL-10 has been shown to a critical regulatory molecule involved in the prevention of asthma (Umetsu and DeKruyff, 1999). Therefore, current therapy for asthma is designed to target inflammatory components of this disease.

MicroRNAs (miRNAs) are a class of endogenous, noncoding small RNAs (22 nucleotides) which regulate gene expression by pairing to the 3` untranslated region (3`-UTR) (Bartel, 2009; Shukla et al., 2011). The main effector mechanisms of miRNA include destabilization of mRNA and inhibition of translation (Djuranovic et al., 2012; Eichhorn et al., 2014). Many major cellular functions including metabolism, development, differentiation and growth are known to be regulated by miRNAs. Dysregulated miRNA expression has been implicated in several kinds of human disease including cancer, cardiac failure, Alzheimer`s disease and inflammatory disease (Ardekani and Naeini, 2010). The dysregulation of miRNA has also been identified in bronchial epithelial cells from asthmatic patients (Martinez-Nunez et al., 2014). In addition, by

Abbreviations: miRNAs, micro-RNAs; HDAC4, histone deacetylase 4; TNF-α, tumor necrosis factor alpha; IL-1β, interleukin 1 beta; IFN-γ, interferon gamma; IL-10, interleukin 10

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using small RNA-seq, the genome wide difference of miRNA expression in bronchial epithelial cells is determined between severe asthma patient and healthy donors (Martinez-Nunez et al., 2018). Total 20 miRNAs are identified to be dysregulated, among them 11 are down-regulated. However, the precise roles of these 20 miRNAs in asthma need to be further elucidated.

In present study, we analyzed the expression of these miRNAs in activated mast cell line HMC-1 cells and found that only miR-20a was down-regulated in PMA/A23187 activated HMC-1 cells. We further explored the role of miR-20a in inflammation and elucidated the underlying mechanisms.

2. Materials and methods

2.1. Cell culture

The human mast cell line HMC-1cells were cultured in IMDM medium supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY), 2 mM L-glutamate, streptomycin (100 mg/ml) and penicillin (100 units/ml). HMC-1 cells were maintained in humidified incubator at 37 °C with 5% CO_2 supply.

2.2. Quantitative real-time PCR

Total RNA from HMC-1 cells was isolated using the RNeasy mini kit (Qiagen, USA) according to the manufacturer's protocols. One microgram of total RNA was reverse-transcribed using SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen, USA). Real-time PCR was performed using QuantiTect SYBR Green PCR Kit (Qiagen, USA) on a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher, USA). The specific target gene primers were purchased from RiboBio Co., Ltd. (Guangzhou, China). The real-time PCR experiment was executed in triplicate and the relative mRNA expression index was normalized with small nuclear RNA U6.

2.3. miRNA transfection

MiR-20a mimics and MiR-20a antagonist were purchased from RiboBio Co., Ltd and transfected using Lipofectamine 2000 (Invitrogen, USA) following supplier's protocols. After 48 h post transfection, cells were treated with 20 nM PMA (Sigma, USA) plus 1 μM A23187 (Sigma, MO, USA) or OVA (5 mg/ml) (Sigma, USA) for another 24 h. The concentration of treatment was selected according to a previous report (Xiao et al., 2018).

2.4. ELISA

Cell supernatant levels of cytokines including TNF-α, IL-1β, IFN-γ and IL-10 were detected using commercial ELISA kits from R&D systems (Minneapolis, USA), according to manufacturer's instructions.

2.5. Western blotting

1 million cells were lysed in RIPA buffer containing cOmplete™ Protease Inhibitor Cocktail (Roche, USA) and protein concentrations were measured using Bradford Protein Assay (Bio-Rad, USA) following manufacture`s protocols. Total 20 μg protein was loaded on SDS-PAGE, and then transferred to PVDF membrane (Bio-rad, Hercules, CA, USA). After blocking with 5% non-fat milk in PBS containing 0.1% tween-20 (Sigma, USA), the blots were incubated with HDAC4, H3k27Ac, H3K27me3 or GAPDH antibodies (Abcam, Cambridge, MA, USA) in 1% non-fat milk with 1:1000 dilution. The membranes were washed and probed with corresponding horseradish peroxidase conjugated secondary antibody. The immunoreactive proteins were detected with an enhanced chemiluminescence reagent (Pierce Biotech, USA). The western blot results were quantitated and analyzed using GS-900™ Calibrated Densitometer and software Image Lab (Bio-Rad, USA) following manufacturer's instructions.

