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Progesterone affects clinic oocyte yields by coordinating with follicle stimulating hormone via PI3K/AKT and MAPK pathways

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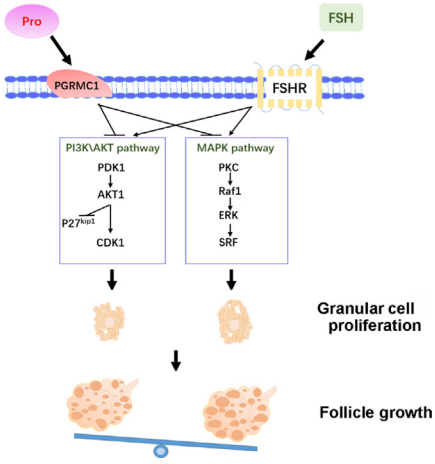
HIGHLIGHTS

- Progesterone reduced oocyte yields in clinic. Yields were rescued by the higher dose of hMG.
- Progesterone downregulated follicle growth and consequently reduced oocyte yields.
- Progesterone inhibited granular cell proliferation via MAPK and PI3K/AKT pathways.
- Progesterone and FSH coordinated follicle growth via signalling crosstalk in granular cells.

GRAPHICAL ABSTRACT

The schematic diagram. Schematic illustration of the signaling cascades by which progesterone coordinates with FSH to regulate the proliferation of granular cells to slow down follicle growth during ovarian stimulation by PI3K/AKT and MAPK pathways.



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ABSTRACT

Introduction: As an effective inhibitor of premature ovulation, progestin was introduced to a novel ovarian stimulation regimen for infertility treatment. However, the local action of progestin on the ovary and its effect on clinical outcomes have not been described.

Objectives: The influence of progesterone administration on clinical oocyte outcomes and the mechanisms involved in the coordination of progesterone and follicle stimulating hormone (FSH) on follicle growth and oocyte yields were investigated.

Methods: Clinical outcomes of patients undergoing ovarian stimulation for *in vitro* fertilization were analyzed. The murine ovarian stimulation model and follicle culture system were used to evaluate the effects of progesterone on oocyte yield, follicle development, granular cell proliferation, and hormone secretion.

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Signal pathway

Phospho-specific protein microarrays were used to explore involved signaling pathways.

Results: Progesterone decreased clinical oocyte yields, and yields were rescued with an increased dose of human menopausal gonadotropin. Administration of progesterone inhibited murine granular cell proliferation and reduced the growth rate of follicles; both of which were rescued by FSH. The phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT) and mitogen-activated protein kinase (MAPK) were identified as pivotal signaling pathways to integrate progesterone into the FSH signaling network in granular cells.

Conclusion: Progesterone inhibited granular cell proliferation and antral follicle growth during ovarian stimulation, and subsequently influenced oocyte outcomes in the clinical setting. Progesterone coordinated with FSH to regulate follicle growth through PI3K/AKT and MAPK signaling pathways. These findings advance our knowledge regarding the ovarian response to gonadotropins during progestin-primed ovarian stimulation and create an opportunity to manipulate individual oocyte yields.

Introduction

An integral component of controlled ovarian hyperstimulation is the prevention of a premature surge of luteinizing hormone (LH) and ovulation; this is essential for the successful outcomes of *in vitro* fertilization (IVF) cycles for infertility treatment. Progestin-primed ovarian stimulation (PPOS) is a new stimulation regimen that uses progestin as an alternative for gonadotropin-releasing hormone (GnRH) analogs to prevent the LH surge [1]. Studies published by our lab and others have demonstrated that PPOS is an effective method to block premature ovulation and obtain clinical outcomes comparable to conventional ovarian stimulation regimes [2–4]. As an easy, flexible, safe, and patient-friendly protocol, it has been widely used for IVF [5–7].

However, in addition to the central inhibition of the LH surge by progesterone [8,9], several *in vivo* and *in vitro* studies have highlighted a local effect of progesterone on follicle growth in rodents [10], rabbits [11], and monkeys [12]. Progesterone was shown to suppress the growth of follicles during pregnancy or in progesterone-treated rabbits [11]. As well, the development of follicles in the monkey ovary following insertion of implanted progesterone was inhibited without altering serum gonadotropin levels, which allowed follicles to continue to grow in the contralateral control ovary [12]. However, in hamsters a high concentration of progesterone stimulated preantral follicles to ultimately grow to ovulation, whereas a low dose of progesterone reduced the number of antral follicles in the ovaries [10]. These studies reporting the effects of progesterone on follicle development in different species using various methods to administer the medication indicated that its effects are relative to the dose and species. Although the precise mechanism by which progesterone regulates the growth of follicles has not been fully elucidated, the actions of progesterone on granular cell function have been reported [13–16].

During ovarian stimulation, the gonadotropin follicle stimulating hormone (FSH) is usually applied in regimens to induce the recruitment of multiple follicles for rapid growth. FSH stimulates a non-linear and complex signaling network involving several G-protein subtypes and interactions with other receptors and proteins [17]. Interestingly, clinical data from our group and other investigators has revealed that the total amount of human menopausal gonadotropin (hMG) applied in the PPOS regimen is generally higher than the amount used in conventional protocols [2,3,5]. However, whether the increase in hMG during ovarian stimulation resulted from the administration of progesterone still needs to be confirmed. The local regulation by progesterone on follicle growth and oocyte yields during ovarian stimulation is not yet clear.

In the current study, the influence of progesterone treatment on clinical outcomes, as well as the signaling mechanisms associated with the coordination of progesterone and FSH on follicle growth and oocyte yields, were investigated.

Materials and methods

Additional details regarding the materials and methods used in this study are available in the Supplementary Materials.

Ethics statement

Approval for human retrospective analysis was obtained from the Institutional Ethics Committee of Shanghai Ninth People's Hospital (No. 2014-94). All participants provided informed consent after counseling for infertility treatments and routine IVF procedures. Animal studies were approved by the Institutional Ethics Committees of Care and Use of Experimental Animals of Shanghai Ninth People's Hospital.

