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Original Article

miR-146b level and variants is associated with endometriosis related macrophages phenotype and plays a pivotal role in the endometriotic pain symptom

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ABSTRACT

Objective: The aim of this study was to explore the effect of miR-146b expression and variants on endometriosis and its associated pain symptom.**Materials and methods:** Genotyping and expression of miR-146b was performed on 74 endometriosis patients and 23 healthy controls. ESCs were subsequently co-cultured with peripheral blood (PB)-derived monocytes (PBMC)-driven macrophages. After overexpression and inhibition of miR-146b, cytokine production from the macrophages were determined by enzyme-linked immunosorbent assay (ELISA). Western blot were done to measure the regulation of IRF5 by miR-146b.**Results:** We found that miR-146b expression was increased in PF supernatant and PF CD14 + monocytes/Macrophages of endometriosis patients, with endometriosis patients with pain (EPWP) showing higher miR-146b expression compared with the endometriosis patients without pain (EPNP). CT/CC genotype of miR-146b rs1536309 was associated with the risk of pain symptom of endometriosis. For the function studies, we found that miR-146b was involved in the negative regulation of inflammation through attenuating IRF5 expression. Macrophages from patients who carries CT/CC genotype of miR-146b rs1536309 showed decreasing miR-146b expression and enhancement of the ability of pro-inflammation.**Conclusions:** Our findings suggest an important role of miR-146b level and variants in endometriosis that helps to regulate the process of endometriosis and its associated pain.© 2019 Taiwan Association of Obstetrics & Gynecology. Publishing services by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Endometriosis is an estrogen-dependent pelvic inflammatory disease characterized by implantation and growth of endometrial tissue (glands and stroma) outside the uterine cavity [1]. This disease affect approximately 10% of women of reproductive age, causing infertility and pain [2]. Medical scientists believed that endometriosis-associated pain was highly related to inflammatory related signaling pathways [3,4]. Growing evidence tend to focus on the role in triggering inflammatory pain in endometriosis through the activation of innate immune cells and peripheral nerve ending

[5,6]. Macrophages are the primary contributor of pro-inflammatory chemotactic cytokines and major source of neuro-angiogenesis among them. The recruitment of macrophages to nerve fibers within the endometriotic lesion have been demonstrated to facilitate the development of inflammatory pain [7]. Further, macrophages can be differentiated into classically activated phenotype (M1) or alternatively activated phenotype (M2) in response cytokine signals. At the transcriptional level, IRF5 is a key transcription factor for M1 macrophage differentiation while IRF4 is necessary for M2 macrophage differentiation [8]. The phenotype alternations are reported to be essential events contributing to the recruitment of macrophages and generation of endometriosis-associated pain [5]. However, it is still not clearly understood how endometriosis affects macrophage polarization and the gene expressions in the development of endometriosis-associated pain.

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MicroRNAs (miRNAs) are a recently discovered class of small noncoding RNAs that are implicated in many physiological and pathological responses as post-transcriptional repressors of gene expression [9]. Mature miRNAs can specifically bind to 3' UTRs of target cellular mRNA in turn triggering mRNA degradation or inhibition of translation [10]. There has been recent evidence regarding novel role of miRNAs in inflammation, and most of them functions through suppressing their target genes [10]. Therefore, we can predict that there might be a possible association between miRNA-mediated inflammation and reproductive diseases such as endometriosis and the related painful symptoms. However, there are very few studies in the literature that have shown a direct relationship between miRNA patterns and endometriosis related pain [11].

Kristeena et al.'s RNA-seq data demonstrated that a differential expression of microRNAs, including miR-146b, in the endometriotic tissues from women with endometriosis with pain compared to those without, suggesting a plausible role for miRNA or epigenetic mechanisms in the etiology of endometriotic pain [12]. Recent publications have indicated that miR-146 family could play a key role in the innate immune response and also participate in the pathogenesis of immune diseases like infection [13,14], cardiovascular disease [15,16], cancer [17–19]. Especially, miR-146b was demonstrated to be closely related to the regulation of various cell functions, such as polarization [8], cell death [20], and autophagy [21]. Considering that many miR-146b regulated cell functions was highly related with endometriosis, we assumed that miR-146b might play an important role in endometriosis. In this study, we demonstrated that miR-146b was significant up-regulated in peritoneal fluid and serum samples of endometriosis patients. Notably, miR-146b rs1536309 C>T polymorphism was associated with the risk of pain symptom of endometriosis. Further molecular mechanisms reveal that this phenotype might because the inhibition of IRF5 mediated macrophage polarization by miR-146b.

