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MSCs exosomal miR-1470 promotes the differentiation of $CD4^+CD25^+FOXP3^+$ Tregs in asthmatic patients by inducing the expression of P27KIP1

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ABSTRACT

Exosomes derived from Mesenchymal Stem Cells (MSCs) possesses similar immunomodulatory effect as MSCs. It had been suggested that MSCs exosomes contain higher level of miR-1470 compared to exosomes derived from fibroblast. Here, we show that MSCs exosomal miR-1470 can elevate the proportion of $CD4^+CD25^+FOXP3^+$ regulatory T cells (Tregs) in asthmatic patients. Moreover, mechanistic studies revealed that miR-1470 can promote the upregulation of P27KIP1 by directly targeting the 3′ region of c-Jun mRNA. Furthermore, miR-1470 mimic transfection could significantly upregulate the proportion of $CD4^+CD25^+FOXP3^+$ Tregs in $CD4^+$ T cells. P27KIP1 knockdown via siRNA silencing significantly inhibited the proportion of $CD4^+CD25^+FOXP3^+$ Tregs with over-expression of miR-1470, which indicates that miR-1470 induces the differentiation of $CD4^+CD25^+FOXP3^+$ Tregs through P27KIP1.

1. Introduction

Due to their immunomodulatory properties, Mesenchymal Stem Cells (MSCs) are widely used for the treatment of immunological diseases such as asthma, graft-versus-host disease (GVHD) and systemic lupus erythematosus (SLE) [1]. MSCs can up-regulate the level of $CD4^+CD25^+FOXP3^+$ regulatory T cells (Tregs), and suppress the proliferative response of the T lymphocytes in the mixed lymphocyte reaction [2]. MSCs can also suppress the function of B lymphocytes, Dendritic Cells (DCs), and NK Cells [3–5]. Studies recently indicated that MSCs can alleviate asthmatic inflammation by up-regulating the proportion of Tregs of asthmatic mice [6,7]. Meanwhile, the deficient proliferation and function of Tregs plays an important role in the immunologic pathogenesis of asthma [8,9].

Exosomes are small vesicles (50–100 nm) released from most cell types, usually consist of protein, RNA, and lipids. Exosomes, as intercellular signalosomes, could allow exchange of RNA (especially miRNA), proteins and lipids between cells [10]. Our recent studies demonstrated that MSC exosomes could promote immunosuppression of Tregs in asthma [11], but the key component in immune suppression remains largely unknown. It was suggested that MSC exosomes contain high level miR-1470 [12]. In this study, we explore whether miR-1470 plays a key role in the process of $CD4^+CD25^+FOXP3^+$ Tregs differentiation induced by MSCs exosomes and its mechanisms.

2. Materials and methods

2.1. Cells

Human bone marrow derived MSCs were purchased from Cyagen Biosciences Inc. (Guangzhou, China). The identity of MSCs were confirmed by morphology, the potency of differentiation into chondrogenic, adipogenic and osteogenic lineages, as well as the surface markers of MSCs. MSCs were cultured in human mesenchymal stem cell growth medium (Cyagen, China) with exosome free fetal bovine serum (FBS) (SBI, America) before the medium was collected for isolation of exosomes. Cell culture supernatants were collected for the isolation of exosomes. MSCs between passages 4–8 were used for all experiments.

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$CD4^+$ T cells were separated from peripheral blood mononuclear cells (PBMCs) of acute asthmatic patients with CD4 MicroBeads (Miltenyi Biotec, Germany). $CD4^+$ T cells were cultured with RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA), 1 ug/ml PHA (Sigma, USA) and 20 U/ml IL-2 (Invitrogen, USA). All experiments included $CD4^+$ T cells from at least 3 donors, and each independent experiment was performed in triplicates.

2.2. Isolation and identification of exosomes

Exosomes were purified from MSCs culture supernatants by differential ultracentrifugation as previously described [13] using Optima L-100XP ultracentrifuge (Beckman Coulter, USA). The supernatant was centrifuged at 300*g* for 10 min, 2000*g* for 10 min and 10,000*g* for 30 min to eliminate large dead cells and large cell debris. The supernatant was then collected and ultracentrifugation at 100,000*g* 70 min was carried out. Exosomes were observed by transmission electron microscope (FEI TECNAI G2, USA). Western Blots were performed to verify the exosome marker CD9, CD63 and CD81.