Human subjects and stimulation protocol

A retrospective analysis was conducted based on the electronic medical records in the Department of Assisted Reproduction of the Ninth People's Hospital of Shanghai Jiaotong University School of Medicine from January 2015 to June 2019. The patients undergoing either a PPOS or GnRH antagonist protocol were selected based on the following criteria: age between 25 and 38 years old, body mass index (BMI) between 16 and 26 kg/m^2, antral follicle count (AFC) of more than three on menstrual cycle day 3, and basal serum FSH concentration of no more than 10 IU/L. Patients were excluded if they had a low ovary reserve (AFC < 3 on menstrual cycle day 3) or the presence of other coexisting diseases, such as polycystic ovary syndrome or history of an ovarian operation. In general, hMG (150–225 IU/d; Anhui Fengyuan Pharmaceutical Co. China) was applied to stimulate follicle growth and medroxyprogesterone acetate (MPA; 4–10 mg/d, Shanghai Xinyi Pharmaceutical Co. China) [2] or Cetrotide (0.125–0.25 mg/d, Merck Serono) was used in PPOS and GnRH antagonist protocols, respectively, to inhibit the LH surge. Follicular monitoring was performed every 2–4 days using a transvaginal ultrasound examination. The dose of hMG was adjusted according to follicle development. When dominant follicles reached $\geq$ 18 mm in diameter, final oocyte maturation was triggered. Hormone measurement, oocyte retrieval, observation of fertilization, cleavage and embryo development was conducted routinely, as previously described [2,18]. For comparison of follicle development and oocyte outcome during ovarian stimulation, patients between the two groups were matched according to baseline hormones: FSH (±20 IU/l), LH (±0.5 IU/l), estradiol (E2; ±20 pg/ml), progesterone (±0.1 ng/ml), AFC (±1), and accumulated hMG doses (±100 IU).

Animals, ovarian stimulation, H&E staining and morphological classification of follicles

Kunming (KM) female mice were maintained with a 12 h light/-dark cycle and given access to food and water *ad libitum*. The diagram of mouse ovarian stimulation is shown in Fig. 2A. In summary, KM mice aged 4–6 weeks were randomly assigned to experimental groups and injected with pregnant mare's serum gonadotropin (PMSG; 10 IU) during the diestrus stage, followed by human chorionic gonadotropin (hCG, 10 IU) to induce superovulation 48 h later. Progesterone (Sigma, USA) was injected at doses of 10 mg/kg [8] or 20 mg/kg at 17:00 for three consecutive days during superovulation in the PPOS group, and the control group was injected with corn oil. At 8:00 AM on day 4, cumulus-oocyte-complexes (COC) were isolated from the ampullae and transferred to human tubal fluid medium (HTF, Millipore) droplets. Total oocytes, including immature oocytes, were denuded and classified. The metaphase II (MII) oocytes were quantified. To measure follicle development, bilateral ovaries were isolated and imaged 48 h after PMSG injection. The length (mm) and width (mm) were measured for index quantification. Ovaries were serially sectioned at 5 μm, and one slice was collected from every six consecutive sections. Ovarian tissue sections were stained with hematoxylin and eosin (H&E) and imaged under an inverted microscope (Olympus, BX53). Follicles at different developmental stages were classified according to the criterion of Myers [19]. The number of corpora luteum was counted per section in each ovary [20].

Mouse follicle culture in vitro, imaging and hormone measurement

Mouse follicle culture was conducted as previously reported [21,22]. Briefly, secondary follicles with a diameter of 110–130 μm were isolated from 13-day-old KM female mice and cultured in 96 well plates (single follicle per well) with MEM Glutamax culture medium (a-MEM, 32571; Gibco) with 5% FBS (10108; Gibco), 10 mIU/mL FSH (Serono), 10 mIU/mL LH (Serono), and 5 μl/mL insulin, 5 μl/mL transferrin, and 5 μl/mL selenium (I1884; Sigma). These follicles were exposed to blank (control), propylene glycol (vehicle), or progesterone (either 2 μM or 4 μM) dissolved in propylene glycol. On day 4, 8, and 12, half of the medium was refreshed, and the follicle morphology was analyzed, and the diameters were measured using a microscope. On day 8, the culture medium from 10 follicles in each group was collected for hormone measurement using chemiluminescence (Abbott Biologicals B.V.). On day 12, follicles were stimulated with 1.5 IU/mL hCG (Serono) and 5 ng/mL epidermal growth factor (EGF; 53003; Gibco) at 4:00 PM. On day 13, the maturation of oocytes was assessed: those with polar body extrusion were identified as MII oocytes; those without a polar body but with germinal vesicles broken down were identified as GVBD oocytes; and those without a polar body but with germinal vesicles were identified as germinal-vesicle (GV) oocytes.

Phospho-specific protein microarray analysis

Granular cells of follicles with or without progesterone treatment on culture day 10 were collected for protein extraction. Phospho-array detection and data analysis were performed in collaboration with Wayen Biotechnology (Shanghai, China) using the system they set up [23]. Briefly, protein samples were labeled with biotin and hybridized to the Phosphorylation ProArray (Full Moon BioSystems, USA) using an Antibody Array Kit (Full Moon BioSystems, CA, USA). The antibody array was composed of 1318 antibodies and most of the antibodies were used to detect the phosphorylated form of the proteins and their unphosphorylated counterparts. Fluorescence intensity was scanned with a GenePix 4000B (Axon Instruments, Houston, TX, USA) using GenePix Pro 6.0. Raw data of were manipulated using Grubbs' method to exclude outliers. The phosphorylation ratio was calculated as follows: phosphorylation ratio = phospho value/nonphosphorylated value. Phosphoproteins that were upregulated or down regulated by more than 20% ($P < 0.05$) were included in the analysis. The key signaling pathways were further analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG).

CCk-8

Granulosa cell proliferation of follicles cultured in 96-well plates on day 8 and day 12 were detected using the Enhanced Cell Counting Kit-8 (CCK-8, C0041, Beyotime) in each experimental group. According to the manufacturer's instructions, granulosa cells were incubated with the enhanced CCK-8 solution for 6 h. Optical density (OD) was measured at 450 nM with a multifunctional enzyme marking instrument (SynergyH1, Biotek, Shanghai). Positive results indicated the number of living cells. In the 96-well plate, the corresponding culture medium and CCK-8 solution were used as the control.

Statistics

Statistical significance was performed using Student's *t*-test and the Mann-Whitney *U* test for normal and non-normal distributions, respectively. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used for comparisons between more than two groups. Proportions were compared using the Chi-square test when appropriate. Grubbs' method was used to exclude outliers in data analysis of protein microarray. The significance level was set at $P < 0.05$.

Results

Progestin-primed ovarian stimulation induced an hMG dose-associated reduction in the number of retrieved oocytes

Progestin-primed ovarian stimulation or GnRH-antagonist (control group) protocols were applied to patients undergoing ovarian stimulation (Fig. 1A). A total of 1200 cycles from 1200 women, 505 cycles in the control group and 695 cycles in the PPOS group, were enrolled. There were no significant differences in the baseline characteristics between the two groups, including age, BMI, AFC, and levels of FSH, LH, E2, and progesterone on day 3 (Table 1). Previous studies reported that patients receiving PPOS had comparative oocyte yields with the higher hMG consumption than the conventional stimulation regimens [2,24]. In the current study, the accumulated hMG doses were similar between the two groups (Fig. 1B), but the PPOS regimen significantly reduced the number of follicles with diameters larger than 10 mm on trigger day, and consequently reduced the number of retrieved oocytes and MII oocytes compared with the control protocol (follicles: 11.7 ± 0.3 *vs* 11.0 ± 0.2, $P < 0.05$; retrieved oocytes: 10.6 ± 0.3 *vs* 9.1 ± 0.2, $P < 0.001$; MII oocytes: 8.7 ± 0.2 *vs* 7.5 ± 0.2, $P < 0.001$, Fig. 1C). Furthermore, the two groups had similar rates of normal fertilization, cleavage, and viable embryos per oocyte retrieved ($P > 0.05$, Fig. 1D). Collectively, these data indicated that PPOS compromised oocyte yields in stimulation cycles if identical doses of hMG were used.

