



## Research paper

## Erythropoietin-producing hepatocellular A7 triggering ovulation indicates a potential beneficial role for polycystic ovary syndrome



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## ABSTRACT

**Background:** The ovulatory dysfunction mechanisms underlying polycystic ovary syndrome (PCOS) are not completely understood. And the roles of EPHA7 and EPHA7-regulated pathway factors in the pathogenesis of anovulation remain to be elucidated.

**Methods:** We used human granulosa cells (hGCs) of PCOS and non-PCOS patients to measure EPHA7 and other target gene expressions. We performed in vitro experiments in KGN cells to verify the molecular mechanisms. Additionally, we conducted in vivo loss- and gain-of-function studies using EPHA7 shRNA lentivirus and recombinant EPHA7-Fc protein injection to identify the ovulation effects of EPHA7.

**Findings:** EPHA7 functions as a critically positive upstream factor for the expression of ERK1/2-mediated C/EBP $\beta$ . This protein, in turn, induced the expression of KLF4 and then ADAMTS1. Moreover, decreased abundance of EPHA7 was positively correlated with that of its downstream factors in hGCs of PCOS patients. Additionally, a 1-week functional EPHA7 shRNA lentivirus in rat ovaries contributed to decreased numbers of retrieved oocytes, and a 3-week functional lentivirus led to menstrual disorders and morphological polycystic changes in rat ovaries. More importantly, we found that EPHA7 triggered ovulation in rats, and it improved polycystic ovarian changes induced by DHEA in PCOS rats.

**Interpretation:** Our findings demonstrate a new role of EPHA7 in PCOS, suggesting that EPHA7 is an effective target for the development of innovative medicines to induce ovulation.

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## 1. Introduction

Polycystic ovary syndrome (PCOS) is a complex endocrine condition characterized by oligo/anovulation, high androgen levels, and polycystic ovaries. It affects at least 10% of females and has become the most common syndrome affecting not only reproduction but also metabolism [1]. High androgen levels and insulin resistance result in metabolic and hormonal dysfunctions in PCOS patients [2]. However, anovulation and menstrual cycle abnormalities are usually the frequent symptoms troubling women of reproductive age. The hyper response of follicles to gonadotropins is thought to induce premature follicles to produce a transient high concentration of circulating estradiol [3]. Subsequently,

higher estradiol suppresses the release of follicle-stimulating hormone (FSH) through negative feedback, leading to abnormal follicle maturation which contributes to the observed disrupted ovulation [3]. Irregular menstrual cycles and anovulation can cause infertility. A Finnish study shows that PCOS leads to difficulty in conception [4]. Therefore, anovulation affects many women of reproductive age with PCOS and remains a common problem.

Ovulation is triggered by a luteinizing hormone (LH) surge; it results in the release of a mature fertilizable oocyte and formation of corpus luteum (CL) [5]. The LH surge triggers the maturation of follicles and ovulation by activating multiple signaling pathways in granulosa cells (GCs), including the cAMP/protein kinase A [6] pathway, p38MAPK $\alpha$  (MAPK14), and epidermal growth factor receptor (EGFR)/RAS/extracellular signal-regulated kinases 1 and 2 (ERK1/2) signaling cascades [5,7]. The ERK1/2 cascade plays vital roles in

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## Research in context

### Evidence before this study

Polycystic ovary syndrome (PCOS) is a complex endocrine condition characterized by oligo/anovulation, high androgen levels, and polycystic ovaries. The ovulatory dysfunction mechanisms underlying PCOS are not completely understood. Ovulation is a complicated process that is influenced by multiple factors. One of the essential transcriptional regulators that impact the ovulation process is CCAAT/enhancer-binding protein  $\beta$  (C/EBP $\beta$ ). ChIP-seq data have hinted that erythropoietin-producing hepatocellular (EPH) A7 (EPHA7) may be closely related to C/EBP $\beta$  in PCOS. The EPH receptor tyrosine kinases (RTKs) and their ephrin (EFN) ligands are crucial for cell signaling pathways linked to various biological outcomes. EPH signaling plays a role in multiple physiological functions involving nervous system development, angiogenesis, insulin secretion, immunity, etc. In addition, EPH signaling influences the activities of integrins and intercellular adhesion molecules that lead to control of cell morphology, adhesion, migration and invasion. Ovulation involves COC expansion and follicular rupture; while the follicular rupture and the formation of CL involve cell adhesion and angiogenesis. Some members of the EPH-EFN family (EPHA2/A4/A7 and EFNA4/B1/B2) have been found to be strongly expressed in human luteinizing GCs. EFNA5 is considered as the ligand of EPHA7, and it is noteworthy that *Efna5*<sup>-/-</sup> female mice produce smaller litters and release fewer oocytes than controls. Hence, EPHA7 is probably related to ovulation. To our knowledge, no study has provided evidence that demonstrates a role for EPHA7 in female fertility and PCOS. The correlation between EPHA7 and ovulation remains unclear and needs to be investigated.

### Added value of this study

Our study firstly demonstrates the role of erythropoietin-producing hepatocellular (EPH) A7 (EPHA7) in the pathogenesis of anovulation in polycystic ovary syndrome (PCOS). EPHA7 functions as a critically positive upstream factor for the expression of ERK1/2-mediated C/EBP $\beta$ . This protein, in turn, induced transcription and translation of *KLF4* and then facilitated the expression of ADAMTS1. Low expression of EPHA7 and EPHA7-regulated pathway factors in human granulosa cells is considered to contribute to PCOS. We performed in vitro experiments in KGN cells to verify the molecular mechanisms and conducted in vivo loss- and gain-of-function studies in rats to identify the ovulatory effect of EPHA7. A 1-week functional EPHA7 shRNA lentivirus in rat ovaries contributed to decreased numbers of retrieved oocytes, and a 3-week functional lentivirus led to menstrual disorders and morphological polycystic changes in rat ovaries. Moreover, we found that EPHA7 triggered ovulation in rats inducing the increase of luteinizing hormone (LH), and it improved polycystic ovarian changes induced by dehydroepiandrosterone (DHEA) in PCOS rats. Also we confirmed the safety of exogenous EPHA7 injection in rats.

### Implications of all the available evidence

Our findings illustrate a new role of EPHA7 in PCOS, suggesting that EPHA7 is an effective and potential target for the development of innovative medicines to induce ovulation and for assisted reproductive treatments. Given the other previous reports, the EPH-EFN family might play a crucial role in female fertility. More experiments about EPH-EFN family members in various animals and clinical trials will be required in the future.

ovulation such as cumulus cell–oocyte complex (COC) expansion, oocyte maturation, and follicle rupture [8–11]. Proteinases exert changes in the extracellular matrix (ECM) to allow the expansion of COC and degradation of the apical follicular walls. Among the different proteinases, a disintegrin and metalloproteinase with thrombospondin-like motifs 1 (ADAMTS1) is thought to be crucial for ovulation and fertilization [12,13].

Ovulation is a complicated process that is influenced by multiple factors. The transcriptional regulators that impact the ovulation process include the CCAAT/enhancer-binding protein  $\beta$  (C/EBP $\beta$ ) [5,8], progesterone receptor (PGR) [14], and liver receptor homolog-1 (LRH1) [15,16], among others. C/EBP $\beta$ , a member of the basic leucine zipper proteins, regulates ovulation and is important for reproduction [5,8]. The translation of C/EBP $\beta$  mRNA gives rise to three isoforms: LAP1, LAP2, and LIP [17]. LAP1 and LAP2 are mostly transcriptional activators, whereas LIP acts as a repressor. In preovulatory follicles, expression of C/EBP $\beta$  is increased by LH and activated in an ERK1/2-dependent manner [8]. Female *Cebp $\beta$ <sup>gsc-/-</sup>* mice are subfertile, and *Cebp $\alpha$ / $\beta$ <sup>gsc-/-</sup>* double-mutant are sterile with follicles failing to ovulate and ovaries lacking CL [5]. Moreover, C/EBP $\beta$  contributes to the differentiation and maturation of GCs as well as to progesterone production through steroidogenic factor 1 (SF1) [18]. Therefore, C/EBP $\beta$  plays essential roles in ovulation, steroidogenesis, and female reproduction.

C/EBP $\beta$  is a transcription factor contributing to the regulation of numerous target genes involved in the formation and maintenance of corpora lutea, steroidogenesis, and vascularization of ruptured follicles [5]. ChIP-seq data have hinted that erythropoietin-producing hepatocellular (EPH) A7 (EPHA7) may be closely related to C/EBP $\beta$  in PCOS [19–21]. The EPH receptor tyrosine kinases (RTKs) and their ephrin (EFN) ligands are crucial for cell signaling pathways linked to various biological outcomes [22]. EPH signaling plays a role in multiple physiological functions involving nervous system development, angiogenesis, insulin secretion and immunity [23–25]. In addition, EPH signaling influences the activities of integrins and intercellular adhesion molecules that lead to control of cell morphology, adhesion, migration and invasion [26,27]. Ovulation involves COC expansion and follicular rupture; while the follicular rupture and the formation of CL involve cell adhesion and angiogenesis [28]. Some members of the EPH-EFN family (EPHA2/A4/A7 and EFNA4/B1/B2) have been found to be strongly expressed in human luteinizing GCs [29,30]. Interestingly, human chorionic gonadotropin (hCG) is not a major determinant of EPH/EFN regulation in human granulosa cells (hGCs) [30]. Whereas FSH can increase the expression of EFNA5 and multiple EPH receptors in GCs of mice and rats, affecting their morphology and adhesion capabilities [31]. It is noteworthy that *Efna5*<sup>-/-</sup> female mice produce smaller litters and release fewer oocytes than controls [32]. Hence, the EPH-EFN family is probably related to ovulation and female fertility. To our knowledge, no study has provided evidence that demonstrates a role for EPHA7 in female reproduction and PCOS. The correlation between EPHA7 and ovulation if any remains unclear and needs to be investigated.

Anovulation affects many PCOS patients, and EPHA7 and other factors may be associated with this abnormality. This study was designed to assess the role of EPHA7 in ovulation in PCOS cases, and to find underlying molecular mechanisms. At the same time, based on our findings we tried to characterize a new feasible approach with EPHA7 for the treatment of anovulation in PCOS.