2.3. Co-culture of $CD4^+$ T cells with MSCs, MSC-derived exosomes

$CD4^+$ T cells were co-cultured with MSCs, MSCs with exosome inhibitor (GW4869, Sigma, USA), MSC-derived exosomes for 48 h, then the expression of miR-1470 of $CD4^+$ T cells was tested by qRT-PCR. Hsa-miR-1470 inhibitors or inhibitor negative control (NC) (Shanghai GenePharma, China) were transfected into MSCs with Lipofectamine IMAX (ThermoFisher Scientific, USA). The supernatants of MSCs transfected with Hsa-miR-1470 inhibitors were collected for exosomes isolation. $CD4^+$ T cells were co-cultured with control, exosomes isolated from MSCs transfected with inhibitor NC ($exosomes^{WT}$) and exosomes isolated from MSCs transfected with hsa-miR-1470 inhibitors ($exosomes^{1470IH}$) for 48 h, the proportion of $CD4^+CD25^+FOXP3^+$ Tregs was tested by Flow Cytometry.

2.4. Flow Cytometry

The proportion of $CD4^+CD25^+FOXP3^+$ Tregs was tested by Flow Cytometry. After staining cell surface antigen by Anti-Human CD4 FITC (Invitrogen, USA) and CD25 APC Cocktail (Invitrogen, USA) for 20 min, $CD4^+$ T cells were incubated with Fixation/Permeabilization working solution (Invitrogen, USA) for 30 min. Then, the cells were washed and resuspended with Permeabilization buffer (Invitrogen, USA). Next, the cells were incubated with Anti-human Foxp3 PE (Invitrogen, USA) for 30 min. Finally, the stained cells were washed by Permeabilization buffer (Invitrogen, USA) and resuspended with Flow Cytometry Staining Buffer (Invitrogen, USA) and acquired on a flow cytometer (BD Biosciences, USA).

2.5. qRT-PCR analysis

For quantification of miR-1470 expression in $CD4^+$ T Cells, RNA was isolated from $CD4^+$ T cells by sequential centrifugation with TRIzol RNA Kit (Invitrogen, USA). The concentration and purity of RNA solution was detected by NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Reverse transcription of extracted RNA was conducted with Mir-X miRNA First-Strand Synthesis Kit (Takara, USA). qPCR reactions were conducted using Mir-X miRNA qRT-PCR SYBR Kit (Takara, USA). The reactions were run on qPCR system (Roche, Switzerland). The level of miR-1470 was determined relative to the level of U6 snRNA.

2.6. Western Blots

$CD4^+$ T cells were subjected to lysis by RIPA buffer (Beyotime, China) with PMSF (Sigma, Germany) for 20 min. The cell lysates were centrifuged at 12,000*g* for 20 min at 4 °C, and the supernatants were transferred into fresh tubes. Cell lysates were subjected for SDS-PAGE electrophoresis, followed by PVDF membrane transfer. Antibodies against P27KIP1 (Cell Signaling Technology, USA), c-Jun (Cell Signaling Technology, USA) were used to visualize the corresponding proteins.

2.7. Luciferase reporter assay

The 3′-Untranslated Region (3′-UTR) of c-Jun was cloned into psiCheck2 (Promega, USA). Plasmids containing mutated miR-1470–binding sites located on the 3′-UTR of c-Jun were generated by site-directed mutagenesis. Wild type (WT) or mutated psiCHECK2 constructs were transfected along with miR-1470 mimic or miR-1470 NC into 293 T cells (ATCC, USA). Forty-eight hours after transfection, luciferase activity was detected with a Dual-Glo Luciferase Reporter Assay System (Promega, USA).

2.8. Statistics

Data was represented as mean ± SD. SPSS 13.0 software was used for statistical analysis. T-test was used when statistical analysis was implemented between two groups. ANOVA was used when statistical analysis was implemented between more than two groups. p-Value ≤ 0.05 was considered to be significant.

2.9. Study approval

The study was approved by the Ethics Committee of Sun Yat-sen Memorial Hospital, Sun Yat-sen University in accordance with the Good Clinical Practice.

