

## Original Article

# Attenuated AMH signaling pathway plays an important role in the pathogenesis of ovarian hyperstimulation syndrome

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**Abstract:** The aim of this study is to investigate the potential role of attenuated anti-Müllerian hormone signaling in the pathogenesis of ovarian hyperstimulation syndrome (OHSS). To analyze the expression of AMH and its receptors in human follicular fluid (FF) and granulosa cells (GCs), this study included consenting patients with moderate to severe OHSS (n = 83) and non-OHSS patients (control population, n = 108) undergoing IVF/ICSI treatment between March 2013 and March 2014. AMH concentrations in single FF samples from the OHSS patients were significantly lower than concentrations in samples from the control group. A negative correlation was found between the E<sub>2</sub> level and the AMH level in single FF samples. Similarly, a negative correlation was found between the FF AMH level and the number of oocytes retrieved. Although the mRNA expression level of AMH was hardly detectable in GCs, the mRNA expression level of AMHR2 in GCs from OHSS patients was significantly lower than the AMHR2 mRNA expression level in the control population. Based on these results, we established a murine model of controlled ovarian hyperstimulation (COH) using AMHR2-down-regulated mice to demonstrate the potential role of AMH signaling in the progression of OHSS. The knockdown of AMHR2 is capable of significantly increasing the ovarian response to exogenous gonadotropins, leading to several major clinical manifestations of OHSS in the murine model. In conclusion, attenuated AMH signaling increases ovarian sensitivity to COH and the incidence of OHSS in individuals undergoes IVF/ICSI.

**Keywords:** AMH, AMHR2, ovarian hyperstimulation syndrome, controlled ovarian hyperstimulation, IVF/ICSI

## Introduction

Controlled ovarian hyperstimulation (COH) is almost always employed during assisted reproductive techniques (ART) in an attempt to retrieve more oocytes. Although this strategy can improve reproductive outcomes, it is accompanied by a risk of the iatrogenic occurrence of ovarian hyperstimulation syndrome (OHSS).

OHSS rarely occurs spontaneously [1], and the majority of OHSS cases occur due to COH in women undergoing ART. Moderate and severe forms of OHSS occur in 3% to 10% of all ART cycles, and the incidence of OHSS reaches 20% among high-risk women [2, 3]. The severe form of OHSS is potentially lethal.

The pathogenesis of OHSS is not completely understood. It is hypothesized to result from the release of vasoactive peptides from granulosa cells (GCs) in hyperstimulated ovaries [4]. The fundamental physiological changes characteristic of OHSS include increased vascular permeability leading to the leakage of fluid from the vascular compartment, third-space fluid accumulation, and intravascular dehydration [5]. However, why the ovaries of OHSS patients are hypersensitive to exogenous gonadotropins and easily hyperstimulated remains unknown.

Anti-Müllerian hormone (AMH) is a member of the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily of growth and differentiation factors [6] that was first studied for its regulatory role in male sex differentiation [7, 8]. During male fetal

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**Table 1.** Clinical characteristics of patients included in studies of mRNA expression and hormone measurement

	Study of mRNA expression			Study of single follicular fluid		
	OHSS (n = 31)	Control (n = 32)	P value	OHSS (n = 52)	Control (n = 76)	P value
Age	29.0±3.4	29.0±3.2	NS	28.2±4.4	29.0±3.3	NS
BMI	21.2±2.0	21.2±2.9	NS	6.3±1.2	6.4±1.0	NS
AFC	14.9± 4.0	13.5± 3.3	NS	16.0± 1.6	15.5± 2.1	NS
Basal FSH (mIU/ml)	6.1±1.2	6.5±1.0	NS	28.2±4.4	29.0±3.3	NS
Duration of infertility (years)	3.8±2.4	3.6±2.5	NS	6.3±1.2	6.4±1.0	NS
Days of gonadotropin (Gn) injection	9.0±1.0	9.7±1.6	P<0.05	10.0±1.0	9.6±1.4	NS
Total dose of Gn (IU) <sup>a</sup>	1564.9±471.4	2188.7±514.4	P<0.01	1783.2±477.9	2070.1±589.0	P<0.01
Daily dose of Gn (IU) <sup>a</sup>	173.9±44.1	226.7±47.1	P<0.01	177.3±40.1	215.1±48.2	P<0.01
E <sub>2</sub> level of hCG per day <sup>a</sup>	6576.0±3463.9	4561.1±1501.2	P<0.01	9454.5±2737.5	4547.0±1711.5	P<0.01
Number of large follicles (>14 mm) <sup>a</sup>	16.7±5.7	11.5±2.9	P<0.01	17.6±4.2	11.3±3.0	P<0.01
Number of oocytes retrieved <sup>a</sup>	20.6±7.4	10.3±3.1	P<0.01	23.2±5.4	11.0±3.2	P<0.01

Data are presented as the means ± SD. <sup>a</sup>Such significant differences between the two groups result from the higher ovarian responsiveness of OHSS patients compared to the control group.

development, AMH is responsible for the regression of the Müllerian ducts, from which the female genital tract develops. While the exact physiological role and clinical utility of this glycoprotein still remain to be established in adult women, several studies show that it may exert crucial regulatory effects on folliculogenesis [9, 10].

