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Original Paper

Inhibition of CXCR4 by MicroRNA-1192 Reduces the Activation of Th17 Cells and Expression of Inflammation Factors in a Mouse Model of Vulvovaginal Candidiasis

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Key Words

Vulvovaginal candidiasis • MicroRNA-1192 • CXCR4 • Th17 cell • Inflammation factors

Abstract

Background/Aims: Vulvovaginal candidiasis (VVC) is a disease commonly occurring in sexually active women. The involvement of microRNAs in several kinds of infectious diseases has been highlighted in a number of researches. Therefore, we conducted the present study in order to investigate whether microRNA-1192 (miR-1192) would significantly target *CXCR4* in Th17 cells as well as inflammatory factors in mouse models suffering from VVC. ***Methods:*** Seventy-five mice were selected as test subjects for this study, of which twenty-five were used as the normal control, while the rest were treated with estradiol or oil-treated in order to establish VVC mouse models (each n = 25). Protein expressions of *CXCR4*, IL-6, IL-17, and IL-23 were all measured using both an immunohistochemistry and ELISA. The Th17 cell percentage in peripheral blood and the expression of RORγt in Th17 cells were detected using a flow cytometry. Mouse vaginal epithelial cells were isolated from normal mice, after which the mice were treated with estradiol to regulate their estrogen, followed by treatments involving the miR-1192 mimic, miR-1192 inhibitor, siRNA-*CXCR4*, and miR-1192 inhibitor + si-*CXCR4*. The cell cycle, apoptosis, and proliferation were all examined by using an additional flow cytometry as well as the employment of the MTT assay. The miR-1192, *CXCR4*, IL-6, IL-17, and IL-23 expressions in tissues and cells were both measured using both RT-qPCR and western blot assay techniques. ***Results:*** The mice treated with either estradiol or oil had presented to us lowered levels in miR-1192 expression as well as higher levels in both Th17 cell percentage and expression of RORγt in Th17 cells, along with mRNA and protein expressions of *CXCR4*, IL-6, IL-17, and IL-23. In cell experiments, the mouse vaginal epithelial cells that had been treated with miR-1192 inhibitor had shown us a decreased cell proliferation rate and contrarily increased expressions of *CXCR4*, IL-6, IL-17, and IL-23 mRNA, protein, and cell apoptosis rate; these results were opposite to the ones found in the mice treated with

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miR-1192 mimic. ***Conclusion:*** Our results provided significant evidence that miR-1192 could directly development and progression of VVC by restraining the *CXCR4* gene in the VVC mice.

Introduction

Vulvovaginal candidiasis (VVC), also referred to as a yeast infection, is a disease popular among sexually active women [1]. The VVC infection has been known to either be uncomplicated or complicated [2]. In uncomplicated VVC cases, sporadic episodes of mild infections commonly occur as a result of an infection due to the Candida albicans [3], whereas a complicated case of VVC classifies as a more severe infection caused by non-Candida albicans species, which remains to be a significant problem especially in women of child-bearing age [4]. Pregnant women are doubly at risk of an increase in the incidence of vaginal colonization due to the Candida species as opposed to non-pregnant women [5]. The major clinical manifestations of VVC include pruritis, discomfort, dyspareunia, and dysuria [6]. The safest and most effective therapeutic method for VVC involves either a Single-dose oral treatment or short-term local therapy, which include miconazole therapy and butoconazole treatment [2]. Certain microRNAs have been identified as having an advantage in the treatment of several diseases, especially in several forms of cancers, such as colorectal cancer, non-small cell lung cancer and prostate cancer [7-9]. However, the involvement of microRNA-1192 (miR-1192) in the treatment of VVC has not been evidently studied.

Specifically, miR-1192 is known to be highly expressed in muscle cells during muscle regeneration [10]. One study suggested that human antigen R (HuR) prevents the mechanism of miR-1192 by interfering with the recruitment of Ago2 to HMGBI 3'UTR [11]. Ago 2 plays a major role in RNA-induced silencing complex, and the binding of HuR to its HuR binding sites (HuRBS) is sufficient enough in order to prevent miR-1192-mediated inhibition [10]. The results from a study involving both miR1192 and *CXCR4* provided evidence that *CXCR4* was in fact the target gene of miR-1192. *CXCR4* is found in a variety of cell types, including breast cancer cells as well as vascularization of the gastrointestinal tract [12, 13]. Low oxygen concentration can trigger an increase in the expression of *CXCR4* gene in a variety of cell types, including monocytes, tumor-associated macrophages, monocyte-derived macrophages, endothelial cells, and cancer cells [14]. RNA interference (RNAi), thought to be a powerful tool for research involving gene function, results in significant inhibition of breast cancer cell migration through inducible down-regulation of endogenous *CXCR4* gene expression in breast cancer cells [15]. However, the exact level of *CXCR4* gene expression in VVC remains unclear, and the role of miR-1192 in the occurrence of VVC has not been abundantly studied in previous previous studies. Therefore, this study was conducted with sole purpose being to evaluate the effect of miR-1192 on the development and progression of VVC by targeting *CXCR4*.

Materials and Methods

Ethical statement

All experiments were approved by the Animal Ethics Committee, and all the research activities of the study subjects met requirements provided by the strict guidelines and principals of the International Association for Study of Pain [16].

Establishment of VVC mouse model and sample collecting

A total of 75 healthy female BALB/C mice (aged 8-10 weeks and weighing 22 ± 4 g) were purchased from animal breeding center of Wuhan University, which were then divided into three groups with 25 mice apart of each group: the estradiol-treated group, the oil-treated group, and the normal group. The estradiol-treated group and the oil-treated group were both inoculated with Candida albicans (purchased from the Department of Microbiology, Tongji Medical College, Shanghai, China). The mice in the estradiol-

treated group were then further inoculated with albicans blastospores (5×10^4 cells/ml, namely 20 μl fugal suspension) through a vaginal administration. Six days prior to the inoculation, 0.1 mL of estradiol containing 0.1 mg benzoate (Tianjin Jinyao Amino Acid Co., Ltd., Tianjin, China) was subcutaneously injected into the posterior region of the mice, once every two days. The mice involved in the oil-treated group were injected subcutaneously with 0.1 mL of olive oil on the 6th day before the inoculation, which was also repeated similarly once every 2 days. The mice in the normal group received no intervention. The vaginal tissues in each group were collected on the 4th day following inoculation and the periodic acid-Schiff (PAS) staining was used in order to observe the presence of either hyphal or yeast phased Candida albicans with the hopes of confirming the successful establishment of VVC mouse model.

Sampling was conducted on the 2nd, 4th, 7th, 14th, and 21st days following inoculation in each group, respectively. At each time interval, five mice were randomly selected, among which three mice were further selected to undergo a vaginal lavage involving a 100 μL of phosphate buffer saline (PBS) solution. The vaginal lavage fluid was collected from each group in a disinfected tube for the subsequent experiments. After the mice had been sacrificed, their vaginal tissues were both dissected and stored in liquid nitrogen for further experimentation. Half of these tissues were used for RNA extraction, while the remaining were used for protein extraction. At each time interval, two mice in each group were sacrificed with their vaginal tissues being dissected and placed with formalin for subsequent histopathological observation.

Hematoxylin-eosin (HE) and PAS staining

Specimens were also placed in 10% neutrally buffered formalin for up to 16 - 18 h. After the tissues had been placed in xylene following dehydration in alcohol (70%, 80%, 90%, and 100%), they were immersed in wax, embedded in paraffin, and then sliced into 4 μm sections for both HE and PAS staining processes. The sections were then both flattened and transferred onto microscope slides at 45°C. Dewaxing was performed using xylene once the slides had been baked for 1 h at 60°C. Following rehydration, the HE staining (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) was conducted. Next, a series of gradient alcohol dehydration along with a round of xylene clearing were performed, after which the slides were sealed off with a neutral balsam. The pathological changes found in the vaginal tissues of mice in each group were observed under an Olympus microscope (CX31-LV320, Beijing, China). PAS staining: slides were prepared for HE staining, after which they were treated with periodic acid solution for 10 min, and stained for 20 min using a schiff reagent. After the sodium metabisulfite solution treatment for 1 min (total 2 times) was performed, Mayer's hematoxylin (Cat. No.: C0105; Beyotime Institute of Biotechnology, Shanghai, China) staining was performed for 10 min. Dehydration and sealing were also both performed following the same procedure in HE staining.