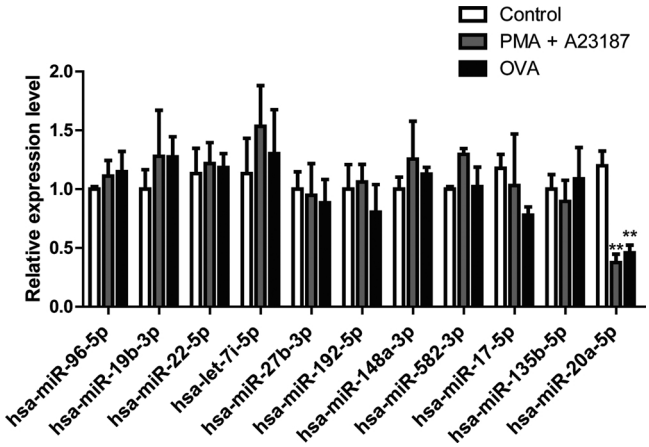

Fig. 1. hsa-mir-20a-5p was down-regulated in HMC-1 cells treated with PMA/A23187 or OVA HMC-1 cells were treated with PMA (20 nM) plus A23187 (1 μM) or ovalbumin (OVA, 5 mg/mL) for 24 h. Then the expression levels of 11 candidate microRNAs, which were identified to be significantly down-regulated in the Frac-seq (Martinez-Nunez et al., 2018), were tested by RT-qPCR. Only hsa-mir-20a-5p was significantly down-regulated by the treatment. Data were represented as mean ± s.d., n = 3 independent experiments. ** indicated $p < 0.001$.

2.6. Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) was performed following previously described (Piunti et al., 2017; Zimmermann et al., 2015). After treatments, HMC-1 cells were fixed with 1% formaldehyde in complete cell medium for 6 min at room temperature followed by 0.125 M glycine for 6 min to stop the crosslinking reaction. Then, cells were washed with ice-cold PBS for three times and scraped on ice, and then lysed for 10 min in L1 buffer (50 mM Tris, pH 8.0, 2 mM EDTA, 0.1% Nonidet P-40 and 10% glycerol) supplemented with proteases and phosphatase inhibitors (Roche, USA). Nuclei were pelleted at 1500 rpm and re-suspended in L2 buffer (50 mM Tris, pH 8.0, 5 mM EDTA and 1% SDS plus protease and phosphatase inhibitors). Chromatin was then sheared by sonication and centrifuged to pellet debris. Finally chromatin was diluted in dilution buffer (50 mM Tris, pH 8.0, 0.5% Nonidet P-40, 0.2 M NaCl and 5 mM EDTA). Nuclear extracts were immunoprecipitated with anti-H3K27Ac, anti-H3K27me3 or anti-H3 antibodies (Abcam, USA). The co-immunoprecipitated material was then subjected to qPCR analysis using IL-10 and TNF-α specific primers (purchased from Life Technologies, Pleasanton, CA, USA). Data from qPCR were expressed as percentage over input DNA and is displayed as means ± SD.

2.7. Statistical analysis

All data were expressed as mean ± SD and one-way ANOVA was used for difference analysis. The difference was considered as significant when $p < 0.05$.

3. Results

3.1. Down-regulation of hsa-mir-20a-5p PMA/A23187 or OVA treated HMC-1 cells

Previous publications have demonstrated the dysregulation of miRNAs in human severe asthma bronchial epithelium in which there were total 11 down-regulated miRNAs (Martinez-Nunez et al., 2018). We further tested these results in our PMA/A23187 or OVA activated