To determine whether this effect was associated with the dose of hMG, patients were grouped according to accumulated hMG dose (low: 1000–1500 IU; middle: 1500–2000 IU; high: 2000–2500 IU) and no differences were observed in basic characteristics between these subgroups (Supplementary Table S1). Indeed, the

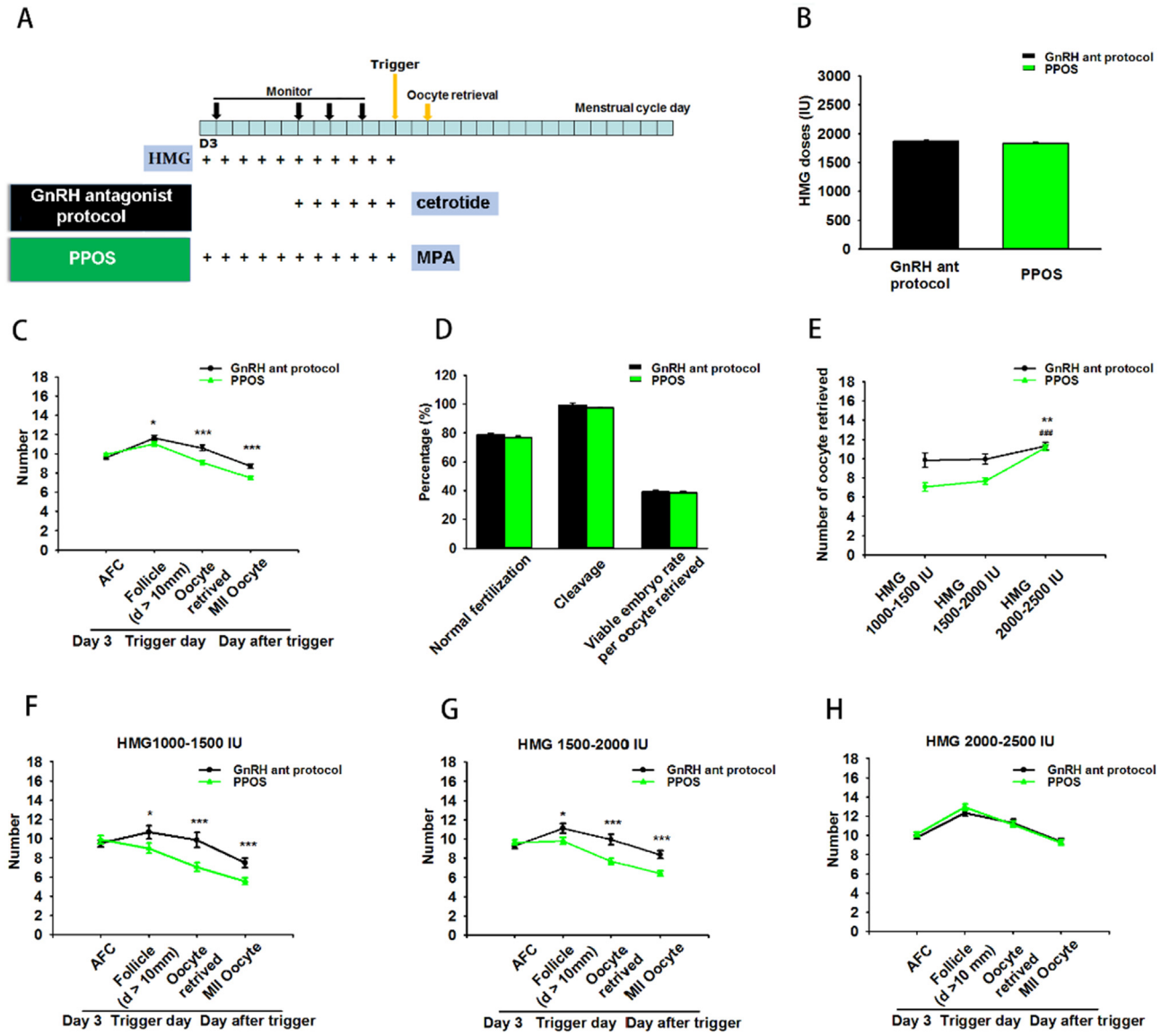

Fig. 1. Human menopausal gonadotropin (hMG) dose associated decrease in oocyte yields in PPOS compared with GnRH antagonist protocol. (A) Procedures of progesterone primed ovarian stimulation (PPOS) protocol and GnRH antagonist protocol. (B) Accumulated HMG doses used in GnRH antagonist protocol (505 cycles) and PPOS (695 cycles). (C) Quantitative analysis of antral follicle count (AFC) on day 3, developing follicles above 10 mm in diameter on trigger day, the total oocytes and the MII oocytes retrieved on the day after trigger in GnRH antagonist protocol and PPOS groups. $^{***}P < 0.001$, $^{*}P < 0.05$. (D) Percentage of normal fertilization, cleavage rate and viable embryo rate per retrieved oocyte were compared between GnRH antagonist protocol and PPOS groups. (E) Quantitative analysis of the retrieved oocytes exposed to high (2000–2500 IU), middle (1500–2000 IU) and low (1000–1500 IU) doses of HMG in GnRH antagonist protocol and PPOS. $^{**}P < 0.01$ in GnRH antagonist protocol, $^{###}P < 0.001$ in PPOS. (F-H), Quantitative analysis of AFC, developing follicles above 10 mm in diameter on trigger day, the total oocytes and the MII oocytes retrieved on the day after trigger in subgroups of 1000–1500 IU HMG (F), 1500–2000 IU HMG (G) and 2000–2500 IU HMG (H) in GnRH antagonist protocol and PPOS. $^{***}P < 0.001$, $^{*}P < 0.05$. Values in (B-H) are represented as mean ± standard error of the mean (SEM).