Data from experimental animals show that AMH not only inhibits the initiation of the growth of primordial follicles [11-13] but also acts as a negative regulator of the FSH-sensitivity of small growing follicles [14, 15]. These findings are in accordance with previous experiments indicating that AMH inhibits the basal and epidermal growth-factor-stimulated proliferation of human granulosa cells [16]. Previous studies have demonstrated a potential role for AMH in decreasing the sensitivity of follicles to FSH-dependent dominance selection during the follicular phase of the human menstrual cycle [17]. A more recent study shows that treatment with AMH significantly reduces FSH-stimulated aromatase expression, E<sub>2</sub> accumulation and intracellular levels of cAMP [18]. Additionally, knockdown of the AMH receptor 2 (AMHR2) was shown to reverse the effects of AMH on aromatase expression [18]. Together, these data add support to the critical role played by AMH in the FSH-stimulated processes of follicular growth and selection. However, women with elevated serum AMH levels tend to be at higher risk for OHSS and usually respond more strongly to exogenous FSH during controlled ovarian

hyperstimulation (COH) [19-23]. These data apparently contradict the fact that AMH attenuates follicular responsiveness to FSH.

Possible explanations for this discrepancy are that the efficiency of the AMH signaling pathway is abnormal in OHSS patients and that the quantitative relationship between AMH levels and the incidence of OHSS may reflect a relatively higher peripheral accumulation of AMH secreted by a larger number of growing follicles in OHSS patients. Thus, we hypothesize that the attenuated efficiency of the AMH signaling pathway in OHSS patients weakens the inhibitory effect of AMH on follicular growth during the process of COH, increasing the sensitivities of ovaries to exogenous gonadotropins during assisted reproductive technology (ART).

To test our hypothesis, we observed the concentrations of AMH in individual follicular fluid (FF) and the expression of AMH and its receptors in the granulosa cells (GCs) of OHSS patients. We also established a murine model of COH in AMHR2 knockdown mice based on previously reported OHSS murine models [24-27] to study the possible relationship between an attenuated AMH signaling pathway and the progress of OHSS.

### Materials and methods

#### Patients

All patients undergoing *in vitro* fertilization/intra-cytoplasmic sperm injections (IVF/ICSI)

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**Table 2.** Primers for reverse transcription qRT-PCR

Gene	Primer sequences 5' to 3'	Length (bp)
h-AMH	F- CGCCTGGTGGTCCTACAC	60
	R- GAACCTCAGCGAGGGTGT	
h-AMHR2	F- TGTGTTTCTCCAGGTAATCCG	164
	R- AATGTGGTCGTGCTGTAGGC	
h-ALK2	F- TCAGGAAGTGCTCTGGTCT	180
	R- CGTTTCCCTGAACCATGACT	
h-ALK3	F- TGATGTGCCCTTGAATACCA	176
	R- ATTCTTCCACGATCCCTCCT	
h-ALK6	F- AAATGTGGGCACCAAGAAAG	171
	R- ACAGGCAACCCAGAGTCATC	
h- $\beta$ actin	F- CATGTACGTTGTATCCAGGC	233
	R- CTCCTTAATGTCACGCACGAT	
m-AMHR2	F- GCAGCACAAGTATCCCCAAC	204
	R- GTCTCGGCATCCTTGCATCTC	
m- $\beta$ actin	F- TGACAGACTACCTCATGAAGATCC	118
	R- TCGAAGTCTAGAGCAACATAGCAC	

between March 2013 and March 2014 at the Reproductive Medicine Center of Tongji Hospital, Huazhong University of Science and Technology, China were eligible for inclusion in this study.

The inclusion criteria for participants in the current study were as follows: (i) patients who had undergone a first cycle of IVF/ICSI using fresh embryos during the study period; (ii) those between 20 and 35 years of age; (iii) those whose body mass indices (BMI) ranged from 18.5-25 kg/m<sup>2</sup>; (iv) non-smokers; (v) those who had not been diagnosed with polycystic ovarian syndrome (PCOS); (vi) those who had been experiencing infertility for fewer than 10 years; (vii) those with normal basal hormone levels (including levels of FSH, luteinizing hormone (LH), E<sub>2</sub>, prolactin (PRL), and testosterone (T)); (viii) those with a normal antral follicular count (AFC); (ix) those undergoing a long gonadotropin releasing hormone agonist (GnRH-a) protocol for pituitary down-regulation; and (x) those taking less than 250 IU of gonadotropin per day. Patients' clinical data are listed in **Table 1**.