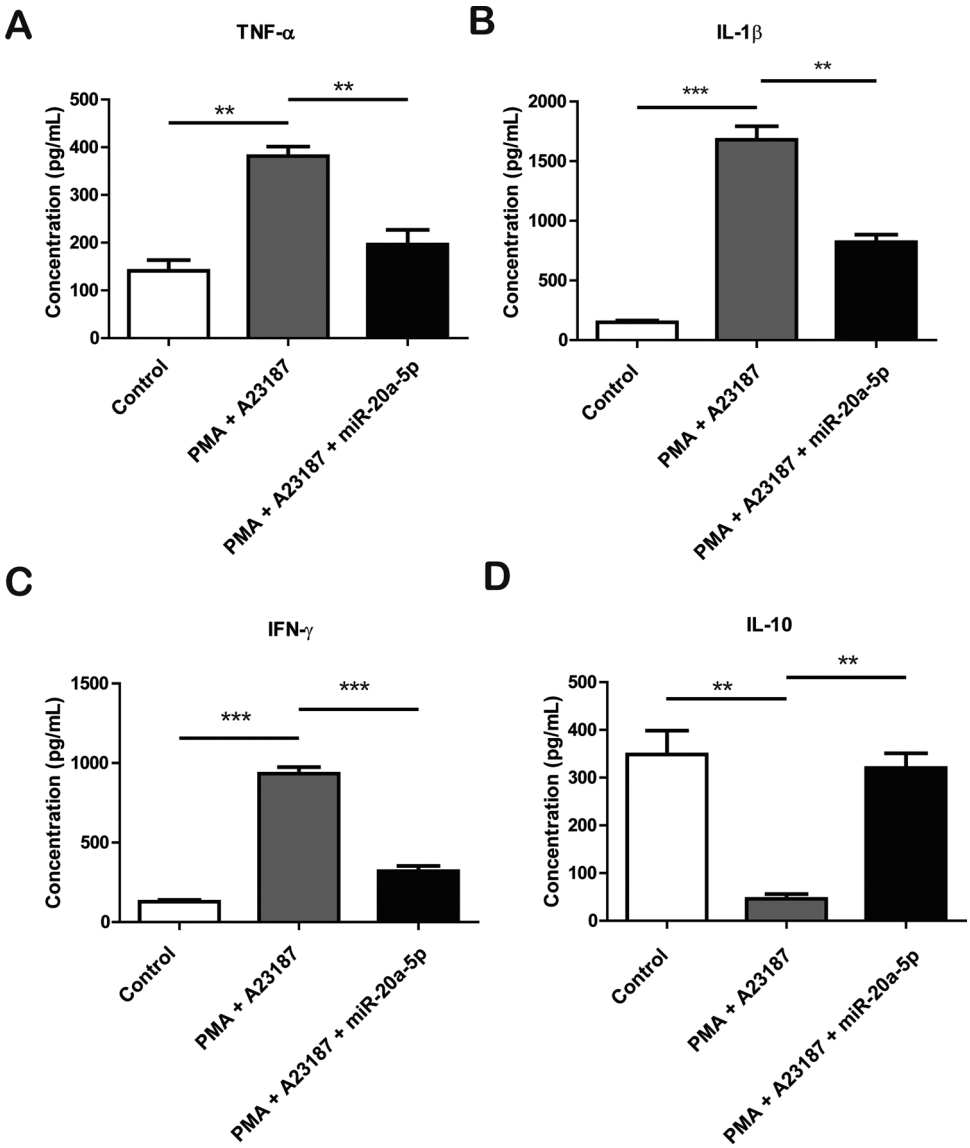

Fig. 2. hsa-miR-20a-5p inhibited inflammatory cytokines and promoted the anti-inflammatory IL-10 in PMA/A23187-treated HMC-1 cells. HMC-1 cell transfected with or without miR-20a were treated as indicated. Released TNF-α (A), IL-1β (B), INF-γ (C) and IL-10 (D) in the supernatant were analyzed by ELISA. Data were represented as mean ± s.d.; n = 3 independent experiments, ** indicated $p < 0.01$ and *** indicated $p < 0.001$.

HMC-1 cell model. As shown in Fig. 1, there was no significant change of expressions for 10 miRNAs including miR-96, miR-19b, miR-22, let-7i, miR-192, miR-148a, miR-582, miR-17 and miR-135b. In contrast, significantly down-regulated expression of miR-20a was identified in both PMA/A23187 and OVA treated HMC-1 cells, suggesting a potential role of miR-20a in activated HMC cells. We also checked other miRNAs which were involved in the regulation of inflammatory stimuli, including miR-125b, miR-16, miR-299-5p, miR-126, miR-206, and miR-133b (Fig. S1). Among them, miR-125b and miR-16 showed an up-regulation pattern, but this up-regulation was quite limited.

3.2. hsa-miR-20a-5p inhibited inflammatory factors and promoted the anti-inflammatory factor in PMA/A23187 induced allergic response

We continued to determine the potential role of miR-20a in PMA/A23187 stimulated inflammation in HMC-1 cells by overexpressing miR-20a mimic in HMC-1 cells before PMA/A23187 stimulation. As shown in Fig. 2, PMA/A23197 treatment significantly induced the expression of pro-inflammatory cytokine TNF-α (Fig. 2A), IL-1β (Fig. 2B) and IFN-γ (Fig. 2C). In contrast, the production of IL-10, an anti-inflammatory cytokine, was significantly reduced in PMA/A23197-treated HMC-1 cells (Fig. 2D). Overexpression of miR-20a prevented PMA/A23187-induced TNF-α (Fig. 2A), IL-1β (Fig. 2B) and IFN-γ (Fig. 2C) productions while rescued the inhibition of IL-10 production by PMA/A23197. Therefore, our data suggested that miR-20a may play an anti-inflammatory role in allergic response.

3.3. Inhibition of miR-20a promoted allergic inflammation in PMA/A23187 treated HMC-1 cells

To further confirm the anti-inflammatory role of miR-20a in allergic response, we inhibited miR-20a expression in HMC-1 cell by transfecting miR-20a antagonist before PMA/A23187 treatment. As shown in Fig. 3, transfection of miR-20a antagonist significantly induced expressions of TNF-α (Fig. 3A), IL-1β (Fig. 3B) and IFN-γ (Fig. 3C), similar to the effects of PMA/A23187 treatment alone. In addition, transfection of miR-20a antagonist further enhanced PMA/A23187-induced TNF-α, IL-1β and IFN-γ production. In contrast, knockdown miR-20a significantly reduced IL-10 production and further enhanced PMA/A23187-induced inhibition of IL-10 production (Fig. 3D). Thus, our