Material and methods

Clinical samples

Totally 74 patients suspected of having endometriosis was referred to the Obstetrics-Gynecology clinic of Shandong provincial third hospital from December 2015 to December 2017. This study was approved by the ethics committees of Shandong provincial third hospital. All patients provided informed written consent before study enrollment. All the experiments were carried out according to principles of Helsinki Declaration. All women completed a gynecologic/infertility history form, a pre-operative quality of life questionnaire and assessment of pain using a visual analog scale for assessment of endometriosis associated pain (adapted from the validated International Pelvic Pain Society's Pelvic Assessment Form). The control group consisted of 23 patients undergoing surgery for uterine leiomyomas, and received regular physiological examinations at the same hospital. Blood samples were drawn at the first outpatient day and routine laboratory examinations, such as blood cell counts and biochemical analysis, were performed.

Peritoneal fluid collection

Peritoneal fluid (PF) was collected from the patients during a performed laparoscopy. Any contamination of the fluid with blood from injured vessels after trocar insertion excluded patients from the analysis. Mononuclear cells were isolated on density gradient medium (Polymorphprep, Nycomed, Norway) by centrifugation at 600×g, for 25 min, at room temperature. After centrifugation, PF supernatant was aspirated and stored until analysis. Monocytes/

Macrophages were obtained through positive selection by CD14 + micromagnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany).

RNA extraction

MicroRNA was extracted and purified from PF supernatant and CD14 + cells of subjects using mirVana™ PARIS™ (Cat #AM1556) kit according to the manufacturer's instructions (Ambion, USA). The quality and quantity of the isolated microRNAs were measured using an ND-2000 NanoDrop spectrophotometer (Thermo-Scientific, USA) and Agilent Bioanalyzer 2100 (Agilent Technologies, USA).

qRT-PCR assay

qRT-PCR analyses for miRNAs were performed by using TaqMan miRNA assays (Ambion) in an iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Reverse transcription reactions were performed using the following parameters: 16 °C for 30 min, 42 °C for 30 min, and 84 °C for 5 min. PCR reactions were performed using the following parameters: 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. U6 small nuclear RNA was used as an endogenous control for data normalization.

Genotyping

Genomic DNA was extracted from peripheral blood samples of patients. The miR-miR-146b-5p rs1536309 genotypes for the patients and the controls were determined using a PCR-restriction fragment length polymorphism assay. The primer sequences used to detect the target were described as following: miR-146b-5p rs1536309 C>T polymorphism were: 5'-TGCCTGGATCGCCTTAGCT-3' (forward) and 5'-AGTCCAGTTTCTCATTTGAAGCA-3' (reverse).

Purification of human endometrial stromal cells (ESCs)

The ESCs were purified as following: The endometrial or endometriotic tissues from the patients were minced into 2 mm pieces and incubated in DMEM/F12 containing collagenase type IV (0.1%; Sigma, San Francisco, CA, USA) and deoxyribonuclease type I (DNase I; 3000 U; Sigma, USA) with constant agitation for 70 min at 37 °C. The resulting dispersion was filtered in turn through 100 µm and 70 µm nylon strainers (Becton Dickinson, Franklin Lakes, NJ, USA). The filtrate was then centrifuged at 800×g for 15 min to further remove the leukocytes and erythrocytes and was washed with phosphate-buffered saline (PBS, Gibco, USA). The ESCs were resuspended in DMEM/F-12 containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), plated on culture flasks, and incubated at 37 °C in 5% CO₂.

Co-culture of human macrophages with ESCs

Peripheral blood mononuclear cell line, THP-1 were purchased from ATCC. The primary peripheral blood mononuclear cells (PBMC) were isolated from the human blood samples by Ficoll-Hypaque density gradient centrifugation. CD14 + cells were obtained through positive selection by CD14 + micromagnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Then the monocytes were cultured with granulocyte macrophage colony-stimulating factor (GM-CSF, 5 ng/mL; Leukine, Genzyme Corporation, UK) and macrophage colony-stimulating factor (M-CSF, 20 ng/mL; R&D systems, USA) in RPMI 1640 medium (Gibco, USA) containing 10% FBS and 2 mM L-glutamine for up to 6 days. Macrophage were co-cultured with ESCs

Table 1
Demographic characteristics of the Study population.