3. Results

3.1. Characterization of MSCs exosomes

Exosomes were isolated by differential ultracentrifugation. Exosomes were cup-shaped membrane vesicles with a diameter of 50–100 nm under electron microscope (Fig. 1A). The molecular marker of exosomes was detected by Western Blots, which was positive for CD9, CD63, and CD81, the typical markers of exosomes (Fig. 1B).

3.2. MSC-derived exosomes elevated the level of miR-1470 in $CD4^+$ T cells

MSCs significantly elevated the expression of miR-1470 in $CD4^+$ T cells compared with control, while the exosome inhibitor GW4869 was added to the co-culture system, the elevation of miR-1470 was significantly inhibited compared with MSCs (Fig. 2A). After co-culturing $CD4^+$ T cells isolated from peripheral blood of asthmatic patients with MSCs exosomes, the expression of miR-1470 in $CD4^+$ T cells found to be significantly elevated (Fig. 2B).

To determine the level of miR-1470 in MSCs-derived exosomes, expression of miR-1470 in exosomes derived from MSCs or fibroblasts was tested by qRT-PCR. As seen in Fig. 3, the level of miR-1470 in MSCs derived exosomes was significantly higher than those derived from fibroblasts ($p < 0.05$), indicating that MSCs-derived exosomes contain high level of miR-1470.

3.3. miR-1470 mediated the induction of $CD4^+CD25^+FOXP3^+$ Tregs induced by MSCs exosomes

$Exosomes^{WT}$ could significantly elevate the proportion of $CD4^+CD25^+FOXP3^+$ Tregs when compared with control group. The proportion of $CD4^+CD25^+FOXP3^+$ Tregs in the $exosomes^{1470IH}$ group was lower than the $exosomes^{WT}$ group, however, the proportion of $CD4^+CD25^+FOXP3^+$ Tregs in the $exosomes^{1470IH}$ group was still

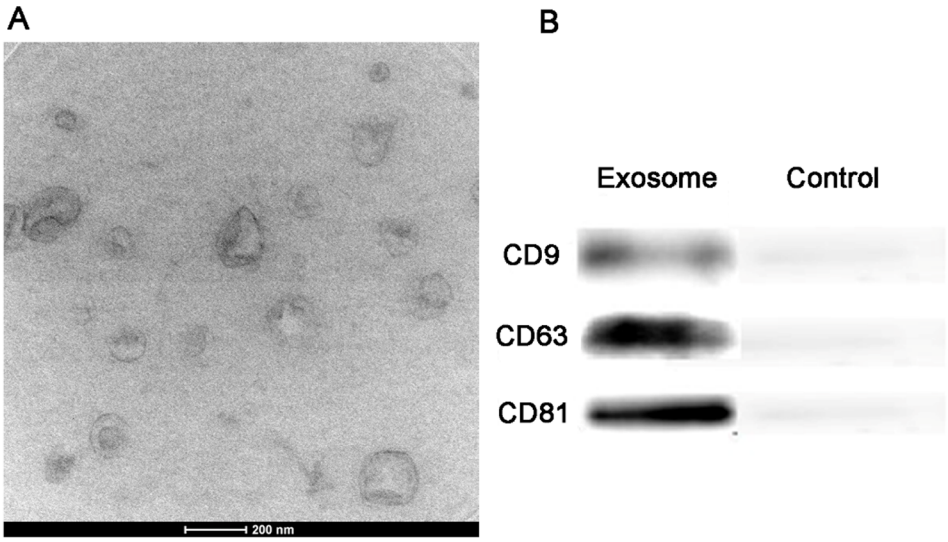

Fig. 1. Characteristics of MSCs exosomes. (A) Transmission Electron-microscopic observation of MSCs exosomes (30000 ×). (B) Protein markers of MSCs exosomes assessed by Western Blots. MSCs = mesenchymal stem cells.

higher than the control group (Fig. 4A & B). This indicated that MSCs exosomes elevate the proportion of $CD4^+CD25^+FOXP3^+$ Tregs partially through miR-1470.