## 2. Materials and methods

### 2.1. Human subjects and granulosa cell samples

Participants were women recruited from patients undergoing in vitro fertilization-embryo transfer (IVF-ET) in the Center for Reproductive Medicine, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine. Appropriate informed consents were obtained from all

**Table 1**  
Demographic features and clinical outcomes of recruited participants with or without PCOS.

	Non-PCOS (n = 32)	PCOS (n = 40)
Age (years)	27.45 ± 6.73	28.48 ± 4.24
BMI (kg/m <sup>2</sup> )	21.17 ± 5.26	24.47 ± 4.49*
Basal FSH (mIU/mL)	6.87 ± 3.22	5.69 ± 2.02
Basal LH (mIU/mL)	4.78 ± 2.83	8.14 ± 6.26*
Basal E <sub>2</sub> (pg/mL)	43.50 ± 12.27	61.54 ± 15.73
Basal T (nmol/L)	0.99 ± 0.63	1.81 ± 0.92*
Basal PRL (μg/L)	17.82 ± 11.77	16.76 ± 12.40
LH/FSH	0.75 ± 1.30	1.52 ± 1.26*
AMH (ng/mL)	5.67 ± 2.70	11.70 ± 6.53*
No. of oocytes retrieved	13.52 ± 7.18	15.63 ± 7.84
High-quality embryos rate	0.53 ± 0.18	0.49 ± 0.25

All data are presented as mean ± SD. \*,  $P < .05$ , vs. Non-PCOS.

BMI: body mass index; FSH: follicle-stimulating hormone; LH: luteinizing hormone; E<sub>2</sub>: estrogen; T: testosterone; PRL: prolactin; AMH: anti mullerian hormone.

patients in this study. Forty PCOS patients fulfilled all three of the Rotterdam criteria [1], and 32 non-PCOS patients were selected due to tubal factor infertility with regular menstrual cycles and normal ovarian morphologies. All subjects, 20–35 years old, were of Han ethnicity and did not undergo hormonal therapy for at least 3 months before the study; all patients underwent a gonadotropin-releasing hormone (GnRH) antagonist protocol with hCG trigger. The clinical information of patients is presented in Table 1. On the retrieval day, hCGs were recovered from the follicular fluid samples aspirated transvaginally from individual follicles under ultrasound guidance during the follicle puncture for IVF procedures. The GC isolation method was followed similar to that described previously using Ficoll-Paque™ PLUS (GE-HealthCare Bio-Science, Uppsala, Sweden) and hyaluronidase (Sigma) [33]. The Institutional Review Board of the Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, reviewed and approved all procedures.

## 2.2. Rats

Female Sprague–Dawley (SD) rats were housed in the animal lab with five rats per cage. All rats were kept under a 12 h light–dark cycle at a temperature of 21 °C ± 2 °C and with a humidity of 65% ± 5%; food and water were provided ad libitum. All experimental protocols were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

## 2.3. Superovulation and sample collection

A total of 20 rats (5-week-old, 150 g) were randomly divided into four groups: phosphate buffer saline (PBS), hCG, EPHA7 and hCG + EPHA7 groups. Each rat was intraperitoneally injected with 30 IU pregnant mare serum gonadotropin (PMSG) (Sansheng Pharmaceutical, Shanghai, China) at 4 p.m. to stimulate follicular development. About 48 h later, control rats were given PBS while EPHA7 group rats were injected with Recombinant Mouse EPHA7-Fc Chimera Protein (0.03 mg/kg BW) (R&D systems, MN, USA) through the caudal vein. Referring to the previous study [34] and considering normal rat serum EPHA7 level detected in the paper (about 3 ng/ml), we finally chose the dose of 0.03 mg/kg BW. At the same time, hCG group rats were intraperitoneally given 30 IU hCG (Sansheng) and hCG + EPHA7 group rats were treated with a combination of hCG (30 IU) and EPHA7 (0.03 mg/kg BW). We collected blood samples from the orbital veins of all rats before, and every 4 h after, PBS, hCG, or EPHA7 injections. Sixteen hours later, all rats were sacrificed by cervical dislocation. All blood samples were immediately centrifuged at 2500 rpm for 15 min to obtain serum samples that were stored at –80 °C. Ovary samples were collected and stored at –80 °C or fixed with 4% paraformaldehyde for further experiments. Oviducts were removed and rinsed in M2 medium

(Sigma). We tore open the ampulla of each rat to release cumulus masses and then treated those with hyaluronidase (Sigma) to separate oocytes under a stereoscope (Olympus, Tokyo, Japan). The retrieved oocytes were carefully numbered and photographed using a microscope (Zeiss, Oberkochen, Germany).

## 2.4. Injection of shRNA lentivirus

The EPHA7 shRNA target sequence (5′-CCGGTCAAAGAAAGATGAA GCTGAACTCGAGTTCAGCTTCATCTTTCTTTTCATTTTTG-3′) and the control shRNA target sequence (5′-CCGGTCTCCGAACGTGTCACGTTTC AAGAGAACGTGACACGTTCCGAGAATTTTTG-3′) were designed at the GeneChem Company (GeneChem, Shanghai, China). The shRNA lentivirus preparation protocol was modified slightly from the published method [35]. Briefly, since the lentiviral vector system included pGCL-GFP, pHelper 1.0, and pHelper 2.0, all three extracted plasmids were transfected into 293 T cells to harvest the active virosomes. The virus titer used was  $1 \times 10^9$  (TU/ml). We cut a small wound on the back of each rat (7-week-old, 200 g), separated the ovary and then directly injected 10 μl shRNA lentivirus into one ovary. The microsyringe needle was carefully held in place for several minutes after the injection. Finally, we sutured the muscle and skin layers. Since the lentiviral vector system included GFP, we fixed rat ovaries with 4% paraformaldehyde and then embedded in paraffin. Then the ovarian sections were directly visualized using a fluorescence microscope to determine the distribution and efficiency of lentiviral transfection. For one part of our study, 12 rats were induced to superovulate with 40 IU PMSG (Sansheng) 1 week after the surgical procedure, and then with 40 IU hCG (Sansheng) 48 h after the PMSG to investigate the role of EPHA7 in the ovulatory process of rats. For the other part of the study, 10 rats were directly sacrificed 3 weeks after the surgical procedure, to explore the long-term impact of EPHA7 shRNA lentivirus on rat ovaries.

## 2.5. Establishment of the PCOS rat model and EPHA7 injection

Four-week-old, female SD rats were injected daily (s.c.) with dehydroepiandrosterone (DHEA) (60 mg/kg BW) (Langchem, Shanghai, China) for 20 consecutive days in the DHEA (n = 6) and in the DHEA + EPHA7 (n = 6) groups. Instead, of DHEA, PBS was used in the control (n = 6) and control + EPHA7 (n = 6) groups according to a report [36]. Estrous cycles were detected during the last eight consecutive days of the rat model building, and the body weights were measured once a week. The successful PCOS rat models were confirmed according to published criteria [37]. Recombinant EPHA7-Fc Chimera Protein (0.03 mg/kg BW) (R&D) was injected through the caudal veins at 4 p.m. at 7 weeks of age, once the PCOS rat model was considered successfully set up. All rats were sacrificed 16 h later and samples were collected.

## 2.6. Glucose tolerance test (GTT)

After DHEA treatment for 20 days, female rats of control and DHEA groups were left to fast for 16 h (5 p.m. to 9 a.m.). Then D-glucose (2 g/kg BW) was intraperitoneally injected into each rat. The blood glucose level was measured before, and 30, 60, 90, and 120 min, after the D-glucose injection with an Accu-Chek glucose monitor (Roche, Basel, Switzerland).

## 2.7. Serum levels of hormones and EPHA7

The concentrations of LH, FSH, progesterone and EPHA7 in rat sera were detected using ELISA Kits for Rat Luteinizing Hormone (LH) (Mybiosource, San Diego, USA), Rat Follicle-stimulating Hormone (FSH) (Biomatik, Canada), Rat Progesterone (PROG) (TSZ, San Francisco, USA) and Rat EPH receptor A7 (EPHA7) (Mybiosource). For the

detection of the LH level we had to perform a 10-fold dilution of the rats' sera. All the procedures were carried out according to standard manufacturers' protocols.

### 2.8. Safety of EPHA7 injection into rats

Recombinant EPHA7-Fc Chimera Protein was injected into 7-week-old SD rats at a dose of 0.03 mg/kg BW through the caudal vein, while PBS was used for controls. Each group had five rats; set up two groups treated with EPHA7 and one group was the control. After 48 h, we recorded the rats' appearances, hair, activity levels, respiratory depths and rates, fecal matters, feeding habits, and local reactions at the injection sites on the tails. Then we collected the ovaries, hearts, livers, spleens, lungs and kidneys of five rats injected with EPHA7 to detect any pathological changes on tissue sections stained with hematoxylin and eosin (H&E). After 14 days, we sacrificed another two groups, repeating this experiment. Meanwhile, we performed the blood routine examinations and measured blood biochemical indexes of the remaining 10 rats.

### 2.9. Histology and immunohistochemistry

Rat tissues were fixed with 4% paraformaldehyde, and then embedded in paraffin. Five  $\mu\text{m}$ -thick tissue sections were deparaffinized and rehydrated through a graded ethanol series. Then the sections were stained in hematoxylin and differentiated by hydrochloric acid. Finally, the sections were incubated in eosin before covering the slides or visualization using a microscope (Zeiss). Total numbers of small antral follicles (oocyte surrounded by greater than five layers of granulosa cells and/or one or two small areas of follicular fluid), large antral follicles (containing a single large antrum) and corpora lutea (CL) were classified and quantified as previously reported [37,38]. To avoid repetitive counting, each follicle was only counted in the section where the oocyte's nucleolus was visible. The thickness of granulosa cell and theca cell layers was respectively measured using ImageJ software (version 1.48; NIH) ( $n = 6$  per group, serial sections of each ovary were used for measurement). For immunohistochemistry, we blocked the ovarian sections with rabbit serum and then incubated them with EPHA7 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:100) overnight at 4 °C in a dark chamber, followed by incubation with secondary antibody. The color reaction was visualized by exposure to diaminobenzidine (DAB).

### 2.10. Cell culture

KGN cells (a human granular carcinoma cell line) were kindly provided by the Center for Reproductive Medicine, Shandong Provincial Hospital. They were cultured in Phenol Red-free DMEM/F-12 medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS) (Gibco) and incubated at 37 °C in a humidified atmosphere, with 5% CO<sub>2</sub>. KGN cells were routinely subcultured every 2 or 3 days.

### 2.11. Transfection of small interfering (si) RNA with liposome

Transfections of siRNA were performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. KGN cells ( $2 \times 10^5$ ) were plated onto six-well plates and cultured until they were 80% confluent. After refreshing the medium, we mixed Lipofectamine 3000 and siRNA (GenePharma, Shanghai, China) against target genes with Opti-MEM (Gibco) and then evenly added the mixture into each well. KGN cells were further incubated for 48 h before detecting the efficiency of knockdown and the abundance of target genes. The specific sequences of target genes were as follows:

EPHA7 siRNA, 5'-GGUAACUAUGUCACUGUUTT-3';  
C/EBP $\beta$  siRNA, 5'-GCACCCUGCGGAACUUGUUTT-3';

KLF4 siRNA, 5'-CCGAGGAGTTCAACGATCT-3';  
nonspecific scrambled siRNA, 5'-UUCUCCGAACGUGACGUTT-3'.