Demographic characteristics	controls (n = 23)	Endometriosis patients (n = 74)	P value
Age (years): mean \pm SD	36.7 \pm 7.3	37.2 \pm 10.8	0.53
BMI: mean \pm SD	24.6 \pm 5.5	23.7 \pm 6.1	0.34
Infertility [n (%)]	6 (26.1%)	24 (32.4%)	0.57
Previous Surgery other than laparoscopy [n (%)]	5 (21.7%)	19 (25.7%)	0.70
Pain symptom [Yes/No]	—	57/17	—
Stage I-II/stage III-IV	—	48/26	—

without direct cell-to-cell contact, using the upper chamber of Transwell inserts (0.4 μ m pores; Costar; USA).

Cell transfection

All oligonucleotides were synthesized from GenePharma (Shanghai, P.R. China) products. Transfections were performed using Lipofectamine 2000 (Invitrogen). Cells were transfected with 50 nM miR-146b mimics, inhibitors, or scrambled miR-control for 24 h.

Chromatin immunoprecipitation (CHIP) assay

CHIP analysis was carried out according to the Simple ChIP Enzymatic Chromatin IP Kit protocol (Cell Signaling) as described [8]. The chromatin was immunoprecipitated with anti-IgG or anti-IRF5 antibodies at 4 °C under rotation for 16 h. The DNA isolated from immunoprecipitated material was amplified by real-time PCR for IL-12p40 promoter.

Statistical analysis

The results are expressed as means \pm SD from at least 3 separate experiments performed in triplicate. The differences between

groups were determined using two-tailed Student's t-test, using SPSS software (Armonk, NY, USA). P values of less than 0.05 were considered significant. The Chi-square test or Fisher's exact test was used to analyze the relationship between rs1536309 C>T polymorphism and the clinicopathological features.

Results

miR-146b expression were significantly increased in PF supernatant and PF CD14 + monocytes/Macrophages of endometriosis patients

The Patient demographics were summarized in Table 1. A total of 74 endometriosis patients (EP group) and 23 controls (CTL group) was enrolled. No significant differences were seen in age and BMI among the patients and controls. Of the 74 patients with endometriosis, 48 had early disease (stage I and II) and 26 had late disease (stages III and IV). Totally 57 patients have pain symptom (EPWP group) according to the visual analog scale (VAS) system, whereas 17 cases were endometriosis patients without pain (EPNP group). Using the samples from these subjects, we found that EP group had considerably miR-146b increased in PF supernatant (Fig. 1A), and PF CD14 + monocytes/Macrophages (Fig. 1B), respectively, comparing with the CTL group. Further, when the endometriosis patients were stratified into subgroups according with the stage and pain symptom, we found that the miR-146b expression markedly increased in the stages III/IV group (Fig. 1C, D) and EPWP group (Fig. 1E, F).

miRNA-146b rs1536309 C>T polymorphism was associated with the endometriotic pain

SNP rs1536309 which was located in the miR-146b promoter, was reported to be highly associated with the miR-146b expression, and was associated with the development and prognosis of several cancers [22]. In this study, the relationship between SNP rs1536309 and the endometriosis development were also determined. We

