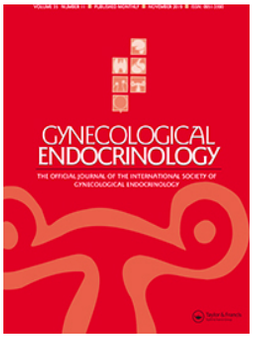

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Abnormal aggregation of myeloid-derived suppressor cells in a mouse model of cyclophosphamide-induced premature ovarian failure

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ABSTRACT

Oocytes are extremely sensitive to radiation and chemotherapy, and premature ovarian failure (POF) is one of the side effects of anti-tumor therapy. The pathogenesis of POF is very complex and still not fully elucidated. A mouse POF model was established after 14 days of cyclophosphamide injection. POF mice presented ovarian atrophy, destroyed follicular structure, a reduction in the number of primordial and mature follicles, and an decrease in the number of corpora luteal along with increased level of follicle-stimulating hormone (FSH), decreased levels of estradiol (E2), and anti-Mullerian hormone (AMH). Additionally, the proportion of bone marrow myeloid-derived suppressor cells (MDSCs) in peripheral blood, spleen, and ovarian tissue increased. MDSCs were mainly distributed around follicles and corpora luteal. Levels of mTOR and p-mTOR increased in ovarian tissue and inhibition of mTOR with rapamycin reduced the aggregation of MDSCs in peripheral blood, spleen, and ovarian tissue. This investigation sheds new light on the modulatory role of mTOR and demonstrates that an increase in MDSC number may play a key role in the pathological reaction during POF. Inhibition of mTOR and reduction of MDSCs in the ovary may represent a novel strategy for the treatment of POF.

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Premature ovarian failure; endocrine system diseases; ovarian diseases; myeloid-derived suppressor cells; mTOR

Introduction

Premature ovarian failure (POF) is characterized by hypoestrogenism, amenorrhea, and elevated gonadotropin levels in women under the age of 40 [1]. POF occurs in approximately 1–3% of women in the general population and causes of amenorrhea, infertility, estrogen deficiency, reduced follicles, and hypergonadotropic hypogonadism. The etiology of POF is complex and needs further elucidation, but it has been reported to be associated with autoimmune reaction, infection, genetic factors, and endocrine dysfunction as well as chemotherapy and radiotherapy [2]. The effects of chemotherapy on the ovaries can be assessed through the measurement of the markers including serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), and anti-Mullerian hormone (AMH) [3,4]. Recent advances in cancer treatment have increased the survival rate of both adult and pediatric patients. However, the adverse effects of anti-tumor therapy on female fertility remain to be resolved. Therefore, we need to find new strategies to restore ovarian function in patients with POF caused by chemotherapy.

Myeloid-derived suppressor cells (MDSCs) are usually composed of heterogeneous populations of cells that are precursors of dendritic cells, macrophages, and granulocytes. MDSCs inhibit the proliferation and function of T cells, block the cytotoxicity of natural killer (NK) cells, and promote the development of Tregs in tumor-bearing hosts [5,6]. The cell population could exhibit potent immunosuppressive capacity by secreting immune suppressive factors such as inducible nitric oxide synthase (iNOS) and arginase-1 (Arg-1) both *in vitro* and *in vivo* [7,8]. Human MDSCs are characterized by the expression of HLA-$DR^{-}CD11b^{+}CD33^{+}$ cell markers, while murine MDSCs are characterized by the expression of CD11b and Gr-1 cell markers [9]. MDSCs do not only have an immunosuppressive effect in cancer, as increasing evidence suggests that MDSCs also play key roles in acute inflammatory responses and tissue damage, such as ischemia-induced acute renal injury, asthma-related airway inflammation, LPS-induced acute lung injury [10], and immunological hepatic injury [11]. Although much has been revealed about the immunosuppressive function of MDSCs in various diseases, less is known about ovarian MDSCs, especially regarding the role of MDSCs in POF.

Recent research has shown that blocking the mTOR signaling pathway of MDSCs can prevent immune-mediated liver injury through an HIF1a-dependent glycolytic pathway [12,13]. However, the role of mTOR in the regulation of MDSCs or POF has not yet been investigated. Therefore, the purpose of this study was to investigate the role of MDSCs in cyclophosphamide-induced POF and the regulatory role of mTOR in this process.

