WNT4 promotes macrophage polarization via granulosa cell M-CSF and reduces granulosa cell apoptosis in endometriosis

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Research Article

Keywords: WNT4, granulosa cell, macrophage, polarization, M-CSF, apoptosis

Posted Date: July 7th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1808461/v1

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Abstract

Background

WNT4 gene polymorphism are common in endometriosis and may functionally link estrogen and estrogen receptor signaling. Previous study confirmed estrogen and estrogen receptor signaling recruit macrophage to promote the pathogenesis of endometriosis. To investigate the effect of WNT4 in endometriosis involved in macrophage polarization and whether WNT4 could reduce the apoptosis of granulosa cells.

Methods

An observational study consisting of 8 cases of women with endometriosis (diagnosed by surgery and histology) and 22 mice of endometriosis animal model was conducted. Granulosa cells were isolated from 16 patients with endometriosis and co-cultured with macrophage under WNT4 treatment using TUNEL assay, quantitative reverse transcription PCR, flow cytometry and ELISA analysis. 22 mice of endometriosis animal model confirmed the WNT4 treatment effects using histology and immunohistochemistry, Western blot and flow cytometry.

Results

We observed that the apoptotic proportion of granulosa cells was significantly decreased and M2 macrophage was significantly increased after WNT4 treatment during the granulosa cell and macrophage co-culture system. To reveal the underlying mechanism for this, we conducted a series of experiments and found that high expression of granulosa cell M-CSF led to the M2 polarization of macrophages. The animal model also suggested that the anti-apoptotic effect of WNT4 on granulosa cells were conducted by the M2 polarized macrophage.

Conclusions

WNT4 promotes macrophage polarization via granulosa cell M-CSF and reduces granulosa cell apoptosis in endometriosis.

Background

Endometriosis (EM) is common gynecological benign disease which acts like malignancy during reproductive age women. Chronic pelvic pain and infertility was the main complains. The overall incidence of EM is about 6%-10% [1] while 35%-50% among infertile patients [2]. Infertile patients with EM seeking assisted reproductive treatment are also associated with poor clinical outcomes [3–5]. Decreased
ovarian reserve and lower fertilization and implantation rates are the main contributors to unpleasant treatment outcomes [6]. During follicular development, the final reproductive outcome of oocyte was affected greatly by the surrounding granulosa cells since there are no direct blood supply to the oocyte. All the nutrition and signals were transmitted through the gap junction of the cumulus oophorus complex and oocytes. There are many pathological changes of granulosa cells of EM patients which compromise the reproductive potency, such as decreased expression of P450 aromatase [7], activation of NF-κB pathway [8], decreased expression of BMP-6 and SMAD4 of cumulus oophorus complex [9] and altered WNT signaling [77].

In recent years, growing interests of dysregulation of immune system are aroused to illustrate the pathogenesis of EM. Decreased phagocytic activity and the lack of adequate immune surveillance [10, 11] were the main concerns of the development and aggravation of EM in the past. Not only the missing part of the normal function of immune cells, but also the misperception to the endometriotic tissue promotes its infiltration [12]. Macrophage plays an important role in this process. Circulating macrophage could differentiate into two different types according to different stimulus in the specific tissue, the classic activated macrophage (M1) and the alternative activated macrophage (M2). Pro-inflammation and killing micro-organisms are the characteristics of M1 macrophage while immunosuppression and promote tissue repair were of M2 macrophage. The polarization of M1/M2 macrophages in the pathophysiology of EM are still controversial. Plenty of researches concerning the endometriotic tissue and the polarization of macrophage [13, 14], rare studies emphasis on the relationship between normal pelvic tissue function and macrophage during EM, for example, the ovary and the granulosa cells.

WNT4 is a member of WNTS family of glycoprotein signal molecules, which plays an important role in normal folliculogenesis, luteogenesis and steroidogenesis [15–18]. Inactivation of WNT4 would result in sex-reversal and oocyte depletion in mice. Emerging evidences from genomic analysis testified the association of WNT4 polymorphism and EM [19], though different population presents controversial results [20, 21]. WNT4 gene polymorphism may functionally link estrogen and estrogen receptor signaling [22]. Previous study confirmed estrogen and estrogen receptor signaling recruit macrophage to promote the pathogenesis of EM [23]. However, whether WNT4 can modulate immune microenvironment components, such as macrophage, to regulate folliculogenesis and granulosa cell apoptosis in EM remains to be elucidated.