numbers of retrieved oocytes increased with increasing doses of hMG, both in the PPOS protocol and the control protocol ($P < 0.001$ and $P < 0.01$ respectively, Fig. 1E). Furthermore, in the low (1000–1500 IU) and middle (1500–2000 IU) dose subgroups, PPOS led to a significant reduction in the number of greater than 10 mm diameter follicles, retrieved oocytes, and MII oocytes, compared with the control subgroups (low dose subgroup: follicles: 10.7 ± 0.4 *vs* 9.0 ± 0.4, $P < 0.05$; retrieved oocytes: 9.8 ± 0.8 *vs* 7.0 ± 0.4, $P < 0.001$; MII oocytes: 7.5 ± 0.5 *vs* 5.5 ± 0.3, $P < 0.001$; middle dose subgroup: follicles: 11.1 ± 0.5 *vs* 9.8 ± 0.4, $P < 0.5$; retrieved oocytes: 9.9 ± 0.5 *vs* 7.7 ± 0.3, $P < 0.001$; MII oocytes: 8.4 ± 0.4 *vs* 6.4 ± 0.3, $P < 0.001$, Fig. 1, F and G). However, there was no significant difference in oocyte yields between high hMG dose subgroups, including the number of 10 mm diameter follicles (12.3 ± 0.3 *vs* 12.9 ± 0.3, $P = 0.17$, Fig. 1H), retrieved oocytes (11.3 ± 0.4 *vs* 11.1 ± 0.3, $P = 0.96$, Fig. 1H), and MII oocytes (9.3 ± 0.3 *vs* 9.2 ± 0.3, $P = 0.92$, Fig. 1H). In addition, no difference was observed with regards to the levels of E2 and progesterone between the subgroups for all doses ($P > 0.5$, Supplementary Fig. S1). These data indicate that the reduction in oocyte yields with the PPOS protocol was dependent on the dose of hMG.

Progesterone reduced the number of retrieved oocytes by slowing down follicle growth in mouse ovaries

To test whether exposure to progesterone suppresses follicle growth and therefore compromises oocyte yields, female mice were used to mimic a clinical scenario (Fig. 2A). Doses of 10 mg/

Table 1
The characteristics of women treated with the PPOS protocol compared with the GnRH antagonist protocol.

	GnRH ant protocol	PPOS	*p*
Cycles (n)	383	247	
Age (years)	33.5 ± 3.1	33.6 ± 2.8	0.60
BMI (kg/m^2)	20.8 ± 2.1	20.7 ± 2.0	0.66
Antral follicle count	9.6 ± 3.3	9.9 ± 4.0	0.15
Baseline hormone (Day 3)			
FSH (IU/l)	5.9 ± 1.3	6.1 ± 1.5	0.05
LH (IU/l)	3.4 ± 1.6	3.5 ± 1.4	0.64
E_2 (pg/ml)	38.5 ± 16.7	38.6 ± 20.2	0.91
Progesterone (ng/ml)	0.3 ± 0.2	0.3 ± 0.1	0.79

Note: The data are presented as the means ± SD. PPOS, progesterone primed ovarian stimulation; GnRH ant protocol, GnRH antagonist protocol; BMI, body mass index; FSH, follicle stimulation hormone; LH, luteinizing hormone; E_2, estrogen.

kg and 20 mg/kg of progesterone were found to significantly reduce the number of retrieved oocytes (34.4 ± 2.9 *vs* 25.2 ± 2.9 *vs* 12.3 ± 3.8, respectively; $P < 0.05$ and $P < 0.001$ compared with the control group, Fig. 2B). Even though there was no difference in ovary size of the animals receiving the two doses of progesterone (Fig. 2, C and D), ovarian histology revealed that the proportion of antral follicles significantly declined in mice exposed to a high dose of progesterone (8.5 ± 1.9 *vs* 5.0 ± 1.5, $P < 0.001$, Fig. 2, E and F). Furthermore, the proportion of preovulatory follicles decreased in both groups treated with progesterone (3.2 ± 1.8 *vs* 1.7 ± 1.0 *vs* 1.6 ± 0.7 respectively, $P < 0.05$, Fig. 2G), while the proportion of corpus luteum per ovarian section were similar when compared across the three groups (Fig. 2H). These findings indicate that the administration of progesterone may reduce the growth rate of follicles driven by gonadotropin, thus leading to a reduction in the number of preovulatory follicles and thus, a reduction in the final number of oocytes retrieved.

Suppression of progesterone on mouse follicle development and antral-like cavity formation in vitro

To evaluate the specific effects of progesterone on follicular development, an *in vitro* follicle culture system was established that allowed the growth of early pre-antral follicles up to the ovulatory stage [22] (Fig. 3A). Follicles exposed to 2 μM and 4 μM of progesterone showed expansion with larger follicle diameters compared to those of the control and vehicle groups on day 4 in culture (Fig. 3, B and D). However, compared with the control group, follicle growth was markedly inhibited by 2 μM progesterone on day 12 (diameter: 693.9 ± 122.1 *vs* 635.4 ± 121.2, $P < 0.001$, Fig. 3D), and 4 μM progesterone on day 8 and 12, thus inducing a significant reduction in follicle diameter (day 8: 426.8 ± 127.2 *vs* 365.6 ± 110.0, $P < 0.001$; day 12: 693.9 ± 122.1 *vs* 519.4 ± 142.8, $P < 0.001$, Fig. 3D). Furthermore, the percentage of follicles with antral-like cavities also declined after treatment with 4 μM progesterone on day 8 (55.6% *vs* 24.7%, $P < 0.05$) and day 12 (99.4% *vs* 72.6%, $P < 0.01$, Fig. 3E). Morphological analysis revealed that the progesterone-treated follicles exhibited small antral-like cavities and a few granular cells, indicating a defective development process (Fig. 3, B and C). It was also found that 4 μM progesterone induced a significant reduction of E2 levels (73.6 ± 16.0 *vs* 36.3 ± 11.8, $P < 0.01$, Fig. 3F), but not testosterone levels (Fig. 3H). The concentration of progesterone remained high in the treatment groups accordingly (Fig. 3G). There were no significant differences in the relative mRNA levels of steroidogenic proteins, such as *cyp11a1*, *cyp17a1*, *cyp19a1*, and *StAR* (Fig. 3I). In addition, there were no significant changes in terms of nuclear maturation in oocytes derived from preovulatory follicles exposed to progesterone treatment (Fig. 3J).

