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ORIGINAL ARTICLE

MiR-217 Inhibits M2-Like Macrophage Polarization by Suppressing Secretion of Interleukin-6 in Ovarian Cancer

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Abstract— Ovarian cancer is one of the most deadly cancers with rapid proliferation and poor prognosis among patients. Therapies focusing on regulation of tumor immunity and microenvironments are developing. MiR-217 was dysregulated in cancer progress and plays important roles in tumorigenesis and metastasis. However, the role of miR-217 in regulation of macrophage polarization and its underlying molecular mechanism remain unclear. The expression of miR-217 in ovarian cancerous tissues and cell lines were assessed by qRT-PCR. And we detected the staining of CD86 and CD206 *via* flow-cytometry and the levels of Arg-1 and CCR2 by western-blot in order to evaluate M2 macrophage polarization. The targeting regulation of miR-217 on pro-inflammatory factor IL-6 was assessed by dual-luciferase reporter assay and western-blot. ELISA assay was used to evaluate the secretion of IL-6 and IL-10 of cells. MiR-217 was found to be downregulated in ovarian cancerous tissues and cell lines. This downregulation correlated with an increased expression of the IL-6, Arg-1, CCR2, and CD206 gene. The overexpression of miR-217 in SKOV3 cells can inhibit the polarization of macrophages towards an M2-like phenotype. We also found that IL-6 was validated to induce M2 macrophage polarization and its secretion in SKOV-3 cells was inhibited by miR-217 directly. Moreover, we revealed that miR-217 suppressed M2 macrophage polarization partly thought JAK/STAT3 signal pathway. Taken together, these findings indicate that miR-217 inhibits tumor-induced M2 macrophage polarization through targeting of IL-6 and regulation JAK3/STAT3 signaling pathway, which may provide a potential therapeutic target for treating ovarian cancer.

KEY WORDS: miR-217; IL-6; M2 macrophage polarization; ovarian cancer; JAK3/STAT3 signaling.

INTRODUCTION

Ovarian cancer was reported as the most fatal gynecological malignancy by a statistical analysis of the American Cancer Society based on cancers [1]. Over 75% of patients diagnosed with ovarian cancer at an advanced stage when it has developed into a widely metastatic disease [2]. For many patients diagnosed with ovarian cancer,

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conduction of aggressive surgery (cytoreduction/tumor debulking) and effective combination of chemotherapies is the common treating strategy to date [3]. However, poor prognosis which resulted from difficulties in diagnosis of ovarian cancer at its early stage as well as formation of chemoresistance makes it important to develop alternative therapies. Immunotherapy is a potentially effective approach of treating ovarian cancer especially of epithelial origin. Current immunotherapy is developed on purpose of preventing mortality caused by cancer relapse following surgery and chemotherapy. Clinical options of immunotherapy to date include therapy with antibodies [4], cytokines [5], active immunization with genetic modified whole tumor cells [6], dendritic cell vaccines [7], peptide-based vaccines [8], and heat shock protein (HSP) vaccines [3]. However, above modalities are still under different phases of clinical investigation; hence, no standardized therapeutic strategies were developed based on immunotherapy.

Tumor microenvironment (TME) refers to cellular environment where tumor exists, including surrounding immune cells, lymphocytes, blood vessels, fibroblasts, bone marrow-derived inflammatory cells, signaling molecules, and the extracellular matrix (ECM) [9]. TME and tumors reciprocally interact. Usually tumors inside TME release extracellular signals, promoting tumor angiogenesis and induce peripheral immune tolerance while TME in turn to affect growth and evolution of tumor cells. Early evidence suggested that immune cell infiltration presents consistently in microenvironments of advanced ovarian cancer [10]. Tumor infiltrating lymphocytes (TILs) such as T cells, B cells, macrophages, and natural killer cells existing in stromal or intraepithelial are thought to control tumor growth of virtually all solid tumors including advanced ovarian cancer [11]. Furthermore, cytokines such as IL-6, IL-10, or TGF-β produced by these TILs promote formation of immunosuppressive environment and are indicated to be involved in the progression of tumor and metastasis [12]. It is noteworthy that in addition to large number of T cells, monocyte/macrophages (MOMA) account to above 50% of the mononuclear leukocytes population in ascetic fluid [13]. Clinically, high percentage of MOMA was also observed in pelvic peritoneal biopsies of patients with advanced stage epithelial ovarian cancer [14]. Specific TME constitutes of ovarian cancer distinguishes it from other solid tumors. Since dissemination of ovarian cancer partially depends on the peritoneal fluid [15], cancer cells hence generate metastatic lesions directly in the peritoneal cavity [2], which results in subsequent accumulation of ascites in peritoneal cavity. The ascites consist of tumor-associated macrophages (TAMs), T cells, extracellular vesicles (EVs), detached cancers, and other host cell populations can collaboratively cause cancer proliferation, metastasis, and chemoresistance [16]. Macrophages and cancer cells affect phenotype and behavior of each other through the exchange of soluble factors; for example, cancer cells can modulate macrophage cytokine/chemokine secretion to facilitate tumor progression in peritoneal cavity [17]. M2 macrophage is also designated as tumor-associated macrophage (TAM), which was demonstrated to drive growth factor release as well as enhancing cell invasion in SKOV-3 ovarian cells [18]. Polarization of macrophages towards an M2 phenotype induced by IL-6 was reported to be associated with the produce of an immune-deficient microenvironment [19]. Therefore, exploring TAM's specific role in immunosuppressive process is meaningful for inhibiting tumor proliferation and treating advanced ovarian cancer.

MicroRNAs are small endogenous non-coding RNAs in length of 19–25 nucleotides that work through binding to 3′-untranslated region (UTR) of mRNA of specific genes, resulting in inhibition or degradation of subsequently translated proteins and ultimate silence of the specific genes [20]. MicroRNAs have been demonstrated to be involved in several biological processes, including cell differentiation, proliferation, invasion, migration, survival, and tumorigenesis. Mounting evidences show microRNAs play a dual-role in tumorigenic progress, as either tumor suppressor or oncogenes [21, 22], shading lights for securing potent therapeutic targets for various types of cancers. MicroRNA-217 (miR-217) was found to be dysregulated in multiple tumors; for example, miR-217 was reported to function as an oncogene in breast cancer through targeting cell fate determination factor (DACH1) [23] while as a tumor suppressor in hepatocellular carcinoma through targeting EZF3 [24]. In epithelial ovarian cancer, miR-217 was previously shown to be downregulated in both of human epithelial ovarian cancer cell lines and tissues and also to play as a tumor suppressor by targeting on insulin-like factor 1 receptor (IGF1R) [25].