In this study, we tested for the first time the hypothesis that WNT4 upregulates macrophage colony stimulating factor (M-CSF) in granulosa cells and thus promote macrophage polarization to reduce the apoptosis of granulosa cells. The findings demonstrate that the immunoregulation role of WNT4 can potentially be used as a therapeutic target for ovarian reserve preservation in EM.

**Materials And Methods**

**Study population and clinical procedures**
Eight patients with EM, undergoing in vitro fertilization (IVF) at Department of Reproductive Medicine Center, the First Affiliated Hospital of Sun Yat-Sen University, were recruited. The diagnosis of EM was confirmed by surgery and staged according to the revised American Society for Reproductive Medicine classification system. Patients suffered from adenomyosis, polycystic ovarian syndrome, hyperprolactinemia, adrenal gland disease, thyroid gland disease and history of irregular menstrual cycles were excluded from this study. Written informed consents were obtained from all the patients enrolled. The ethics committee of the First Affiliated Hospital of Sun Yat-Sen University approved this study ([2021]399-1).

The controlled ovarian stimulation procedure of the participants was following the standard agonist or antagonist protocol. When at least three follicles had reached 17 mm or two follicles had reached 18 mm in diameter, an intramuscular injection of 5,000 IU-10000 IU of human chorionic gonadotropin (hCG) was administrated to trigger oocyte maturation. Oocyte retrieval was scheduled approximately 34–36h after hCG injection guided by transvaginal ultrasonography. Clear follicular fluid containing granulosa cells was collected and centrifuged at 626 ×g for 10 min. The cells were resuspended in hyaluronidase and digested at 37 °C for 20 min. Finally, the lymphocyte separation solution was added and centrifuged at 626 ×g for 10 min. The white turbid clusters of cells in the middle were granular cells. Demographics, clinical characteristics and outcomes of patients are listed in Supplementary Table 1.

Animals model of EM

Twenty-two 6-week-old female Balb/c mice used in this study were purchased from Guangdong Medical Laboratory Animal Center. All the mice were maintained under controlled conditions with a light/dark cycle of 12/12 h, stable temperature of 25 ± 1°C and a relative humidity of 55% (±10%). All animals had free access to laboratory food and water. We established a mice EM model by i.p. injection of allogeneic endometrial segments as described in the previous study [24]. Briefly, 6-week-old mice were initially treated with estradiol benzoate (200μg/ kg/d/mouse, APExBIO, China). Seven days later, the mice treated with estradiol benzoate were sacrificed and their uterus were collected in a petri dish containing warm 0.01 M phosphate-buffered saline (PBS; pH 7.2–7.4). All the uterus were dissected identically, including isolation of endometrial tissue, and dividing into small fragments which were consistent and smaller than 1 mm. Then we injected these fragments intraperitoneally into recipient mice with a 5-ml syringe. To eliminate any potential bias, we mixed endometrial fragments from 8 donor mice, and injected the mixture fragments to 14 recipient mice equally for about 1ml. Two mice were sacrificed on Day-7 to identify whether the EM model was successfully established and collect their endometriotic lesions. The other 12 mice were randomly and equally distributed into two groups and received i.p. injection of WNT4(100ng/ml, Abcam, British) or PBS 100ul/d for 9 days. on Days 9, all mice were sacrificed and their ovarian tissue were collected.

Cell culture
KGN (luteinized granulosa cell line) and THP-1 human monocyte cell line were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). KGN and primary granulosa cells were cultured in Dulbecco's modified Eagle's medium while THP-1 cells were grown in RPMI 1640 containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA, Cat. # 16140071) and 1% gentamicin at 37 °C in 5% CO2.