Immunohistochemistry

The fixed mice vaginal tissues were selected for an immunohistochemistry process on the 14th day following inoculation, were subsequently embedded in paraffin, and sliced into 3 - 4 μm sections, after which were routinely dewaxed and hydrated at room temperature for a total of 20 min. Next, the sections were placed in a 3% H_2O_2 environment in order to block the endogenous peroxidase activity. The antigen retrieval was then conducted for these tissues with a 5-min placement at a 90°C microwave with 80% power. Then, the rabbit anti-mouse *CXCR4* (1: 100, ab124824, Abcam, Inc., MA, USA) monoclonal antibody was added in, and the incubation was done at conditions of approximately 4°C overnight. Afterwards, a biotinylated sheep anti-rabbit IgG antibody (1: 1000, ab6789, Abcam, Inc., MA, USA) was added to the mice vaginal sections followed by a 30-min incubation at 37°C. Following a wash with PBS, we once again performed hematoxylin staining for 30 s. Diaminobenzidine (DAB) (No., P0202; Beyotime Institute of Biotechnology, Shanghai, China) was used in order to develop color. Hydrochloric acid ethanol was used for further cycles of dehydration and clearing, with the sections finally being sealed off with neutral balsam. The sections were further observed and photographed using an Olympus microscope (CX31-LV320). The results were then categorized as having either a positive expression or negative expression. A positive expression was reflected by presentation of a brown color change in the cell cytoplasm and membrane. The cells that were found without brown color staining in the cytoplasm and membrane were marked as having a negative expression.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of interleukin-6 (IL-6), interleukin-17 (IL-17), and interleukin-23 (IL-23) in vaginal lavage fluid of mice were all detected by employment of an ELISA, which was conducted strictly in accordance with the instructions found on the ELISA kit (Bender MedSystems, Burlingame, CA, USA). The biotin-labeled antibody (50 μL) was also added into the wells of the microplate pre-coated with antibody, followed by the addition of 50 μL samples into their appropriate wells. The mixture was mixed by gentle tapping and was subsequently sealed. After incubating for 2 h at a temperature of 37°C, a freshly prepared tetramethylbenzidine (TMB) substrate solution (0.1 mL) was added into the wells and incubated for 10 min at 37°C before the reactions were terminated. The optical density (OD) values were measured at 450 nm using a Microplate Reader (MultiSkan FC, Thermo Fisher Scientific Inc., Waltham, MA, USA). A standard curve was generated using the standard solution that was included in the kit, and the concentration of IL-6, IL-17, and IL-23 were subsequently calculated.

Measurement of the ratio of Th17 and the expression of RORγt by flow cytometry

Blood anticoagulated with heparin (200 μL) was added into test tubes, and mixed with the same amount of RPMI1640 medium (without calf serum, No.: 11875093; Thermo Fisher Scientific Inc., Waltham, MA, USA). After an addition of 50 μL phorbol myristate acetate (PMA, 1 μg/mL), 16 μL ionomycin (50 μg/mL), and 13.6 μL monensin (0.1 mg/mL), the cells were incubated for a total of 5h in a 5% CO_2 incubator at the temperature of 37°C. Then, 100 μL of treated blood was transferred into a test tube, which was then added with 10 μL of CD3-PE-Cy5 (ab25453; Abcam, Inc., MA, USA) as well as 10 μL CD8-FITC (ab25676, Abcam, Inc., MA, USA), while incubating the mixture under dark conditions for 15 min. At room temperature, the blood was incubated with little to no exposure to light for 15 min with the an addition of a 100 μL Reagent A (fixative) of the FIX&PERM kit (No.: GAS001S100; Thermo Fisher Scientific Inc., Waltham, MA, U.S.A). Subsequently, 3 mL of saline solution was added and the mixture was centrifuged at a rate of 1200 r/min for 5 min, followed by removal of the supernatant. After adding an additional 100 μL Reagent B (DMA) along with 10 μL of the IL-17 antibody, and Alexa Fluor® 647-RORγt antibody (BD Biosciences, Franklin Lakes, NJ, USA), the sample was continuously incubated at room temperature for 15 min with little to no exposure of light. Afterwards, a 3 mL saline solution was added, followed by a centrifugation at the rate of 1200 rpm for 5 min in order to collect the sediment. The corresponding isotype control was added to the control tube. Cells were resuspended with an additional 500 μL resuspension solution and were then measured by the Becton Dickinson Immunocytometry Systems (FACSCalibur analyzer, Beckman Coulter, Fullerton, CA, USA).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from 100 mg frozen vaginal tissues in strict accordance to the instructions provided by the TrizolTM reagent kit (No.: 16096020; Thermo Fisher Scientific Inc., Waltham, MA, USA). The PCR primers were designed by several softwares including Oligo 6 and Primer Premier 5, and synthesized by TaKaRa Company (Dalian, China). The primer sequences are shown in Table 1. U6 was used as the internal reference for miR-1192, and β-actin was used as the internal reference for *CXCR4*, IL-6, IL-17, and IL-23. Afterwards, 10 μL RNA samples were diluted 20 times with the use of ultra-pure RNase-free water, with the OD value measured at 260 nm and 280 nm collectively, using an ultraviolet spectrophotometer, respectively, in order to detect both the purity and concentrations of RNA samples [Total RNA = 1.25 / OD 260 (μL)]. Afterwards, a 5 μL mixture (No.: 4368702; Tideradar

Table 1. Primer sequences of RT-qPCR. Notes: RT-qPCR, reverse transcription quantitative polymerase chain reaction; F, forward; R, reveres; miR-1192, microRNA-1192; CXCR4, C-X-C motif chemokine receptor 4; IL-6, interleukin-6; IL-17, interleukin-17; IL-23, interleukin-23

Genes	Sequences
miR-1192 RT	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAATTTGG-3'
miR-1192	F: 5'-ACACTCCAGCTGGGAAACAAAACAAACAGACCA-3'
	R: 5'-CTCAACTGGTGTCGTGGA-3'
IL-6	F: 5'- TCCTTAGCCACTCCTTCTGT-3'
	R: 5'-CTCATTCTGTCTCGAGCCCACCA-3
IL-17	F: 5'-CAGACTACCTCAACCGTTCCA-3'
	R: 5'-ACAATCGAGGCCACGCAGGTGCAGC-3'
IL-23	F: 5'-GACTCAGCCAACTCCTCCAGCCAG-3'
	R: 5'-TTGGCACTAAGGGCTCAGTCAGA-3'
CXCR4	F:5'-GGCTGACCTCTTTGT-3'
	R: 5'-GTTTCCTTCGCCTTTGAC -3'
β-actin	F: 5'-CTAGGAACCAAGGTGGTAT-3'
	R: 5'-CAAACATGATCTGGGTCATC -3'
U6	F: 5'-TCGCTTCGGCAGCACATATACTA-3'
	R: 5'-TAGTATATGTGCTGCCGAAGCGA-3'

Beijing Technology Co., Ltd, Beijing, China) was added into an Eppendorf (EP) tube, along with 5 μL total RNA as well as 10 μL RNase free H_2O. The PCR reaction conditions for reverse transcription were 37°C for 15 min, followed by 85°C for 5 s; the reaction was terminated at 4°C. The cDNA obtained was then stored at a -20°C refrigerator. The reaction conditions for the RT-qPCR were as follows: pre-denaturation for 10 min at 95°C, 40 cycles of denaturation at 95°C for 10 s, annealing for 20 s at 60°C, and extension for 34 s at the temperature of 72°C. The fluorescent dye SYBR Green kit (No.: RR091A; Takara Bio Company, Japan) was used. The same procedure was used in order to detect gene expressions in cells.