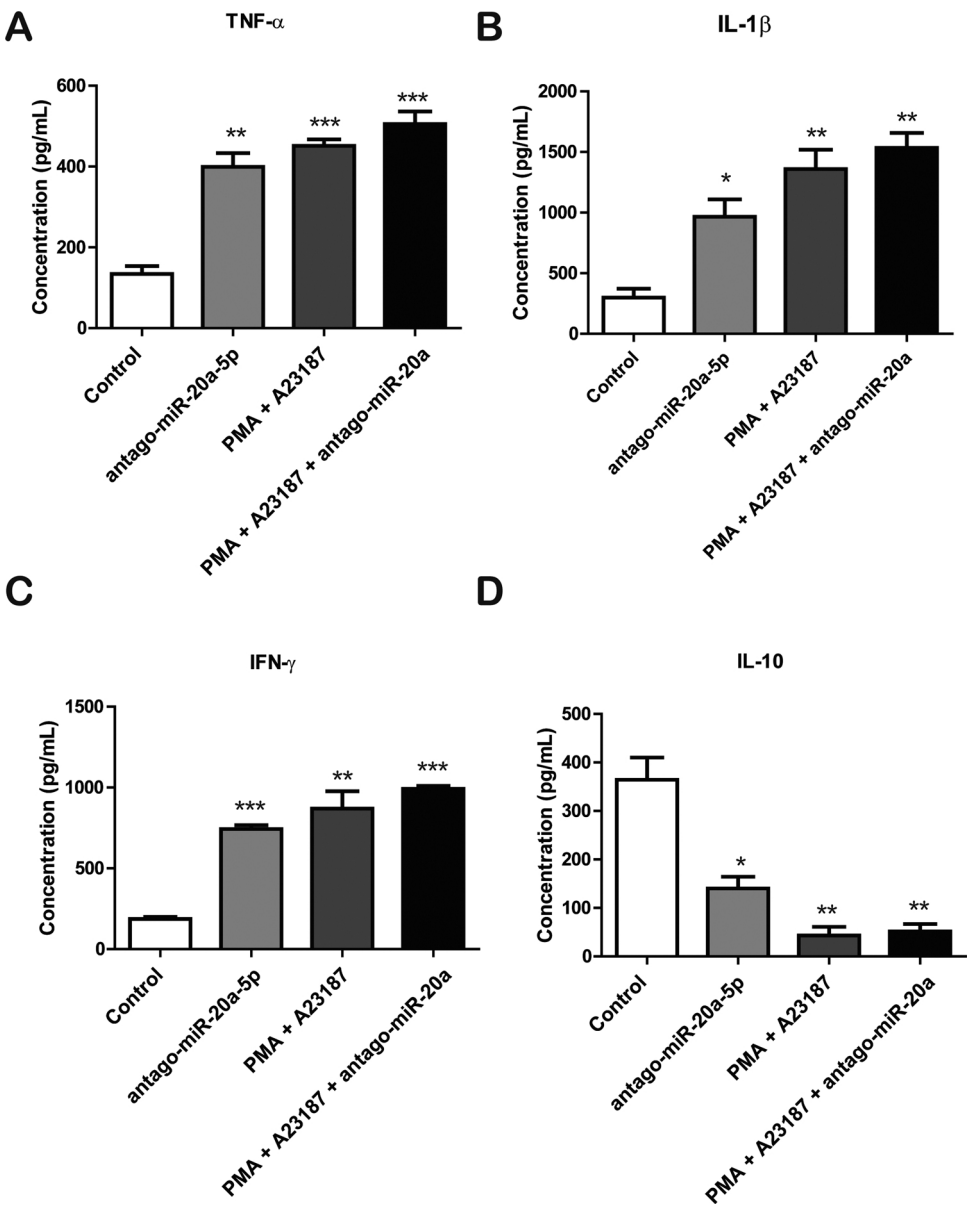

Fig. 3. Down-regulation of hsa-miR-20a-5p promoted the allergic response induced by PMA/A23187. HMC-1 cell transfected with or without antago-miR-20a were treated as indicated. Released TNF-α (A), IL-1β (B), INF-γ (C) and IL-10 (D) in the supernatant were analyzed by ELISA. Data were represented as mean ± s.d.; n = 3 independent experiments, * indicated $p < 0.05$, ** indicated $p < 0.01$ and *** indicated $p < 0.001$.

data demonstrated that miR-20a played an important role in allergic inflammation.

3.4. hsa-miR-20a-5p targeted HDAC4

To explore the underlying mechanisms of anti-inflammatory effect of miR-20a, we searched for the potential target gene of miR-20a in HMC-1 cells by using the miRNA prediction software (TargetScan 7.0, www.targetscan.org). As shown in Fig. 4A, miR-20a may potentially target the 3'UTR of histone deacetylase 4 (HDAC4). We also used the dual luciferase assay to demonstrate that the 3' UTR of HDAC4 was the target of miR-20a (Fig. S2). HDAC4 possessed histone deacetylase activity and was responsible for the deacetylation of lysine residues on the N-terminal part of the core histones (H2A, H2B, H3 and H4). We detected the expression levels of HDAC4 after different treatments. As shown in Fig. 4B, PMA/A23187 treatment promoted HDAC4 expression. Although transfection of miR-20a did not affect its expression, overexpression of miR-20a prevented PMA/A23187-induced expression of HDAC4. In addition, knocking down miR-20a promoted the expression of HDAC4 in HMC-1 cells. Therefore, our data indicated that miR-20a regulated HDAC4 expression. As HDAC4 was responsible for histone deacetylation, we continued to detect the histone acetylation level of H3 under different conditions. Although there were different expressions of HDAC4, there was no obvious change of H3K27Ac levels. In addition, neither PMA/A23187 nor miR-20a affected H3 methylation in HMC-1 cells.