Although miR-217 was previously identified to play regulatory roles in many cancer types, few studies have revealed its impacts on tumor immunity and the surrounding microenvironments. Therefore, this study focused on investigating the role of miR-217 *in vitro* by using ovarian cancer cell lines and TPH-1 monocytes/macrophages. The overexpression of miR-217 was demonstrated to suppress M2 macrophage polarization and this function was found to be partly performed by directly targeting on the expression and secretion of IL-6 by SKOV-3 ovarian cancer cells.

Further biological evaluation confirmed that miR-217 regulates macrophage polarization through attenuating the activation of JAK2/STAT3 signaling pathway *via* inhibiting IL-6. This study may share insights into securing novel therapeutic target in treating ovarian cancer.

METHODS

Patients and Tissue Samples

Tumor and the corresponding adjacent tumor tissues (more than 5 cm from the tumor site without cancer cell infiltrations) were obtained from 15 epithelial ovarian cancer patients who underwent surgery at the Third Xianya Hospital of Central South University. All samples were frozen in liquid nitrogen immediately and stored at − 80 °C condition for further RNA extraction. The exclusion criteria included those who received chemotherapy or radiotherapy before surgery. All patients provided written informed consent for the use of their tissues. This project was approved by the ethics committee of Xianya Hospital affiliated to Central South University.

Cell Culture and Transfection

The human ovarian surface epithelial cell line (HOSEpiC) and four human ovarian cancer cell lines (SKOV-3, CAOV3, OVSCAR-3, H08910) were obtained from (ATCC, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco BRL) at 37 °C in a humidified atmosphere containing 5% CO_2. Oligonucleotides including miR-217 mimics, miR-217 inhibitor, corresponding miRNA-217 negative control, siRNA targeting human IL-6 (si-IL-6), and scrambled siRNA negative control were purchased from GenePharm Co., Ltd. (Shanghai, China). Briefly, the cells were seeded on a 6-well plate (2×10^6/well) and cultured for 24 h. And then, cells were transfected with 100 pmol oligomer mixed with lipofectamine 2000 reagent in serum-free DMEM according to the manufacturer's instructions. Medium was changed to complete culture medium 6 h later, and the cells were incubated at 37 °C in a CO_2 incubator for another 48–96 h before harvest.

Cell Treatments and Co-Culture

THP-1 cells were seeded in 6-well plates and induced into M0 macrophages by adding 100 ng/ml phorbol 12-myristate 13-acetate (PMA, Abcam, USA) in transwell inserts for 48 h. Briefly, the PMA containing media was aspirated out gently and the inserts containing activated THP-1 cells were placed into a new 6-well plate adding with 2 ml of fresh RPIM/FCS media left for 1 h before treatment. The differentiated M0 type macrophages were subsequently co-cultured with SKOV-3 cells or SKOV-3 supplementary anti-IL-6 neutralizing antibody (20 ng/ml) or IL-6 (0.250 ng/ml) to assess the impacts of IL-6 on M2 polarization of macrophages.

Dual-Luciferase Reporter Assay

The human IL-6 3'-UTR containing wild-type or mutant miR-217 binding site was amplified using PCR, and subsequently cloned into the pMIR-REPORT vector (Ambion, TX, USA), constructing pMIR-IL-6-WT and pMIR-IL-6-MUT. The constructed reporters were co-transfected with miR-217 or miR-217 negative control into SKOV-3 cells by Lipofectamine 2000 (Invitrogen, CA, USA). The pRL-TK vector (Promega, WI, USA) encoding Renilla luciferase gene was used as an endogenous control to normalize transfection efficiency. The luciferase activity was detected with the Dual-Luciferase Reporter Assay kit (Promega, WI, USA) 48 h after transfection according to the manufacturer's protocols.

ELISA

To assess the M2 polarization of macrophages, the expression of IL-10 was detected by ELISA assay with the cell supernatants from control, miR-NC, and miR-217 mimics groups. To assess the impacts of miR-217 on secretion of IL-6 in ovarian cancer cells, the expression of IL-6 was detected in supernatants from control, miR-NC, miR-217 mimics, inhibitor NC, and miR-217 inhibitor groups by ELISA. All cell supernatants were collected 96 h later and centrifuged to remove dead cells. The supernatants were then analyzed using IL-6 or IL-10 specific enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, CA, USA). Standards were assayed in duplicate and the value was normalized to the total protein content.

RNA Extraction and Real-Time PCR Analysis

Total RNA of cells was extracted with TRIzol solution (Invitrogen, CA, USA) according to manufacturer's protocols. Equal amount total RNA was transcribed into the first-strand cDNA by using PrimeScript RT reagent kit (TaKaRa, Tokyo, Japan). SYBR Premix Ex taq reagent (TaKaRa) was employed to measure the relative level of

IL-6 quantitatively. The expression level of miR-217 was evaluated by mirVana qRT-PCR miRNA Detection Kit (Thermo Fisher Scientific, MA, USA) based on manufacturer's protocols. U6 snRNA and β-actin were deployed as internal reference of miR-217 and IL-6/IL-10/Arg-1/CCR2, respectively. Primer sets used for amplification were as follows:

IL-6, 5′- ACAGGGAGAGGGAGCGATAA-3′ (forward) and
5′- GAGAAGGCAACTGGACCGAA-3′ (reverse);
IL-10, 5′- TACGGCGCTGTCATCGATTT-3′ (forward) and
5′- TAGAGTCGCCACCCTGATGT-3′ (reverse);
Arg-1, 5′- CCAAGGTCTGTGGGAAAAGCA-3′ (forward) and
5′- TACAGGGAGTCACCCAGGAG-3′ (reverse);
CCR2, 5′- TAGTTGCCCTGTATCTCCGC-3′ (forward) and
5′- TGGGGAAATGCGTCCTTGTT-3′ (reverse);
β-actin, 5'-GCATGGGTCAGAAGGATTCCT-3′ (forward) and
5'-TCGTCCCAGTTGGTGACGAT-3′ (reverse);
miR-217, 5′- CGGCTACTGCATCAGGAACTG-3′ (forward) and
5′- CGGCCCAGTGTTCAGACTAC-3′ (reverse);
U6, 5′- TGCGTTCCCTTTGTCATCCT-3′ (forward) and
5′- AACGCTTCACGAATTTGCGT-3′(reverse).