Western blot assay

The vaginal tissues in each group were ground into homogeneous fine powder in liquid nitrogen, followed by the addition of a protein lysis buffer (No. C0481; Sigma-Aldrich, St. Louis, Missouri, USA). Tissues were then centrifuged for 20 min at a temperature of 4°C at the speed of 12000 rpm, allowing the supernatant to be collected. Protein concentrations were then measured using the bicinchonininc acid (BCA) assay kit, and adjusted to the same loading volume with de-ionized water. Subsequently, the stacking gel and separation gel (10% respectively) were prepared. The protein samples were then mixed with the help of the loading buffer, and then boiled for 5 min, placed in ice, and centrifuged before they were once again loaded onto the gel for protein separation. After electrophoresis, the proteins currently on the gel were transferred into a nitrocellulose membrane and blocked with 5% skim milk overnight. The primary rabbit antibodies anti-*CXCR4* (1: 10000; ab124824; Abcam, Inc., Cambridge, MA, USA), rabbit anti-IL-6 (1: 10000; ab46100; Abcam, Inc., Cambridge, MA, USA), rabbit anti-IL-17 (1: 10000; ab79056; Abcam, Inc., Cambridge, MA, USA), and rabbit anti-IL-23 (1: 10000; ab45420; Abcam, Inc., Cambridge, MA, USA) were all added to the proteins for incubation at room temperature for 1h, with β-actin (1: 2000; ab8226; Abcam, Inc., Cambridge, MA, USA) used as their internal reference. The membrane was then washed by using the PBS buffer three times, 5 min each wash. Then, the secondary antibody (goat anti-IgG; 1: 1000; Boster Co., Ltd., Wuhan, China) was added and incubated for 1 h at room temperature, followed by three washes with PBS, 5 min each time. Following the immersion of the membrane into the electrochemiluminescence (ECL, Pierce Chemical Company, IL, USA) solution for 1 min at room temperature, the excessive liquid was removed and the membrane was covered using saran wrap for a convenient X-ray film process; β-actin was considered as the internal reference. The ratio of gray values between targeted band and the internal reference band was used as the relative expression of protein. The same procedure was performed in order to detect the gene expressions in cells

Cell culture in vitro

The vaginal tissues involved in the mice in both the estradiol-treated and the normal groups were obtained. After the vaginal mucosa had been separated and sheared to pieces under aseptic conditions, the VM was treated with 2% trypsin (Gibco, Gaitherburg, MD, USA) for 30 min at room temperature, centrifuged, and digested with a type II collagenase (0.06%) for 15 min at room temperature; the cells were filtered soon after. The filtrates (vaginal epithelial cells) were cultured with RPMI 1640 (Gibco, Gaitherburg, MD, USA) containing a 10% fetal bovine serum (FBS) (Hyclone, USA) and were then incubated in a 5% CO_2 incubator at 37°C. After a treatment involving 0.25% trypsin, RPMI 1640 containing 10% (v/v) FBS was used in order to resuspend the cells into single cell suspensions with a routine culture being performed. The cells undergoing the logarithmic growth phase were used for further experimentation. The purity of vaginal epithelial cells was detected using an anti-keratin antibody. Following a PBS washing cycle, the cells were added with both a Fluor 488-cytokeratin antibody (53-9003-82; Thermo Fisher Scientific Inc., Waltham, MA, USA) and 4',6-diamidino-2-phenylindole (DAPI) (D21490; Thermo Fisher Scientific Inc., Waltham, MA, USA). Afterwards, the purity of the epithelial cells was observed and calculated under a fluorescence microscope [17].

Cell grouping and transfection

To begin our next section, the mice vaginal cells would be divided into the following groups: normal group (vaginal cells of mice in the normal group with no transfection), the blank group (cells of mice in the estradiol-treated group that didn't receive transfection), the negative control (NC) group (cells of mice in the estradiol-treated group transfected with the NC sequence in the transfection kit), the miR-1192 mimic group (cells of mice in the estradiol-treated group transfected with miR-1192 mimics sequence), the miR-

1192 inhibitor group (cells of mice in the estradiol-treated group transfected with miR-1192 inhibitors sequence), the si-*CXCR4* group (cells of mice in the estradiol-treated group transfected with si-*CXCR4* sequence), and the miR-1192 inhibitor + si-*CXCR4* group (cells of mice in the estradiol-treated group transfected with si-*CXCR4* and miR-1192 inhibitors sequences). All the transfected sequences were synthesized by Shanghai Kehua Bio-Engineering Co., Ltd., (Shanghai, China) (Table 2). Cell transfection processing went as such: the cells (1×10^5 cells/well) were sub-cultured in a 6-well plate the day prior to the transfection, reaching a 70%-80% confluence on the day of transfection. The cells underwent transfection using Lipofectamine 2000 in accordance with the manufacturer's protocol (Invitrogen, Carlsbed, CA, USA). All transfected cells were obtained from the vaginal epithelial cells found in the estradiol-treated group. The sequences (100 pmol) of the NC, miR-1192 mimic, miR-1192 inhibitor, si-*CXCR4,* and miR-1192 inhibitor + si-*CXCR4* groups had all been diluted with 250 μL serum-free Opti-MEM (Gibco Company, USA) (final concentration: 50 nM), mixed, and incubated at room temperature for a total of 5 min. The reagent of Lipofectamine 2000 underwent the process of dilution using 250 μL serum-free Opti-MEM, mixing, and incubating at room temperature for 5 min. The two aforementioned solutions were then both mixed and incubated at room temperature for 20 min, after which the mixture was added into the cell culture plate. The transfected cells were then further cultured at a temperature of 37°C with 5% CO_2 for up to 6 - 8 h, followed by another cycle of culturing with a complete medium between 24 to 48 h.

Table 2. Transfection sequences. Notes: miR-1192, microRNA-1192; CXCR4, C-X-C motif chemokine receptor 4

Vector	Sequence
miR-1192 mimics	5′-AAACAAACAAACAGACCAAAUU-3′
miR-1192 inhibitors	5′-AAUUUGGUCUGUUUGUUUGUUU-3′
si-CXCR4	5′-CCGACCUCCUCUUUGUCAUTT-3′

Dual-luciferase gene reporter assay

The Bioinformatics website, microRNA.org was used to collect the analysis of the targeting gene of miR-1192, in order to predict the targeting relationship between both *CXCR4* and miR-1192. A dual-luciferase reporter assay was performed for further verification of the targeting relationship. *CXCR4* 3'UTR was synthesized, digested with *Spe*I and *Hind*III, and introduced into the pMIR-Reporter plasmid (Promega, Madison, WI, USA). Using the wild-type (WT) sequence and *CXCR4* as the reference, we were able to design the mutated complementary sequence of the seed sequence. Following digestion by restriction endonucleases, the mutated fragment was then inserted into the pMIR-Reporter plasmid using T4 DNA ligase. Both the cell number and transfection efficiency were normalized using a pRL-TK (TaKaRa Biotechnology Ltd., Dalian, China), with Renilla luciferase being used as an internal reference. The vaginal epithelial cells in the MiR-1192 mimics and negative control groups were respectively co-transfected with the luciferase reporter vector. After transfection had been completed for a duration of 48 h, the cells were collected and lysed. Luciferase activities were determined by the Luciferase Reporter Assay System (Promega, Madison, WI, USA).

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

The vaginal epithelial cells of the mice involved in the logarithmic growth phase were once again selected and inoculated into a 96-well plate with 200 μL per well containing 2×10^3 cells. The cells were then further ran through a culturing cycle in a 5% CO_2 incubator for 48 h at a temperature of 38°C. Following the addition of the medium without FBS, the cells were again further cultured in an incubator with 5% CO_2 at 37°C for 24 h, 48 h, and 72 h, respectively. Subsequently, 20 μL of 5 mg/mL MTT (Sigma-Aldrich Chemical Company, St Louis, USA) was added into the cells, with the cells cultured for 4h. Subsequent to the culturing, the supernatant was extracted and discarded. Each well then added with 100 μL dimethyl sulphoxide (DMSO) (Sigma-Aldrich Chemical Company, St Louis MO, USA) and gently shaken for 5 min to produce a thorough mixture. Next we measured the optical density (OD) at 590 nm using a microplate reader. With 3 parallel wells in each group, the average OD value was recorded and the experiments would be repeated a total of three times. The cell survival curve was then drawn with the time intervals used as the X-axis and the OD values used as Y-axis.

Measurement of cell cycle and apoptosis by flow cytometry

Following 48 h of transfection, the culture medium was discarded and the cells were washed once in PBS and detached by 0.25% trypsin. Once the cells were presenting a round shape when observed under a microscope, the digestion solution was discarded and the medium with serum was used in order to re-suspend cells. Cell suspension would be centrifuged for 5 min at a rate of 1000 rpm, with the sediment preserved. The cells were then washed in PBS and fixed in a pre-cooled 70% ethanol for 30 min and later centrifuged. After washing with a PBS solution, the cells were stained with propidium iodide (PI) for 30 min; the excess PI was washed away using a Hank's balanced salt solution (HBSS). Cell volume was then be adjusted to 1 mL with PBS solution. The flow cytometry (FACSCalibur analyzer, Beckman Coulter EPICS XL, USA) method was performed for the purpose of detecting the cell cycle. Three samples were set for each group, and each experiment was repeated three times. Approximately 48 h following transfection, the cells were detached by means of an EDTA-free trypsin and collected in a flow tube, centrifuged, with the supernatant discarded soon after. Next, the cells would go on to be washed three times, centrifuged, and the supernatant was once again removed afterwards. Cell apoptosis was measured with the assistance of an Annexin V-FITC apoptosis detection kit (No., C1065, Shanghai Beyotime Biotech. Co., Ltd., Shanghai, China). The solutions of Annexin-V-FITC, PI, and HEPES were all prepared with the proportion set at 1:2:50 to obtain the Annexin V-FITC/PI dye. The cells were then resuspended in 100 μL (1×10^6 cells in 100 μL) of the Annexin V-FITC/PI dye and incubated at room temperature for 15 min; 1 mL HEPES was added after incubation. The excitation wavelength was measured at 488 nm, and the fluorescence emissions were measured using both 525 nm and 620 nm band pass filters (corresponding to FITC and PI fluorescence) in order to detect cell apoptosis.