3.5. hsa-miR-20a-5p epigenetically regulated IL-10 expression

Although we did not detect any change of total H3K27Ac and H3K27me3, the changes of protein expression enabled us to further explore the histone modification in specific genes locus. As shown in Fig. 5A, by using CHIP-qPCR, we found that PMA/A23187 decreased H3K27Ac level while increased H3K27me3 level at regions of IL-10 gene locus, suggesting PMA/A23187repressed IL-10 transcription. Knocking down miR-20a by overexpression of miR-20a antagonist

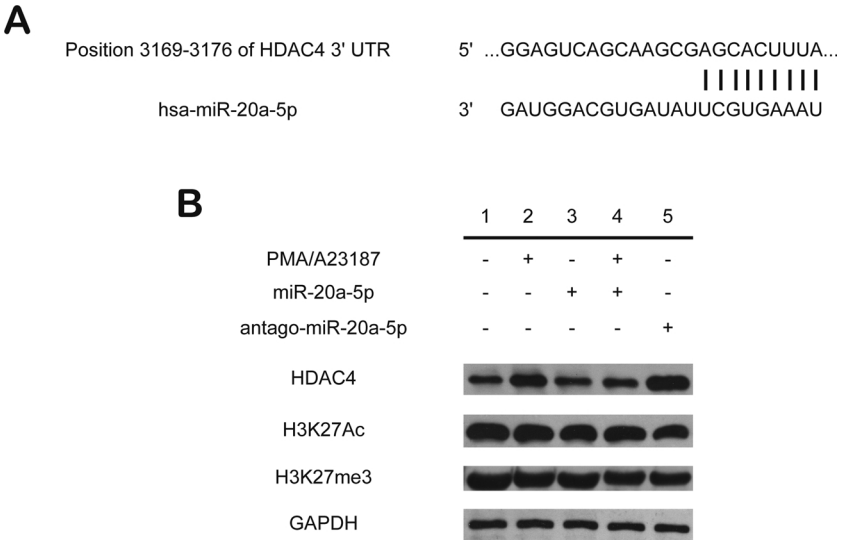

Fig. 4. hsa-miR-20a-5p targeted HDAC4 in PMA/A23187-treated HMC-1 cells. (A) Predicted interaction between hsa-miR-20a-5p and the 3' UTR of HDAC4. B. HMC-1 cells were treated with PMA/A23187 in the presence or absence of miR-20a-5p as indicated. (B) The protein level of HDAC4, H3K27Ac and H3K27me3 were determined by Western blot with corresponding antibodies. GAPDH was used as loading control.

displayed similar effects. In contrast, overexpression of miR-20a rescued H3K27Ac level and decreased H3K27me3 level at IL-10 gene locus in PMA/A23187-treated HMC-1 cells, indicating miR-20a rescued the repression of IL-10 transcription. H3 was used as control and all the treatment did not change its level. Therefore, our data demonstrated that miR-20a prevented PMA/A23187-induced repression of IL-10 transcription in HMC-1 cells.

We also explored the H3 modification in TNF-α locus. As shown in Fig. 5B, PMA/A23187 treatment increased H3K27Ac level in TNF-α locus while miR-20a did not affect the H3K27Ac in TNF-α locus in PMA/A23187-treated HMC-1 cells. Expression of miR-20a antagonist did not affect H3K27Ac when compared to control HMC-1 cells. In addition, all the treatment did not affect H3K27me3 level and H3 level in TNF-α locus. Thus, our data demonstrated that miR-20a did not affect pro-inflammatory cytokine TNF-α transcription.

4. Discussion

miRNAs are non-coding RNAs which are known to function as epigenetic regulators. miRNAs have been implicated in pathogenesis of many diseases including inflammatory disease (Dissanayake and Inoue, 2016). Various inflammatory diseases have been reported to be associated with altered miRNA expression. For example, miR-145, miR-223 and miR-494 are up-regulated in the bronchial epithelium of cystic fibrosis patients (Oglesby et al., 2010). Down-regulated miR-149 is identified in osteoarthritis (Santini et al., 2014). miR-155, which is considered as a master regulator of pro-inflammatory response, is reported to be dysregulated in numerous inflammatory diseases including rheumatoid arthritis, endotoxemia and multiple sclerosis (O'Connell et al., 2012).

Asthma is an inflammatory disease associated with chronic inflammation and tissue remodeling. miRNAs have been implicated in pathogenesis of asthma. Liu and colleagues reported that miRNA-221 and miRNA-485-3p were upregulated in pediatric asthmatics and in an ovalbumin (OVA)-induced murine asthma model (Liu et al., 2012). Administration of miRNA let-7a mimic to lungs of mice with allergic inflammation resulted in alleviation of airway inflammation, attenuation of mucus metaplasia and subepithelial fibrosis (Kumar et al., 2011). Inhibition of miR-126 effectively suppressed Th2 response and led to anti-inflammatory treatment for allergic asthma (Collison et al., 2011). Therefore, elucidating the function of miRNAs is with great importance for treating allergic diseases including asthma. Recently Marinez-Nunez and colleagues described the genome-wide differences in microRNA levels between severe asthma patients and healthy donor (Martinez-Nunez et al., 2018). They identified total 21 dysregulated miRNAs in severe asthma, among them 11 miRNAs were down-regulated. These miRNA may play potential role in pathogenesis of asthma. In another study, by comparing the miRNA expression pattern among healthy people, asthmatic patients and nonasthmatic patients with AR, Panganiban et al identified that circulating miR-125b, miR-16, miR-299-5p, miR-126, miR-206, miR-133b levels were predictive marker of allergic and asthmatic status (Panganiban et al., 2016).