3.4. *miR-1470 elevated the proportion of $CD4^+CD25^+FOXP3^+$ Tregs through P27KIP1*

The target prediction of miR-1470 was carried out via prediction programs of microRNA (TargetScan, RNA22, and mirtarbase). 111 target proteins were predicted by TargetScan, RNA22, and mirtarbase (Fig. 5A). Then, the target proteins of miR-1470 were analyzed by GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis (Fig. 5B–E). With the literature review of target proteins in the pathogenesis of asthma, c-Jun mRNA transcription was predicted as the target protein of miR-1470 in this study. To confirm the prediction, we did the luciferase reporter assay, which indicates that miR-1470 target the 3′ region of c-Jun mRNA (Fig. 5F–G). miR-1470 mimics, miR-1470 inhibitors, mimic NC and inhibitors NC were transfected into $CD4^+$ T cells. With the overexpression of miR-1470 in $CD4^+$ T cells, the expression of c-Jun was inhibited, while the expression of P27KIP1 was up-regulated (Fig. 6A). When the effect of miR-1470 was inhibited, the expression of c-Jun in $CD4^+$ T cells was up-regulated, while the

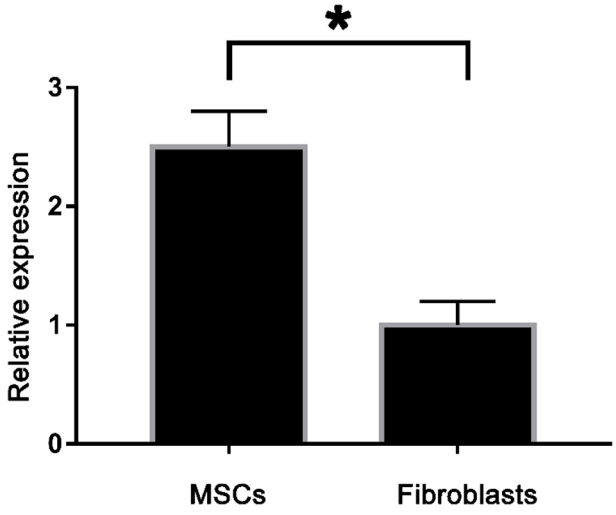

Fig. 3. Expression of miR-1470 in exosomes derived from MSCs or fibroblasts. qRT-PCR for the expression of miR-1470 in the same number of exosomes derived from MSCs or fibroblasts, *P < 0.05. *MSCs = mesenchymal stem cells.*

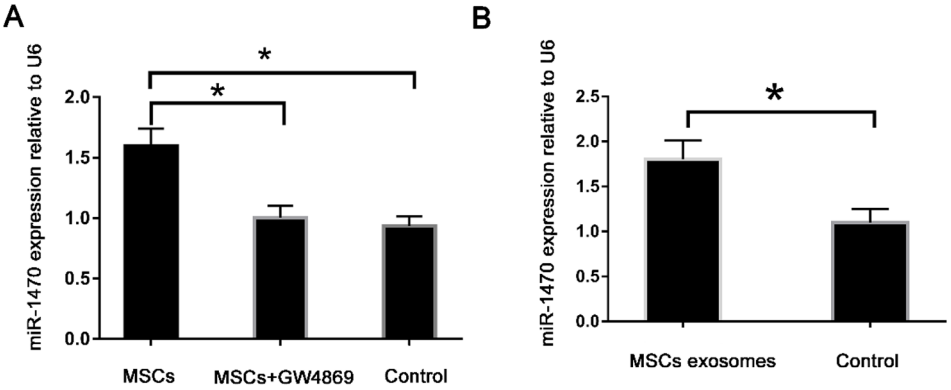

Fig. 2. The expression of miR-1470 in $CD4^+$ T cells was elevated by MSCs exosomes. (A) $CD4^+$ T cells were co-cultured with MSCs, MSCs + GW4869. (B) $CD4^+$ T cells were co-cultured with MSCs exosomes. The level of miR-1470 was determined by qRT-PCR relative to U6. MSCs = mesenchymal stem cells, *P < 0.05.