FSH rescued the progesterone-induced inhibition of granular cell proliferation in growing follicles

It was then investigated whether FSH could rescue the effect of progesterone on follicular development. We found that both 20 mIU/mL and 40 mIU/mL FSH could fully rescue the reduction in follicle diameters induced by progesterone treatment on day 8 and day 12 ($P < 0.001$, Fig. 4, A and B). In comparison to the control group, there was a significant increase in the follicle diameters in the group treated with 20 mIU/ml FSH on day 8 ($P < 0.01$), whereas a decrease in 40 mIU/ml of FSH treated group on day 12 ($P < 0.01$), indicating a dose relative recovery. Furthermore, the proportion of follicles with antral-like cavity formation also increased to a level similar to that in the control group after treatment with 20 mIU/mL or 40 mIU /mL of FSH (day 8: 58.2 ± 14.1 *vs* 70.8 ± 0.1 *vs* 56.5 ± 3.7, $P > 0.5$; day 12: 98.4 ± 1.6 *vs* 96.3 ± 5.2 *vs* 96.4 ± 1.4, $P > 0.5$, Fig. 4, A and C). Next, cell proliferation in the growing follicles was examined using a CCK-8 assay. These results revealed that progesterone significantly reduced the proliferative ability of granular cells (day 8: 0.50 ± 0.25 *vs* 0.39 ± 0.24, $P < 0.01$; day 12: 0.60 ± 0.23 *vs* 0.49 ± 0.20, $P < 0.01$, Fig. 4D) and this effect could be rescued by 20 mIU/mL of FSH (day 8: 0.45 ± 0.21; day 12: 0.63 ± 0.22, Fig. 4D). These results indicated that progesterone and FSH most likely coordinate during follicle development via the regulation of granular cell proliferation.

PI3K/AKT and MAPK pathways mediated crosstalk between progesterone and FSH in the granular cells in growing follicles

To understand the mechanisms that mediate the effects observed in the previous experiments, granular cells were selected from growing follicles exposed to progesterone and a phosphoantibody array was performed to identify protein phosphorylation events at specific sites and downstream effectors that modulate follicular growth (Fig. 5A). When compared with the control granular cells, progesterone treatment was associated with 104 proteins whose phosphorylation levels were increased more than 20%, and 97 proteins whose phosphorylation levels were reduced by more than 20% (Fig. 5A). Of these proteins with altered phosphorylation levels, a total of 36 and 19 proteins were enriched in the PI3K-AKT and MAPK signaling pathways, respectively, as determined by the KEGG analysis (Fig. 5B). Both these pathways play a pivotal role in the proliferation and survival of granular cells and are known to be activated by FSH [25–28].

Next, the established signals associated with the PI3K/AKT and MAPK signaling pathways were analyzed using the phosphoantibody array data. Among the altered signals in the PI3K/AKT pathway, the activity of pyruvate dehydrogenase kinase 1 (PDK1) and its substrate AKT, which are closely linked to the cell cycle [29], were significantly suppressed. Furthermore, there was an increase in the phosphorylation of the downstream signal $p27^{kip1}$ (Fig. 5C). On the other hand, the molecular cascades associated with the MAPK pathway, protein kinase C (PKC) alpha, Raf1, and its substrate MEK1, were all downregulated in response to progesterone. Moreover, phosphorylation of the downstream targets serum response factor (SRF) and Myc were also significantly reduced. The phosphorylation of these two targets is known to play a positive role in cell proliferation (Fig. 5D). Results were also validated by western blotting. The most well-characterized targets of PI3K/AKT and MAPK signaling pathways, p-AKT and p-Erk, were found to be suppressed in granular cells following progesterone treatment but were rescued by a higher dose of FSH (Fig. 5, E and F). Collectively, these results indicated that the PI3K/AKT and MAPK pathways might mediate the crosstalk between progesterone and FSH during the regulation of granular cell function in growing follicles.