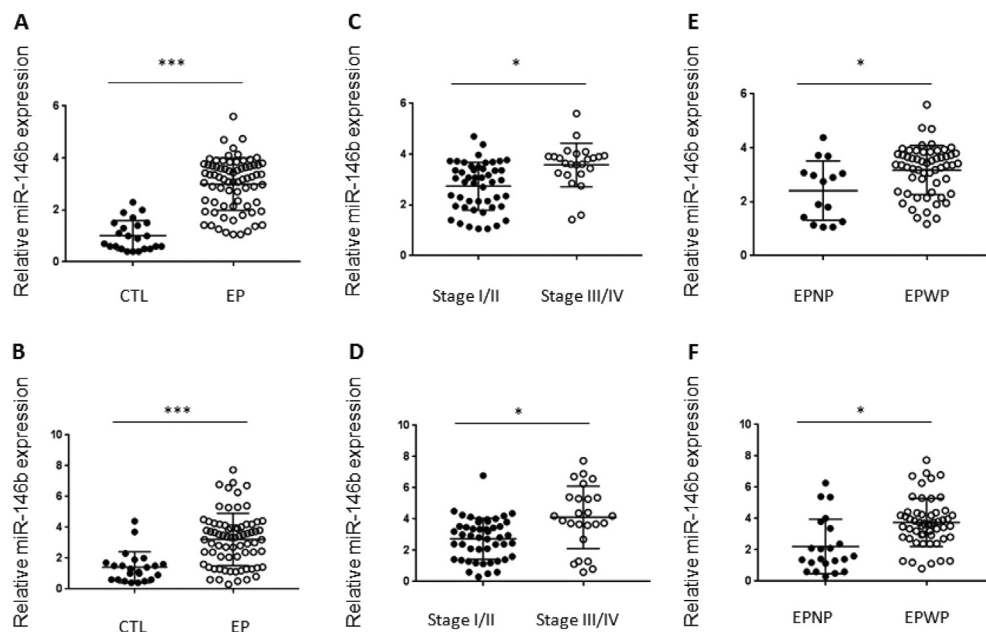


Fig. 1. miR-146b expressing patterns in endometriosis patients. (A–B) miR-146b levels in (A) PF supernatant and (B) PF CD14 + monocytes/Macrophages of endometriosis patients were significantly higher compared with controls. (C–D) miR-146b levels in (C) PF supernatant and (D) PF CD14 + monocytes/Macrophages of stage III/IV endometriosis patients were significantly higher compared with the stage I/II cases. (E–F) miR-146b levels in (E) PF supernatant and (F) PF CD14 + monocytes/Macrophages of EPWP group were significantly higher compared with the EPNP cases. *P < 0.05; **P < 0.01; ***P < 0.001. CTL: control; EPWP: endometriosis patients with pain; EPNP: endometriosis patients not with pain.

Table 2
Genotype distributions of miR-146b rs1536309 in patients with endometriosis and controls.

All subjects divided into:				EP patients divided into:				EP patients divided into:				EP patients divided into:																	
EP (n = 74)				Stage I/II (n = 48)				Stage III/IV (n = 26)				Infertility (n = 24)				not Infertility (n = 50)				EPWP (n = 57)				EPNP (n = 17)					
		CTL (n = 23)	P	OR (95% CI)																									
TT	48	16		1	28	20		1	17	31		1	33	15		1													
CT	21	6	0.78	0.86 (0.29–2.50)	16	5	0.15	0.44 (0.14–1.39)	7	14		0.87	1.10 (0.37–3.24)	19	2		0.06												
CC	5	1	0.65	0.60 (0.07–5.53)	4	1	0.35	0.35 (0.04–3.37)	0	5		0.11	0.65 (0.52–0.80)	5	0		0.14												
TT	48	13		1	28	20		1	17	31		1	33	15		1													
CT + CC	26	10	0.47	1.42 (0.55–3.68)	20	6	0.11	0.42 (0.14–1.23)	7	19		0.46	1.49 (0.52–4.25)	24	2		0.02												