### 2.12. Transfection of plasmids with electroporation

KGN cells ( $6 \times 10^6$ ) were mixed with 10  $\mu\text{g}$  pCMV3-C-Flag-EPHA7, pCMV3-C-Flag-KLF4 (Transheep, Shanghai, China) or LAP-overexpression plasmid (gift from the Institute of Health Sciences, Shanghai Institutes for Biological Sciences), respectively, in Opti-MEM (Gibco). We added the mixture into 2-mm gap cuvettes. The cell suspensions were electroporated at 170 V for 5 ms using a NEPA21 electroporator (Nepa Gene). After diluting with DMEM/F-12 containing 10% FBS, KGN cells were transferred into a six-well plate and were prepared for the treatment after further incubation for 72 h before detecting the efficiency of overexpression and abundance of target genes.

### 2.13. Real-time polymerase chain reaction

Total RNA was extracted from cells and rat ovaries specimens by using a total RNA kit (FOREGENE, Chengdu, China). RNA concentration and quality were determined by measuring optical density at 260 nm (OD260) and OD260/OD280 ratio with a NanoDrop ND-2000. mRNA from total cellular RNA was reverse-transcribed to cDNA using PrimeScript RT Master Mix Perfect Real-time kit (TaKaRa, Dalian, China). Real-time polymerase chain reaction, also known as quantitative PCR (qPCR), was used to detect the abundance of target genes. The ratio of the target gene over  $\beta$ -ACTIN was calculated as the target mRNA level. The primer sequences used of targeting genes were as follows:

EPHA7 (human), 5'-GTGAAGATGGGTATTACAGGGC-3' (forward) and 5'-CAACTGCACCGCTTACACAAT-3' (reverse);  
Epha7 (rat), 5'-TGTTTTTCAGATCCGGCGT-3' (forward) and 5'-TTCACTGGACACCGCTGTTG-3' (reverse);  
C/EBP $\beta$  (human), 5'-CTTCAGCCCGTACTGGAG-3' (forward) and 5'-GGAGAGGAAGTCGTGGTGC-3' (reverse);  
C/ebp $\beta$  (rat), 5'-CCACGACTTCTTCCGACC-3' (forward) and 5'-GTAGTCGGACGGCTTCTTGC-3' (reverse);  
KLF4 (human), 5'-CGGACATCAACGACGTGAG-3' (forward) and 5'-GACGCCTTCAGCACGAAT-3' (reverse);  
Klf4 (rat), 5'-GGGCAAGTTGTGCTGAAGG-3' (forward) and 5'-GGGACGGCTCTTGCTTAAT-3' (reverse);  
ADAMTS1 (human), 5'-CAGAGCACTATGACACAGCAA-3' (forward) and 5'-AGCCATCCAAGAGTATCACA-3' (reverse);  
Adamts1 (rat), 5'-CGTAATCCAAGGGGTCCAG-3' (forward) and 5'-TGAGTGGACTAAAGCTGCGG-3' (reverse);  
Cyp11a1 (rat), 5'-CCAAAACACCACGCACTCC-3' (forward) and 5'-AACTTGGTCCCAACATCACG-3' (reverse);  
Star (rat), 5'-GAAGAACTGGTGGACCGCAT-3' (forward) and 5'-GTGGAACCTCTACGCTTGGT-3' (reverse);  
 $\beta$ -ACTIN (human), 5'-GGGAAATCGTGCCTGACATTAAG-3' (forward) and 5'-TGTGTTGGCGTACAGGTCCTTG-3' (reverse);  
 $\beta$ -Actin (rat), 5'-GGCCAACCGTGAAGATGACC-3' (forward) and 5'-AACCTCATAGATGGGCACAG-3' (reverse).

### 2.14. Western blotting

Total cellular proteins were extracted from cells and rat ovaries specimens using ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer (Cowbiotech, Beijing, China) containing a protease inhibitor cocktail (Roche), and a phosphatase inhibitor (Roche). The abundance of target genes was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, Massachusetts, USA). Thirty  $\mu\text{g}$  of protein from each sample were electrophoresed in 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Millipore, Billerica, MA). After blocking with 5% nonfat milk, the membranes were incubated

with EPHA7 antibody (1:500; SAB, Maryland, USA), T-ERK1/2 antibody (1:500; Cell Signaling Technology, Massachusetts, USA), P-ERK1/2 antibody (1:500; Cell Signaling Technology), C/EBP $\beta$  antibody (1:200; Santa Cruz Biotechnology), KLF4 antibody (1:500; Abcam, Cambridge, UK), KLF4 antibody (1:1000; Cell Signaling Technology), ADAMTS1 antibody (1:1000; Abcam), CYP11A1 antibody (1:1000; Cell Signaling Technology) or StAR antibody (1:1000; Cell Signaling Technology), overnight at 4 °C. After incubation with the appropriate diluted peroxidase-conjugated secondary (SAB), the enhanced chemiluminescent detection system (Millipore, Billerica, MA) was used to detect the bands with peroxidase activity. The bands were visualized using a G-Box iChemi Chemiluminescence image capture system (Syngene, Haryana, India). The ratio of band intensity of a target protein to that of the intensity of  $\beta$ -ACTIN (Proteintech, Wuhan, China) or GAPDH (Proteintech) was obtained as each target protein level.

### 2.15. Chromatin immunoprecipitation (ChIP) assay

The binding of C/EBP $\beta$  to the *KLF4* promoter after treatment with or without *EPHA7* was measured with ChIP assay referring to previous protocol [39]. The lysed KGN cells were sonicated and pre-cleaned with Protein A Agarose/Salmon Sperm DNA (Millipore). Then the sheared chromatin DNA was immunoprecipitated with C/EBP $\beta$  antibody (Santa Cruz Biotechnology) or negative control IgG, followed by washing with Magna ChIP Protein A Agarose Magnetic Beads (Millipore). After reverse cross-linking, RNA contamination and protein digestion, sheared DNA was extracted using a DNA extraction kit for next quantitative analysis. The primer sequences of *KLF4* used for qRT-PCR were 5'-CGAACGCCGAATCCAAAGT-3' (forward) and 5'-TCCCCTGGTTGTGA TCAGT-3' (reverse), which amplified a region between -879 bp and -865 bp spanning the putative C/EBP $\beta$  binding site. The ratio of DNA precipitated by C/EBP $\beta$  antibody over input control indicated the amount of bound transcription factor.

### 2.16. Statistical analysis

Results are presented as mean  $\pm$  SEM or SD. Each experiment was repeated 3 to 5 times. The data were initially subjected to Kolmogorov-Smirnov tests to assess deviation from Gaussian distribution. For normally distributed data, we used paired Student's *t*-test or one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test. For data not normally distributed, we applied Kruskal-Wallis test followed by Dunn's multiple comparison test. For all tests, a two-tailed *P* value <.05 was considered as statistically significant. Statistical significance is shown as \**P* <.05, \*\**P* <.01, or \*\*\**P* <.001.

## 3. Results

### 3.1. Decreased mRNA expression of *EPHA7* and target genes in granulosa cells of PCOS patients

We examined the relative mRNA expression profiles of *EPHA7*, *C/EBP $\beta$* , *KLF4*, and *ADAMTS1* using hGCs collected from PCOS (*n* = 40) and non-PCOS (*n* = 32) patients who underwent IVF-ET. As shown in Fig. 1, decreased mRNA abundances of *EPHA7*, *C/EBP $\beta$* , *KLF4*, and *ADAMTS1* were clearly demonstrated in hGCs of PCOS patients compared with those of non-PCOS patients. These results suggested that low expression levels of *EPHA7*, *C/EBP $\beta$* , *KLF4*, and *ADAMTS1* in hGCs are related to the lack of ovulation in PCOS patients and in the development of PCOS itself.

### 3.2. Effect and mechanisms of *EPHA7* on ovulation-related molecules in KGN cells

The mechanisms by which *EPHA7* contributes to ovulation abnormalities remain unclear. Based on our findings of hGCs in PCOS patients,

we decided to use human granulosa-like KGN cells for experiments to probe possible specific molecular mechanisms of *EPHA7* in ovulation.

After treatment with *KLF4* siRNA, we examined the mRNA and protein abundances of ADAMTS1, a human ovulatory marker [40,41], and observed that ADAMTS1 expression tended to decrease (Fig. 2A). On the contrary, overexpression of *KLF4* using vector transfection led to increased mRNA and protein expressions of ADAMTS1 (Fig. 2B). Hence, *KLF4* positively regulated ADAMTS1 expression in KGN cells.

We found a C/EBP $\beta$  putative binding site on the *KLF4* promoter. After transfection of *C/EBP $\beta$*  siRNA into KGN cells, we found that the mRNA and protein abundances of *KLF4* and ADAMTS1 had decreased (Fig. 2C). In addition, *C/EBP $\beta$*  overexpression led to increased expression of *KLF4* and ADAMTS1 mRNA and protein abundances (Fig. 2D). Therefore, our evidence showed that *C/EBP $\beta$*  induces the expression of *KLF4* and ADAMTS1 in KGN cells.

Next, we found that knockdown of *EPHA7* in KGN cells resulted in reduced levels of phosphorylated ERK1/2 and decreased expressions of *C/EBP $\beta$* , *KLF4*, and ADAMTS1 at both mRNA and protein levels (Fig. 2E). Concurrently, treatment of cells with the ERK1/2 inhibitor PD98059 (Sigma Chemical, St. Louis, MO) for 24 h hindered the expression of downstream factors after overexpressing *EPHA7* (Fig. 2F).