Western Blot Analysis

Cells were lysed in RIPA buffer with protein inhibitors cocktail (Complete Mini; Roche Diagnostics, Basel, Switzerland). BCA assay kit (Pierce, IL, USA) was used to measure the concentrations of total cellular protein based on the manufacturer's instructions. Thirty micrograms of proteins from each sample were electrophoresed on 10% sodium dodecysulfate-polyacryl-amide gels (SDS-PAGE) and then transferred to polyvinylidene difluoride membrane (PVDF; Bio-Rad, CA, USA). The membranes were blocked in 5% non-fat dry milk for 1 h at room temperature and incubated at 4 °C overnight with primary antibodies against IL-6, Arg-1, CCR2, phosphorylated (p)-JAK2 or JAK2, phosphorylated (p)-STAT3 or STAT3, β-actin and GAPDH (Santa Cruz Biotechnology, TX, USA). The membranes were then washed and incubated with the secondary antibody against rabbit at 37 °C for 1 h and washed with TBST for 10 min. The intensity of protein bands was analyzed using the SmartChemTM Image Analysis System (Sagecreation, China). All results were reproduced in three independent experiments using different samples.

Flow Cytometry

M0 macrophages were co-cultured with SKOV-3 cells transfected with miR-217 mimics or miR-217 negative control; with or without IL-6. Cells were then washed with cold PBS and detached with 5 mM EDTA and 0.5×10^6 cells were counted to resuspend in 1 ml PBS. Cell suspension was incubated with FITC, PE- and allophycocyanin (APC)-conjugated Abs anti-human CD14, CD206, and CD86 (BD Pharmingen, USA) for 30 min at 4 °C. Cells were also incubated with the control isotype corresponding to each primary antibody. Then cells were washed and resuspended in 1 ml PBS containing 5% his 0.1% NaN_3. Prepared cells were analyzed by flow cytometry with a FACScalibur (Beckman Coulter, USA). Macrophages were gated on the basis of forward and side light scatter and by using FITC-$CD14^+$.

Statistical Analysis

All quantitative data was expressed as mean ± SD for at least three separate experiments using SPSS 20.0 and GraphPad Prism 6.0. The difference between two groups was analyzed by unpaired two-tailed Student's *t* test. One-way ANOVA was used for comparison among multiple groups and multiple comparisons were further performed using *post hoc* Turkey's test. Western blot analysis was quantified by Image J. P values < 0.05 were considered as statistically significant.

RESULTS

MiR-217 Was Downregulated in Ovarian Cancer Cell Lines and Tissues

The expression profile of miR-217 was evaluated using qRT-PCR in tumor tissues and their corresponding non-cancerous tissues in 15 cases of patients diagnosed with ovarian cancer. The expression level of miR-217 was significantly downregulated in tumor tissues compared with that in normal ovarian tissues adjacent to the tumor (Fig. 1a, $P < 0.01$). Also, the expression level of miR-217 in ovarian cancer cell lines and human ovarian epithelial cell (HOSEpiC) was measured using qRT-PCR, as Fig. 1b suggested, miR-217 was downregulated in all ovarian cancer cell lines compared with that in HOSEpiC. Notably, the

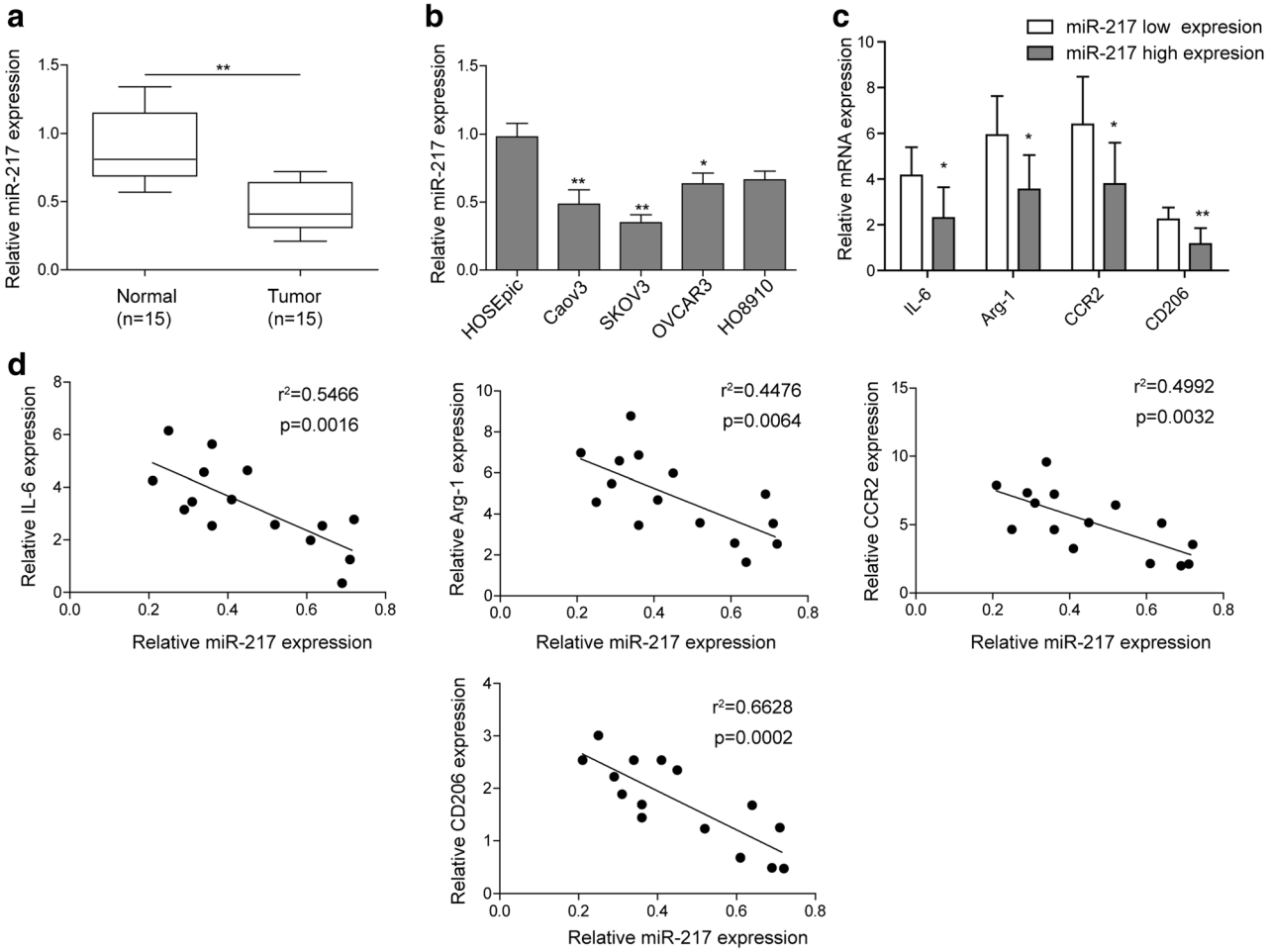


Fig. 1. miR-217 was downregulated in ovarian cancer tissues and cancerous cell lines. **a** Relative expression level of miR-217 in 15 pairs ovarian cancer tumor tissues and corresponding non-cancerous tissues were measured using qRT-PCR. Data were normalized to U6 and expressed relative to adjacent tumor tissues. **b** Relative expression level of miR-217 was tested in HPSEpiC, CAOV3, SKOV-3, OVSCAR-3, and H08910 ovarian cancer cell lines. Data were normalized to U6 and expressed relative to HPSEpiC. **c** Histogram of expressions of M2-associated markers Arg1, CCR2, CD206, and IL-6. The mRNA was extracted from 15 ovarian cancer tissue sample. β-Actin was used as an internal reference. **d** Correlation analysis on relevance between expressions of miR-217 and M2-associated markers. The mRNA was extracted from 15 ovarian cancer tissues. All data was expressed as mean ± SD. $*P < 0.05$, $**P < 0.01$.