Mast cells played important role in inflammatory disease by modulating immune response and were shown to be important during the development of asthma (Reuter et al., 2010). In present study, we tested the miRNA dysregulation using mast cell model. Ideally it is best to monitor the miRNA expression on a genome wide level in our mast cell model. Due to experimental limitation, we followed the information from previous reports and tested the expression of these identified miRNAs in the mast cell model. We identified that among these miRNAs, miR-20a was the only significantly down-regulated miRNA in PMA/A23187 treated HMC-1 cells, suggesting miR-20a may function in mast cells and asthma pathogenesis. Although we identified slightly up-regulated miR-125b and miR-16, the up-regulation was not significant. Therefore, we only focused on miR-20a and continued to explore its role in inflammation and asthma. Interestingly, the dysregulation of miRNA expression data from our mast cell model was not identical to that in previous reports. For example, miR-16 and miR-125b were significantly up-regulated in asthma patients in previous study (Panganiban et al., 2016). In current study, we only detected slightly up-regulation of miR-16 which was not significant and there was no change of miR-125b expression level. The difference of results could be due to the difference of detection models used. It is also possible that miR-20a could be the miRNA specifically regulated in mast cells in asthma while miR-16 and miR-125b are regulated only in other cells which function in asthma.

Our studies demonstrated that miR-20a regulated cytokine expression. Overexpression of miR-20a in PMA/A23187-treated HMC-1 cells resulted in inhibiting production of pro-inflammatory cytokines TNF-α, IL-1β and IFN-γ, all of which had been shown to contribute to airway inflammation and asthma pathogenesis. In contrast, overexpression of miR-20a promoted IL-10 production in PMA/A23187-treated HMC-1 cells. IL-10 counteracted the inflammatory effects and was capable to inhibiting synthesis of pro-inflammatory cytokines (Grimbaldeston et al., 2007). Similarly, knocking down miR-20a by expressing miR-20a antagonist promoted pro-inflammatory cytokines production and inhibited IL-10 proudction. Thus, our data demonstrated the inhibitory effect of miR-20a on allergic inflammation.