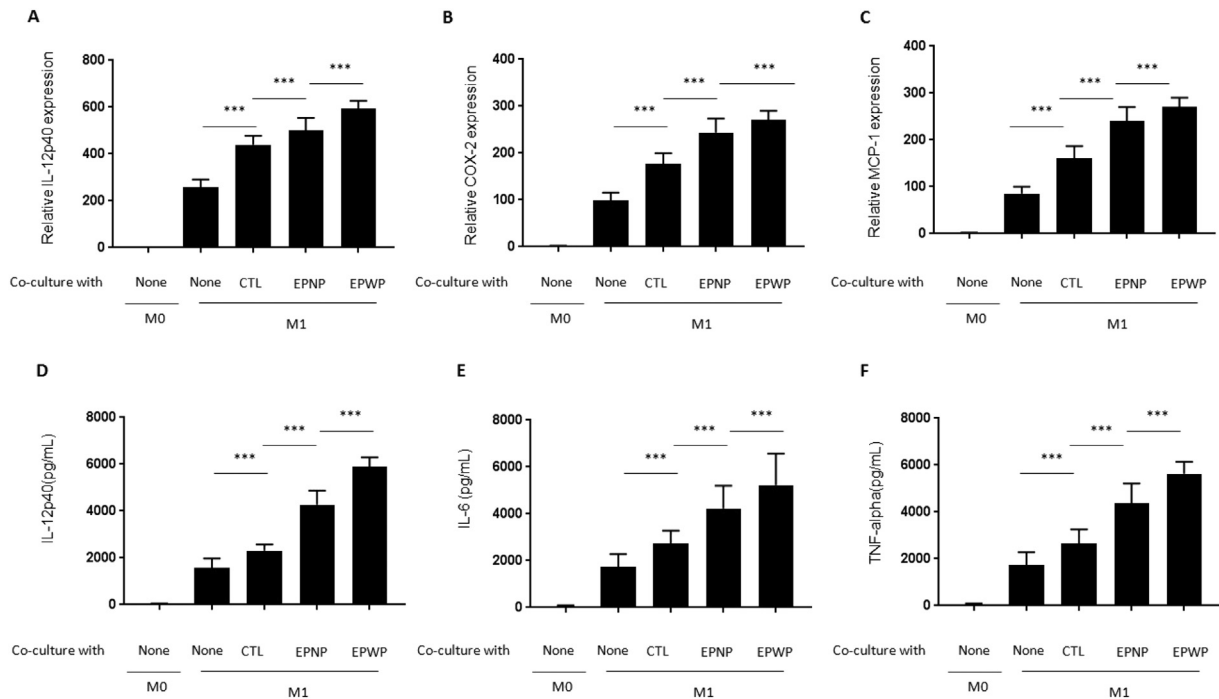


Fig. 3. Co-cultured with the ESCs enhanced the macrophage M1 polarization. THP-1 cells were pretreated with 200 ng/mL LPS(M1) or not(M0), and then co-cultured with ESCs from HC group, EPNP group and EPWP group. (A–C) The relative mRNA expressions of (A) IL-12p40; (B) COX-2; (C) MCP-1 were assayed by qRT-PCR. (D–F) The secretion levels of (A) IL-12p40; (B) IL-6; (C) TNF- α were determined by ELISA assay. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

cultured THP-1 cells with ESCs from CTL, EPNP and EPWP group, respectively. Macrophages co-cultured with ESCs from EPWP group showed highest miR-146b expression, followed by EPNP group, and CTL group showed significant lower miR-146b expression than EP cases (Fig. 2A). We pretreated the macrophages with NF- κ B inhibitor (BAY-117082) or not, followed by exposure to ESCs, and found that BAY-117082 significantly suppressed the miR-146b expression, suggesting that the NF- κ B pathway was involved in regulating miR-146b expression (Fig. 2A). To further assess the possible contribution of NF- κ B in miR-146b expression, the miR-146b promoter reporter plasmid (miR-146b-Luc) and mutant plasmid for NF- κ B binding sites (mNF- κ B -Luc) were respectively transfected into THP-1 cells. Then the THP-1 cells were co-cultured with ESCs for 24 h and analyzed by luciferase reporter assay. As shown in Fig. 2B, mutation of NF- κ B sites significantly decreased miR-146b basal promoter activity. Above data indicated NF- κ B pathway is required for the induction of miR-146b in ESC co-cultured macrophages.

miR-146b reverse the M1 polarization of ESCs co-cultured macrophages by inhibiting IRF5/IL-12p40 axis

M1/M2 phenotype switch of macrophages in endometrial tissue was demonstrated to be the important factors for pain development. THP-1 macrophages were induced to M1 polarization by 200 ng/mL LPS treatment, and then co-cultured with the ESCs. The mRNA of IL-12p40, MCP-1 and COX-2, which reflect macrophage M1 polarization, was highest in the EPWP co-cultured macrophages, followed by EPNP groups, and HC group showed significant lower levels of M1 polarization markers than EP cases (Fig. 3A–C). Meanwhile, the secretion levels of IL-6, IL-12p40 and TNF- α were also significant increased in EP subjects, especially in EPWP (Fig. 3D–F).