level of miR-217 was decreased about 60% in SKOV-3 cell line compared with that in HOSEpiC, which was the reason for using SKOV-3 as the target of interest in the subsequent experiments.

To investigate the relevance of miR-217 in M2 type macrophage polarization *in vivo*, we detected the mRNA levels of Arg1, CCR2, CD206, and IL-6 in miR-217 low expression (< median; 0.410) group and miR-217 high expression (> median) group of ovarian cancer tissues. The result showed that Arg1, CCR2, CD206, and IL-6 were increased in miR-217 high expression group (Fig. 1c). Moreover, the relationship between miR-217 expression and M2 macrophage-associated markers including Arg1, CCR2, IL-6, and CD206 was summarized in Fig. 1d. The results demonstrated that the expression of miR-217 was negatively associated with the levels of IL-6 and M2 markers.

MiR-217 Overexpression in Ovarian Cancer Cells Inhibits M2 Macrophages Polarization

To further investigate influences of miR-217 on process of ovarian cancer cells regulate polarization of macrophages, THP-1 cells polarization into M0 macrophage was induced *in vitro* by sequentially adding PMA. Differentiated M0 macrophages were then co-cultured with

SKOV-3 ovarian cells that transfected with miR-217 mimics or miR-217 negative control. Firstly, transfection efficiency of miR-217 mimics was assessed by qRT-PCR and it was shown that expression of miR-217 was significantly boosted in SKOV-3 cells after induction of miR-217 mimics (Fig. 2a). Flow-cytometry was employed to evaluate population of M2 macrophages in co-culture cell suspension. The result showed that $CD206^+$ and $CD86^+$ cells accounted for about 35% and 20% in co-cultures containing untreated SKOV-3 group or SKOV-3 cells transfected with miR-217 negative control (miR-NC), respectively, while overexpression miR-217inhibited the amount of $CD206^+$ cells (decreased to 20%) and promoted the amount of $CD86^+$ cells (increased to 40%) (Fig. 2b). The results of qRT-PCR on quantitative expressions of M2 macrophage-associated factors (Arg-1, CCR2, IL-10) demonstrated that SKOV-3 cells overexpressing miR-217 significantly suppressed the expression of Arg-1, CCR2, and IL-10 compared to that in negative control groups (Fig. 2c). The following western blot analysis on protein levels of Arg-1 and CCR2 again confirmed the downregulation of these M2 macrophage-associated factors (Fig. 2d). In addition, The ELISA test showed that protein level of IL-10 was also decreased in co-culture of M1 macrophage and SKOV-3 cells transfected with miR-217 mimics compared to those transfected with negative control as well as untreated SKOV3 groups (Fig. 2e). These results indicated significant lower proportion of M0 macrophages was differentiated into M2 macrophages when cells were co-cultured with ovarian cancer cells which overexpressed miR-217.

MiR-217 Targeted Against of IL-6 in Ovarian Tumor Cells

Complementary binding site for miR-217 on IL-6 3'-UTR was predicted using TargetScan (http://www.targetscan.org/) and complementary region was demonstrated in Fig. 3a. To validate the complementary region on IL-6, luciferase reporter vectors were constructed with wild type or mutant IL-6 3'-UTR region and subsequently co-transfected with miR-217 mimics or negative control into SKOV-3 cells. As Fig. 3b demonstrated, miR-217 suppressed luciferase activity of reporter system associated with IL-6 wild type 3'-UTR region, while no distinct difference was observed in IL-6 mutant 3'-UTR region. Collaboratively, IL-6 was suggested as the direct target of miR-217 in SKOV-3 ovarian cells. To further elucidate regulatory effects of miR-217 on IL-6 secreted by ovarian tumor cells, qRT-PCR was used to measure the expression level of IL-6 in cells transfected with miR-217 mimics, miR-217 inhibitor, miR-217 negative control, and inhibitor negative control. The results confirmed that IL-6 expression in SKOV-3 cells was suppressed when miR-217 was overexpressed compared to that in control, it was inversely enhanced when miR-217 was inhibited (Fig. 3c). The protein level of IL-6 was also downregulated in SKOV-3 cells overexpressing miR-217 according to results of western blot and ELISA test (Fig. 3d–e).

MiR-217 Inhibits M2 Macrophages Polarization Through Suppressing Secretion of IL-6 in Ovarian Cancer Cells

To determine whether M2 macrophage polarization in ovarian cancer cells are induced by secretion of IL-6 and the impacts of miR-217 on M2 polarization, anti-IL-6 neutralizing antibody, IL-6 knocked down, and miR-217 overexpressed SKOV-3 cells were employed to treat M0 macrophages. The efficiency of knock down of IL-6 in SKOV-3 was validated using RT-qPCR and ELISA assay. The results showed IL-6 expression was significantly decreased in si-IL-6 group compared to that in si-NC group and also corresponding protein level of IL-6 was downregulated significantly in si-IL-6 group (Fig. 4a, b). The results also demonstrated that the proportion of $CD206^+$cells measured by flow-cytometry was upregulated to around 35% in SKOV-3 and IL-6 groups but was downregulated to 23% in SKOV-3 plus anti-IL-6 group. The trend was similar in SKOV-3-si-IL-6 group. In addition, IL-6 rescued the inhibition effect of miR-217 on the rate of $CD206^+$ cells (Fig. 4c). Moreover, the mRNA and protein levels of Arg-1, CCR2, and IL-10 were increased in both SKOV-3 and IL-6 groups. However, the expressions of Arg-1, CCR2, and IL-10 at both mRNA and protein levels were suppressed in SKOV-3 + anti-IL-6, SKOV-3-si-IL-6, and SKOV3 + miR-217 mimics + IL-6 group compared with SKOV-3, SKOV3-si-NC, and SKOV3 + miR-217 mimics groups (Fig. 4d–f). These data suggested ovarian cancer cells induce M2 macrophage polarization *via* secretion of IL-6, while miR-217 inhibits M2 macrophages polarization through suppressing secretion of IL-6 in ovarian cancer cell lines.