Materials and methods

Animal model

All animal experiments were carried out in accordance with the approval of the animal ethics committee of Nanjing Medical University (Nanjing, China, ethics committee approval number: K2014001). C57BL/6 mice were obtained from the Suzhou University Experimental Animal Center (Suzhou, China). Six- to eight-week-old female mice were weighed and injected once daily with cyclophosphamide (75 mg/kg, $n = 12$) or an equal volume of saline solution (vehicle) for the control group ($n = 12$). A mouse POF model was established after

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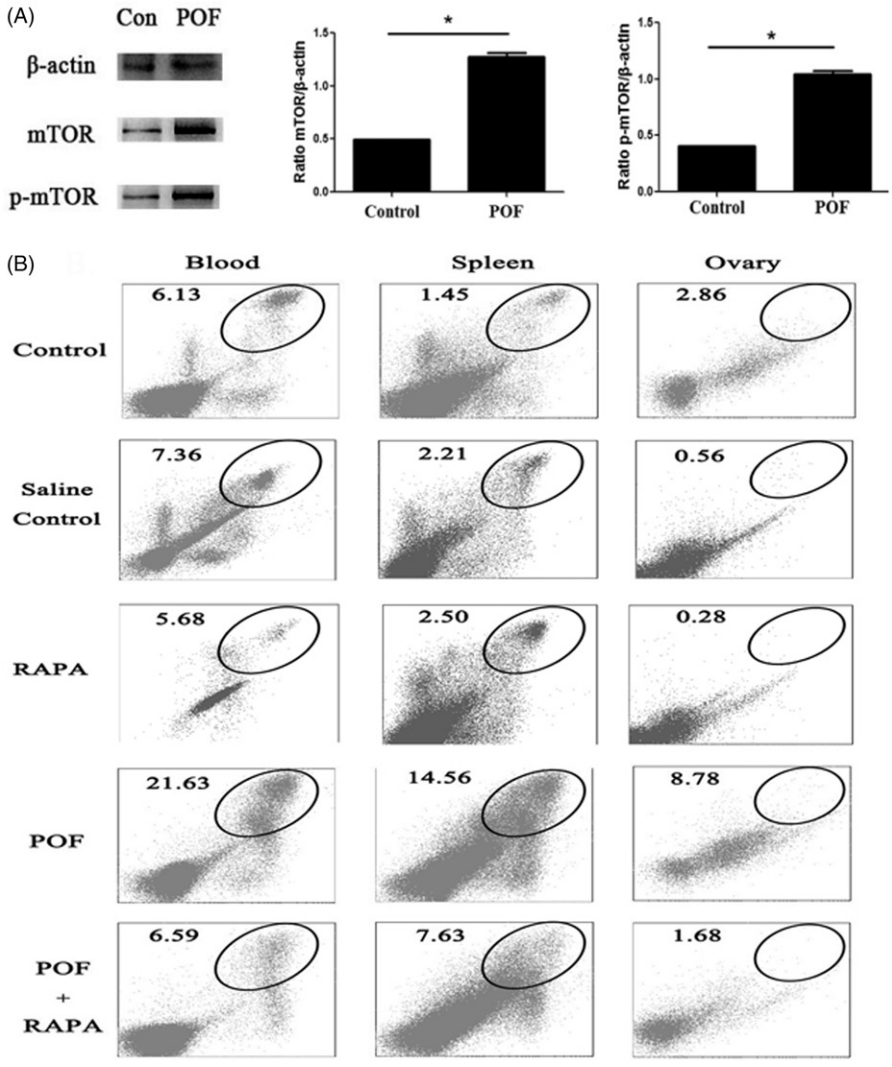

Figure 1. mTOR is required for the aggregation of MDSCs in the ovary. POF mice were induced by intravenous injection of cyclophosphamide. Extraction of total protein from the ovaries of POF or control mice and the phosphorylated mTOR and mTOR expressions were determined by Western blotting. Relative quantification of the protein was carried out by densitometry (A), $^{*}p < .05$. The experimental groups were divided into control group, saline solvent control group, POF group, POF + RAPA group, and RAPA group ($n = 8$). Peripheral blood, spleens, and ovaries of mice were obtained and the distribution of MDSCs in various tissues and organs was detected by flow cytometry (B).