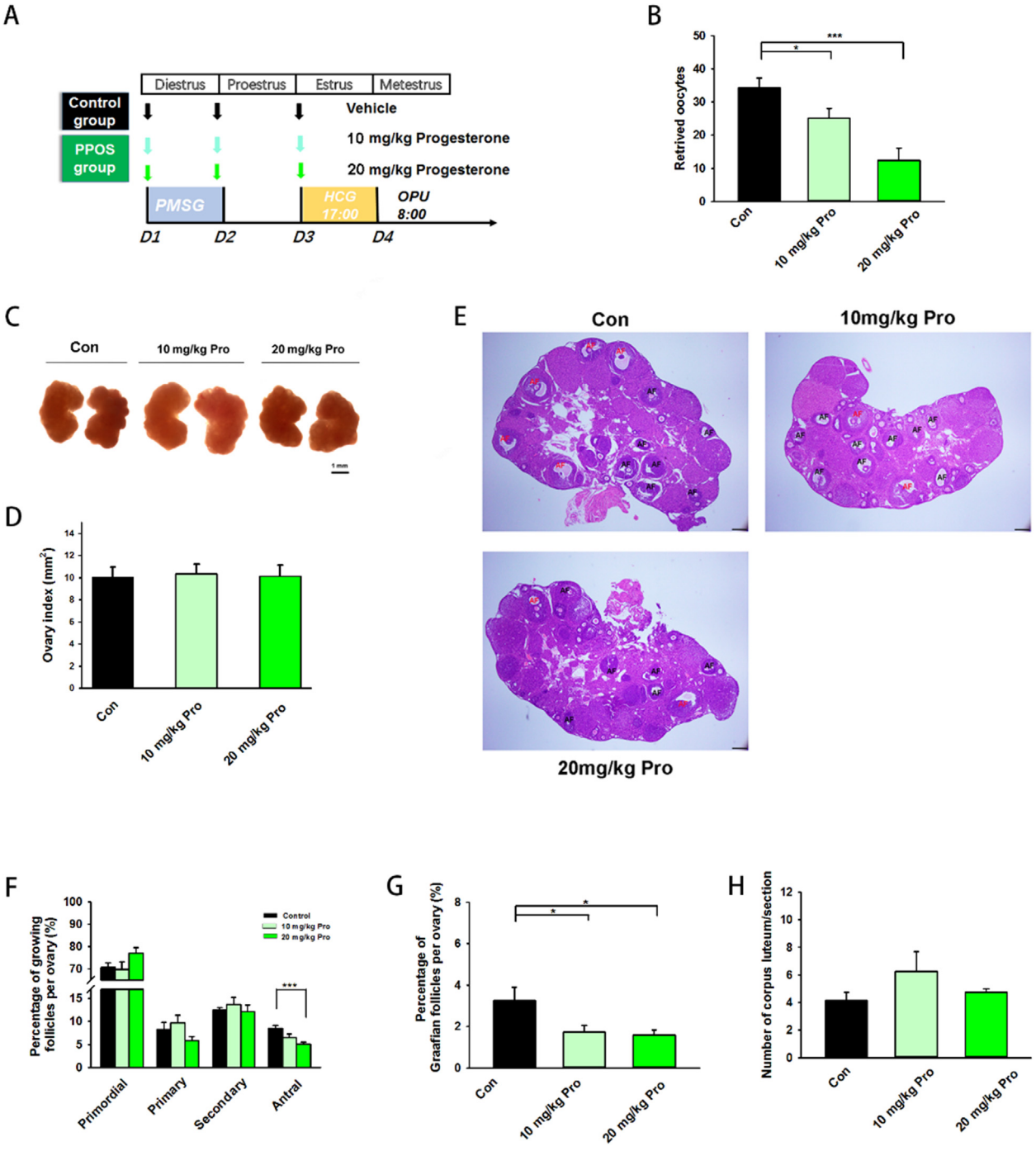

Fig. 2. Progesterone slows down follicle development during mouse ovary stimulation. (A) Schematic of experimental design: vehicle (control group) or two doses of progesterone (PPOS group) were administered. (B) Quantitative analysis of retrieved oocytes in control and progesterone treated groups (Number of mice: control, n = 13; 10 mg/kg progesterone, n = 16; 20 mg/kg progesterone, n = 17). (C) Representative ovary morphology subjected to ovary stimulation. Scale bar: 1 mm. (D) Ovary sizes were quantified by ovary index (width X length, mm) (Number of ovaries: control, n = 14; 10 mg/kg progesterone, n = 14; 20 mg/kg progesterone, n = 12). (E) Representative photomicrographs of ovaries stained with hematoxylin-eosin from control and progesterone treatment groups. Scale bar: 200 μm. AF in black: antral follicle. AF in red: Graafian follicle. (F-H), Quantitative analysis for the percentages of primordial, primary, preantral, and antral follicle (F) and Graafian follicle ratio per ovary (G), and the mean number of corpora luteum per section (H) from complete serial ovary sections in control and each treatment groups (n = 9 ovaries in each group). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. Data are displayed as mean ± standard error of the mean (SEM).

Discussion

As a new and simple ovarian stimulation regimen for IVF, the PPOS protocol has received increasing levels of attention over the past five years. Progestin-primed ovarian stimulation protocols are advantageous in that they can be administered orally, are flexible and economic for patients, and can easily gain control over the LH surge [1,2,7]. However, the influence of progesterone on clinical outcomes has yet to be determined. The current study demonstrated that PPOS reduced the number of follicles and mature oocytes, although this could be restored by using a higher dose of hMG. Furthermore, data derived from an *in vivo* mouse model, and an *in vitro* follicle culture system, strongly support the notion that progesterone inhibited granular cell proliferation and thus

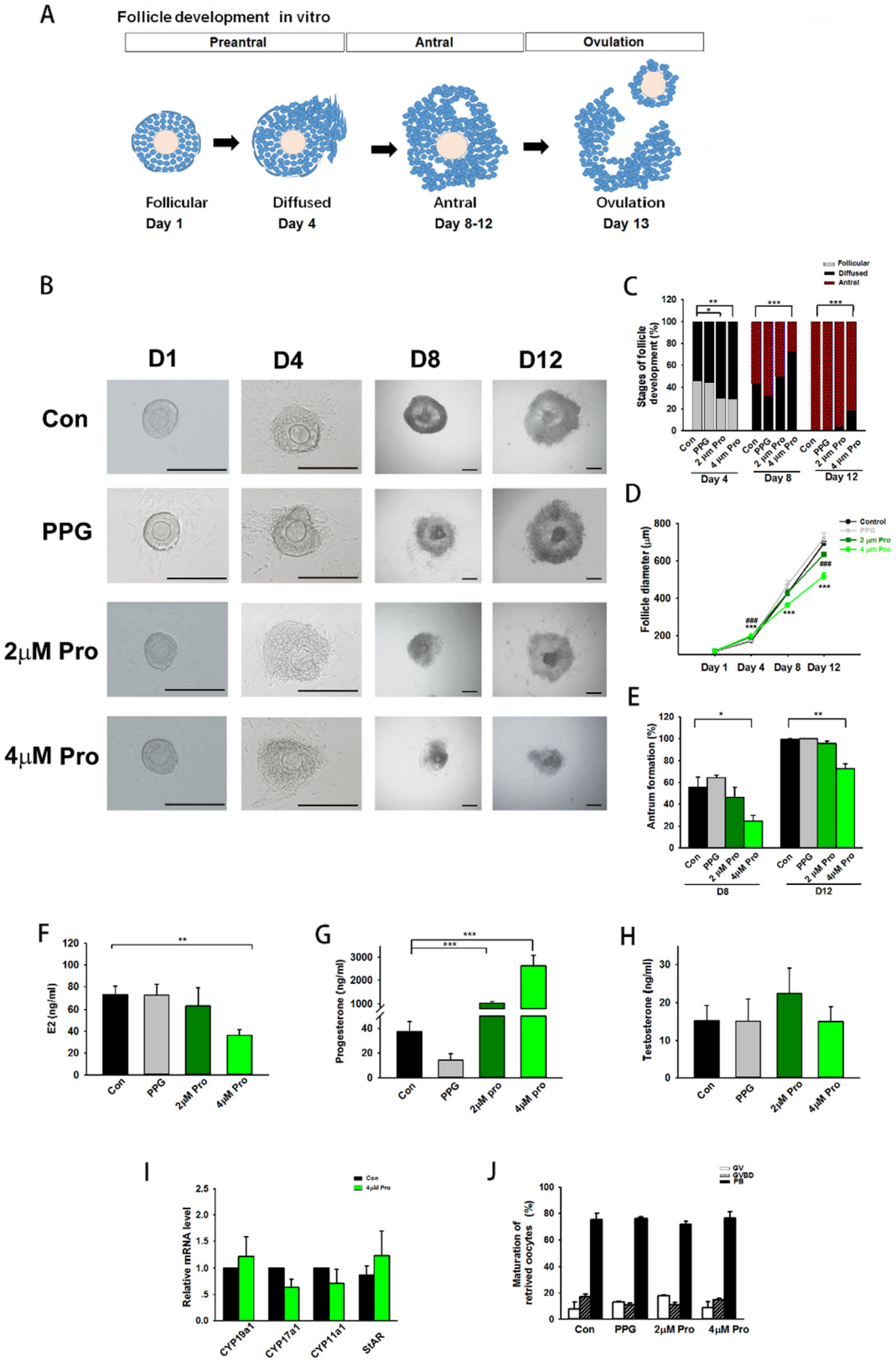

Fig. 3. Effect of progesterone on mouse follicle development *in vitro*. (A) Schematic of *in vitro* follicle development. Isolated second follicles grow up to preovulatory follicles, experiencing follicular, diffused, antral stage orderly and then ovulation induced by HCG. (B) Representative photomicrographs of follicle development without or with different doses progesterone treatment. PPG: propylene glycol (vehicle). Scale bar: 200 μm. (C) The process of follicle development was analyzed through the distribution of follicular, diffused and antral stages on culture day 4, day 8 and day 12. Data are displayed as the ratios in each group: control (n = 122), PPG (n = 64), 2 μM progesterone (n = 106) and 4 μM progesterone (n = 129). Chi-square test, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$. (D) Follicle diameters were measured in each group: control (n = 122), PPG (n = 36), 2 μM progesterone (n = 107), 4 μM progesterone (n = 126). $^{\#\#\#}P < 0.001$, comparison between control and 2 μM progesterone group; $^{***}P < 0.001$, comparison between control and 4 μM progesterone group. (E) Percentage of follicles with antrum formation in each group. $^{*}P < 0.05$, $^{**}P < 0.01$. (F-H), E2 (F), progesterone (G) and testosterone (H) levels secreted from single follicle in each group. $^{***}P < 0.001$. (I) Real-time PCR analysis for *CYP19a1*, *CYP17a1*, *CYP11a1*, *StAR* mRNA in follicular granular cells on culture day 12. (J) Maturation of oocytes derived from preovulatory follicles was calculated. GV: germinal vesicle; GVBD: germinal vesicle broken down; PB: polar body, MII. Data in (D-J) were pooled from at least 3 independent experiments. Data are displayed as mean ± standard error of the mean (SEM).