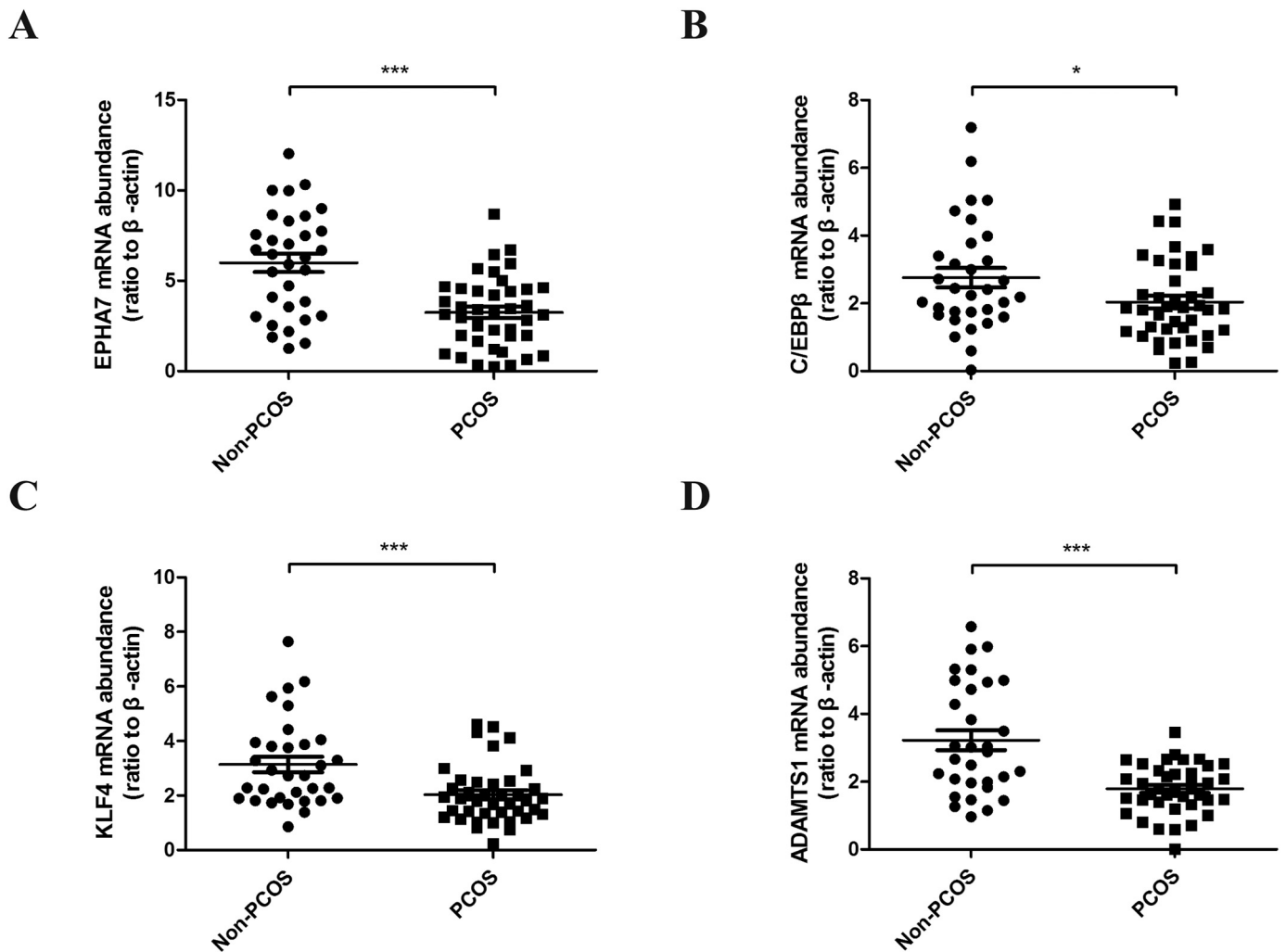
Furthermore, using ChIP assays, we found that overexpression of *EPHA7* actually increases the binding of C/EBP $\beta$  to the *KLF4* promoter region containing the C/EBP $\beta$  binding site (Fig. 2G). All these data indicated that *EPHA7* is a critical upstream factor of ERK1/2-mediated C/EBP $\beta$ , which stimulated the transcription and translation of *KLF4* and the downstream ADAMTS1 responses in KGN cells. Taken together, *EPHA7* stimulates *KLF4* expression via C/EBP $\beta$ , thus inducing ADAMTS1 expression in KGN cells. *EPHA7* and its downstream factors should be considered as essential factors in ovulation.

### 3.3. Ovulatory dysfunction in rats due to *EPHA7* gene silencing by shRNA lentivirus injection

Given our findings of hGCs in PCOS and non-PCOS patients along with the in vitro data in KGN cells, we reckoned that declined expression of *EPHA7* might contribute to anovulation. We injected either *EPHA7* shRNA or control shRNA lentivirus preparations into the ovaries of SD rats to verify the role of *EPHA7* in ovulatory disorders. GFP expression of ovarian slices was observed under fluorescence microscopy after injection to determine the distribution and efficiency of lentiviral transfection (Fig. S1). Each group included six rats. One week after the injection, we induced superovulation and then sacrificed all rats. Immunohistochemical results show that *EPHA7* is strongly expressed in GCs, theca cells and the CL of the ovary (Fig. 3A). We found no ovarian weight differences between two groups of rats (Fig. 3B). As expected, *EPHA7* shRNA-injected ovaries displayed remarkably reduced numbers of oocytes retrieved per ovary ( $9.0 \pm 1.581$  oocytes) than the control shRNA-injected ovaries ( $17.0 \pm 1.291$  oocytes) (Fig. 3C and D). Moreover, the mRNA and protein abundances of *EPHA7*, *C/EBP $\beta$* , *KLF4* and ADAMTS1 were significantly decreased after the treatment with *EPHA7* shRNA lentivirus, which points to *EPHA7* and its downstream factors as being players in the ovulatory process of rats (Fig. 3E).

### 3.4. Effect of treatment duration of *EPHA7* shRNA lentivirus on rat ovaries

The *EPHA7* shRNA lentivirus injections had given rise to reduced ovulatory ability 1 week later; hence, we wondered whether the long-term effects of *EPHA7* shRNA would include pronounced phenotypic changes in SD rats in vivo that are similar to those observed in PCOS cases (regarding estrus cycle, hormones, and ovarian morphology). We formed two groups of four rats each. This time we sacrificed the rats 3 weeks after the lentivirus injections. We observed irregular estrus cycles during the last 8 days in the *EPHA7* shRNA lentivirus injection rats (Fig. 4A), but found no body or ovarian weights differences between the two groups (Fig. 4B). Also, the serum FSH levels presented a decreasing



**Fig. 1.** Decreased mRNA expression of *EPHA7*, *C/EBP $\beta$* , *KLF4*, and *ADAMTS1* in granulosa cells of PCOS patients. Relative mRNA abundances of *EPHA7* (A), *C/EBP $\beta$*  (B), *KLF4* (C) and *ADAMTS1* (D) in ovarian granulosa cells from 32 non-PCOS patients and 40 PCOS patients calculated by qRT-PCR analysis.  $\beta$ -actin values were used for normalization. \*  $P < .05$ , \*\*\*  $P < .001$ .

tendency, the LH levels remained unchanged, and the LH/FSH ratio exhibited an increasing trend in the rats injected with *EPHA7* shRNA lentivirus compared to those treated with control shRNA lentivirus (Fig. 4C). Of note, we found fewer corpora lutea and more antral follicles in rats treated with *EPHA7* shRNA lentivirus than in control rats (Fig. 4D). Thus, we concluded that the 3-week effects of *EPHA7* shRNA lentivirus are able to bring about an irregular estrus cycle and lead to polycystic ovaries in rats.

### 3.5. A triggering role of *EPHA7* in ovulation induction of rats

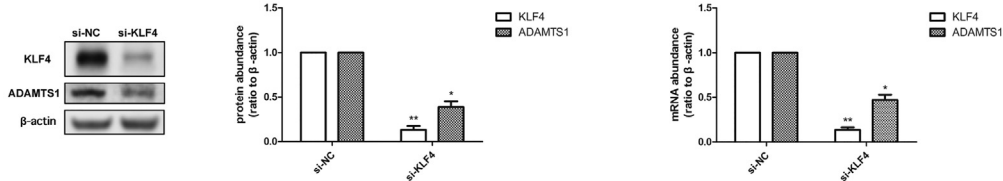
We used a PMSG-induced superovulation model of immature SD rats for in vivo experiments to verify the vital role of *EPHA7* in ovulation taking place of hCG. Recombinant *EPHA7*-Fc protein (or an equivalent volume of PBS) was injected through the caudal vein at a dose of 0.03 mg/kg of body weight. We formed four groups of five rats each. Three rats in the PBS treatment group and three in the *EPHA7* treatment group ovulated; we counted the numbers of oocytes retrieved per ovary ( $13.67 \pm 1.202$  and  $21.67 \pm 1.453$ , respectively), and found that a larger amount of COCs were stimulated and ovulated in the *EPHA7* group. All rats in the hCG and hCG + *EPHA7* groups ovulated, and the numbers of oocytes retrieved were  $27.60 \pm 1.030$  and  $31.20 \pm 1.463$ , respectively (Fig. 5A). We found no mRNA expression differences in terms of *Epha7*, *C/ebp $\beta$* , *Klf4* or *Adamts1* among the rat ovaries of all the groups (Fig. 5B), which is consistent with the rats having indeed ovulated. Since we

injected *EPHA7*-Fc through the caudal vein into rats, we made efforts to explore the systemic role of *EPHA7* not only just in the ovary. Thus we examined the concentration of a soluble splice variant of *EPHA7* in the serum to observe whether *EPHA7* could mimic hormone change in the serum or not. This soluble splice variant has been previously reported [42–44]. As observed, serum *EPHA7* levels reached a peak value 4 h after recombinant *EPHA7*-Fc protein injection and returned to the normal level 12 h later (Fig. 5C). Serum LH levels started to rise 12 h after *EPHA7* injection and reached a peak 16 h later, suggesting that *EPHA7* triggered an LH surge before ovulation (Fig. 5D). Serum E2 levels significantly decreased 16 h after *EPHA7* injection due to increased LH levels (Fig. 5E). Based on this, *EPHA7* is involved in ovulation induction and is able to (at least partially) trigger ovulation.