14 days of cyclophosphamide injection [14,15]. In Figure 1(B), the experimental groups were divided into control group (without any treatment), saline solvent control group, POF group (injection of cyclophosphamide), POF + RAPA group (pretreatment with RAPA for seven days followed by injection with RAPA + cyclophosphamide for 14 days) and RAPA group. The RAPA group received rapamycin (Re-Bio, Hangzhou, China) at a dose of 2 mg/kg for 21 days.

Flow cytometry

Blood, spleen, bone marrow, and ovary cells were prepared as single cell suspension and incubated with fluorescein-conjugated antibodies (CD11b-FITC, Gr-1-APC, HLA-DR-FITC, CD33-PE, and CD11b-APC) (Biolegend, San Diego, CA) for 20 min at 4 °C. The cells were washed twice with PBS. Cells were acquired with a flow cytometer (Navios, Beckman Coulter, Brea, CA) and data were analyzed using Kaluza software (Beckman Coulter, Brea, CA).

Immunofluorescence staining and ELISA

Tissue sections were incubated for 1 h in 1% BSA and then incubated overnight at 4 °C with the mouse CD11b-FITC (Biolegend, San Diego, CA) antibody. Finally, the tissue sections were rinsed twice in PBS and observed and photographed with a fluorescence microscope (Olympus, Tokyo, Japan). The contents of FSH, LH, E2, and AMH in serum of each group were determined by an ELISA kit (Gu Yan, Shanghai, China).

Q-PCR analysis

Total mRNA was extracted from the peripheral blood and spleen and bone marrow tissue using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and reverse-transcribed into cDNA (Bioer Technology, Hangzhou, China). Real-time PCR amplification was carried out in the presence of 2 µl of cDNA template and 12.5 µl of SYBR master mixture (Bioer Technology, Hangzhou, China). The expression of target genes was determined using

the $2^{-\triangle\triangle CT}$ method. The primers used were as follows: GAPDH (forward 5′-CGGTGCTGAGTATGTCGT-3′, reverse: 5′-CTTCTGGGTGGCAGTGAT-3′), Arg-1 (forward 5′-ATTGG CAAGGTGATGG-3′, reverse: 5′-AGTCCCTGGCTTATGG-3′), and iNOS (forward 5′-CAGGCAACCAGACC-3′, reverse: 5′-GGCTTCAAGATAGGGA-3′).

Western blotting

Lyses of mouse ovarian tissues were collected by a protein extraction kit (Beyotime, Nanjing, China). The protein concentration was measured with a BCA protein assay kit (Beyotime, Nanjing, China). Ovarian samples were separated in 8% SDS-denatured polyacrylamide gel and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% skim milk at RT for 2 h. Specific primary antibodies (1:1000) targeted against mTOR (7C10) and p-mTOR (Ser2448) (CST, Danvers, MA) were used. HRP-conjugated secondary antibodies and ECL HRP chemiluminescent substrate reagent kit were obtained from Invitrogen (Carlsbad, CA).

Statistical analysis

Each experiment was performed at least three times. The Mann–Whitney *U*-test and Student's *t* test were used for non-parametric and parametric values, respectively. One-way analysis of variance (ANOVA) was used to determine significant differences among the groups (SPSS, GraphPad Prism version 5.0, La Jolla, CA). A p value of $<.05$ was considered statistically significant.

Results

Weight loss and ovarian damage after injection of cyclophosphamide in mice

To explore whether cyclophosphamide can induce POF effectively, C56BL/6 mice were divided into a vehicle-treated group and a cyclophosphamide-treated group. Weight loss, spleen weight, and ovarian weight was reduced in cyclophosphamide-treated mice compared with vehicle-treated mice (Figure 2(A) and Table 1), and the volume of these tissues also decreased after cyclophosphamide treatment (Figure 2(B)). In cyclophosphamide-treated mice, ovarian atrophy was observed with H&E staining. Follicle structure was destroyed, the numbers of primordial and mature follicles were reduced, while the number of corpora luteal decreased (Figure 2(C)). Compared with the control group, the serum level of FSH was significantly increased ($p< .05$), while the serum levels of AMH and E2 were significantly decreased in the cyclophosphamide-treated group ($p< .05$) (Table 1).