MiR-217 Repressed Macrophage Polarization Through Inhibiting IL-6 Secretion and JAK2/STAT3 Pathway

To reveal the underlying mechanism of how miR-217 affects macrophage polarization, we investigated activation of IL-6 relevant signaling pathway in this study. The

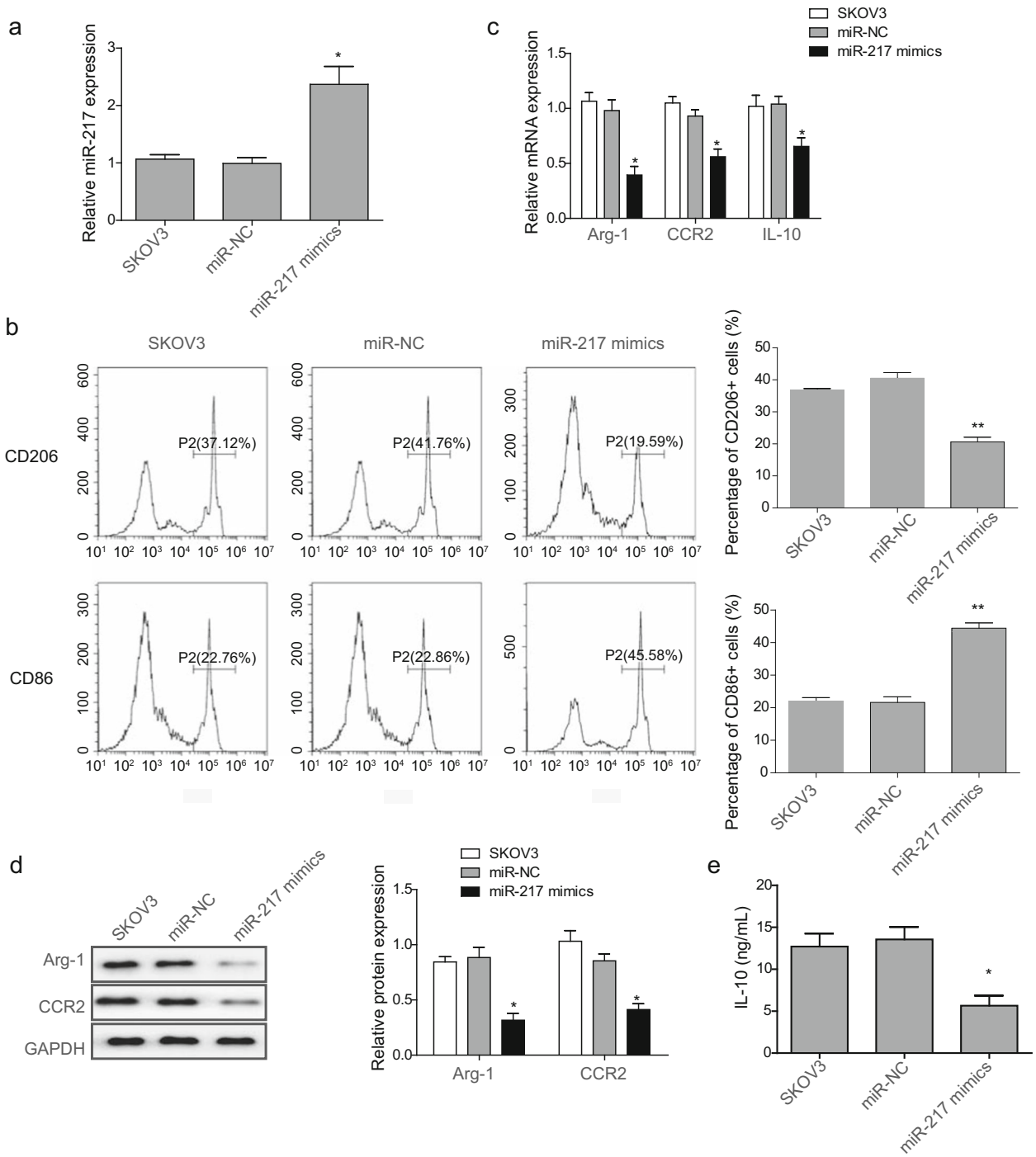


Fig. 2. Ovarian cancer cells overexpressing miR-217 inhibited M2 macrophage polarization. **a** Efficacy of transfection was tested by relative expression level of miR-217 in co-culture of macrophages and untreated SKOV-3 cell (SKOV3), transfected with miR-217 negative control (miR-NC) and transfected with miR-217 mimics (miR-217 mimics) using qRT-PCR. **b** M2 polarization in macrophage and percentages of $CD206^+$ and $CD86^+$ cells were presented in SKOV3, NC, and miR-217 mimics groups. **c** Relative expression levels of Arg-1, CCR2, and IL-10 in SKOV3, miR-NC, and miR-217 mimics groups. **d** Protein levels of Arg-1 and CCR2 were measured in SKOV3, NC, and miR-217 mimics groups. GAPDH was used as an internal control. **e** The expression of IL-10 in con, NC, and miR-217 mimics groups was tested using ELISA. All data was expressed as mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$.