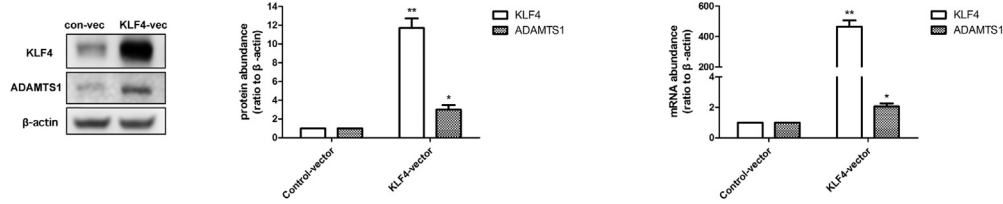
### 3.6. Successful establishment of the PCOS rat model via DHEA injection

We used DHEA injection to disrupt the normal 4-day estrus cycle of control SD rats as verified by daily examination of vaginal smears (Fig. 6A). The DHEA-treated rats maintained irregular estrus cycles throughout the examination period. We found no difference in body weights between the DHEA-treated and the control rats, but the DHEA-treated rats showed lighter ovarian weights than the control rats (Fig. 6B). In addition, GTT results showed that DHEA injection alters glucose tolerance in rats. While the fasting glucose levels were similar across groups, the DHEA-treated rats displayed marked increased glucose levels

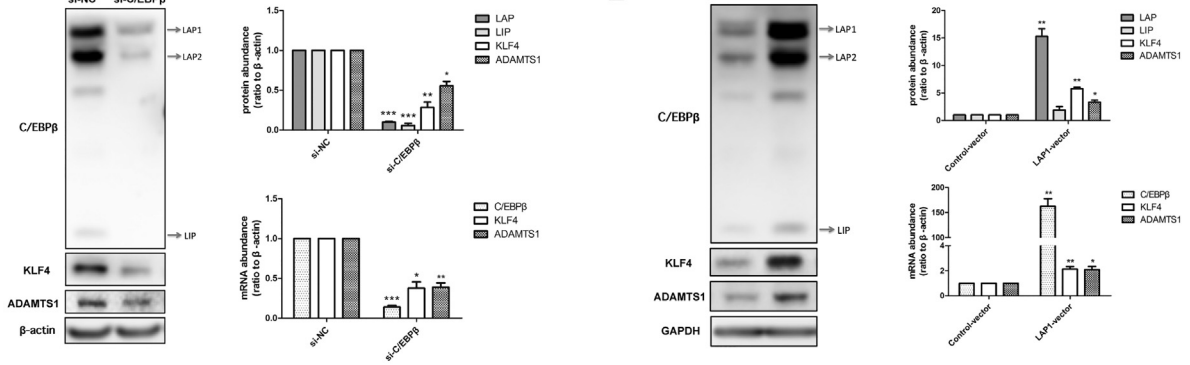
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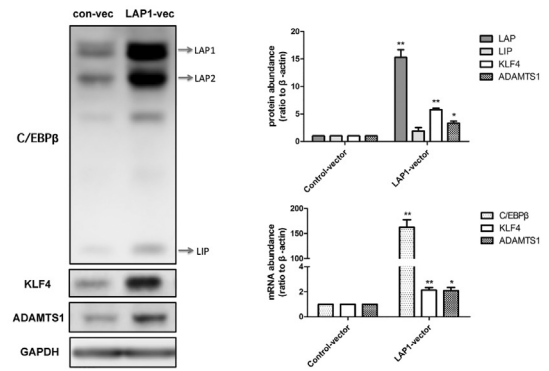
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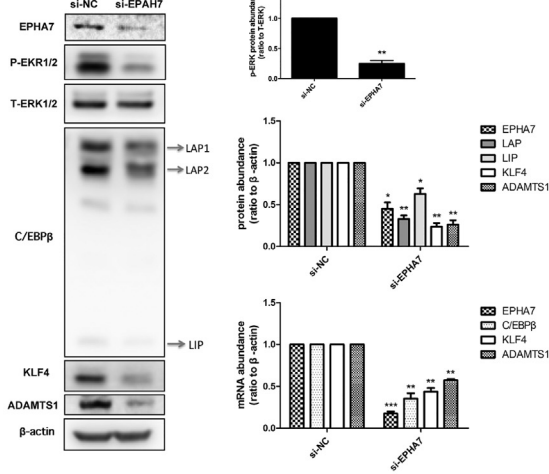
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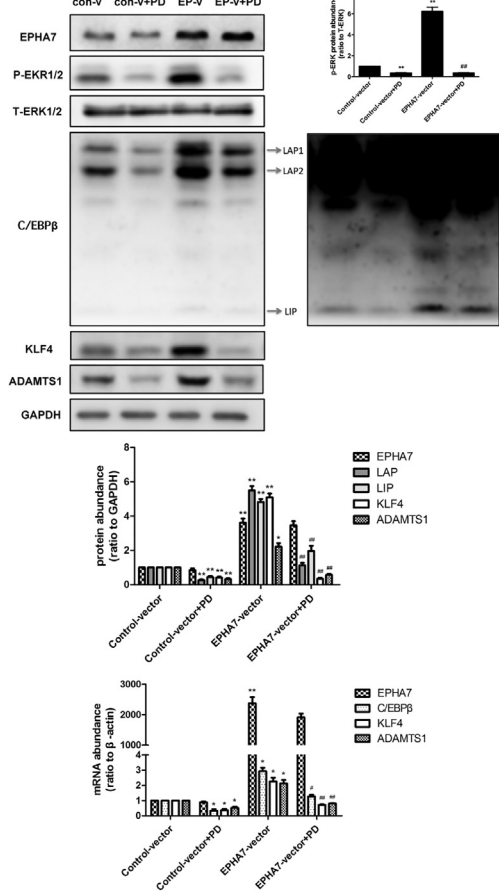
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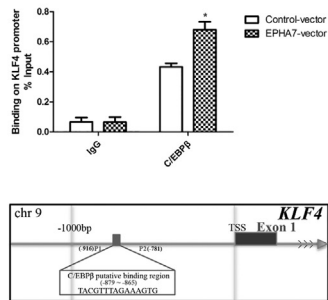
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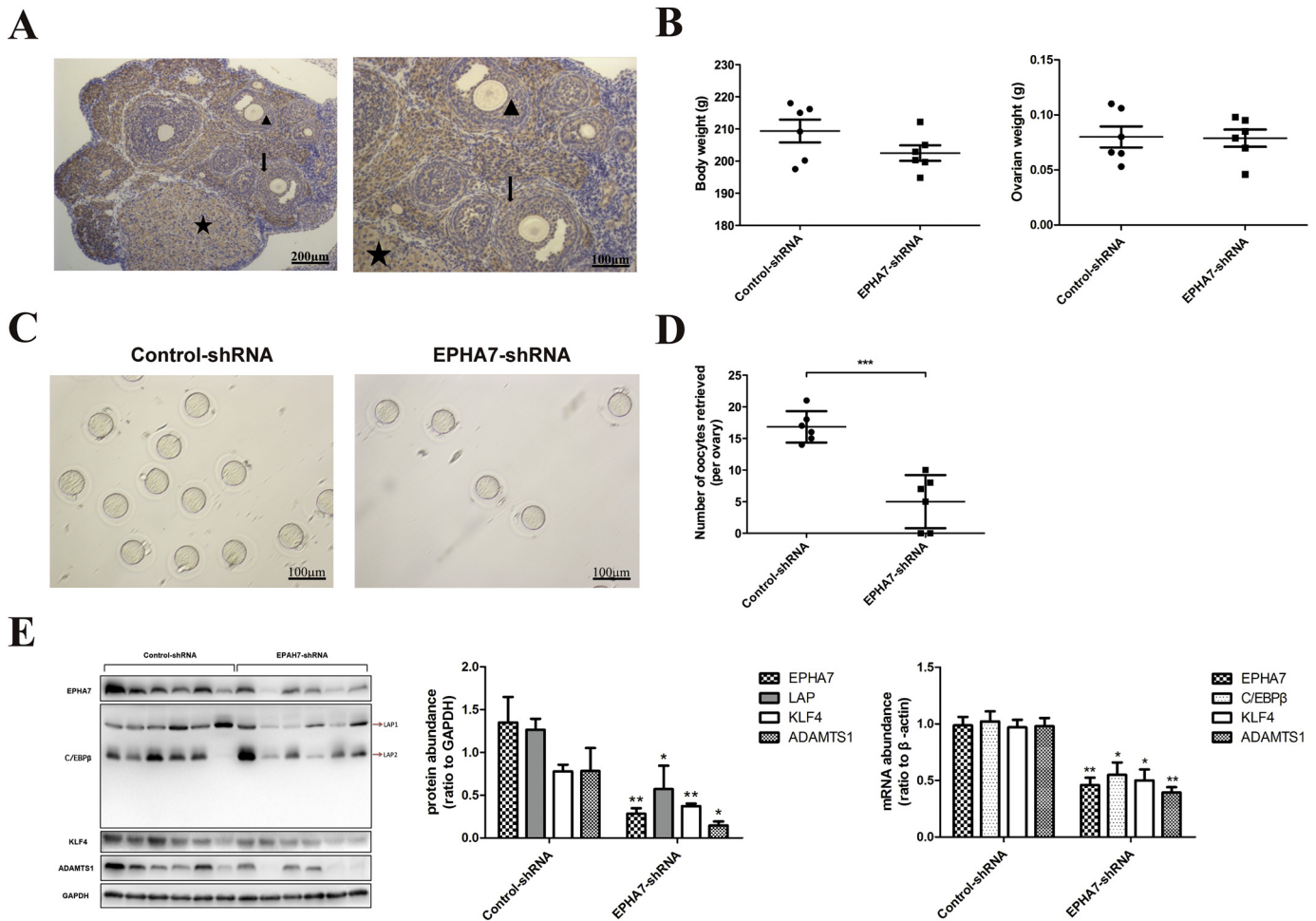


**F**



**G**





**Fig. 3.** Ovulatory dysfunction in rats due to low expression levels of EPHA7 and its downstream factors. (A) Immunohistochemistry demonstrates the distribution of EPHA7 in granulosa cells (triangle), theca cells (arrow) and corpus luteum (asterisk) of the rat ovaries. (B) Body weight (left) and ovarian weight (right) of rats. (C) Representative images of oocytes retrieved per rat. (D) Number of oocytes retrieved per rat. (E) mRNA and protein whole-ovary abundances of EPHA7, C/EBP $\beta$ , KLF4 and ADAMTS1 in rats, detected by western blot and qPCR analyses. The panel (left-to-right) shows representative images of western blot assays, immunoreactive bands were quantified by densitometry; and relative mRNA abundances by qPCR.  $\beta$ -actin or GAPDH were used as loading controls.  $N = 6$  per group. Data are presented as means  $\pm$  SEM. \*  $P < .05$ , \*\*  $P < .01$ , \*\*\*  $P < .001$ .

after 30, 60 and 90 and 120 min of glucose administration, and had a bigger area under the curve [45] compared with the control group rats (Fig. 6C). Although the serum EPHA7 level remained constant (Fig. 6D), the DHEA injection induced prominent increases in the serum LH level and in the LH/FSH ratio, but a distinct decrease in the serum FSH level compared to those in the control rats (Fig. 6E). All these data indicate a successful establishment of a PCOS rat model using the DHEA injections.