Statistical analysis

All data was analyzed using the SPSS 21.0 statistical software (IBM Corp. Armonk, NY, USA). The measurement data was expressed using the mean ± standard deviation. The comparisons among multiple groups were conducted by means of an analysis of variance, while the comparisons between two groups were analyzed through the least significant difference (LSD) *t*-test. The count data was expressed as both frequencies and rates. The Chi-square test was used in order to compare the categorical data, and the hierarchical data was compared by the rank sum test. A probability value of $p < 0.05$ indicated a statistically significant difference.

Results

Positive protein of CXCR4 is highly expressed in vaginal tissues of VVC mice following the successful establishment of VVC mouse models

HE staining was employed in order to observe histological changes in the vaginal tissues of the mice. The results obtained showed that the epithelial cell layer of vaginal mucosa in the estradiol-treated group had become significantly thickened, with a large number of inflammatory cells detected in the vaginal cavity as opposed to mice involved in the normal group (Fig. 1A-C). In the oil-treated group, the inflammatory cells infiltrated into the superficial layer of vaginal mucosa and submucosa, with even a few superficial abscesses being observed in the superficial layer of mucosa when compared with the normal group. PAS staining results went on to show that, in comparison with the normal group, Candida albicans blastospore as well as pseudohyphae appeared in both the vaginal cavity and superficial layer of vaginal mucosa in the oil-treated group, with a large number of mycelium visible in the estradiol-treated group, lasting up until the end of the observation (Fig. 1D-F).

Immunohistochemical staining was performed in order to detect the positive protein expression rate of *CXCR4*. The positive expression of CXCR4 protein was indicated by the appearance of brown particles. On the 4th day, the *CXCR4* protein in vaginal tissues of both the estradiol-treated and oil-treated groups had been mainly expressed in keratinocyte cell membrane and cytoplasm (Fig. 1G-I). In addition, *CXCR4* was also expressed in the cytoplasm of some inflammatory cells detected in both lamina propria and vascular endothelial cells.

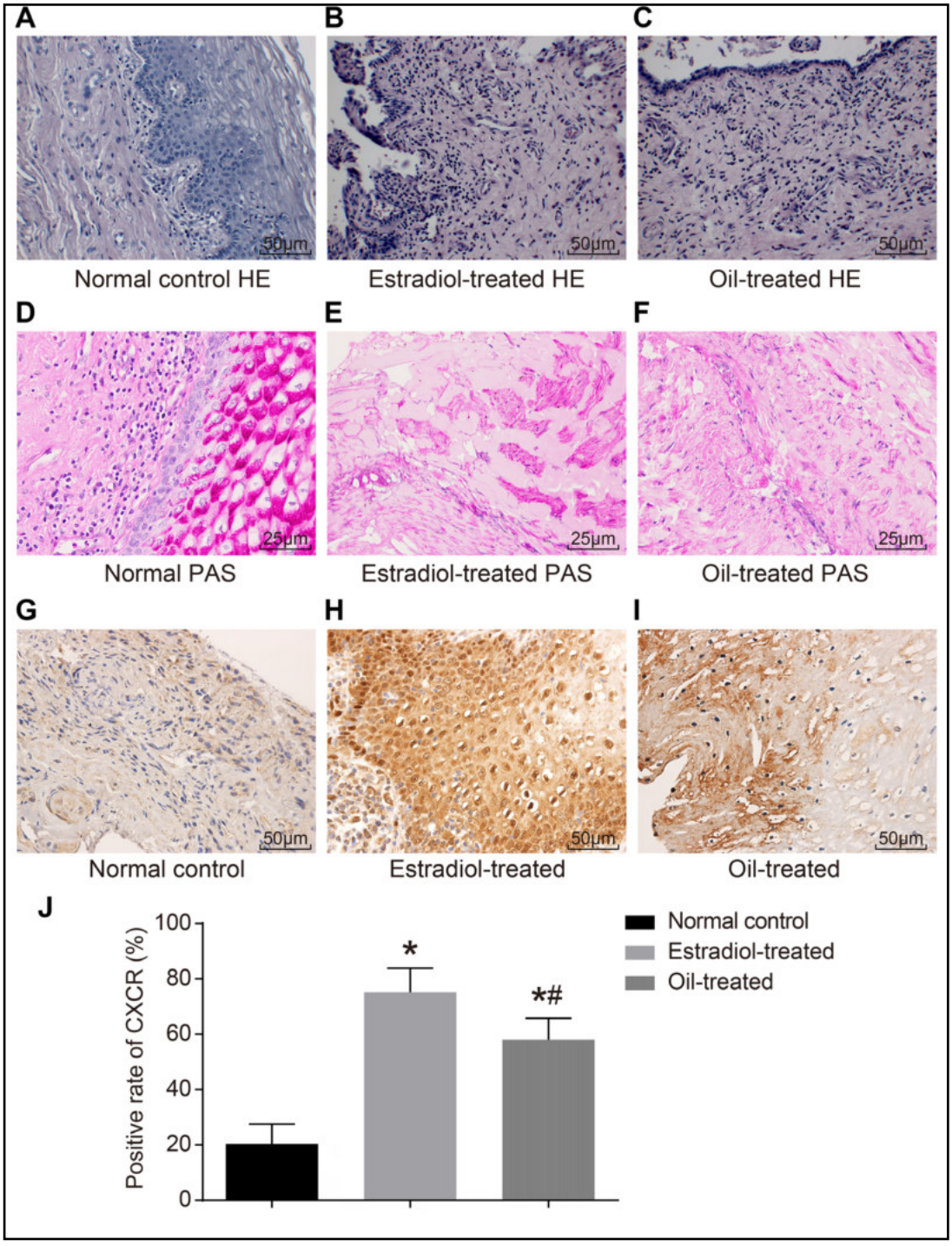

Fig. 1. Positive protein of CXCR4 is highly expressed in vaginal tissues of VVC mice following the successful establishment of VVC mouse models. Note: A-C, histological changes of vaginal tissues of mice in each group using HE staining x 200; D-F, histological changes of vaginal tissues of mice in each group using PAS staining; G-I, immunohistochemical staining images for vaginal tissues of mice in each group x 200; J, positive protein expression of CXCR4 vaginal tissues of mice in each group. Two mice were detected in each group, and here the image for one mouse is presented. The histogram indicates the average value of 10 visual fields in two mice. *, $p<0.05$, compared with the normal group; #, $p<0.05$, compared with the estradiol-treated group. CXCR4, C-X-C motif chemokine receptor 4; VVC, vulvovaginal candidiasis; HE, hematoxylin-eosin; PAS, periodic acid-schiff; IHC, immunohistochemical.

There was no *CXCR4* expression found in the vaginal mucosa of the mice in the normal group. Based on the analysis of the positive *CXCR4* cells (Fig. 1J), there were more cells with positive *CXCR4* in estradiol-treated and oil-treated mice than those found in the normal mice ($p < 0.05$). In comparison with mice in the estradiol-treated group, mice in the oil-treated group presented with a reduced number of cells that had a positively-expressed *CXCR4* ($p < 0.05$). Judging on the aforementioned information, it can be concluded that *CXCR4* might be overexpressed in VVC.

miR-1192 is downregulated in VVC mice while CXCR4, IL-6, IL-17 and IL-23 are reciprocal

ELISA was adopted in order to evaluate the protein expressions of IL-6, IL-17, and IL-23. The results suggested that, the protein expression of IL-6, IL-17, and IL-23 in the vaginal lavage fluid of both oil-treated and estradiol-treated mice from 2nd to 21st day had become elevated when compared with the expression found in the normal group ($p < 0.05$). The oil-treated group showed reduced expressions of both IL-17 and IL-23 protein as opposed to the expression found in the estradiol-treated group ($p < 0.05$, Fig. 2A-C). The aforementioned results suggested that IL-6, IL-17, and IL-23 expressions were upregualted in the vaginal lavage fluid.