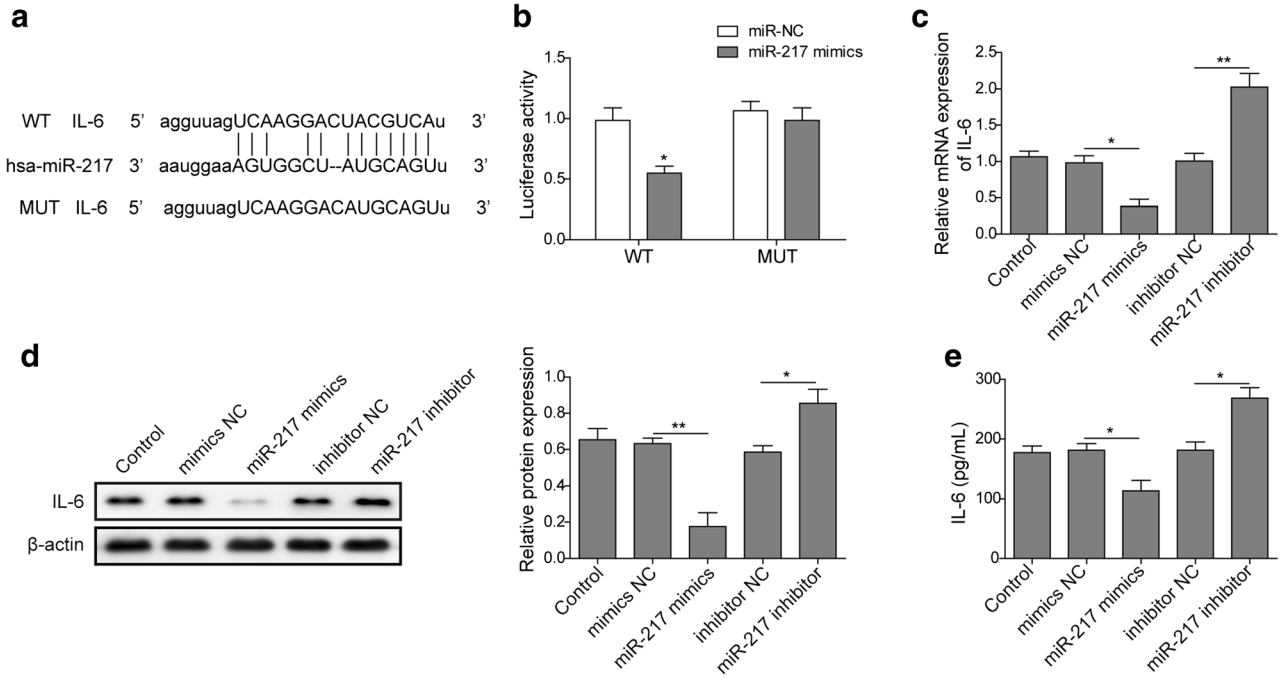


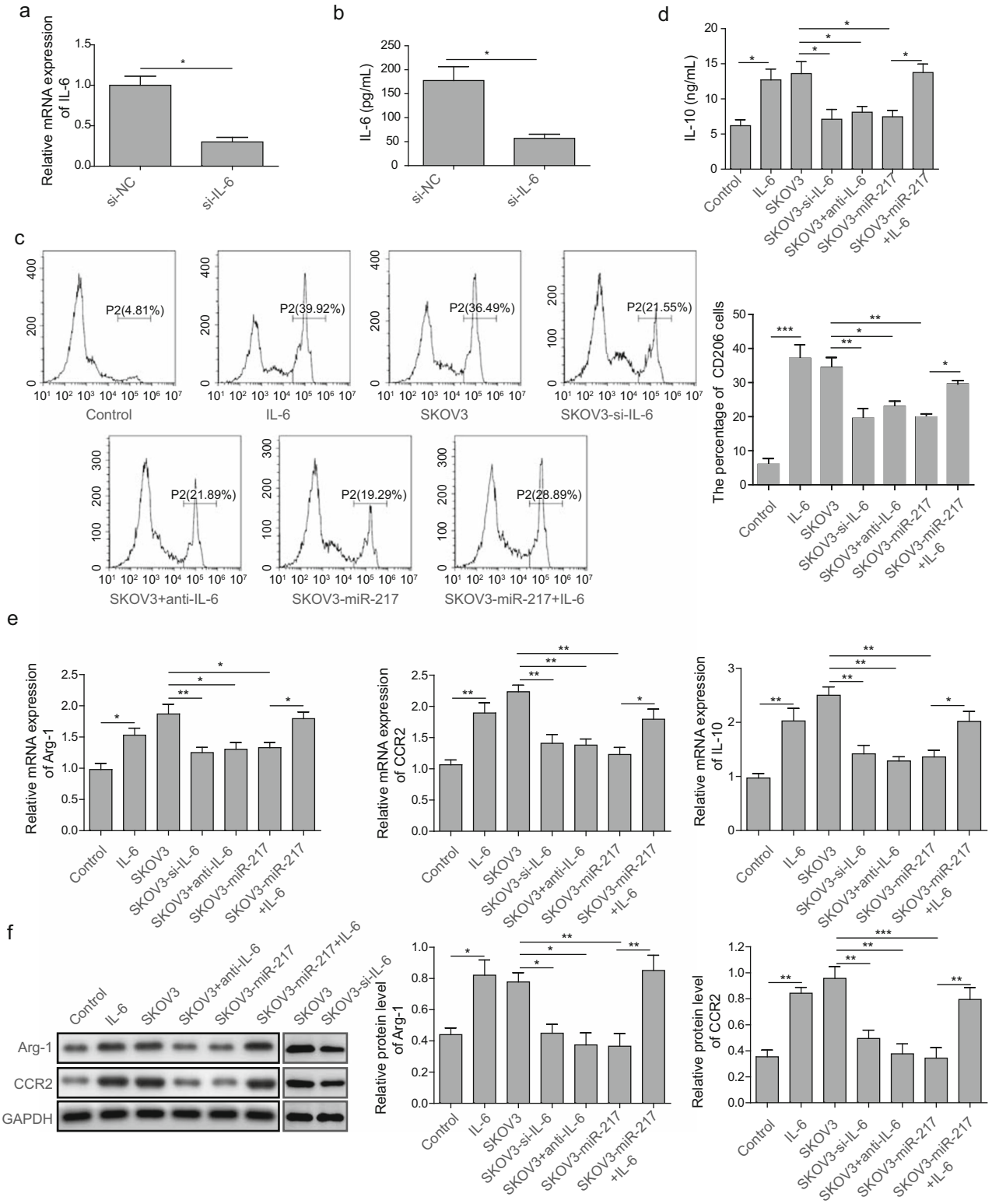
Fig. 3. miR-217 regulated secretion of IL-6 in ovarian cancer cells by directly targeting on it. **a** Complementary binding region of has-miR-217 on 3'-UTR of IL-6 was predicted. **b** Relative luciferase activity in SKOV-3 cells co-transfected with NC or miR-217 mimics and wild-type 3'-UTR-IL-6 (WT-3'UTR) or mutant 3'-UTR-IL-6 (Mut-3'UTR) reporter plasmid. **c** Relative expression level of IL-6 in untreated SKOV-3 cell control (con), transfected with miR-217 negative control (mimics-NC), transfected with miR-217 mimics (miR-217 mimics) was tested using qRT-PCR. **d** Protein level of IL-6 in con, mimics-NC, and miR-217 mimics groups was measured with western blot. B-Actin was used as internal control. **e** Protein level of IL-6 in con, mimics-NC, and miR-217 mimics groups was tested using ELISA. All data was expressed as mean ± SD. $*P < 0.05$, $**P < 0.01$.

results showed IL-6R/JAK2/STAT3 signaling was activated when macrophages were co-cultured with SKOV-3 ovarian cancer cells as the bands of western blot showed in SKOV-3 group compared with that in control group (Fig. 5). The overexpression of miR-217 markedly down-regulated levels of phosphorylated JAK2 (p-JAK2) and STAT3 (p-STAT3) as it was shown in SKOV-3 + miR-217 mimics group compared with that in control group. However, the suppressed phosphorylation of JAK2 and STAT3 under the overexpression of miR-217 was partially recovered as IL-6 was adding into the co-culture system, which was shown in the group where macrophages were co-culture with SKOV-3 transfected with miR-217 and adding IL-6.