### 3.7. EPHA7 improved the polycystic ovarian changes and induced ovulation in PCOS rats

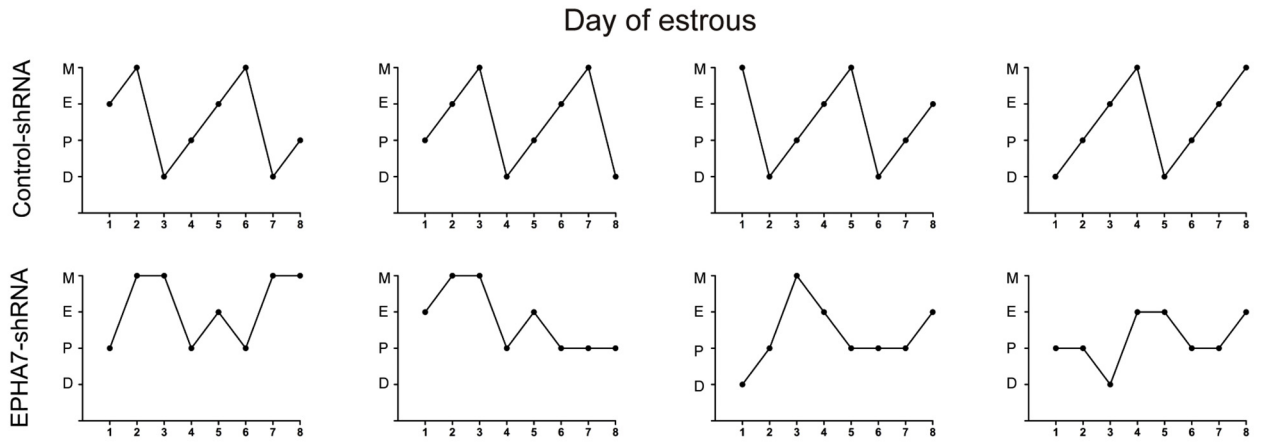
After confirming the relationship between EPHA7 and PCOS and verifying the significant positive role of EPHA7 in ovulation, we set up to

investigate whether exogenous EPHA7 can improve the DHEA-induced phenotypic changes in PCOS rats. Recombinant EPHA7-Fc protein was injected through the caudal vein at a dose of 0.03 mg/kg at 7 weeks of age, once the PCOS rat model was considered successfully set up. Rats were divided into four groups of six each. As seen in the figures, 16 h after exogenous EPHA7 injection, the estrus cycles (Fig. 6A), body and ovarian weights (Fig. 6B), and glucose tolerance results (Fig. 6C) of PCOS rats were not affected compared with those in control rats. The serum EPHA7 level remained unchanged, which was consistent with our previous result (Fig. 6D). Although the EPHA7 injection had no effect on the serum FSH level, it significantly increased the serum LH level and the LH/FSH ratio, not only in CONTROL+EPHA7 rats but also in DHEA+EPHA7 rats (Fig. 6E). We found the EPHA7

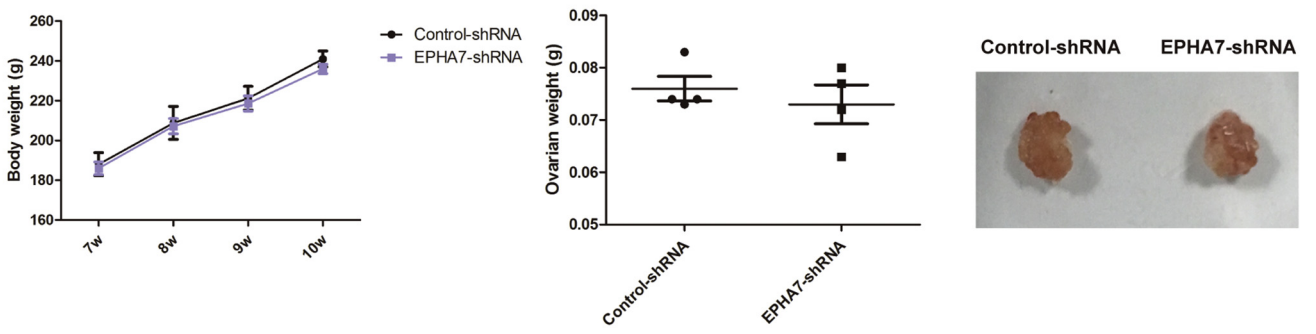
**Fig. 2.** EPHA7 promotion of KLF4 expression via C/EBP $\beta$ , leading to induction of ADAMTS1 expression in KGN cells. (A) mRNA and protein abundances of KLF4 and ADAMTS1 after KLF4 knockdown in KGN cells detected by western blot analysis and qPCR analysis. The panel (left-to-right) shows representative images of western blot assays, we quantified protein abundances by measuring the densitometry of the immunoreactive bands. (B) mRNA and protein abundances of KLF4 and ADAMTS1 after KLF4 overexpression in KGN cells. (C) mRNA and protein abundances of C/EBP $\beta$ , KLF4 and ADAMTS1 after C/EBP $\beta$  knockdown in KGN cells. Left, a representative western blot is shown. Right, the immunoreactive bands were densitometrically quantified (above); and mRNA abundance is presented (below). (D) mRNA and protein abundances of C/EBP $\beta$ , KLF4 and ADAMTS1 after LAP overexpression in KGN cells. (E) mRNA and protein abundances of EPHA7, C/EBP $\beta$ , KLF4 and ADAMTS1 after EPHA7 knockdown in KGN cells. (F) mRNA and protein abundances of EPHA7, C/EBP $\beta$ , KLF4 and ADAMTS1 after EPHA7 overexpression and further incubation with PD98059 (ERK1/2 inhibitor) in KGN cells. Above, a representative western blot is shown (left), and the immunoreactive bands for ERK1/2 phosphorylation were quantified densitometrically (right). Middle, the immunoreactive bands for other proteins were also quantified densitometrically. Below, mRNA abundance is presented. (G) Above, we used a ChIP assay to detect the enrichment of C/EBP $\beta$  at the KLF4 promoter in KGN cells in response to EPHA7 overexpression. IgG served as the negative control. Below, sequence of the KLF4 promoter spanning -879 to -865 base pairs (bp). Boxed letters indicate putative transcription factor binding sites. TSS, transcription start site.  $\beta$ -actin or GAPDH were used as loading controls for western blot and for qPCR analyses. Blots are representative and data are presented as means  $\pm$  SEM from 3 to 5 experiments. \*  $P < .05$ , \*\*  $P < .01$ , \*\*\*  $P < .001$  against si-NC cells or against Control-vector cells; #  $P < .05$ , ##  $P < .01$  against EPHA7-vector cells.



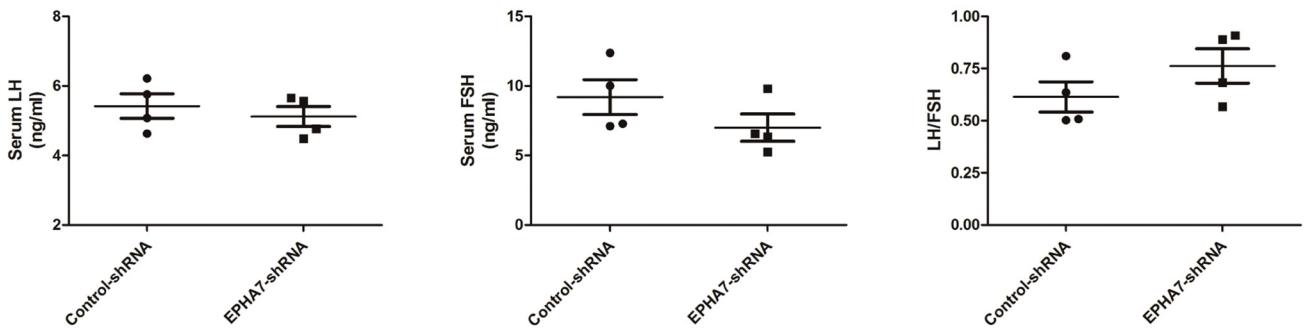
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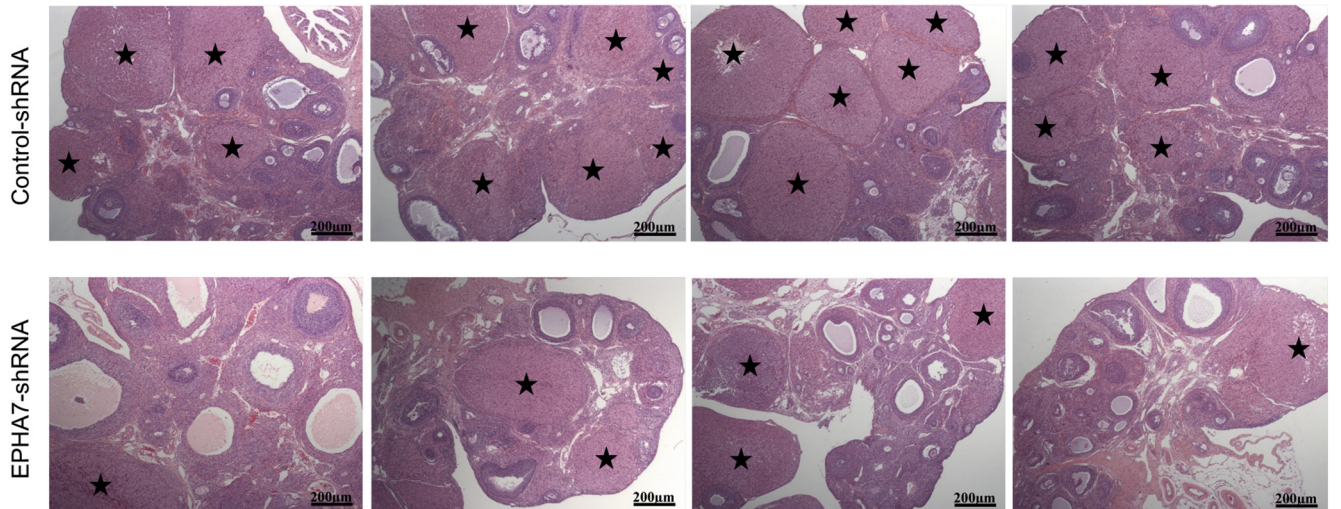
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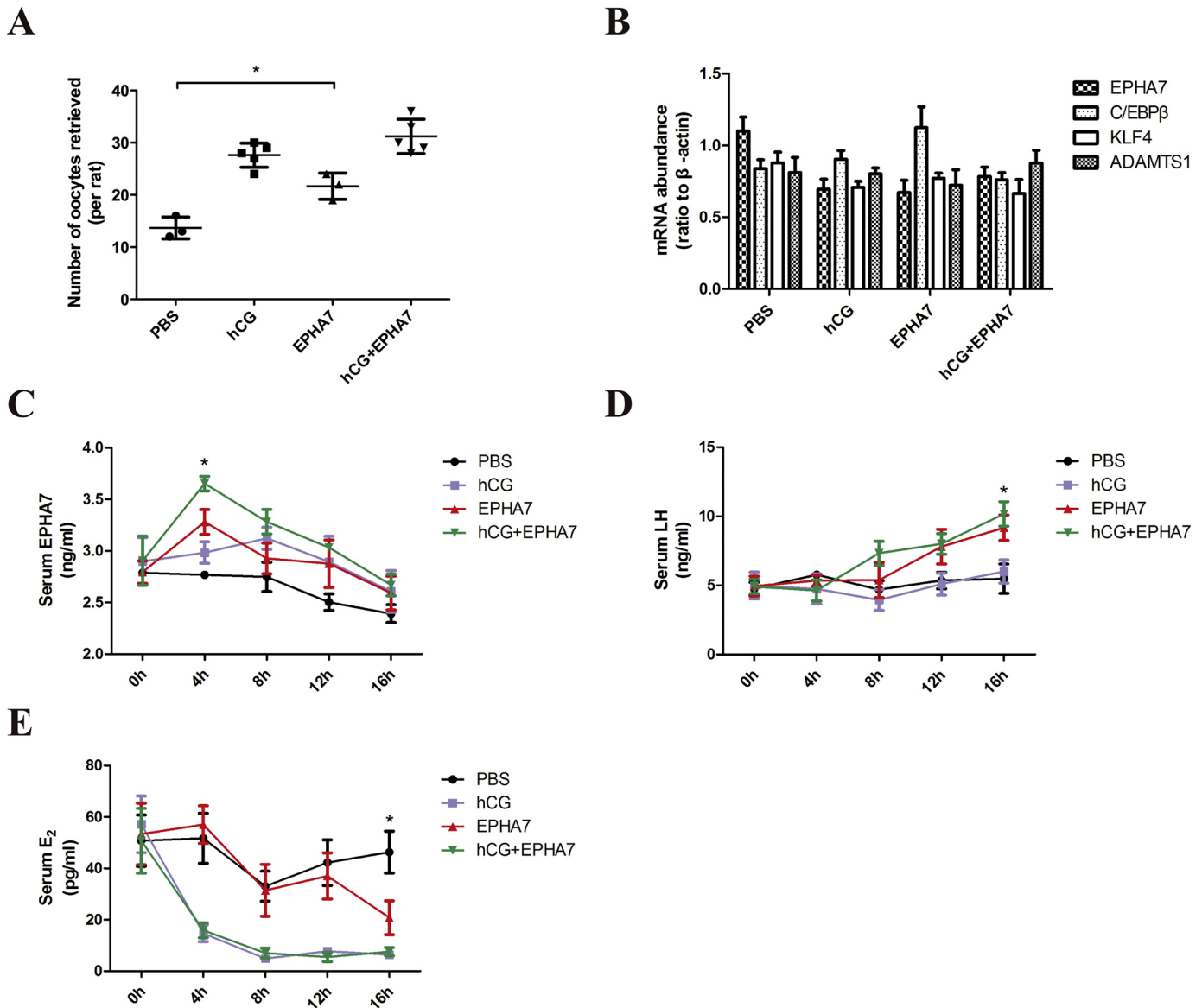