RT-qPCR was conducted with the purpose of detecting both the miR-1192 expression and the mRNA expressions of *CXCR4*, IL-6, IL-17, and IL-23. The RT-qPCR demonstrated that, down-regulated miR-1192 expression and up-regulated mRNA expressions of *CXCR4* of the vaginal tissues existed in both oil-treated and estradiol-treated groups than in the normal group ($p < 0.05$, Fig. 2D-E). In comparison with the normal group, the expressions of IL-6, IL-17, and IL-23 mRNA in both the oil-treated group and estradiol-treated group were elevated significantly from the 2nd to the 21st day ($p < 0.05$), in which the mRNA expressions of both IL-6 and IL-17 showed a tendency of initial increase and then a decrease with the highest level of reduction observed on the 14th day. When comparing with the estradiol-treated group, the *CXCR4*, IL-6, and IL-23 mRNA expressions in the mouse vaginal lavage fluid of oil-treated group all had decreased between the 14th to 21st day, whereas the IL-17 mRNA expression declined between the 7th to 21st day (all $p < 0.05$, Fig. 2F-H). The above results suggested that the miR-1192 expression and the mRNA expressions of *CXCR4*, IL-6, IL-17, and IL-23 might be overexpressed in VVC tissues.

We employed a western blot assay in order to detect the protein expressions of *CXCR4*, IL-6, IL-17, and IL-23 in vaginal tissues of mice in each group. Western blot results reflected that the protein expressions detected in the *CXCR4* of vaginal tissues in both the oil-treated and estradiol-treated groups were increased (Fig. 2I-M) and had reached their peak on the 14th day, as opposed to the normal group ($p < 0.05$). The collective protein expressions of IL-6, IL-17, and IL-23 were elevated remarkably in the two treatment groups ($p < 0.05$, Fig. 2J-L), also presenting an increase followed by a slow decrease while reaching the highest point on the 14th day, when compared to the normal group (Fig. 2N-P). In comparison with the estradiol-treated group, the protein expressions of IL-23 and *CXCR4* between the 14th and 21st day and protein expressions of IL-17 between the 7th and 21st day of mice in the oil-treated group were both reduced ($p < 0.05$). Judging on the previously reported information, it was revealed that the protein expressions of *CXCR4*, IL-17, and IL-23 might be expressed at a high level in VCC.

Percentage of Th17 cells in peripheral blood of mice and the RORγt expression in Th17 cells are higher in VVC mice

Flow cytometry was applied in order to measure the percentage of Th17 cells in the peripheral blood of mice. Based on the results mentioned in the previous section, VVC mouse models at the 14th day were sacrificed and the peripheral blood was collected to detect the percentage of Th17 cells using flow cytometry. The results showed us that the percentages of Th17 cells in both the peripheral blood in the oil-treated and estradiol-treated groups were significantly elevated when compared with those of the normal group ($p < 0.05$). However, no statistical differences were detected between both the oil-treated mice and estradiol-

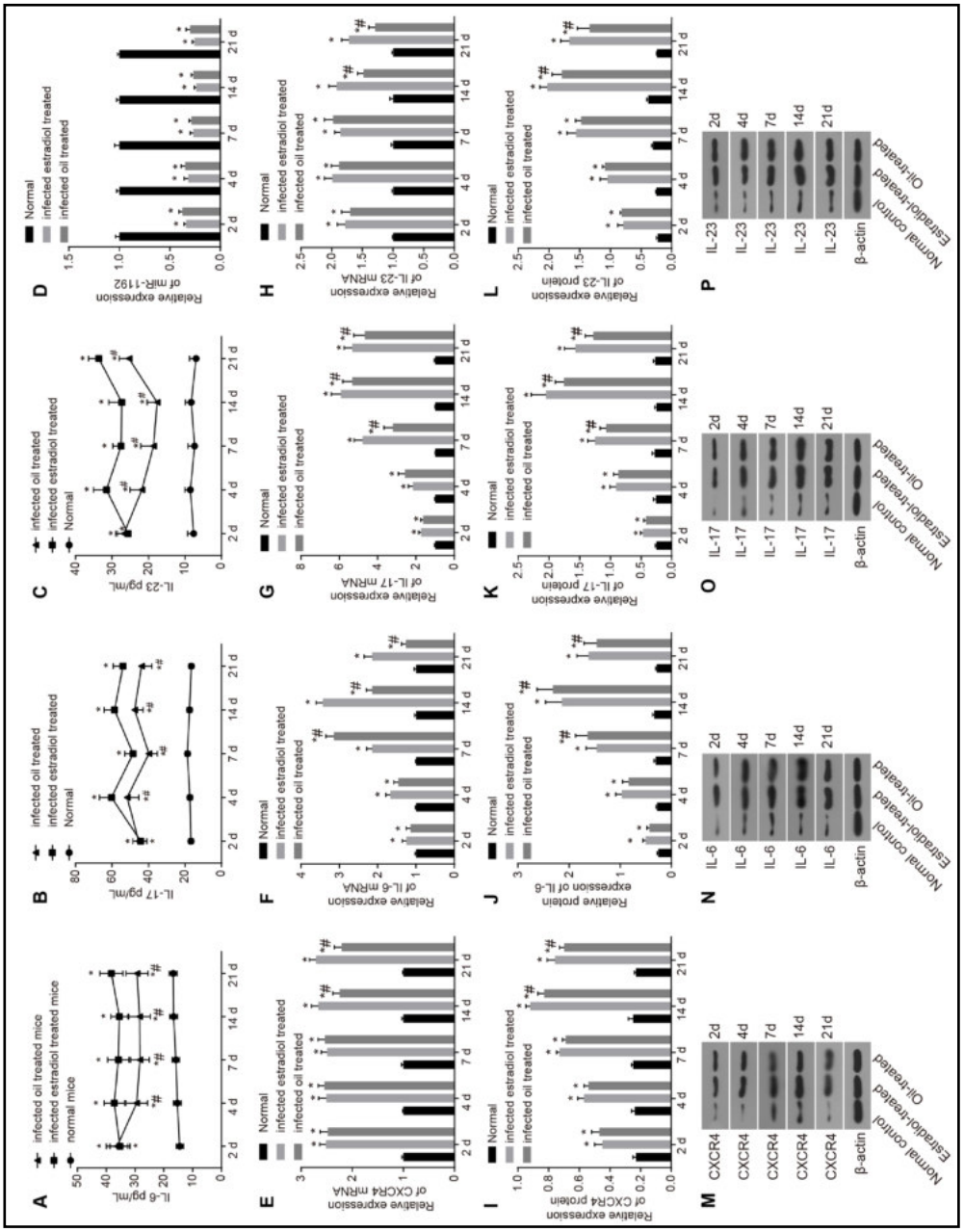

Fig. 2. miR-1192 is downregulated in vaginal tissues of VVC mice while CXCR4, IL-6, IL-17 and IL-23 are upregulated as determined by ELISA, RT-qPCR and western blot assay. Note: A, protein expression of IL-6 in vaginal lavage fluid of mice in each group; B, protein expression of IL-17 in vaginal lavage fluid of mice in each group; C, protein expression of IL-23 in vaginal lavage fluid of mice in each group; D, expression of miR-1192 in vaginal tissues of mice in each group; E, mRNA expression of CXCR4 in vaginal tissues of mice in each group; F, mRNA expression of IL-6 in vaginal tissues of mice in each group; G, mRNA expression of IL-17 in vaginal tissues of mice in each group; H, mRNA expression of IL-23 in vaginal tissues of mice in each group; I, protein expression of CXCR4 in vaginal tissues of mice in each group; J, protein expression of IL-6 in vaginal tissues of mice in each group; K, protein expression of IL-17 in vaginal tissues of mice in each group; L, protein expression of IL-23 in vaginal tissues of mice in each group; M-P, protein bands of CXCR4, IL-6, IL-17 and IL-23. A-L indicate a mean value obtained from three repeated experiments for three mice; M-P indicate protein band image for one mouse. *, p<0.05, compared with the normal group; #, p<0.05, compared with the estradiol-treated group. RT-qPCR, reverse transcription quantitative polymerase chain reaction; miR-1192, microRNA-1192; CXCR4, C-X-C motif chemokine receptor 4; IL-6, interleukin-6; IL-17, interleukin-17; IL-23, interleukin-23; VVC, vulvovaginal candidiasis.