Cyclophosphamide induces MDSC aggregation in various organs and tissues

Cyclophosphamide is used clinically as a chemotherapeutic agent because of its strong immunosuppressive effect. In order to explore the relationship between POF and MDSCs, we defined MDSCs as CD11b+, Gr-1+ cell population and observed MDSCs in peripheral blood, spleen, and ovary of mice. The proportion of MDSCs increased in peripheral blood, spleen, and ovarian tissues of cyclophosphamide-treated mice (Figure 2(D)). After CD11b-FITC immunofluorescence staining, the number of CD11b + cells in cyclophosphamide-treated mice increased significantly (Figure 2(E)). Interestingly, CD11b + cells were mostly distributed around follicles and corpora luteal. This may indicate that cells of this group play an important role in the development of follicles. Finally, we examined the expression of functional cytokines of MDSCs, such as Arg-1 and NOS2, in peripheral blood, spleen, and bone marrow. These cytokines were up-regulated in the spleen and bone marrow (Figure 2(F)). Arg-1 and NOS2 can inhibit the function of T cells by decomposing arginine in the micro-environment.

mTOR is associated with the aggregation of MDSCs in the ovary

Recent studies have found a close relationship between mTOR and ovarian lifespan. To investigate the function of mTOR in $CD11b^{+}Gr1^{+}$ cells, we monitored mTOR activity of ovarian tissue in mouse POF models. Levels of mTOR and p-mTOR in ovarian tissue increased in cyclophosphamide-treated mice (Figure 1(A)). This finding suggests that mTOR activity might be involved in MDSC aggregation in cyclophosphamide-induced ovarian injury.

mTOR causes MDSCs aggregation in the ovary

As cyclophosphamide treatment results in abnormal accumulation of MDSCs and increased levels of both mTOR and p-mTOR, we attempted to block the above effects using rapamycin, a specific mTORC1 inhibitor. We first pretreated mice with rapamycin for seven days following administered to mice with intraperitoneal injections of both cyclophosphamide and rapamycin for 14 days. Treatment with rapamycin caused a reduction of MDSCs in peripheral blood, spleen, and ovaries (Figure 1(B)), which indicates that mTOR can mediate MDSC aggregation in mouse ovaries.

Discussion

Common side effects of chemotherapy on female survivors include early menopause, ovarian damage, and infertility. The etiology of POF is complex and there is currently no effective method for treating POF [16]. Stem cell transplantation for the treatment of POF is mainly limited to preliminary animal experiments, and few clinical studies have been reported [17,18]. Therefore, it is currently impossible to determine the safety and effectiveness of stem cell therapy. One of the problems is that it is difficult to explain the cause of POF. Cyclophosphamide is a commonly used chemotherapeutic agent in cancer treatment and inhibits tumor cell division by inducing cross-linking between intra- and inter-strand DNA. In particular, cyclophosphamide causes severe ovarian damage, including ovarian atrophy as well as the destruction of growing follicles and thus a reduction in follicle number [19].

Research on MDSCs is mainly focused on their ability to exert immunosuppression in tumor microenvironments [20–22]. Zöller et al. [23] have demonstrated that MDSC exosomes have important immunomodulatory effects in a mouse model of autoimmune alopecia areata. It has also been demonstrated that MDSCs can prevent immune-mediated liver injury via an HIF1a-dependent glycolytic pathway [12] and that neonatal infection of *Escherichia coli* reduces the immunosuppressive activity of MDSCs [24]. These studies indicate that MDSCs play important roles in the regulation of autoimmune diseases and acute inflammation. Our study has demonstrated for the first time that the ovaries of cyclophosphamide-treated mice exhibit