Macrophages were transiently transfected with miR-146b mimics, inhibitors, or negative control, together with LPS treatment, and then co-cultured with the ESCs, we found the macrophage M1 markers were decreased in miR-146b group, whereas

miR-146b inhibitor reversed this function (Fig. 4A–C). IRF5 has been identified as a novel target of miR-146b, which play an important role in M1 macrophage differentiation. In this study, we found that miR-146b mimic suppressed IRF5 protein expression in LPS induced M1 macrophages, whereas miR-146b inhibitor affected little in IRF5 expression (Fig. 4D). IRF5 was regarded as a key regulator of IL-12p40, and CHIP assay demonstrated that IL-12p40 transactivation was down-regulated in transfecting miR-146b mimic, and was enhanced in miR-146b inhibitor treatment (Fig. 4E). Further, overexpression of IRF5 rescued miR-146b mimic-induced suppression of IL-12p40 expression (Fig. 4F), together with other M1 polarization markers (Fig. 4G and H). Collectively, above data indicated that miR-146b reverse the M1 polarization of ESCs co-cultured macrophages by inhibiting IRF5/IL-12p40 axis.

miR-146b rs1536309 C>T polymorphism was associated with the M1 polarization of macrophages from the primary PBMC

To address the relationship between miRNA-146b rs1536309 C>T polymorphism and the macrophage polarization, we isolated the primary PBMC from the 12 healthy women volunteers (miR-146b rs1536309 pattern: 6 cases of TT and 6 cases of CT/CC) and then induced them into M1 macrophages. RT-PCR assay demonstrated that miR-146b expression was significantly decreased in the macrophage with CT/CC genotype (Fig. 5A), whereas M1 markers were significantly enhanced in this group (Fig. 5B), comparing with the macrophage with TT genotype. Furthermore, the M1 markers of TT genotype macrophages was significantly decreased when treated with miR-146b mimics, reaching the same level as CT/CC macrophages (Fig. 5B).

Discussion

Several group used miRNA microarrays to identify differentially expressed miRNAs between the endometriotic tissues and the