**C**



**D**





**Fig. 5.** EPHA7 triggers ovulation after induction in rats. (A) Number of oocytes retrieved per rat. (B) mRNA abundances of *EPHA7*, *C/EBP $\beta$* , *KLF4*, and *ADAMTS1* in the rats' ovaries were quantified by qPCR analyses. (C) Serum EPHA7 levels in rats before and every 4 h after PBS, hCG, or EPHA7 injection detected by ELISA. (D) Serum LH levels in rats before and every 4 h after PBS, hCG, or EPHA7 injection detected by ELISA. (E) Serum E<sub>2</sub> levels in rats before and every 4 h after PBS, hCG, or EPHA7 injection detected by ELISA.  $\beta$ -actin was used as a loading control.  $N = 5$  per group. Data are presented as means  $\pm$  SEM. \*  $P < .05$ .

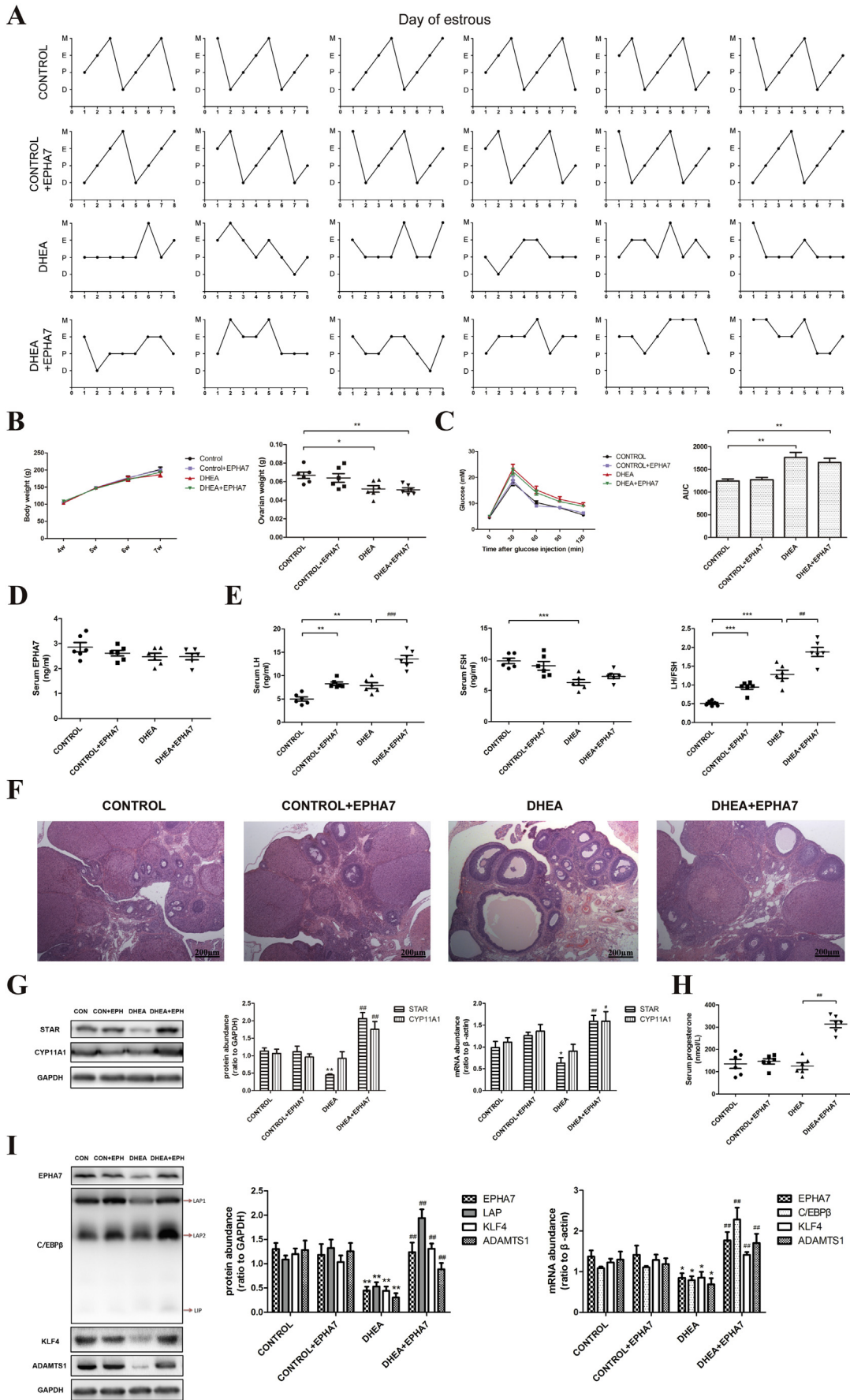
injection dramatically improves the polycystic ovarian changes in PCOS rats. We observed more corpora lutea and fewer large antral follicles in the DHEA+EPHA7 rats than in the DHEA rats (Fig. 6F, S2). At the same time, the mRNA and protein abundances of steroidogenic acute regulatory protein (StAR) [3] and cytochrome P450 family 11 subfamily A member 1 (CYP11A1) (Fig. 6G), as well as the serum progesterone level (Fig. 6H), were also much higher in DHEA+EPHA7 rats compared with those in DHEA rats. According to these phenotypes, we asserted that EPHA7 might promote the progress of ovulation and the generation of the CL in PCOS rats. In addition, we detected the mRNA and protein abundances of EPHA7, C/EBP $\beta$ , KLF4 and ADAMTS1 in the ovaries of these rats. The evident lower EPHA7, C/EBP $\beta$ , KLF4 and ADAMTS1 abundances in DHEA rats compared with those in control rats (Fig. 6I) are

consistent with the differences we observed in hCGs between PCOS and non-PCOS patients. Most importantly, the EPHA7 injection induced the expression of C/EBP $\beta$ , KLF4 and ADAMTS1 in rat ovaries (Fig. 6I). We also performed a similar experiment in which all rats were sacrificed 48 h after the EPHA7 injections (Fig. S3) and got very similar results. In conclusion, our results suggest EPHA7 together with its downstream factors improves the polycystic changes of ovaries and induces ovulation in rats with DHEA-induced PCOS.

#### 4. Discussion

Ovulation is a dynamic and rather complicated process initiated by LH, leading to breakdown of the follicular wall and extrusion of the

**Fig. 4.** Long-term effects of EPHA7 shRNA lentivirus contributing to irregular estrus cycles and polycystic ovaries in rats. (A) Estrus cycles were detected in rats injected with control shRNA (above) or EPHA7 shRNA lentivirus (below). D, diestrus; P, proestrus; E, estrus; M, metestrus. (B) Body weight changes (left), ovarian weight (middle), and representative image of rats' ovaries (right). (C) Rats' serum LH (left) and FSH (middle) levels, and LH/FSH ratio (right) detected by ELISA analysis. (D) Histology of microscopic ovarian structures of rats injected with control shRNA (above) or EPHA7 shRNA lentivirus (below). Asterisk stands for corpus luteum.  $N = 4$  per group. Data are presented as means  $\pm$  SEM.



oocyte. Substantial evidence has pointed to an ovulatory dysfunction in PCOS, which is directly associated with female infertility. Therefore, a better understanding of the molecular underpinnings affecting ovulation defects will aid the development of effective treatments for ovulation induction during assisted reproductive technology (ART) approaches.

C/EBP $\beta$ , stimulated by LH and regulated by the ERK1/2 signaling cascade, plays an essential role in ovulation and luteinization [5,8]. We discovered that the C/EBP $\beta$  mRNA abundance in the hGCs of PCOS patients was significantly lower than that in non-PCOS patients, which suggested a connection between C/EBP $\beta$  and PCOS. We analyzed previous ChIP-seq data and focused on *EPHA7* that was possibly associated with C/EBP $\beta$  in PCOS [19–21]. The EPH-EFN family genes are widely expressed during embryonic development and its proteins function in various physiological processes during organ development. *EPHA7* has also been shown to participate in neural and pronephros development [46]. *EPHA7* has also attracted attention in human malignancy studies on prostate, small cell lung, and colorectal cancers; on human germinal center B-cell and T-cell lymphoblastic lymphomas; on oral squamous cell and head and neck carcinomas; and in melanomas, among others [47]. Although a few members of the EPH-EFN family have confirmed ovarian functions [29–32], the role of *EPHA7* in female fertility, let alone its involvement in PCOS, has never been demonstrated. In this study, we showed that the *EPHA7* mRNA expression levels in hGCs of PCOS patients were decreased in comparison with those in non-PCOS patients. The same trend was demonstrated in the ovaries of rats with DHEA-induced PCOS. We confirmed wide *EPHA7* expression in the rat ovaries, especially in the GCs, theca cells, and CL. Additionally, the injection of *EPHA7* shRNA lentivirus in rats' ovaries gave rise to declined ovulatory abilities (as seen after 1 week), and to menstrual disorders and morphological changes of polycystic ovaries (as seen after 3 weeks). In here, we have presented these *in vivo* and *in vitro* results as the first evidence for an important role of *EPHA7* in ovulation and PCOS.