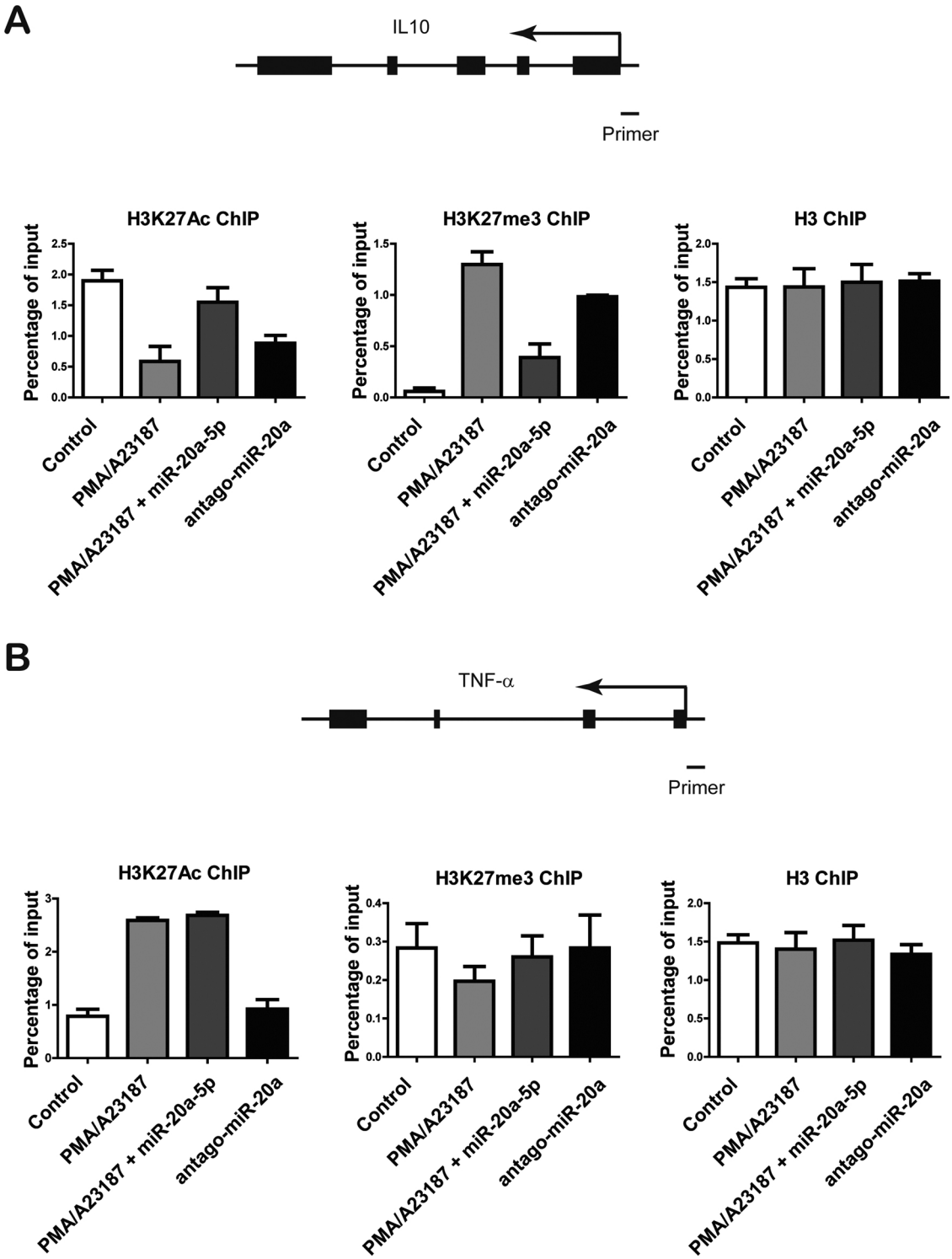

Fig. 5. hsa-miR-20a-5p epigenetically regulated IL-10 expression. ChIP-q-PCR analysis showing the enrichment of H3K27Ac (left panel) H3K27me3 (middle panel) and H3 (right panel) as control in the IL-10 gene (A) and TNF-α gene (B) locus in the HMC-1 cells with different treatment as indicated. The genes and the primer locations were presented on the top of the panel. Data were represented as mean ± S.D.; n = 3 independent experiments.

To determine the underlying mechanism by which enable miR-20a to inhibit inflammation in HMC-1 cells, we used TargetScan to predict potential targets of miR-20a and identified that histone deacetylase 4 (HDAC4) was the potential target. We further used dual luciferase reporter assay and confirmed that miR-20a targeted HDCA4. Consistent with prediction, miR-20a regulated HDAC4 expression by inhibiting PMA/A23187-induced HDAC4 expression. Histone deacetylase (HDAC) was responsible for histone deacetylation which induced chromatin condensation, and transcriptional repression. The reverse reaction was mediated by histone acetyltransferase (HAT), which resulted in modification in the structure of chromatin and transcriptional activation. The acetylation and deacetylation of histones play an important role in the regulation of gene transcriptions (Wang et al., 2014). Collectively, our data suggested that miR-20a may affect inflammation through regulating HDAC4 expression.

Although miR-20a did not affect global level of histone acetylation,

the regulations of pro-inflammatory cytokine and IL-10 by miR-20a enable us to look the histone acetylation at specific gene locus. We demonstrated that in PMA/A23187-treated HMC-1 cells, the histone acetylation level in IL-10 locus was decreased, indicating a repression of IL-10 expression in the cells. Knocking down miR-20a also resulted in decreased histone acetylation in IL-10 locus. In contrast, miR-20a rescued histone acetylation level in IL-10 locus, indicating miR-20a directly activated IL-10 expression by enhancing histone acetylation, possibly through repressing HDAC4 expression. However, miR-20a did not affect histone acetylation in TNF-α locus, suggesting miR-20a did not epigenetically regulated IL-10 expression. As miR-20a inhibited TNF-α expression, it may regulate inflammatory cytokine production in an indirect way, possibly through IL-10 (Denys et al., 2002; Murray, 2005).

5. Conclusion

In present study, we demonstrated that miR-20a prevented pro-inflammatory cytokines and HDAC4 expression, promoted IL-10 expression in activated HMC-1 cells. miR-20a attenuated allergic inflammation by targeting HDAC4.

Funding

None.

Conflicts of interest

None.

Acknowledgement

None.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2019.01.010.