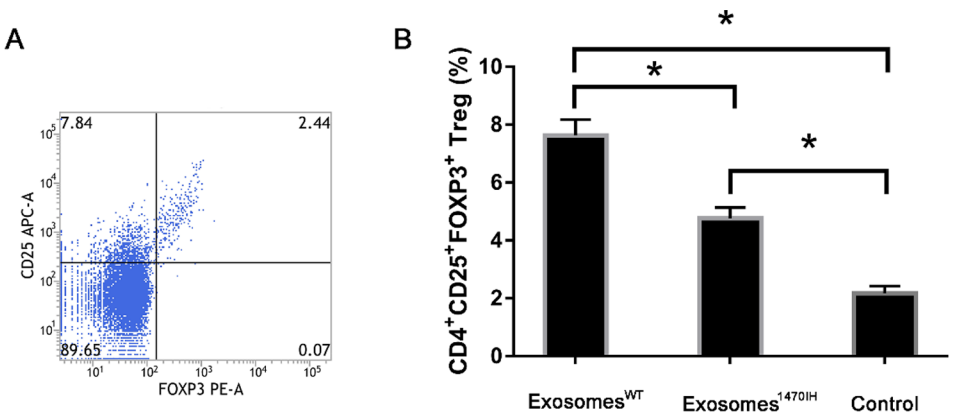

Fig. 4. miR-1470 mediated the elevation of $CD4^+CD25^+FOXP3^+$ Tregs induced by MSCs exosomes. (A) $CD4^+CD25^+FOXP3^+$ Tregs detected by Flow Cytometry. (B) The proportion of $CD4^+CD25^+FOXP3^+$ Tregs in $exosomes^{WT}$, $exosomes^{1470IH}$, and control group, *P < 0.05. *MSCs = mesenchymal stem cells, Tregs = regulatory T cells, $exosomes^{WT}$ = exosomes isolated from the supernatant of MSCs transfected with inhibitor NC, $exosomes^{1470IH}$ = exosomes isolated from the supernatant of MSCs transfected with miR-1470 inhibitor, NC = negative control.*

expression of P27KIP1 was inhibited (Fig. 6A). These results indicated that miR-1470 up-regulates the expression of P27KIP1 through targeting c-Jun. To explore whether miR-1470 up-regulated the proportion of $CD4^+CD25^+FOXP3^+$ Tregs through P27KIP1, $CD4^+$ T cells were transfected with either miR-1470 mimics, miR-1470 mimics + P27KIP1 siRNA, mimics NC or mimics NC + Si-NC. Through Western Blot analysis, the expression of P27KIP1 in miR-1470 mimics transfection group was higher than control group, while the expression of P27KIP1 in miR-1470 mimics + P27KIP1 siRNA group was lower than control (Fig. 6B). The proportion of $CD4^+CD25^+FOXP3^+$ Tregs in miR-1470 mimics transfection group was higher than control group, while the proportion of $CD4^+CD25^+FOXP3^+$ Tregs in miR-1470 mimics + p27kip1 siRNA group was lower than control group (Fig. 6C). These results indicated that miR-1470 elevate the proportion of $CD4^+CD25^+FOXP3^+$ Tregs through P27KIP1.

4. Discussion

Here we report that MSCs exosomal miR-1470 promote the differentiation of $CD4^+CD25^+FOXP3^+$ Tregs in asthmatic patients by inducing the expression of P27KIP1.

MSCs are being widely explored for treating immune related diseases on the basis of its' immunological effect. Exosomes isolated from MSCs culture supernatants are also immunologically active for