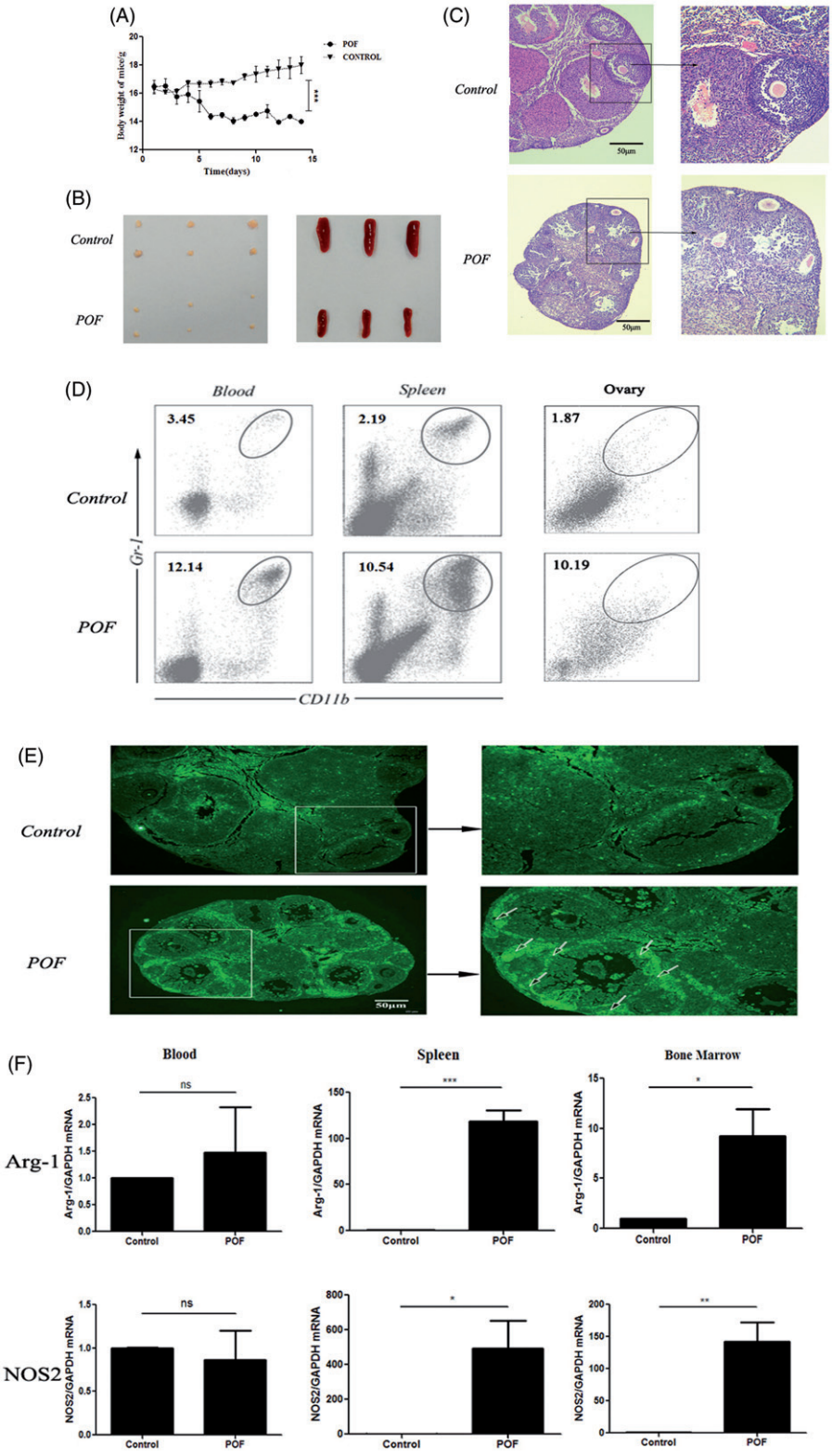

Figure 2. Cyclophosphamide induces MDSC aggregation in various organ tissues. Mice were injected with cyclophosphamide for 14 days and weighed daily. The change of weight in control ($n = 12$) and POF mice ($n = 12$) (A). Representative images of the spleen and ovaries of control and POF mice (B). Ovarian sections of control and POF mice were stained with H&E (C). Peripheral blood, spleen, and ovarian single cell suspension was prepared from POF and control mice. Flow analysis after incubation with CD11b, Gr-1 antibodies (D). Representative images of histological sections of ovaries from POF and control mice that were stained with CD11b-FITC immunofluorescence. Arrows indicate positive cells (E). Quantitative PCR was used to detect the relative expression of Arg-1 and NOS2 in peripheral blood, spleen, and bone marrow (F). $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

Table 1. FSH, LH, E2, AMH levels of POF and control groups.