**Macrophage–granulosa cells interaction**

THP-1 cells were differentiated into macrophages using supplementation, following established protocols [25,26]. Briefly, THP-1 cells were differentiated into non-polarized macrophages (M0) using 10 ng/ml of 12-O-tetradecanoylphorbol13-acetate (PMA, Sigma-Aldrich) for 24 h. M0 macrophages were polarized into M1 macrophages by treating with 15 ng/ml of lipopolysaccharide (LPS, Sigma-Aldrich), or M2 macrophages by treating with 25 ng/ml of IL4 (Millipore) for 24 h. When most monocytes were attached to the plate and appeared to be stretching out, the stimulation was suspended by replacing culture medium back to RPMI1640 medium. Primary granulosa cells were co-cultured with M1, or M2 for 48 h, and fresh media were added for 24 h. Both cells and culture media (CM) were collected from the co-culture system for further analysis.

**Identification of apoptotic nuclei by TUNEL assay**

Apoptotic nuclei were identified in primary granulosa cells using the TUNEL apoptosis Assay kit (Solabio) according to the manufacturer's protocol. Briefly, add 50 µL of TUNEL working solution to each sample after remove the cell media and then incubated in a humidified chamber at 37°C for 1 h. The cells were rehydrated in PBS (pH 7.5) after removing the TUNEL working solution for 2 times and then 100 uL reaction buffer were added to each sample. Slides were washed with PBS and then incubated with 4% formaldehyde fixative buffer to each well and underwent solution for 30 min at room temperature. Wash the cells with PBS 2-3 times and then monitor the fluorescence intensity with a fluorescence microplate reader at Ex/Em = 550/590-650 nm (Cutoff = 570 nm).

**Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis**

Trizol reagent (Life Technologies, Carlsbad, CA, USA) was used to isolate and extract total RNA from cells. SYBR Premix Ex Taq II kit (Takara Bio, Dalian, China, Cat. # RR036A) was then used to synthesize cDNA. The primer pair sequences were listed in supplementary table 2. SYBR Green PCR Master Mix (Takara Bio, Dalian, China, Cat.# RR420A) was used to amplify the target RNA on the CFX96 Real-time PCR System (Bio-Rad, Hercules, CA, USA), and 18S was selected as the internal control. The expression change of the target gene was calculated using the 2-ΔΔCt method.

**Histological studies and Immunohistochemistry**

Ovaries of the model mice were fixed in Bouin's solution. The samples were dehydrated in a graded series of ethanol and embedded in paraffin. Sections of 5 mm in thickness were obtained using a Microm HM 325 rotation microtome and stained with hematoxylin-eosin. Histological analysis was carried out using...
a computer-assisted image analysis system consisting of an Olympus BX-40 binocular microscope. Ovary tissue were immunostained using antibodies targeting CD163, and CD206. The paraffin sections were rehydrated, boiled for 20 minutes in 10 mM citrate buffer (pH 6.0), and then incubated with antibodies against CD163 (1:100, Cell Signaling Technology), CD206 (1:100, Proteintech) or normal rabbit IgG (1:100, sc-2027, Santa Cruz Biotechnology, Dallas, TX, USA) as a negative control, overnight at 4°C. Subsequently, the sections were incubated with Histofine Simple Stain MAX-PO (Nichirei Biosciences Inc., Tokyo, Japan), developed using DAB (Fujifilm Wako Pure Chemical Corp.), and counterstained using hematoxylin.

**Flow cytometry**

Conditioned media from granulosa cells were collected and then co-cultured with M0 for 48h. Flow cytometry (FCM) were used to analyze macrophage proportion and polarization. In this study, HLA-DR/ TNF-α and CD163/CD206 were detected as markers of M1 and M2 macrophages, respectively [27]. The macrophages were inactivated by incubation in 2% paraformaldehyde for 10 min on ice before the antibody staining to maintain the original polarization state. After centrifugation (300g), The cells were washed twice with 1× Perm/wash buffer (BD Biosciences, USA), and then incubated with anti-human HLA-DR/ TNF-α (Ichorbio, UK) at a concentration of 1:1000 and PE-conjugated anti-human CD206/CD163 (AAT Bioquest, USA) at a concentration of 1:50 for 45 min in the dark. After washing with 1× Perm/wash buffer, cells were resuspended in PBS for FCM analysis. HLA-DR/ TNF-α-positive and CD206/CD163-negative cells were identified as M1 macrophages, whereas HLA-DR/ TNF-α-negative and CD206/CD163-positive cells were identified as M2 macrophages.