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Supplementary materials

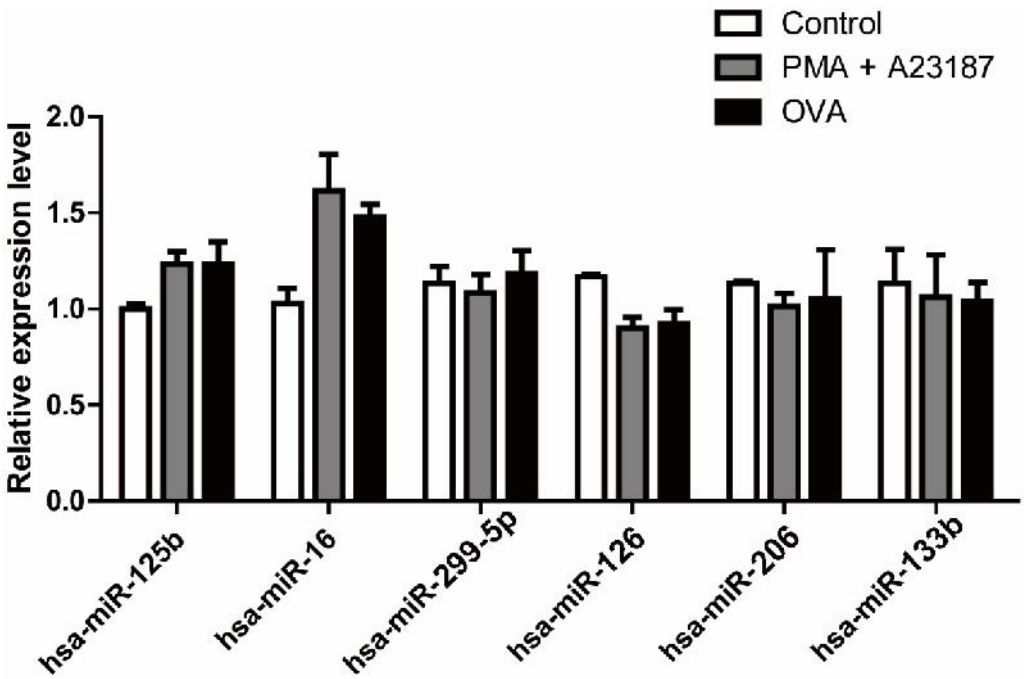

Figure S1. miRNAs involved in inflammatory stimuli did not changed in HMC-1 cells with the treatment of PMA/A23187 treatment or OVA. The expression levels of miR-125b, miR-16, miR-299-5p, miR-126, miR-206, and miR-133b were tested by RT-qPCR. HMC-1 cells were treated with PMA (20 nM) plus A23187 (1 μM) or ovalbumin (OVA, 5 mg/mL) for 24 hours. Data were represented as mean ± s.d., n = 3 independent experiments.

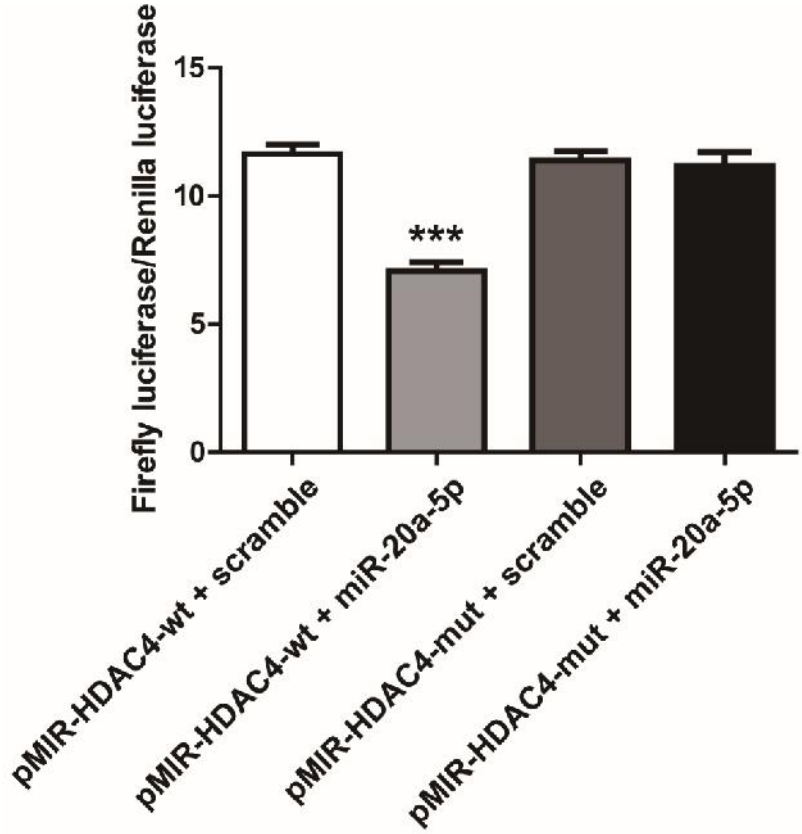

Figure S2. HDAC4 was the direct target of miR-20a-5p. Dual-Reporter assay was used to determine whether miR-20a-5p directly target the mRNA of HDAC4. Wild type or mutated 3' UTR of HDAC4 replaced the 3' UTR of Firefly luciferase in pMIR plasmid and miR-20a-5p or the scramble miRNA were transfected together with the luciferase plasmid. Renilla luciferase activity in pRL-CMV plasmid was used as the internal control. The Ratio of Firefly luciferase/ Renilla luciferase was used to determine the luciferase activity in different groups as indicated. Data were represented as mean ± s.d., n = 4 independent experiments. *** indicated $p < 0.001$.

3' UTR luciferase reporter assay

The 3' UTR of HDCA4 was amplified by PCR using following primers: Forward: 5'-ACCTCTAGAAGATTCTATTTTCAGAAAGTGAGAG-3', Reverse: 5'-ACCTCT AGAAGGGTTTCCCTCAAAGGGATATAA -3' and cloned into pMIR reporter vector (Thermo Fisher) downstream of the luciferase gene. The mutated 3' UTR was generated using QuikChange II Site-Directed Mutagenesis Kit (Aligent, Santa Clara,

CA, USA) using following primers: Forward: 5'- AGCTTCACAAATGTGC TGAGCTGTGTAGCCTTTTCTTTGA -3', Reverse: 5'- TCAAAGAAAAGGCTAC ACAGCTCAGCACATTTGTGAAGCT -3'. For luciferase assays, HMC cells were seeded in 48-well plate and then co-transfected with 50nM miR-20a-5p and 0.2μg luciferase reporter plasmid using Lipofectamine 2000 (Invitrogen). 48h post transfection, Dual-Glo Lucierase assay system (Promega) was used to measure the luciferase activities.