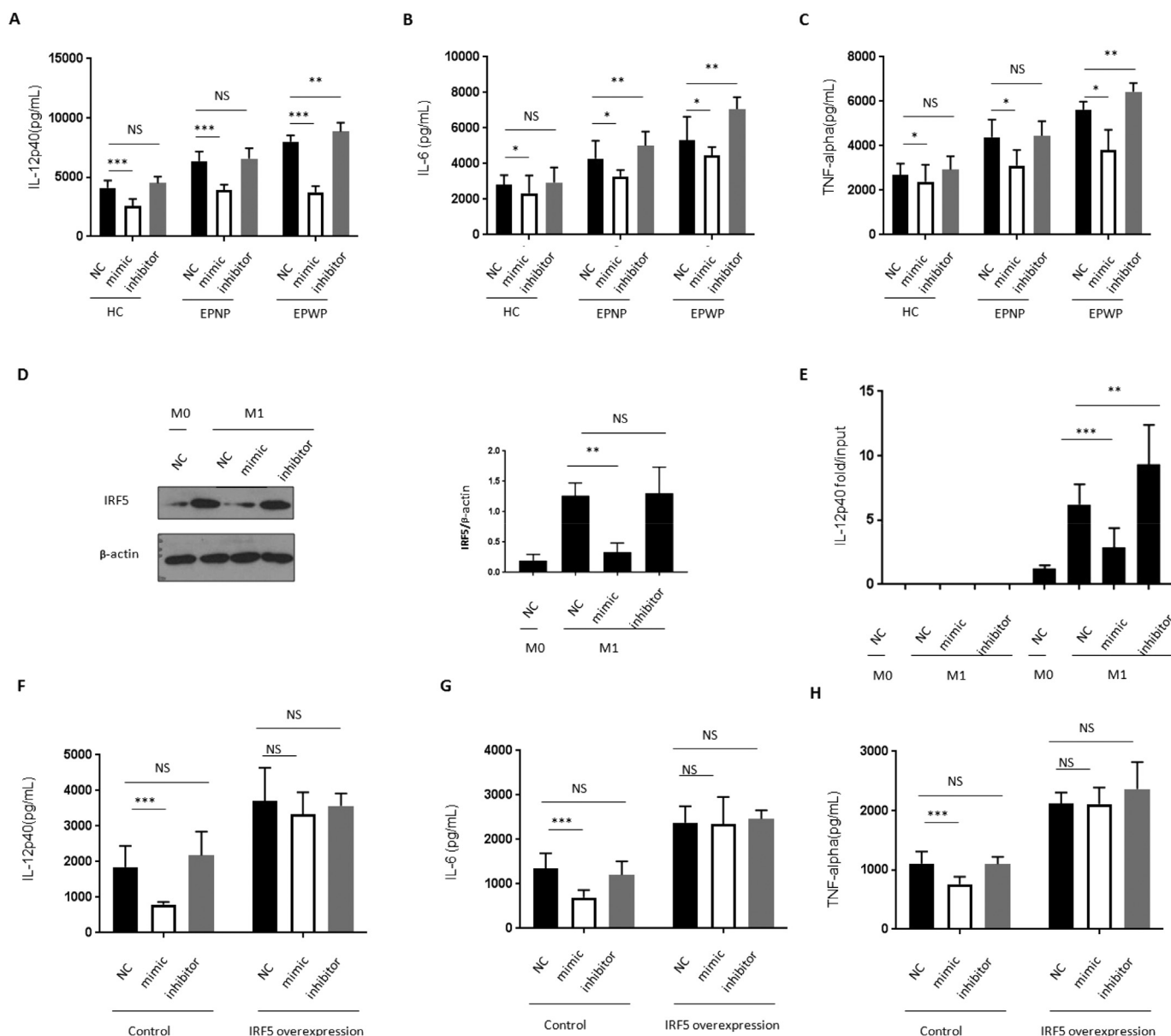


Fig. 4. miR-146b reverse the M1 polarization of ESCs co-cultured macrophages by inhibiting IRF5/IL-12p40 axis. (A–C) THP-1 cells were pretreated with NC, miR-146b mimic or miR-146b inhibitor, together with 200 ng/mL LPS, and then co-cultured with ESCs from HC group, EPNP group and EPWP group. The secretion levels of (A) IL-12p40; (B) IL-6; (C) TNF- α were determined by ELISA assay. (D) THP-1 cells were pretreated with NC or miR-146b mimic, together with 200 ng/mL LPS or not, and then IRF5 were determined by western blotting assay. (E) THP-1 cells were pretreated with NC, miR-146b mimic or miR-146b inhibitor, together with 200 ng/mL LPS or not. The ChIP assay was performed using anti-IgG and anti-IRF5 antibodies. The immunoprecipitated chromatin was amplified by real-time PCR using IL-12p40 primers. (F–H) THP-1 cells were transfected with IRF5 overexpressing plasmid, and then treated with NC, miR-146b mimic or miR-146b inhibitor, together with 200 ng/mL LPS or not. The secretion levels of (F) IL-12p40; (G) IL-6; (H) TNF- α were determined by ELISA assay.

normal endometrial tissues, in order to development biomarkers for diagnosing and treating women afflicted with endometriosis. From these studies, we found the relationship between miR-146a/b and endometriosis are inconsistent and controversial [12,23,24]. Some groups reported that the endometriotic tissues showed significantly lower levels of miR-146a [23], whereas several other studies exhibited an opposite expression trend [24]. Meanwhile, among the endometriosis patients, miR-146b was reported to highly expressed in patients with endometriosis associated pain [12]. Above data indicated that the relationship between miR-146b expression and endometriosis development was complex and should be further determined. In this study, we found the miR-146b expression was increased in endometriosis patients, and was

positive related with stage and pain. Furthermore, CT/CC genotype of miR-146b rs1536309 SNP site was associated with pain development. These results promote our further study of the mechanism of miR-146b increasing and its functions on the pathological of endometriosis and its associated pain.