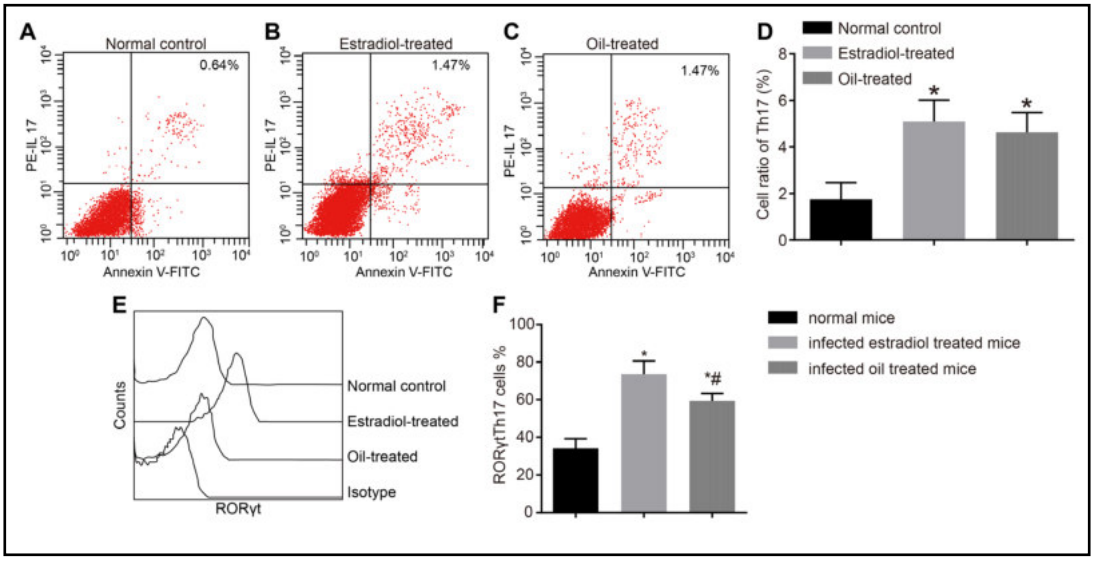

Fig. 3. Percentage of Th17 cells in peripheral blood of mice and the RORγt expression in Th17 cells are higher in VVC mice as detected by flow cytometry. Note: A-C, flow cytometry for percentage of Th17 cells in peripheral blood in each group; D, histogram of percentage of Th17 cells in peripheral blood in each group; E, flow cytometry for the RORγt expression in Th17 cells in each group; F, statistical plot for the RORγt expression in Th17 cells in each group. Three mice were detected in each group and the experiment was repeated three times. A, B, C, and E show the result of one mouse; D and F show the average value of each group. *, p<0.05, compared with the normal group; #, p<0.05, compared with the estradiol-treated group. VVC, vulvovaginal candidiasis.

treated-mice ($p > 0.05$, Fig. 3A-D). Based on the aforementioned findings, it can be concluded that the percentage of Th17 cells in peripheral blood of mice might be markedly higher in both the oil-treated and estradiol-treated groups, as opposed to the normal group.

The RORγt antibody can detect the RORγt expression in Th17 cells. In comparison with the normal group, the expression of RORγt found in the Th17 cells of the mice increased both in the oil treated and estradiol-treated group ($p < 0.05$), and the expression of RORγt in the oil-treated group was lower than that detected in the estradiol-treated group ($p < 0.05$) (Fig. 3E-F).

CXCR4 is a target gene of miR-1192

The dual-luciferase gene reporter assay was used in order to verify whether or not *CXCR4* is the target gene of miR-1192. The binding sites between *CXCR4* gene and miR-1192 were confirmed by a biological prediction website (www.microRNA.org). These results suggested that *CXCR4* is a target gene of miR-1192. The sequence of 3'-UTR region of *CXCR4* gene binding to miR-1192 is illustrated in Fig. 4A. The luciferase reporter vectors were then constructed using either mutated or wild type *CXCR4* 3'-UTR in order to confirm that the predicted binding sites for miR-1192 could lead to a change in the luciferase activity. The mouse vaginal epithelial cells were then transfected with miR-1192 mimics, Wt-miR-1192/*CXCR4*, and Mut-miR-1192/*CXCR4* plasmid using double luciferase activity assay. The findings of the dual luciferase reporter assay showed that the luciferase signal in the miR-1192 mimic group with Mut-miR-1192/*CXCR4* presented with no significant change, whereas the luciferase signal in miR-1192 mimic group with Wt-miR-1192/*CXCR4* had significantly decreased by approximately 65% ($p < 0.05$, Fig. 4B). These results suggested that *CXCR4* is a target gene of miR-1192.

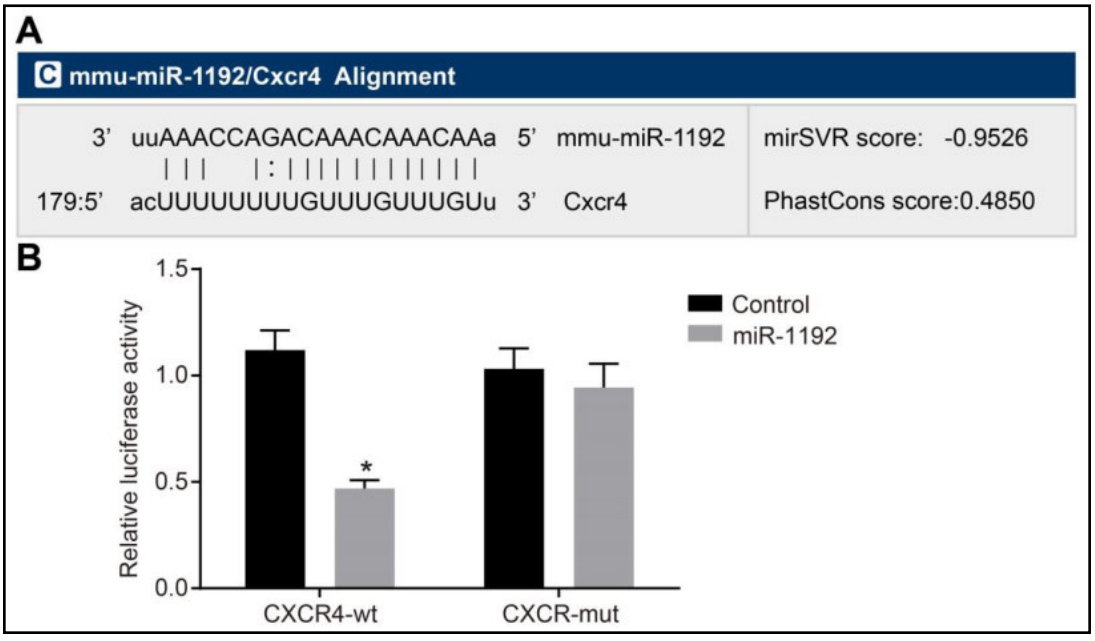

Fig. 4. The target relationship between miR-1192 and CXCR4 is identified. Note: A, the binding sites of miR-1192 in the CXCR4 3'UTR; B, relative luciferase activity results. The data are average values obtained from three repeated experiments. miR-1192, microRNA-1192; CXCR4, C-X-C motif chemokine receptor 4; wt, wild type; mut, mutant.

miR-1192 reduces mRNA and protein expressions of CXCR4, IL-6, IL-17 and IL-23 in vaginal epithelial cells

The vaginal epithelial cells of mice were isolated from the vaginal tissues of mice involved with the normal and the estradiol-treated group, after which the purity was detected using a keratin antibody expressed in the epithelial cells. As shown in Fig. 5A, B, the results demonstrated that the purity of the epithelial cells was between 98 - 99%.

After the vaginal epithelial cells were transfected, an RT-qPCR was used with the purpose of detecting miR-1192 expression as well as the *CXCR4*, IL-6, IL-17, and IL-23 mRNA expressions (Fig. 5C). The results determined from the RT-qPCR suggested that the miR-1192 expression in vaginal epithelial cells was evidently increased in the miR-1192 mimic group, contrarily being significantly reduced in the blank, NC, si-*CXCR4*, miR-1192 inhibitor, and miR-1192 inhibitor + si-*CXCR4* groups as opposed to the normal group ($p < 0.05$), while the mRNA expressions of *CXCR4*, IL-6, IL-17, and IL-23 were all significantly increased (all $p < 0.05$). There were no significant changes observed in both the miR-1192 expression and mRNA expressions of *CXCR4*, IL-6, IL-17, and IL-23 between the blank group and the NC group ($p > 0.05$). In comparison with the NC group, the miR-1192 expression had shown an evident increase in the miR-1192 mimic group ($p < 0.05$), while the expression in the si-*CXCR4* group provided no significant difference ($p > 0.05$). However, there was a significant down-regulation visible in the expressions of *CXCR4*, IL-6, IL-17, and IL-23 mRNA in the miR-1192 mimic and si-*CXCR4* groups as opposed to the NC group ($p < 0.05$). When comparing with the expressions of the NC group, no significant differences in the miR-1192 expression in the miR-1192 inhibitor group were detected ($p > 0.05$) while the expressions of *CXCR4*, IL-6, IL-17, and IL-23 mRNA ($p < 0.05$) had all increased. In the miR-1192 inhibitor + si-*CXCR4* group, down-regulation of the miR-1192 expression had been observed when comparing with the NC group ($p < 0.05$), while the mRNA expression of *CXCR4*, IL-6, IL-17, and IL-23 showed no significant difference between these two groups ($p > 0.05$). These findings made the following indication to us: miR-1192 could reduce the mRNA expressions of CXCR4, IL-6, IL-17, and IL-23 in vaginal epithelial cells.