In addition to *EPHA7*, Krüppel-like factor 4 (KLF4) had also been identified via ChIP-seq analyzes as a putative C/EBP $\beta$ -target gene in PCOS [19–21]. KLF4 is a zinc-finger transcription factor suggested to play various roles in cell differentiation, proliferation, cycle regulation, and adipogenesis as well as to function as a tumor suppressor or an oncogene depending on its molecular context [48–52]. Moreover, KLF4 is expressed in several epithelia of the male and female murine reproductive tracts and participates in spermatogenesis [48,53]. Notably, the regulation of KLF4 expression in ovarian GCs by LH is associated with steroidogenic genes comprising the low-density lipoprotein receptor (LDLR), StAR and CYP11A1 [54]. Our results showing rather low levels of *KLF4* mRNA abundance in the hGCs of PCOS patients were expected. We thought it likely that KLF4 played a part in PCOS and might be involved in the functions of hGCs, although only limited amounts of molecular and genetic data were available. *ADAMTS1* has been identified as a new human ovulatory gene and a cumulus marker for fertilization capacity [40]. Female rats with *Adamts1* disruption are subfertile with mature oocytes trapped in follicles—the rats' ovulation rates are reduced by 77% and the fertilization of ovulated oocytes is further reduced by 63% [13,55]. *ADAMTS1* is indispensable for extracellular matrix remodeling during ovarian folliculogenesis and lymphangiogenesis [56]. What's more, the decreased expression of *ADAMTS1* in hGCs of PCOS

patients compared to that in normally ovulating women is consistent with our results, and explains the abnormal findings in oocyte recovery, oocyte maturity, and fertilization rates [57]. Using KGN cells, we obtained evidence implicating *EPHA7* as a critical positive upstream factor of ERK1/2-mediated C/EBP $\beta$  expression, which further binds to the *KLF4* promoter stimulating its transcription and translation, and then leads to the downstream expression of *ADAMTS1*. Likewise, the rat ovaries that were injected with *EPHA7* shRNA lentivirus and produced fewer oocytes, also displayed the mRNA and protein abundances of *EPHA7*, C/EBP $\beta$ , KLF4 and *ADAMTS1* that were all distinctly reduced. Based on our data, we concluded that *EPHA7* together with its downstream factors is vitally involved in ovulation.

To further investigate the role of *EPHA7* in the treatment of ovulation induction for PCOS patients, we used the Recombinant Mouse EphA7-Fc Chimera Protein. The alignments of *EPHA7* nucleotide and protein sequences between mice and rats are respectively 96% and 99%. After stimulating follicle development with PMSC, we injected *EPHA7* through the caudal vein instead of the usual hCG. The number of oocytes retrieved was larger than that in control rats injected with PBS, which indicated a triggering role of *EPHA7* for ovulation induction in rats. Four hours after *EPHA7* injection, serum *EPHA7* levels reached the peak and then gradually returned to normal. Serum LH levels began to rise 12 h after the injection and  $E_2$  levels decreased 16 h later. Generally, hCG is regarded as an upstream activator of ovulatory genes, but it has not been shown to be a regulator of EPH/EFN in GCs [31]. Here we propose that the exogenous *EPHA7* probably contributed to the rise of serum LH levels in the female rats, and that this preovulatory LH surge initiated and synchronized a series of biochemical events before ovulation. Since serum  $E_2$  levels were not increased after *EPHA7*-Fc administration, we excluded the role of positive feedback of  $E_2$  in upregulating LH secretion. The decrease of serum  $E_2$  levels resulted from the increase of LH levels. As known, the serum estrogen binds ER $\alpha$  on the pituitary gland and stimulates LH secretion [58]. Similarly, insulin could also facilitate LH secretion via the pituitary insulin receptor [59]. EFNA5 has always been considered as the ligand of *EPHA7* [47,60] and it is strongly expressed in the pituitary of mice [61] and in the brain of rats [62]. Thus, we suspect that the injected recombinant *EPHA7*-Fc protein reached the pituitary gland via the blood circulation and reacted with EFNA5, to then stimulate LH secretion. Another possibility is that the existing crosstalk between EPHB2 and EFNA5 in the EPH-EFN interaction network [63,64] had indirect effects on the availability of other molecules in the system. In other words, the binding of *EPHA7* and EFNA5 in the peripheral blood, would free up EPHB2 molecules that would presumably interact with other factors. For example, the increased level of unbound EPHB2 might bind to EFNB2, expressed on the rat pituitary gland [65,66], and would promote the secretion of LH. Alternatively, *EPHA7* may directly modulate EFNB2 activity via lateral *cis* interactions on the same cell [67], just like the reported attenuation by EFNB2 of the trans ligand-binding capacity of *EPHA3* and its activation via lateral *cis* interactions [68]. However, the exact molecular mechanism about the effect of *EPHA7*-Fc systemic administration on upregulating LH secretion is not yet understood and warrants further research.

We successfully established a PCOS rat model using DHEA according to a publication [36]. Sixteen hours after *EPHA7* injection, more corpora lutea and fewer antral follicles were observed in PCOS rat ovaries. At the

**Fig. 6.** Improvement of the 16-h function of *EPHA7* in PCOS rats. (A) Estrous cycles were detected during the eight last days after the establishment of the PCOS rat model. The panel (top-to-bottom) shows the CONTROL, CONTROL+*EPHA7*, DHEA, and DHEA+*EPHA7* groups. D, diestrus; P, proestrus; E, estrus; M, metestrus. (B) Body weight (left) and ovarian weight (right) changes in rats. (C) Glucose tolerance test and AUC values were obtained after each daily injection (s.c.) with DHEA for 20 consecutive days. (D) Rats' serum *EPHA7* levels detected by ELISA. (E) Rats' serum LH (left) and FSH (middle) levels, and LH/FSH ratio (right) detected by ELISA. (F) Rats' histological microscopic ovarian structures. The panel (left-to-right) shows the CONTROL, CONTROL+*EPHA7*, DHEA, and DHEA+*EPHA7* groups. (G) mRNA and protein abundances of StAR and CYP11A1 in rats' ovaries detected by western blot and qRT-PCR analyses. The panel (left-to-right) shows representative images of western blot assays (the immunoreactive bands were densitometrically quantified), and relative mRNA abundances. (H) Rats' serum progesterone levels detected by ELISA. (I) mRNA and protein abundances of *EPHA7*, C/EBP $\beta$ , KLF4 and *ADAMTS1* in the rats' ovaries.  $\beta$ -actin or GAPDH were used as loading controls. N = 6 per group. Blots are representative, and data are presented as means  $\pm$  SEM. \*  $P < .05$ , \*\*  $P < .01$ , \*\*\*  $P < .001$  against the group marked in the figure or against control rats; ##  $P < .01$  against DHEA rats.

same time, the serum LH and progesterone levels increased significantly. That is to say, EPHA7 promoted the progress of ovulation in PCOS rats. And during this process, the mRNA and protein abundances of EPHA7, C/EBP $\beta$ , KLF4 and ADAMTS1 in rat ovaries were all significantly increased. Therefore, we concluded that EPHA7 and its downstream factors directly helped trigger ovulation in PCOS rats. At the same time, EPHA7 facilitated LH secretion further contributing to ovulation. Based on our results, these two functions of EPHA7 dramatically improved the polycystic changes in PCOS rat ovaries induced by DHEA. This improvement of polycystic ovarian changes was clearer in the 48-h experiment than in the 16-h one. The levels of serum progesterone secreted by corpora lutea were also much higher in the 48-h experiment, suggesting the presence of more corpora lutea in rat ovaries. Since the serum EPHA7 level came back to normal only 16 h after the EPHA7 injection, we asserted that the resulting raised LH levels generated the ovulation after 16 h.

Considering a future clinical application for EPHA7, a protein molecule already existing in the body, we tried to confirm the safety of exogenous EPHA7 injections. We observed no changes in appearance, characteristics, or life habits of treated rats after 48 h or 14 days. We also found similar body weights (Fig. S4A), ovarian weights (Fig. S4B) and ovary appearances (Fig. S4C) between EPHA7-treated and PBS-control rats. Meanwhile, all tissue sections were free of visible acute or chronic toxicities (Fig. S4D). Finally, the negative results of routine blood examinations and blood biochemical indexes of rats implied a lack of bone marrow inhibition, or hepatic or renal toxicities (Table S1). Hence, our preliminary results indicate that exogenous EPHA7 injections can be considered safe.

Among the limitations of our study is the fact that we assessed only the number of oocytes retrieved in order to evaluate the role of EPHA7 in ovulation. The reduced fertility of PCOS patients could be attributed not only to a failure to release mature oocytes, but also to ovum growth obstacles. After all, oocyte maturity and fertilization rate are both closely related to female fertility, which would be further investigated to demonstrate the truly imperative role of EPHA7 in ovulation. Secondly, due to the technical difficulties for maintaining sufficient numbers of primary hGCs and the complexity of the EPHA7-mediated pathway studied, we decided to perform our experiments using the KGN cells. However, we obtained convincing evidence for a probable regulatory relationship between EPHA7 and downstream factors in human ovaries. Given the differences among species, more experiments in various animals and clinical trials will be required in the future.

Our study revealed that EPHA7 is a positive upstream factor of C/EBP $\beta$  contributing to the transcription and translation of KLF4, promoting ovulation via ADAMTS1. We demonstrated the functional role of EPHA7 in PCOS, which explains how the loss of EPHA7 underlies the ovulatory dysfunction. Our findings suggest EPHA7 may be a crucial and useful target for developing drugs for ovulation induction in ART treatment.

Findings from this study have been used to apply for a patent (China patent No. 201810166416.9).

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## Declaration of interests

The authors declare that they have no competing interests.

## Author contributions

Shang Li, Junyu Zhai, Jiansheng Liu, Fangfang Di, Yun Sun, Weiping Li, Zi-Jiang Chen and Yanzhi Du designed the study. Fangfang Di collected patient specimens and related information. Shang Li, Junyu Zhai, Fangfang Di and Yanzhi Du contributed to conducting the experiments and analyzing the data. Shang Li, Zi-Jiang Chen and Yanzhi Du drafted and revised the paper. All authors reviewed the results and approved the final version of the manuscript.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ebiom.2018.09.046>.

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