Macrophages are the dominant immune cells that are recruited to the peritoneal cavity to remove the retrograded endometrial debris in patients with endometriosis. Macrophages M1 polarization markers, such as TNF- α , IL-12 and IL-6, have been associated with the evolution of endometriosis. TNF- α has potent inflammatory, cytotoxic and angiogenic potential. IL-6 promotes endometrial cell growth and is a potent stimulator of the vascular endothelial growth factor gene [25]. Therefore, identification of factors which

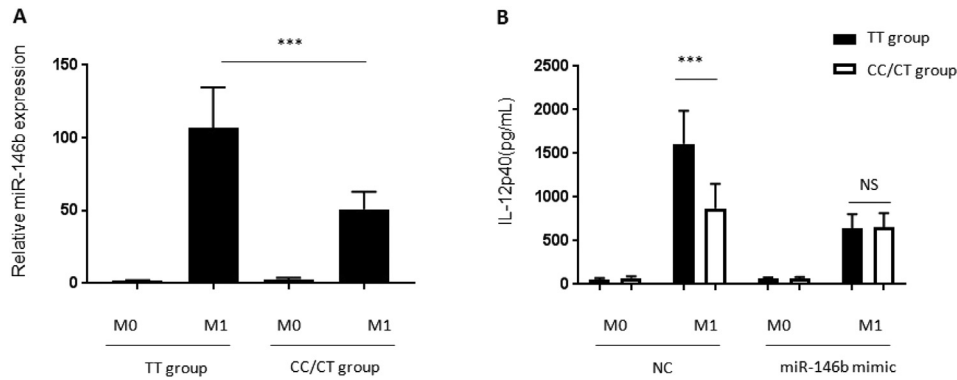


Fig. 5. miR-146b rs1536309 C>T polymorphism was associated with the M1 polarization of macrophages by regulating miR-146b expression. (A) Primary PBMC were isolated from 12 healthy women (6 cases of rs1536309 TT and 6 cases of rs1536309 CC/CT) and were induced to M1 polarized macrophages. The miR-146b expressions were assayed by qRT-PCR. (B) The macrophages in (A) were pretreated with miR-146b mimics or NC, and were induced to polarized macrophages. The secretion levels of IL-12p40 were determined by ELISA assay.

can block the pelvic macrophage M1 polarization, might suppress the endometriosis development. To our best knowledge, this is the very first work to determine the immune mechanism of miR-146b upon endometriosis. Using the cell based model, we found that EPWP-ESCs induced miR-146b expression in macrophage play a negative role in M1 polarization process, which could help decrease the expression of TNF- α , IL-12 and IL-6. Numbers of miR-146b targets have been identified in previous reports, and many of them are key regulators of macrophage functions. For example, miR-146b has been proved to inhibited IRAK1 and TRAF6 expression by binding to the 3'UTR of IRAK1 or TRAF6, and therefore block the TLR mediated pathway [26]. Also, He et al. demonstrated that miR-146a/b could blocks the activation of M1 macrophage by targeting STAT1 [27]. In Peng's report, they proposed that IRF5 was one of the target of miR-146b, and play the key role in mediating M1 polarization [8]. In our study, we overexpressed these targets, together with miR-146b mimics treatment, and found that IRF5 could offset the miR-146b effects, whereas little influence could be observed in other targets overexpression (such as IRAK1, TRAF6 or STAT1, data not shown in this study). These data indicated that miR-146b negatively regulate the macrophage M1 polarization mainly by targeting IRF5, and this physiological function might play key role in endometriosis development.

Further, more and more studies focus on the relation between macrophage M1/M2 polarization switch and endometriosis pain experience [28]. The macrophage M1 polarization has been confirmed to generate inflammatory cytokines, such as TNF- α and MCP-1 (CCL2), which can induce the sensory nerve to produce a sustained induction of action potential via transient receptor potential vanilloid 1 (TRPV1) [29]. Also, there have been many studies confirming that some M1 related pro-inflammatory factors have the ability to modulate the voltage-gated sodium channels [30,31], which may lead to endometriosis associated pain. Besides, preoperative pain sensitivity has significant individual differences, which related to the genetic factors of patients [32–34]. Previous studies have shown that various genetic polymorphisms were correlated with pain sensitivity, such as KCNJ6 gene [32], COMT gene [33] and CYP2D6 [34]. Our results demonstrated that some patients have higher pain sensitivity might because that they carry the miR-146b rs1536309 CC/CT phenotype. Additional, we found that miR-146b rs1536309 CC/CT phenotype decreased the miR-146b expression, and therefore make the immune system more prone to proinflammatory state. That might be the main reason why patients carrying this phenotype have higher pain sensitivity.

Conflicts of interest

No authors has any potential conflict of interest.

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