**Elisa**

The concentration of IL-6, IL-10, M-CSF in the CM was measured using an ELISA kit IL-6, IL-10, M-CSF (R&D Systems), according to the manufacturer's protocol. One hundred microliters of CM were used in each well. Three independent cultures were performed for each condition and each sample was measured in duplicate.

**Results**

**Culture media from WNT4 treated granulosa cells-macrophage coculture system reduces the apoptosis of granulosa cells**

Granulosa cells from EM patients were isolated and co-cultured with macrophages and treated with 200 ng/mL recombinant human WNT4 (rhWNT4). After 72 hours incubation, the CM were collected to treat the granulosa cells to confirm whether the CM of WNT4 treated granulosa-macrophage could prevent the EM-induced apoptotic granulosa cells. The level of apoptosis and apoptotic-related protein of granulosa cells were analyzed. The results showed the proportion of apoptotic granulosa cells was decreased when cultured with CM collected from granulosa-macrophage treated with WNT4 (Fig1A-B). Meanwhile, the
mRNA expression level of apoptotic-related protein: caspase3, Fas, FasL were significantly lower than the PBS treated granulosa-macrophage group (Fig1C-E).

**WNT4 induces polarization of M2 macrophage phenotype in the granulosa-macrophage interactions**

M0 cells were cultured with the culture medium of rhWNT4 treated granulosa cells for 72 hours while the control group were treated with PBS to testify whether the effects of rhWNT4 treated granulosa cells could modulate M1/M2 polarization of macrophages. After incubation for 72h, PBMC cells were extracted and total RNA was collected to assess the expression of gene markers of both M1 (HLA-DR, TNF-α) and M2 (CD206, CD163) phenotypes. We observed that incubated with the CM of rhWNT4 treated granulosa cells induced significant upregulation of CD206, a marker of M2 macrophages of PBMC cells (Fig 2A). Meanwhile, mRNA level of TNF-α, a marker of M1, synchronously decreased (Fig 2D). No statistical significance was observed in the mRNA levels of CD163(Fig 4B) and HLA-DR (Fig 2C). For further evaluation, the distribution of CD206 and HLA-DR in PBMC cells were analyzed by flow cytometry. Significant upregulation of CD206, while no statistical difference of HLA-DR expression was observed (Fig 2E-G).

**M2 macrophage inhibit the apoptosis of granulosa cells**

To further verify the influence of M2 macrophages on the apoptosis of granulosa cells, we co-cultured the M1/M2 phenotype with granulosa cells of EM patients. The results showed that the proportion of apoptotic granulosa cells was decreased when co-cultured with M2 phenotype. On the contrary, the apoptotic granulosa cells were increased when co-cultured with M1 phenotype. (Fig.3)

**Secretory modification of anti-inflammatory factors and pro-inflammatory in PBMC Cells treated with the culture medium of rhWNT4 treated granulosa cells**

Concentration of anti-inflammatory and pro-inflammatory factors were evaluated to further detect the impact of CM of rhWNT4 treated granulosa cells on PBMC Cells. The expression of IL-6, the pro-inflammatory factor, was significantly decreased in PBMC cells (Fig 4A, C), while the expression of IL-10, the anti-inflammatory factor, was significantly increased (Fig 4B, D).

**Identification of M-CSF as a mediator of WNT4 and granulosa cell interaction**

Previous studies have shown that macrophage is critically important in the progress of EM and M-CSF was critically important in activation of M2, we hypothesized that WNT4 may improve the production of M-CSF of granulosa cells which could recruit macrophages. In the present study, we found that cultured granulosa cells of EM patients got increased M-CSF mRNA in response to rhWNT4 treatment (Fig. 5B) and the concentration of M-CSF protein in the media of KGN cell line was increased accordingly (Fig. 5C). However, the concentration of M-CSF protein in the media of cultured macrophage was not increased (Fig. 5D). rhWNT4 treatment of in the granulosa-macrophage coculture system increased the M-CSF protein concentration in the media compared to the PBS treatment group (Fig. 5E). These results
suggesting that, in the coculture condition, M-CSF is primarily induced in and secreted in the granulosa cells but not macrophage.