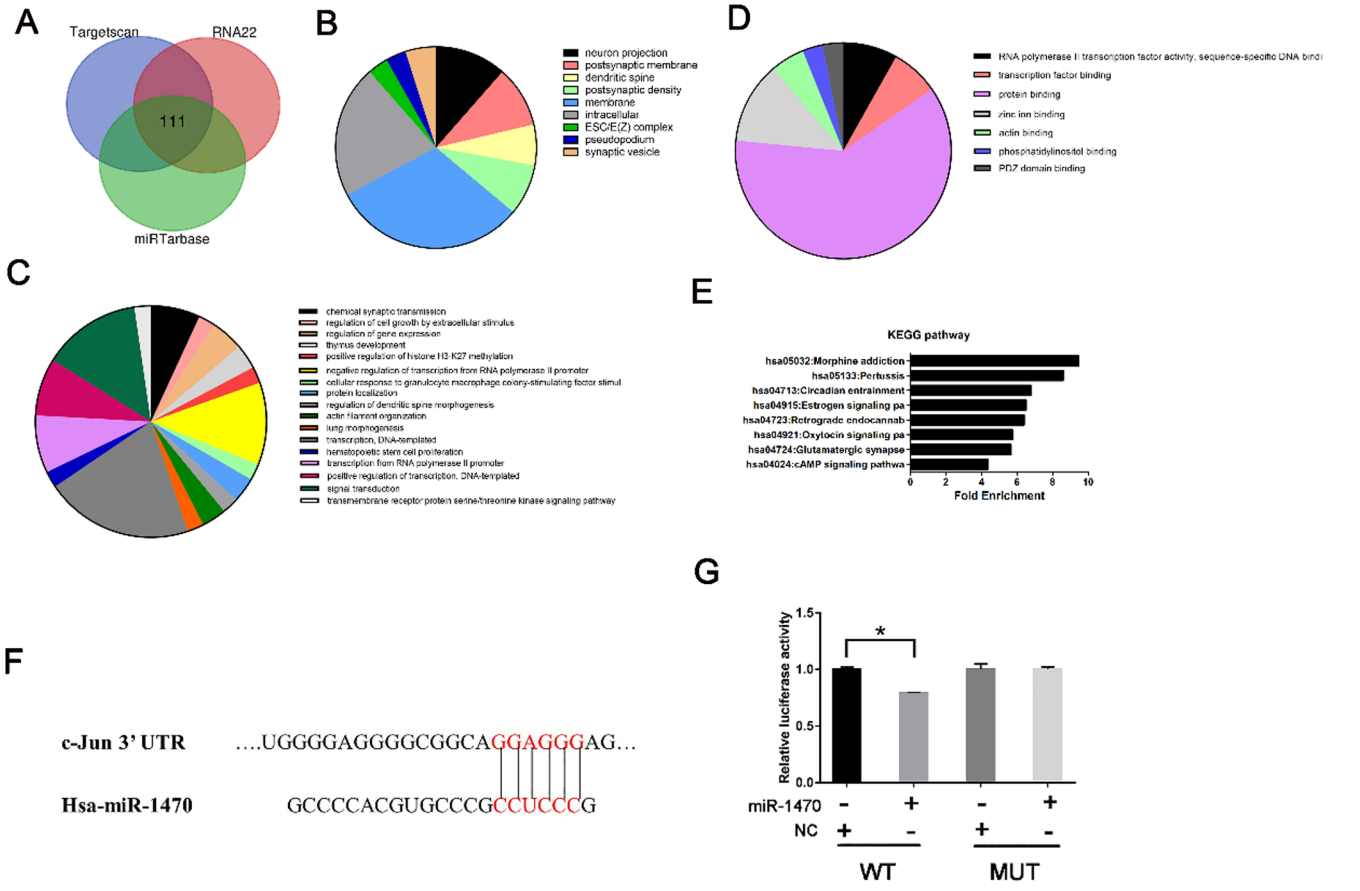

Fig. 5. The target protein of miR-1470 was c-Jun. (A) Target proteins predicted by TargetScan, RNA22, and mirtarbase. (B) GO (Gene Ontology) analysis: cellular component. (C) GO analysis: biological process. (D) GO analysis: molecular function. (E) KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis. (F) Schematic description of the hypothesized duplexes of the miR-1470 binding site within the c-Jun mRNA 3′-UTR. (G) Luciferase reporter assay showed that miR-1470 could bind to the 3′-UTR of c-Jun, *P < 0.05. *UTR = Untranslated Region, NC = negative control.*