Western blot assay was further employed in order to detect the protein expressions of *CXCR4*, IL-17, and IL-23 in vaginal epithelial cells (Fig. 5D, E). The result of the western blot assay showed that the protein expressions of *CXCR4*, IL-6, IL-17, and IL-23 in the mouse vaginal epithelial cells had all increased in all the remaining groups as opposed to the normal group ($p < 0.05$). There were also no significant differences observed in expressions of *CXCR4*, IL-6, IL-17, and IL-23 proteins among the blank, NC, and miR-1192 inhibitor + si-*CXCR4* groups ($p > 0.05$). Both the miR-1192 mimic and si-*CXCR4* groups revealed evidently reduced protein expressions of *CXCR4*, IL-6, IL-17, and IL-23 in comparison with the NC group ($p < 0.05$), whereas a remarkable increase in protein expression was observed in the miR-1192 inhibitor group than the NC group ($p < 0.05$). Based on these findings, we can confidently conclude that miR-1192 has the potential of reducing the protein expressions of *CXCR4*, IL-6, IL-17, and IL-23 in vaginal epithelial cells.

miR-1192 promotes cell proliferation, alters cell cycle distribution and inhibits cell apoptosis of vaginal epithelial cells

An MTT assay was performed in order to determine the vaginal epithelial cell proliferation. The results went onto demonstrate that the cell proliferation had decreased in the remaining six groups, when compared with the results of the normal group. No significant differences were found in the cell proliferation among the blank, NC, and miR-1192 inhibitor + si-*CXCR4* groups $p > 0.05$). In comparison with the NC group, the miR-1192 mimic, and si-*CXCR4* groups had all shown faster cell growth, whereas the miR-1192 inhibitor group had presented with a slower cell growth (all $p < 0.05$). This data collectively indicated to us that the over-expression of miR-1192 along with the lower-expression of *CXCR4* could work to improve the cell proliferation of the subject mouse vaginal epithelial cells, while the inhibition of miR-1192 expression could result in opposite outcomes (Fig. 6A).

The flow cytometry along with PI staining was performed in order to detect the cell cycle distribution. The results would go on to show us that there was an increase in the vaginal epithelial cells during the G1 phase as well as a decrease in the S phase in all groups with the exception of the normal group ($p < 0.05$). No significant differences were found in the cell cycle among the blank, NC, and miR-1192 inhibitor + si-*CXCR4* groups ($P > 0.05$). In comparison with the NC group, the vaginal epithelial cell count had decreased in G1, while opposingly increasing in the S phase in both the miR-1192 mimic and si-*CXCR4* groups; however, in the miR-1192 inhibitor group, there were more cells in G1 phase, while fewer cells were arrested in S phase ($p < 0.05$, Fig. 6B-I).

The results obtained from the Annexin V-FITC/PI staining (Fig. 6J-Q) revealed that the apoptosis rate of mouse vaginal epithelial cells in all the groups was remarkably up-regulated when compared with that of the normal group (8.3 ± 0.90)% (all $p < 0.05$). Again, no significant differences were detected in the apoptosis rate among the blank [(48.1 ± 4.00)%), NC [(47.9 ± 5.00)%] and miR-1192 inhibitor + si-*CXCR4* [(45.7 ± 3.90)%] groups ($p > 0.05$). The miR-1192 mimic [(24.3 ± 1.80)%] and si-*CXCR4* [(23.7 ± 1.73)%] groups however, presented a down-regulated apoptosis rate, while the miR-1192 inhibitor group [(63.7 ± 6.70)%] showed an up-regulated apoptosis rate when comparing with the NC group ($p < 0.05$). This data would collectively provide us evidence that the over-expression of miR-1192 coupled with the silencing of *CXCR4* expression had the potential of leading to the inhibition of apoptosis of vaginal epithelial cells, with the suppression of miR-1192 expression also being able to enhance the apoptosis of vaginal epithelial cells.

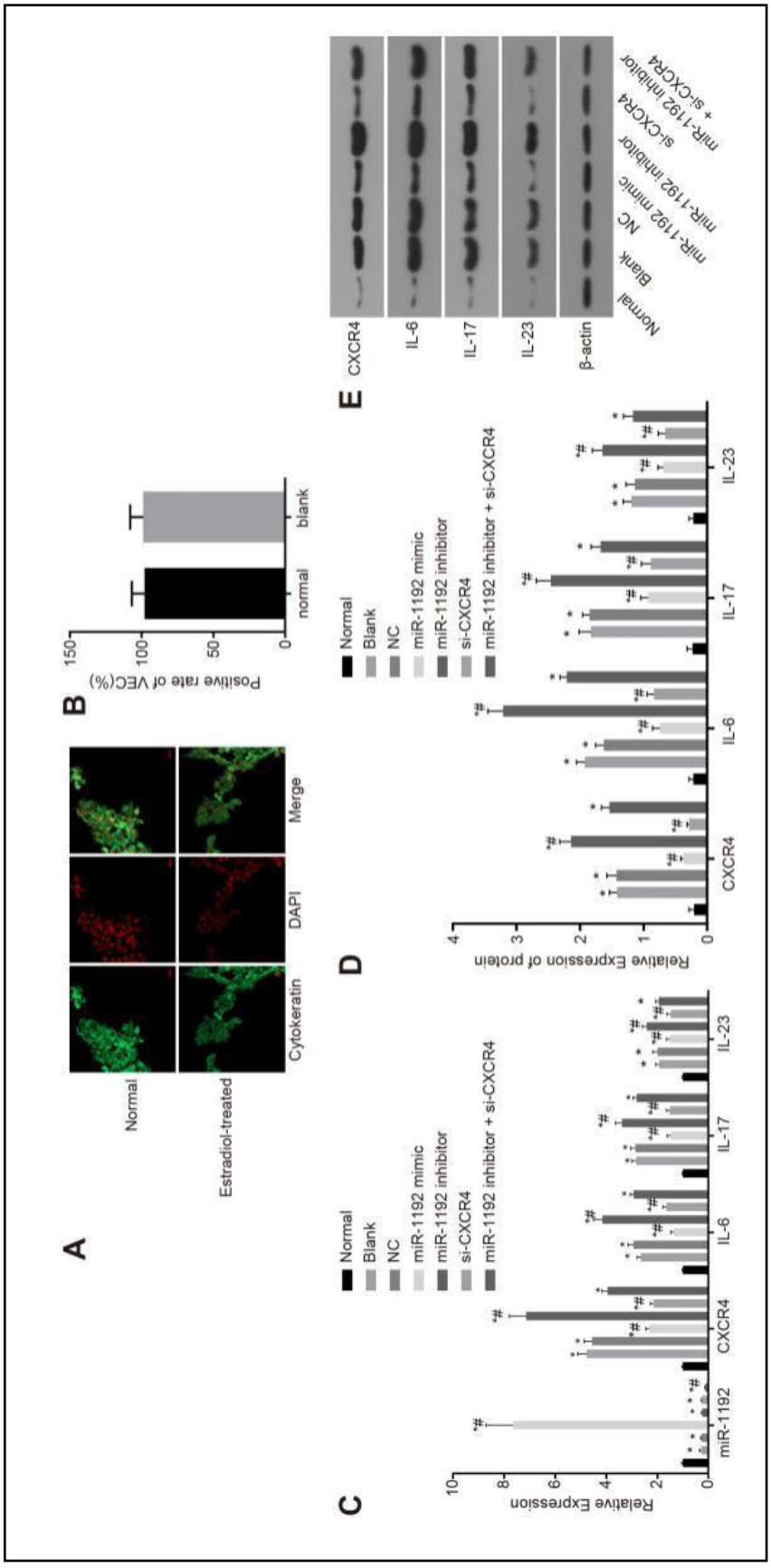

Fig. 5. miR-1192 reduces the mRNA and protein expressions of CXCR4, IL-6, IL-17 and IL-23 in vaginal epithelial cells, determined by RT-qPCR and western blot assay. Note: A, staining image (× 400) for keratin antibody in isolated vaginal epithelial cells; DAPI is used to label the cell nucleus; scale bar = 25 um; B, the purity of vaginal epithelial cells in each group; C, miR-1192 expression and mRNA expressions of CXCR4, IL-6, IL-17 and IL-23 in seven groups; D, protein expressions of CXCR4, IL-6, IL-17 and IL-23 in seven groups; E, electrophoretogram for CXCR4, IL-6, IL-17 and IL-23 in seven groups; the image indicates one western blot assay. The results presented are average values from three repeated experiments. *, p<0.05, compared with the normal group; #, p<0.05, compared with the NC group. NC, negative control; RT-qPCR, reverse transcription quantitative polymerase chain reaction; miR-1192, microRNA-1192; CXCR4, C-X-C motif chemokine receptor 4; IL-6, interleukin-6; IL-17, interleukin-17; IL-23, interleukin-23 DAPI, 4′,6-diamidino-2-phenylindole.