**M-CSF as a mediator of Granulosa Cell-Macrophage interactions after rhWNT4 treatment**

To further investigate the effect of M-CSF on Granulosa Cell-Macrophage interaction, M-CSF-neutralizing antibodies (anti-M-CSF) and M-CSF receptor inhibitor (GW-2580) were added to the co-culture system together with rhWNT4, and the collected CM were added to the only granulosa cells (KGN cells) culture system. The apoptotic effects were detected by TUNEL. Results showed that treatment of CM with anti-M-CSF and GW-2580 profoundly promoted the apoptosis of granulosa cells (KGN cells) while CM without those still inhibited this process. And M-CSF add to the co-culture CM and KGN cells, the inhibiting effect still existed. The presented results indicate that the anti-apoptotic effect of macrophage on granulosa cells were mediated by M-CSF. (Fig. 6)

**WNT4 reduces granulosa cell apoptosis in the KGN–macrophage interactions require M-CSF in KGN and M-CSFR in macrophage**

In order to further verify that the anti-apoptotic effect of M-CSF on granulosa cells originated from granulosa cells themself rather than from macrophage after the treatment of rhWNT4, we established M-CSF-knock-down KGN cell line and M-CSF-knock-down THP-1 cell line, and co-cultured them with or without rhWNT4. rhWNT4 inhibited the apoptosis of KGN cells in the WT-KGN cells and WT-THP-1 cells cocultured group, while the effect diminished in the M-CSF-knock-down KGN cells and WT-THP-1 cells cocultured group. Meanwhile, WT KGN cells co-cultured with M-CSF-knock-down-THP-1 cells brought about lower apoptotic rate of KGN cells. This indicates that KGN M-CSF, but not M-CSF generated from macrophages, plays an essential role in mediating the KGN-macrophage interactions after the treatment of rhWNT4. We also set up cocultures of M-CSFR-deficient-KGN and WT macrophages and found a lower apoptotic rate of the KGN cells. However, the CM from the reverse condition (WT KGN and M-CSFR-deficient-macrophages) promotes the apoptosis of KGN cells after the treatment of rhWNT4. Together, these data support the idea that the rhWNT4 promotes the KGN-derived M-CSF binds to M-CSFR receptors on macrophages, thus initiating signaling pathways that inhibit the apoptosis of KGN cells (Figure. 7).

**WNT4 improves ovarian function in mice with EM**

Figures 8A-B represents prototypical appearance of EM-like lesions established on mouse peritoneal membrane. Ovaries from control mice had few follicles with thin theca and granulosa cell layers (Fig.8C) with microscopic inspection. The ovaries from rmWNT4 treated mice showed more mature follicles, a higher granulosa cell layer, compared to control group (Fig.8D-E). As presented in Figure 8F, we observed robust CD206 staining in the ovary treated with rhWNT4. These present data demonstrated that WNT4 treatment led to M2 macrophage phenotype in the ovaries of EM mice and decreased the EM-induced ovarian damage in mice (Fig.8G-H). rhWNT4 treatment increased the expression of serum AMH and ovarian AMHR expression (Fig.8I-L), indicating WNT4 treatment could improve the ovarian function in EM.
Many factors take part in the pathogenesis of EM, retrograde menstruation/transplantation, coelomic metaplasia, altered cellular immunity, metastasis, genetic basis, environmental basis, multifactorial mode of inheritance with interactions between specific genes and the environment \[2\]. In recent years, genetic predisposition and altered immunity gain more and more attention. Angioni et al. \[20\] reported in different ethnic groups, it is possible that specific risk alleles could act differently in the pathogenesis of EM. Marfa et al. \[19\] reported rs16826658 and rs3820282 polymorphisms on \textit{WNT4} gene might be involved in the pathogenesis of EM in the infertile women in a Brazilian population. Wu et al. \[21\] reported \textit{WNT4} rs2235529 is associated with EM in Chinese Han women, which may result in aberrant expression of \textit{WNT4}, leading to the pathogenesis of EM. Since \textit{WNT4} plays an important role in normal folliculogenesis, luteogenesis and steroidogenesis \[15\] and \textit{WNT} pathway is vital in regulating granulosa cell apoptosis \[7\], we assume \textit{WNT4} might improve folliculogenesis by inhibit granulosa cell apoptosis in EM. Sanchez et al. \[28\] reported granulosa cells derived from EM patients had significantly higher transcript levels of the \(\beta\)-catenin-independent molecules \(\textit{WNT4}\) and \(\textit{WNT5a}\) and lower levels of the \(\beta\)-catenin-dependent molecule \(\textit{WNT1}\). A decrease of total \(\beta\)-catenin as well as of its dephosphorylated active form, together with an aberrant gene expression of the downstream targets \textit{survivin} and \textit{BMP4}, two genes involved in human granulosa cell survival. And Flow cytometry analysis confirmed an enhanced apoptosis of luteinized granulosa cells from patients with EM. This study presented the connection of \textit{WNT} pathway and granulosa cell apoptosis; however, their results seem contradicted to the role of \textit{WNT4} in normal folliculogenesis, luteogenesis and steroidogenesis. It gives us a hint that the positive role of \textit{WNT4} playing on granulosa cell apoptosis in EM might not through the direct granulosa cell \textit{WNT} pathway.