DISCUSSION

Ovarian cancer is one of the most deadly cancers for human due to difficulty in diagnosis of cancer at its early stage and poor prognosis. The unique biology of ovarian cancer's primary dissemination within peritoneal cavity and superficially invasive property results in over 75% of patients were diagnosed with ovarian cancer at metastatic

Fig. 4. MiR-217 inhibits M2 macrophages polarization though suppressing secretion of IL-6 in ovarian cancer cells. **a–b** The SKOV3 cells were transfected with IL-6 siRNA or scrambled siRNA for 48 h. The mRNA and protein expression of IL-6 was determined by RT-qPCR (**a**) and ELISA (**b**). **c** M2 polarization in macrophage and percentage of $CD206^+$ was presented in co-cultures of macrophages cultured alone (control), with recombinant IL-6 (IL-6), with SKOV-3 cells (SKOV-3), with SKOV-3, and anti-IL-6 neutralizing antibody (SKOV-3 + anti-IL-6), with IL-6 knocking down SKOV-3 (SKOV-3-si-IL-6), with miR-217 mimics (SKOV-3-miR-217), and with miR-217 mimics and IL-6 (SKOV-3-miR-217 + IL-6) groups. **d** Protein level of IL-10 was measured in control, IL-6, SKOV-3, SKOV-3 + anti-IL-6, SKOV-3-si-IL-6, SKOV3-miR-217, and SKOV3-miR-217 + IL-6 groups using ELISA. **e** Relative expression levels of Arg-1, CCR2, and IL-10 were measured using qRT-PCR in control, IL-6, SKOV-3, SKOV-3 + anti-IL-6, SKOV-3-si-IL-6, SKOV3-miR-217, and SKOV3-miR-217 + IL-6 groups. **f** Protein levels of Arg-1 and CCR2 in control, IL-6, SKOV-3, SKOV-3 + anti-IL-6, SKOV-3-si-IL-6, SKOV3 + miR-217, and SKOV3-miR-217 + IL-6 groups were measured using western-blot. GAPDH was used as internal control. All data was expressed as mean ± SD. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.


a
Relative mRNA expression of IL-6
si-NC
si-IL-6
b
IL-6 (pg/mL)
si-NC
si-IL-6
d
IL-10 (ng/mL)
Control
IL-6
SKOV3
SKOV3-si-IL-6
SKOV3+anti-IL-6
SKOV3-miR-217
SKOV3-miR-217 +IL-6
c
P2(4.81%)
P2(39.92%)
P2(36.49%)
P2(21.55%)
Control
IL-6
SKOV3
SKOV3-si-IL-6
P2(21.89%)
P2(19.29%)
P2(28.89%)
SKOV3+anti-IL-6
SKOV3-miR-217
SKOV3-miR-217+IL-6
The percentage of CD206 cells
e
Relative mRNA expression of Arg-1
Relative mRNA expression of CCR2
Relative mRNA expression of IL-10
f
Control
IL-6
SKOV3
SKOV3+anti-IL-6
SKOV3-miR-217
SKOV3-miR-217+IL-6
SKOV3
SKOV3-si-IL-6
Arg-1
CCR2
GAPDH
Relative protein level of Arg-1
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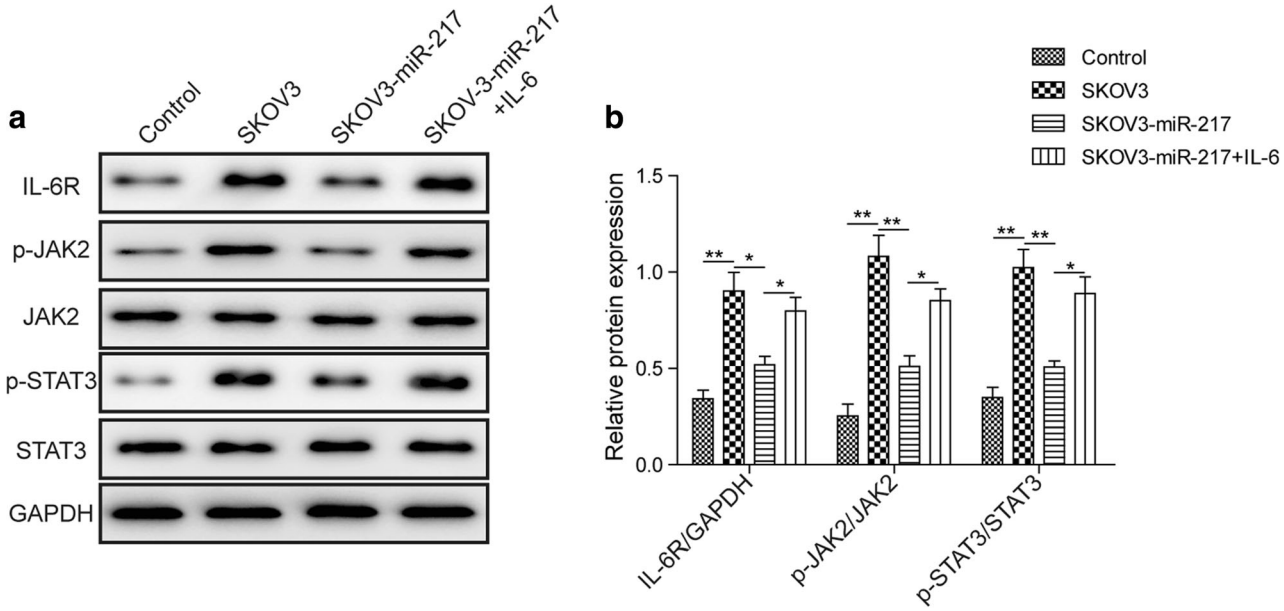