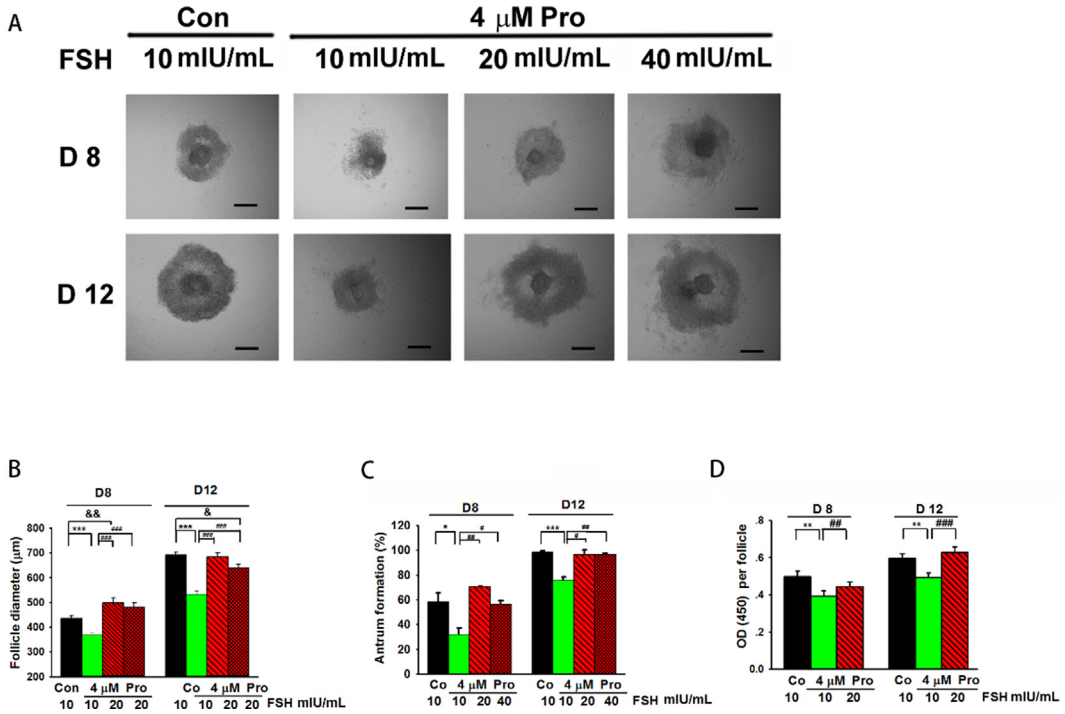

Fig. 4. FSH rescues the inhibition of progesterone on granular cell proliferation. (A) Representative photomicrographs of mouse follicle development exposed to progesterone with 10 mIU/mL, 20 mIU/mL and 40 mIU/mL FSH. The basal medium of the control group also contained 10 mIU/mL FSH. Scale bar: 200 μm. (B-C) Follicle diameters (B) and percentages of follicles with antrum formation (C) on culture day 8 and day 12 in each group (control: n = 142, progesterone: n = 123, 20 mIU/mL FSH: n = 59, 40 mIU/mL FSH: n = 61). $^{*}P < 0.05$, $^{***}P < 0.001$, compared with control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with the progesterone treatment group. $^{\&\&}P < 0.01$, $^{\&}P < 0.05$, compared with the control group (D) Quantification of cell proliferation by CCK-8 assay. $^{**}P < 0.01$, compared with the control group. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with the progesterone treatment group. Data are displayed as mean ± standard error of the mean (SEM).

reduced follicular growth. It is likely that this mechanism is responsible for the reduction in oocyte yields following PPOS stimulation. These findings also advance our understanding of the alterations of ovarian response to gonadotropins during PPOS and provide an explanation for the higher hMG consumption compared to the traditional protocol in clinical practice if similar oocyte yields were obtained.

Although the actions of progesterone on granular cell mitosis, apoptosis, and steroid synthesis have been reported [13–16], the precise mechanism by which progesterone is able to regulate the growth of follicles has not been fully elucidated. For the first time, the current study illustrates the critical role of the MAPK and PI3K/AKT signaling pathways in the regulation of progesterone-mediated inhibition of granular cell proliferation and follicular growth. It is well known that the PI3K/AKT and MAPK pathways activated by FSH play critical roles in the mitosis and cell cycle of granular cells [25,26,30,31]. In granular cells, unphosphorylated FOXO functions to repress the cell cycle via inhibition of cyclin-cyclin-dependent kinase (cdk) complexes and/or the activation of $p27^{Kip}$ [32,33]. FSH is able to phosphorylate AKT and FOXO, and dissociate the suppression of FOXO on cyclin D, leading to the proliferation of granular cells [34,35]. According to the data from the phosphorylation array and western blot, it is possible that progesterone led to a significant suppression in the phosphorylation of AKT and CDK1, thus leading to the activation of $p27^{kip1}$ during PI3K/AKT signaling. Meanwhile, the signal PKC, Raf1/MEK/ERK, and SRF in MAPK-related signaling cascades were also extensively inhibited by progesterone treatment. These results revealed that signaling alterations in granular cells of growing follicles are responsive to progesterone treatment and reveal a critical role for PI3K/AKT and MAPK signaling pathways in the coordination of progesterone and FSH to influence follicular growth.

Importantly, the local inhibition of progesterone on follicle growth has been shown in previous studies [10–12] and our data provided additional consistent evidence from *in vivo* and *in vitro* systems. The inhibition of progesterone on follicle growth was found to be dose-dependent in our results. Although the stimulation of high-dose progesterone on follicle development in hamsters was observed [10], these results were not repeated in the current study or others; this might be due to a species-specific difference. Interestingly, the current data revealed that the secondary follicles exposed to progesterone exhibited a larger diameter and more rapid migration of the surrounding cells than the control follicles. These findings indicated that the effects of progesterone on follicle development were dynamic and relative according to the developmental stage. Furthermore, the mRNA levels of key sterol synthesis enzymes showed no obvious alterations in response to progesterone treatment in mouse granular cells. These results exclude the possibility that the effect of progesterone on follicle growth stems from the regulation of steroidogenesis.