	Control	POF	*p*
Spleen (g), mean ± SD	0.101 ± 0.048 ($n = 12$)	0.031 ± 0.007 ($n = 12$)	<.0001***
Ovary (g), mean ± SD	0.0031 ± 0013($n = 12$)	0.0016 ± 0.0010 (n = 12)	<.0001***
FSH (U/L), median (min–max)	10.42 (5.31–11.29)	12.00 (10.52–14.38)	.04*
LH (pg/ml), median (min–max)	1818.56 (1263.88–2328.88)	2115.88 (1743.13–2488.63)	N.S.
E2 (pmol/L), median (min–max)	175.57 (168.56–190.71)	112.03 (73.60–123.79)	.002**
AMH (pg/ml), median (min–max)	150.27 (136.46–178.28)	124.69 (92.28–149.88)	.04*

FSH: follicle-stimulating hormone; LH: luteinizing hormone; E2: estradiol; AMH: anti-Mullerian hormone.
Spleen and ovary weight changes in POF group and control group.
POF group ($n = 6$), control group ($n = 6$).
*$p < .05$.
**$p < .001$.
***$p < .0001$.

an increased proportion of MDSCs, which are aggregated around follicles. These results indicate that MDSCs may be involved in cyclophosphamide-induced ovarian primordial follicle over-consumption and luteal production.

Since the mTOR signal controls the response of cells to nutrient supply, this pathway is important for regulating cell life span [25]. However, whether mTOR signaling affects the aggregation of MDSCs remains largely unexplored. One study shows that MDSC accumulation correlates with the MAPK and AKT-mTOR pathways, which indicate that the mTOR pathway may be the driver of MDSC accumulation [26]. Treatment with rapamycin (a mTOR inhibitor) can reduce MDSCs in P53N-C tumor-bearing mice [27]. Our results have also demonstrated increased mTOR expression in mouse ovaries containing aggregated MDSCs. A multi-centric study by the National Institute on Aging indicated that the use of rapamycin in young or old mice (600 days) can extend life span [27]. Another report indicated that short-term rapamycin treatment can increase ovarian longevity both in young and middle-aged female mice [28]. It has been suggested that mTORC1 inhibition is effective in treating age-related diseases, even when treatment starts in middle-aged subjects. Recent studies using specific knock out of TSC1 or TSC2 (suppressor genes of mTOR) in oocyte and granulosa cells have demonstrated the importance of the mTOR signaling pathway in regulating ovarian follicle development [29]. Continuous activation of mTOR in germ cells leads to the recruitment of primordial follicles, leading to POF and infertility [30]. A study of the dynamic changes in p-rpS6 in neonatal ovaries demonstrated the role of the mTOR signaling pathway in follicular formation. When ovaries begin follicle assembly, basal levels of p-rpS6 are detected in the germ cells undergoing cyst destruction, and levels are higher in the somatic cells surrounding the oocyte. Once the formation of primordial follicles is complete, the expression of p-rpS6 is lost in oocytes. Significant expression of p-rpS6 in oocytes was found only in the center of the ovary, where follicles are activated, indicating that mTORC1 is inhibited in the inactivated follicles [31]. In contrast, Rictor (a key component of mTORC2) conditional knockout mice also exhibited a POF phenotype, including a large number of follicular death, excessive loss of functional follicles, abnormal gonadotropin secretion, and subsequent secondary infertility [29]. Our study suggests that POF is caused by mTORC1 activation leading to excessive consumption of primordial follicles and that MDSCs may be involved in this process. However, the role of mTOR in the aggregation of MDSCs remains to be further elucidated.

Our results may provide novel insights toward the etiology of POF by indicating the potential role of mTOR in the abnormal accumulation of MDSCs in the pathological processes of POF, but the underlying mechanisms still need further investigation. Besides, although the present study was only conducted in the mouse models, we speculate that our results can also have clinical relevance in POF patients because of the structural and functional similarities between human and mouse ovaries. Hence, upon the approval of the ethics committee, we will further explore our study using human peripheral blood samples which are grouped by age, treatment period, treatment dosage, and ovarian reserve markers of POF patients before and after cyclophosphamide therapy. Hopefully, we can provide new understanding into the role of MDSCs in POF.

In conclusion, POF mouse models in this study exhibited an increased percentage of MDSCs and an upregulation of mTOR1 expression in ovaries. This effect was reversed by rapamycin, which suggests that rapamycin may play a protective role in POF by down-regulating MDSCs. Although further research is needed to confirm the effectiveness of rapamycin in humans, treatment with mTORC1 inhibitors prior to cyclophosphamide treatment and subsequent oral administration may be a promising strategy to preserve fertility in women with cancer whose reproductive function is threatened. Our results demonstrate that the roles of MDSCs and mTOR1 warrant further research to better understand the molecular mechanisms of POF.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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