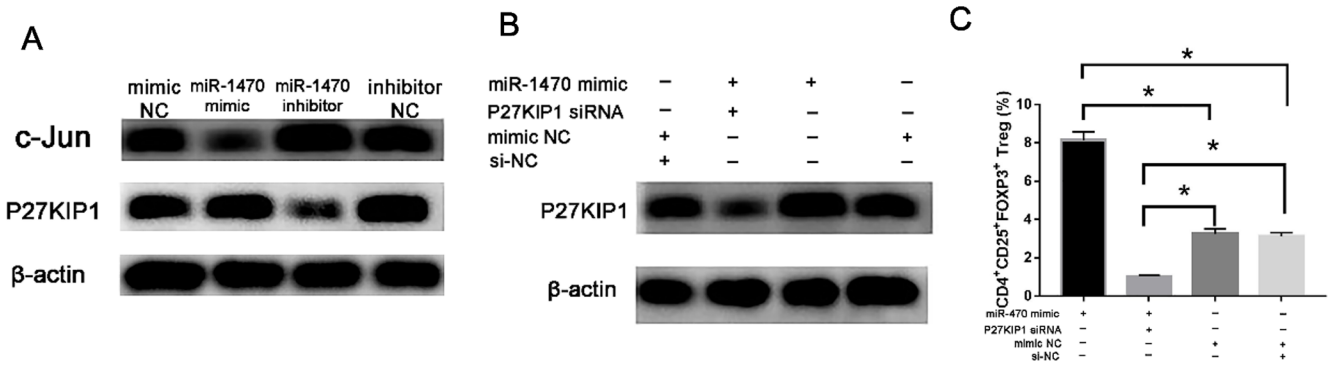

Fig. 6. miR-1470 elevated the proportion of $CD4^+CD25^+FOXP3^+$ Tregs through P27KIP1. (A) Western Blot analysis of the expression of c-Jun and P27KIP1 in $CD4^+$ T cells transfected with miR-1470 mimics or miR-1470 inhibitor. (B) Western Blot analysis of the expression of P27KIP1 in $CD4^+$ T cells transfected with miR-1470 mimics or miR-1470 mimics + P27KIP1 siRNA. (C) Flow Cytometry analysis of the proportion of $CD4^+CD25^+FOXP3^+$ Tregs in $CD4^+$ T cells transfected with miR-1470 mimics or miR-1470 mimics + P27KIP1 siRNA, *P < 0.05. *Tregs = regulatory T cells, NC = negative control.*

suppressing a variety of immune reactions [14]. Our previous studies indicated that MSC exosomes can up-regulate and promote the immunological suppression effect of $CD4^+CD25^+FOXP3^+$ Tregs [11]. MSC exosomes can suppress the proliferation of T cells co-cultured with CD3 and CD28 antibodies [15]. A study show that MSC exosomes can promote the survival of allogenic skin grafts in mice by inducing regulatory T cells proliferation [16].

Exosomes composition analyses have demonstrated that exosomes contain miRNA, mRNA, protein, and lipid [10]. However, the specific role of each compartment in immunological modulation of MSCs exosomes remains largely unknown. Studies have indicated that miRNA plays an important role in MSCs exosomes biological effects [17,18]. It was reported that MSCs exosomes can transfer miR-133b to neural cells and contribute to neurite outgrowth [19]. MSCs exosomes miRNA analysis showed that exosomes isolated from MSCs have 2.5-fold higher level of miR-1470 compared with those isolated from adult fibroblasts [12]. In this study, we showed for the first time, that MSCs exosomes induce the differentiation of $CD4^+CD25^+FOXP3^+$ Tregs through miR-1470, which could represent a new pathway for MSCs exosomes to exert their immunological effects.

As negative gene modulators, miRNAs can inhibit the expression of their target genes through post-transcriptional silencing of complementary mRNA targets [20]. It was reported that miR-1470 can indirectly up-regulate the level of P27KIP1 by directly inhibiting the expression of c-Jun [21]. Studies indicated that P27KIP1 plays an important role in the process of $CD4^+CD25^+FOXP3^+$ Tregs differentiation [22,23]. In our studies, we demonstrated that MSC exosomal miR-1470, which targeted c-Jun, induce the differentiation of $CD4^+CD25^+FOXP3^+$ Tregs through indirect up-regulation of P27KIP1 in $CD4^+$ T cells isolated from peripheral blood of asthmatic patients.

Asthma is a chronic inflammatory airway disease which is characterized by T helper cell 2 (Th2) inflammation, the characteristics of which are airway hyperresponsiveness and remodeling [24]. Tregs are indispensable in the maintenance of immunological homeostasis in the airways. Studies indicated that impaired function of $CD4^+CD25^+FOXP3^+$ Tregs plays a critical role in the pathogenesis of asthma [8]. MSC exosomal miR-1470, which plays an important role in the up-regulation of $CD4^+CD25^+FOXP3^+$ Tregs in our studies, is a promising therapeutic target of asthma.

Together, this study implies that MSCs exosomal miR-1470 promotes the differentiation of $CD4^+CD25^+FOXP3^+$ Tregs in asthmatic patients by inducing the expression of P27KIP1. Moreover, our finds highlight a novel mechanism by which MSC-derived exosomes exert their immunological effect.

Declaration of competing interest

None.

Acknowledgements

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