Combined with 'freeze all' strategies, PPOS protocols may avoid the potentially harmful effects of ovarian stimulation on endometrial receptivity, and thus improve IVF outcomes [1]. On the other hand, the number of oocytes retrieved per aspiration can be compromised if the dose of hMG used is similar to that of conventional protocols. In the future, it is likely that standard, heavy-handed ovary stimulation protocols will give way to simple, effective, feasible, and individualized strategies. It will be fascinating to tailor oocyte yields by balancing the doses of hMG and progesterone according to each patient's individual characteristics and needs. It is also conceivable that understanding the mechanisms underlying these effects will provide more targets in signaling pathways to individually regulate clinical oocyte yields.

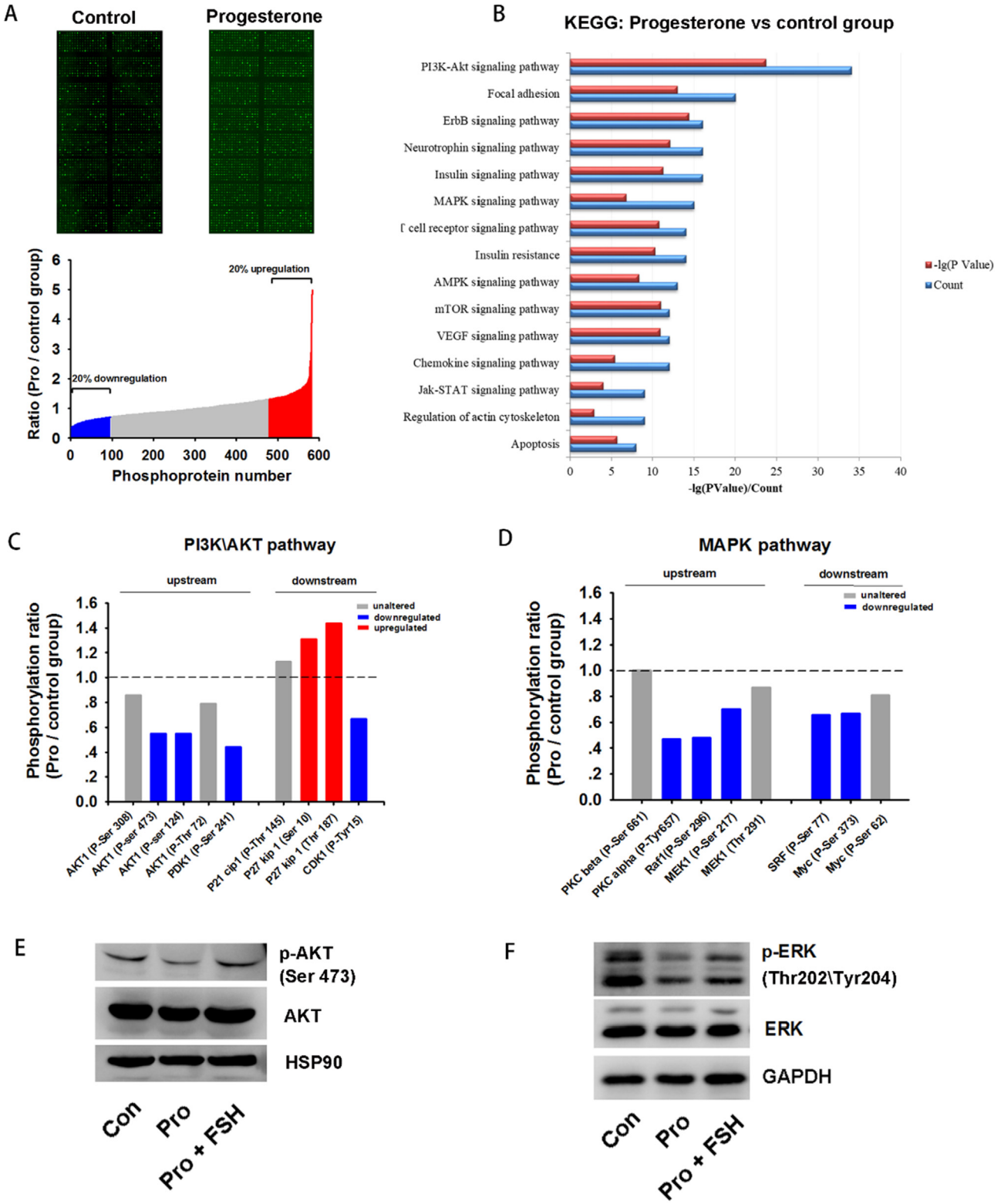

Fig. 5. PI3K/AKT and MAPK pathways mediates the coordination between progesterone and FSH in mouse granular cells within growing follicles. (A) Phospho-antibody array analysis of the expression changes of phosphoproteins in granular cells from the growing mouse follicles with or without progesterone treatment. The levels of the individual proteins were normalized to total protein levels. The phosphorylation ratio was used as the modulation difference of phosphorylation sites between two groups. Phosphoproteins whose levels increased or decreased by more than 20% were labeled red and blue, respectively. (B) KEGG pathway analysis of differential phosphoproteins in progesterone treated granular cells compared with the control group. Red bar = −log (P value); blue bar = numbers of genes. (C-D) Expression changes of up and downstream phosphoproteins in PI3K (C) and MAPK (D) pathways in progesterone treated granular cells compared with the control group indicated from the phospho-array. (E-F) Western blot analysis of granular cells extracts in control (con), progesterone (Pre) group. Phosphorylation of AKT (Ser 473) in PI3K (E) pathway and ERK (Thr 202/Tyr 204) in MAPK (F) pathway were measured. HSP90 and GAPDH were used as loading control.

CREDIT Author Statement

HL designed the study, performed experiments, analyzed data, and wrote the manuscript. WY performed experiments, interpreted the results, and helped to prepare the manuscript; SY analyzed the clinical data and helped to prepare the manuscript. MY performed experiments and interpreted the results. LW performed experiments and interpreted the results. QC and RC helped to analyze and interpret the clinical results. LS helped to interpret the results. LW, QL, and YK were involved with study design, the interpretation of results, and helped to prepare the manuscript.

Compliance with Ethics statement

Approval for human retrospective analysis was obtained from the institutional Ethics Committee of Shanghai Ninth People's Hospital (No.2014-94). All participants provided informed consent after counseling for infertility treatments and routine IVF procedures. Animal studies were approved by the institutional Ethics Committees of Care and Use of Experimental Animals of Shanghai Ninth People's Hospital.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2021.02.008.

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