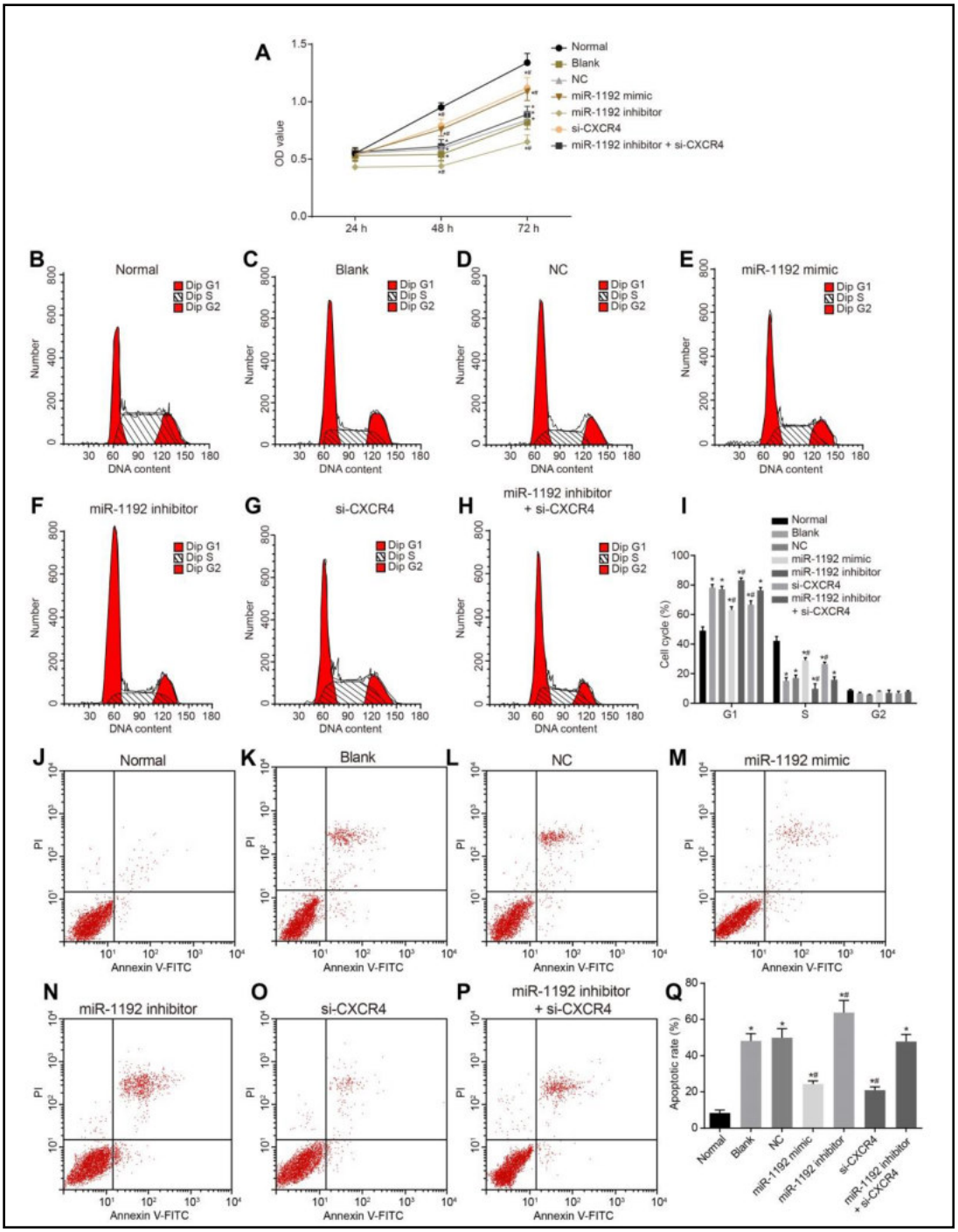

Fig. 6. miR-1192 promotes cell proliferation, alters cell cycle distribution and inhibits cell apoptosis of vaginal epithelial cells as detected by MTT and flow cytometry. Note: A, cell proliferation of vaginal epithelial cells in mice of seven groups detected by MTT assay; B-H, cell cycle of vaginal epithelial cells in seven groups using flow cytometry with PI staining; I, histogram of cell cycle of vaginal epithelial cells in seven groups; J-P, cell apoptosis rate of vaginal epithelial cells in seven groups using flow cytometry with Annexin V-FITC/PI staining; Q, histogram of cell apoptosis of vaginal epithelial cells in seven groups. A, I, Q indicate a mean value obtained from three repeated experiments; the image indicates the result from one of the three repeated experiments. *, $p<0.05$ compared with the normal group; #, $p<0.05$ compared with the NC group. OD, optical density; NC, negative control; miR-1192, microRNA-1192.

Discussion

VVC, especially in its recurrent form, is still an intractable problem for clinicians, microbiologists, and patients [18]. Since there is an insufficiency in data associated with the relationship between miR-1192 and VVC, we dedicated our entire study in order to evidently illustrate that *CXCR4* gene can be a target gene for miR-1192 along with the idea that miR-1192 could reduce the activation of both Th17 cells and inflammation factors in a mouse model with VVC by inhibiting the expression of *CXCR4*.

Th17 cells have been detected with having abundant levels of VVC in their composition [19]. Th17 cell responsiveness plays a major role in the prevention of VVC with the role of protection against vaginal candidiasis infection, possibly via the effect it has on antimicrobial peptide production by vaginal epithelial cells [20]. According to the results obtained from the current study, the proportion of Th17 cells located in the peripheral blood of mice infected with VVC in the estradiol-treated group had presented a significantly up-regulated expression, as opposed to the normal group. Both IL-17 and IL-23 are types of pro-inflammatory cytokines that aid with the activation of immune cells in order to produce cytokines, chemokines, or cell adhesion molecules [21, 22]. In this study the concentrations of IL-6, IL-17, and IL-23 detected in mouse vaginal lavage fluid from the estradiol-treated group had all increased from the 2nd all the way through to the 14th day when comparing the equivalent found in the normal group. Both the mRNA and protein expressions of the two inflammatory factors presented with a sharp rise in the estradiol-treated group.

CXCR4 gene has frequently been found to be expressed in a variety of diseases such as breast cancer and organ vascularization [12, 13]. However, little evidence has been provided regarding whether or not *CXCR4* gene was expressed in VVC. Our findings have examined that the positive expression rate for *CXCR4* gene in VVC-infected groups was much higher than the expression detected in the normal mice group. The down-regulation of *CXCR4* by inducing small interfering RNA inhibits breast cancer cell invasion [15]. As for VVC, our study initially investigated miR-1192 could down-regulate *CXCR4* to inhibit the proliferation of Th17 cells and to promote the expression of IL-6, IL-17, and IL-23.

Several kinds of microRNAs have demonstrated important roles in cell proliferation in a variety of diseases such as colorectal cancer and hepatocellular carcinoma [23, 24]. The mRNA and protein expressions of miR-1192 presented low expression in the estradiol-treated group. When comparing with the normal group however, cell proliferation rate had lowered in all groups. When comparing with the NC group, the growth rate of cells was faster in vaginal epithelial cells of miR-1192 mimic and si-*CXCR4* groups, while the growth rate in the miR-1192 inhibitor group was proven to be significantly slower. This data collectively indicated to us that the over-expression of both miR-1192 and *CXCR4* gene silencing could potentially improve cell proliferation in the vaginal epithelial cells of mouse, while the silencing of miR-1192 expression could present a contrary result in producing a decline in cell proliferation in the mice vaginal epithelial cells.

Conclusion

In conclusion, the aforementioned results evidently showed that miR-1192 could potentially inhibit cell apoptosis and promote cell growth by suppressing the *CXCR4* gene, which was the confirmed target gene of miR-1192. This suggests to us that miR-1192 has therapeutic potential, while also providing novel prognostic markers for VVC. However, due to both the limited sample size and experimental conditions, further studies are needed in order to define the detailed mechanisms of miR-1192 in VVC.

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Disclosure Statement

All authors declare that they have no existing conflicts of interest concerning this article.

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