Pitzer et al. \[22\] reviewed that single nucleotide polymorphisms at the \textit{WNT4} gene locus may functionally link estrogen and estrogen receptor signaling. And many studies reported the estrogen/estrogen receptors worked with the macrophage to play the immunity modulation role during EM \[23, 29\]. We make a hypothesis that \textit{WNT4} might influence the function of macrophage to reduce the apoptosis of granulosa cells. In our study, we treated granulosa cells derived from EM patients with rh\textit{WNT4} and co-cultured with macrophage, the proportion of apoptotic granulosa cells was significantly decreased, as well as the apoptotic-related protein: caspase3, Fas, FasL, compared with the PBS treated co-culture system. This result validates our hypothesis in the first step. Circulating macrophage could differentiate into M1 or M2 macrophage according to different stimulus in the specific tissue. Pro-inflammation and killing microorganisms are the characteristics of M1 macrophage while immunosuppression and promote tissue repair were of M2 macrophage. We further analyzed the M1 and M2 macrophage proportion during the rh\textit{WNT4} treated granulosa cell and macrophage interaction. We found the M2 macrophage proportion was increased significantly during this treatment, which means rh\textit{WNT4} treated granulosa cell could promote macrophage polarization into the M2 macrophage. As we know, M2 macrophage could promote
tissue repair. We deem the anti-apoptosis effect during rhWNT4 treated granulosa cell and macrophage interaction mainly counts on M2 macrophage. So, we divided the M1 and M2 macrophage, and co-cultured with granulosa cell separately to test this hypothesis. As we presented, the apoptotic proportion of granulosa cell co-cultured with M2 macrophage was significantly decreased. And the pro-inflammation M1 macrophage increased the granulosa cell apoptosis. Concordantly, the expression of IL-6, the pro-inflammatory factor, was significantly decreased while the expression of IL-10, the anti-inflammatory factor, was significantly increased.

The alternatively activation of macrophage (M2 macrophage polarization) is a complex process, which consists of differentiation, priming and activation steps \[30\]. Many factors involved in this process, such as M-CSF, IL-4, IL-13, Toll-like receptor stimuli, and so on \[30-32\]. To figure out the mediator of rhWNT4 treated granulosa cell and macrophage interaction, we measured these mentioned factors and found out M-CSF expression was significantly increased in this co-culture system. And the origins of M-CSF were granulosa cells. To further testify the role of M-CSF, we put M-CSF-neutralizing antibodies(anti-M-CSF) and M-CSF receptor inhibitor (GW-2580) into this co-culture system. As we presented in the result, without M-CSF function, the anti-apoptosis effect of co-cultured macrophage was significantly decreased. With genetic modified cell lines, we depicted the whole regulation pathway of this process. As presented in the scheme chart, WNT4 increases M-CSF secretion of granulosa cell, and the increased M-CSF promotes macrophage polarization, therefore, more M2 macrophage exist in the co-culture system, which reduces the granulosa cell apoptosis as a result. The detrimental effect of an increased granulosa cell apoptosis has indeed been correlated with poor oocyte quality and ovarian function \[33\]. Decrease the apoptosis of granulosa cell of EM might improve the ovarian function of EM patients. With animal model, we confirmed the effect of WNT4 treatment during EM. As we can see, the total follicular count and AMH secretion were significantly increased after WNT4 treatment. In individual follicle, the granulosa cell layer was significantly thicker than the control group. The worst-case scenario of EM is ovarian reserve destruction, apart from the pelvic pain. Many previous studies and review demonstrated this, especially for the stage III/IV patients, which would decrease the fecundity severely \[34\]. The findings demonstrate that the immunoregulation role of WNT4 can potentially be used as a therapeutic target for ovarian reserve preservation in EM.