Fig. 5. miR-217 inhibited macrophage polarization through suppressing JAK2/STAT3 pathway activation by targeting IL-6 secretion in ovarian cancer. Protein levels of IL-6R, phosphorylated JAK2 (p-JAK2), JAK2, phosphorylated STAT3 (p-STAT3), and STAT3 were measured using western blot in co-cultures of macrophages cultured alone (con), macrophages with SKOV-3 cells (SKOV-3), with SKOV-3 cells transfected with miR-217 mimics (SKOV-3-miR-217), and with SKOV-3 cells transfected with miR-217 mimics with supplementary IL-6 (SKOV-3-miR-217 + IL-6) groups. Relative expression of IL-6, p-JAK2/JAK2, and p-STAT3/STAT3 were measured. All data was expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

stage [2]. Although chemotherapeutic regimens have been employed as the standard of care for over 4 decades worldwide, it is difficult to achieve progress beyond platinum-based therapy as different patients may present various tumor histology or molecular features [26]. However, findings on aberrant expression of microRNAs have shared new insights into pathways of ovarian cancer progress and ovarian tumorigenesis for its potential use in detection and surveillance of epithelial ovarian cancer [27]. MiR-217 that previously reported to act as a tumor suppressor in human epithelial ovarian cancer hence came to the first place of our interest in this study. It is interesting that evidence also indicates miR-217 plays a crucial role in suppressing tumor-associated macrophages (TAMs) polarization [28]. However, little information revealed the influence of miR-217 on TAM polarization in ovarian cancer. Here, we demonstrated the potential impacts of miR-217 in attenuating M2 type polarization of macrophages in ovarian cancer TMEs, further revealed its directly suppressive function on IL-6 expression and secretion in ovarian cancer cells as well as inhibiting the subsequent IL-6R/JAK2/STAT3 signaling pathway.

Accumulative evidences show miR-217 plays a vital role in carcinogenesis, including its downregulation and functions as anti-oncogene in colorectal cancer [29], pancreatic cancer [30], hepatocellular carcinoma [24] as well as upregulation and functions as carcinogen in cancers including cutaneum carcinoma [31], and breast cancer [32]. It is noteworthy that evidence also revealed miR-217 is dysregulated and acting as a tumor suppressor in human epithelial ovarian cancer by regulating its target gene IGF1R [25], which suggests the tissue-specific function of miR-217. In the present study, miR-217 was validated to be downregulated in ovarian cancer tissues and cell lines, which is in accordance with the previous study [25].

The current results collaboratively suggested the potentially repressive role of miR-217 in blocking polarization towards M2 macrophages in ovarian cancer. According to our results, co-culture of M0 macrophage with SKOV-3 cells that overexpressed miR-217 significantly decreased the percentage of $CD206^{+}$ cells but increased proportion of $CD86^{+}$ cells. Moreover, the overexpression of miR-217 in SKOV-3 markedly inhibited the expressions of M2 marker Arg-1, CCR2, and IL-10 proteins. It was reported that polarization towards M2 phenotype can be induced by several stimuli including IL-10 [33], M2-associated gene Arg-1,

CCR2, and surface marker CD206 [34]. CD206 has been used as a reliable marker to detect the M2 macrophages [35]. The latest research reports that IL-17 induces M2-like phenotype in THP-1-derived macrophages, which can present a high expression of CD206 [36]. However, it has also been noted that upregulation of M2 surface marker CD206 was undetectable in THP-1 macrophage compared to that in monocyte-derived macrophage (MDM) [37]. This discrepancy can be caused by different approaches that were employed to induce macrophage polarization. For example, M0 cells were polarized towards M1 or M2 macrophage by incubation with INF-γ and LPS or with IL-4, IL-3 or IL-7 as the previous studies mentioned [36, 37]. In our study, CD206 was detected in ovarian cancer cells by inducing THP-1-derived M2 phenotype polarization. Apart from surface marker CD206, mRNA expressions and protein levels of IL-10, CCR2, and Arg-1 were measured in the current study as well and these results were considered collectively to identify THP-1-derived M2 macrophages polarization that induced by ovarian cancer cells. Moreover, since M0 to M2 macrophage polarization can be considered as a tumor-prone biomark in TMEs [19], here, based on our findings, it is the first time miR-217 was reported to play a regulatory role in ovarian tumor immunity.

MiR-217 was found to directly inhibit the expression of IL-6 in ovarian cell lines through binding to its 3'-UTR region in the present study. This finding was consistent to the previous study that miR-217 directly targeted on IL-6 to suppress proliferation and enhanced apoptosis in cardiac myxoma [38]. Evidence demonstrates that IL-6 present at a high concentration in ovarian cancer ascites skews monocyte differentiation into TAM-like cells by increasing macrophage colony-stimulating factor consumption [19, 39]. Interestingly, IL-6 can be secreted in a huge amount by M1 macrophages and in turn induce M2 polarization [40]. Our result in Fig. 4 is in accordance with the previous studies that ovarian cancerous cell lines promoted M2 macrophage polarization by secreting IL-6. Therefore, it is indicated that SKOV-3 can facilitate M2 macrophage polarization *via* secreting IL-6, and the overexpression of miR-217 could lead to attenuated M2 polarization by directly suppressing expression and secretion of IL-6 by ovarian cancer cells.

Moreover, the underlying mechanism of how miR-217 regulates alternative macrophage polarization was investigated in this study. The current study suggests IL-6R/JAK2/STAT3 signaling pathway in macrophage is activated when cells were co-cultured with SKOV-3 ovarian cancer cells, and the overexpression of miR-217 was indicated to attenuate this pathway through directly targeting against IL-6. As a pro-inflammatory factor, the role of IL-6 and its receptor was defined in the activation of Janus activated kinase (JAK) and the subsequent phosphorylation of signal transducer and activator of transcription 3 (STAT3) [41]. Since membrane-bound IL-6 receptor is frequently expressed in hepatocytes and leukocytes, IL-6R plays critical roles in many biological processes *via* its trans-signaling function. To note, it was earlier reported in gastric cancer that IL-6 can induce M2 macrophage polarization through STAT3 signaling pathway, which was positively correlated with disease progression and poor patient prognosis clinically [42]. In addition, IL-6 was reported to induce or enhance M2 macrophage polarization by activating STAT3 pathway [42, 43] and is involved in tumorigenesis by promoting JAK2 activity *in vitro* [44]. Our results supported the previous findings and further revealed the suppressive role of miR-217 in the expression and secretion of IL-6 by ovarian cancer cells, which may cause the attenuation of IL-6R/JAK2/STAT3 signaling pathway in M1 macrophages and eventually blocks M2-type macrophage polarization.

CONCLUSIONS

To summary, we did not only confirm the important role of dysregulation of miR-217 in the progress of human epithelial ovarian cancer, but also revealed its potential functions on treating ovarian cancer *via* regulating the corresponding TMEs in the present study. MiR-217 acts to inhibit directly the expression and secretion of pro-inflammatory factor IL-6 by ovarian cancer cells, which is an important step for the activation of M2 macrophage polarization that promotes the development of tumor growth. Furthermore, we here confirmed the suppressive function of miR-217 is partly by attenuating IL-6R/JAK2/STAT3 signaling pathway which explains the results from molecular basis. According to our study, novel immunotherapies targeting on miR-217 are worthy investigating and future study is needed regarding the underlying mechanism.

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COMPLIANCE WITH ETHICAL STANDARDS

Competing Interests. The authors declare that they have no competing interests.

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