To our knowledge, our study is the first one to illustrate the immune regulation role of WNT4 in EM. There were also some limitations in our study. First, we didn’t depict the concrete regulation pathway of WNT4 on granulosa cell M-CSF secretion. Second, the anti-apoptotic pathway of granulosa cell during M2 macrophage co-culture was also not elucidated. Further studies would be needed to illustrate the specific regulation pathway of this process.

**Conclusion**

our study testified that WNT4 upregulates M-CSF in granulosa cells and thus promote macrophage polarization to reduce the apoptosis of granulosa cells for the first time. Apart from the modulation role in
folliculogenesis, luteogenesis and steroidogenesis, we found WNT4 also plays an important role in immune modulation during EM, which could be used as ovarian reserve preservation method.

**Abbreviations**

EM  
endometriosis  
M-CSF  
macrophage colony stimulating factor  
IVF  
in vitro fertilization  
hCG  
human chorionic gonadotropin  
qRT-PCR  
quantitative reverse transcription polymerase chain reaction  
FCM  
flow cytometry  
CM  
culture media  
rhWNT4  
recombinant human WNT4  
anti-M-CSF  
M-CSF-neutralizing antibodies.

**Declarations**

**Ethics approval and consent to participate:**

Written informed consents were obtained from all the patients enrolled. The ethics committee of the First Affiliated Hospital of Sun Yat-Sen University approved this study ([2021]399-1).

**Consent for publication:**

All the authors authorized the publication at Journal of Ovarian Research.

**Availability data and materials:**

The authors declare that all data supporting the results of the present study are available within the article.

**Competing interests:**

The authors declare that they have no competing interests.
Funding:
The National Key Research and Development Program of China (2016YFC1000205), the Guangdong Provincial Key Laboratory of Reproductive Medicine (2012A061400003), the National Natural Science Foundation of China (32000589) and the Guangdong Basic and Applied Basic Research Foundation (2114050h000636).

Authors’ contributions:
Y.Y., W.Z. and C.Z. participated in study design, execution, acquisition of data, analysis. Y.H. and Y.L. were responsible for critical discussion and experiment execution. Y.Y. and W.Z. participated in writing and revising article. Y.Y. and Y.L. conducted experiment execution and identification of patients. L.L. and T.L. participated in study design, execution, analysis, critical discussion and final approval of the version to be published.

Acknowledgement:
The authors wish to acknowledge all the stuffs of Reproductive Medicine Center, the First Affiliated Hospital of Sun Yat-Sen University for their help of the patients’ recruitment.

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References


Figures

Figure 1

WNT4 and granulosa cell apoptosis co-cultured with macrophage
Figure 2

WNT4 treated granulosa cells induce M2 macrophage polarization
Figure 3

M2 macrophage inhibits granulosa cell apoptosis
Figure 4

Altered inflammatory factor secretion of macrophage after interaction with WNT4 treated granulosa cell
Figure 5

M-CSF was increased of granulosa cell after WNT4 treatment
Figure 6

MSCF mediates granulosa cell and macrophage interaction after WNT4 treatment
M-CSF originated from granulosa cell and acts on macrophage
Figure 8

WNT4 improves the ovarian function of WNT4 treated EM mice

**Supplementary Files**

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- SupplementaryTables.zip