We divided patients according to their clinical characteristics into two groups: the OHSS group and the non-OHSS group. Diagnoses and classifications of OHSS were performed according to the latest criteria [28].

### Animals

Eight-week old female ICR mice were obtained from the Center of Experimental Animals, Tongji Hospital (Wuhan, China). Animals were kept in groups of two mice per cage with free access to water and chow. All animal studies were conducted according to protocols approved by the ethical committee of Tongji hospital, Tongji medical college, Huazhong University of Science and Technology.

### Ethical approval

This study was approved by the medical ethics board of the Tongji Hospital of Huazhong University of Science and Technology, and we received informed consent from all participants.

### Ovarian stimulation protocol

Patients undergoing IVF/ICSI received a lengthy treatment protocol using triptorelin acetate (Diphereline, IPSEN Pharma Biotech, France) for pituitary down-regulation. Ovarian stimulation was performed using recombinant FSH (Gonal-F, Serono, Switzerland or Puregon, Organon, Netherlands) beginning at 150-225 IU/day; dosages were individually adjusted according to ovarian responses as assessed by B-ultrasound and serum hormone levels on subsequent days. When at least two leading follicles reached a mean diameter of 18 mm, patients received recombinant HCG (Serono, Switzerland). Oocyte retrieval was performed 34-36 hours after HCG triggering. The oocytes were fertilized using routine techniques for IVF/ICSI, and two embryos were typically transferred on the third day following oocyte retrieval.

### FF and GC collection

Single FF and GCs were collected by ovarian puncture.

*Single FF collection:* To avoid blood contamination and dilution, only the first follicle from each 17-18 mm ovary was punctured, and the FF was centrifuged and collected in a separate tube.

*GC extraction and purification:* Total FF was collected and centrifuged at 2000 × g for 5 min. Then, the supernatant was aspirated, and the cell slurry was diluted two-fold with Hank's bal-

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anced salt solution (HBSS) and gently layered on top of 5 ml of 50% Percoll solution (Pharmacia, Uppsala, Sweden) in a 15 ml centrifuge tube. The tubes were centrifuged at  $5000 \times g$  for 20 min, and the GC layer at the interface of the Percoll solution and the HBSS was collected. The cells were then washed in HBSS three times and stored at  $-80^{\circ}\text{C}$ .

### *Measurements of AMH and $E_2$ in single FF*

The concentrations of AMH in the FF were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D, USA) according to the manufacturer's instructions. All samples were diluted 10 times in phosphate buffered saline (PBS) with 30% FBS and assayed in duplicate. The AMH sensitivity was 0.1 ng/ml. Assays were independently repeated on a random selection of 20% of the samples to confirm the intra-essay and inter-assay variations, which amounted to <15%. The hormone concentration was measured at 450 nm and corrected at 570 nm according to the manufacturer's instructions. The concentrations of  $E_2$  in the FF were determined by an electrochemiluminescence immunoassay using the ADVIA Centaur XP immunoassay system (Siemens, Germany) according to the manufacturer's instructions.

### *Reverse transcription quantitative real-time PCR (qRT-PCR)*

Total RNA was extracted from the GCs and reverse transcription was performed using a mix kit (total RNA extraction reagent) (Takara, Japan) according to the manufacturer's protocol. RT-PCR was performed with a SYBR master mix kit (Takara, Japan) on a Roche LC480 (Roche, Basel, Switzerland) RT-PCR System. The initial denaturation step was performed at  $95^{\circ}\text{C}$  for 15 s followed by 40 cycles of amplification at  $95^{\circ}\text{C}$  for 10 s and at  $55^{\circ}\text{C}$  for 30 s. Each sample was measured in triplicate. A mean value was used to determine mRNA levels using the comparative Ct method using the  $\beta$ -actin housekeeping gene as a reference and the formula  $2^{-\Delta\Delta\text{Ct}}$ . The sequences of the primers used are listed in **Table 2**.

### *AMHR2 down regulation in vivo*

SiRNA of mouse AMHR was purchased from RiboBio, Guangzhou, China. A Cy3-labelled

non-silencing siRNA control was used to determine *in vivo* uptake in ovarian tissue. AMHR siRNA [5'-GCCUACAGCAUGACCAUAUdT-3' (sense) and 3'-dTdT CGGAUGUCGUACUGGUAUA-5' (antisense)] and negative control siRNA with no sequence homology to any known mouse mRNA sequences were dissolved in siRNA buffer (Atelogene, Japan) and then mixed with atelocollagen (AteloGene Local Use, Koken, Tokyo, Japan). The siRNA-atelocollagen complexes were prepared according to the manufacturer's guidelines.

Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (2%, 50 mg/kg). A laparotomy was then performed to expose the ovarian bursa and 10  $\mu\text{l}$  of the siRNA/Atelocollagen complex, with a total dose of 1 ng siRNA per ovary, was injected into the bursa at the base of the oviduct using a NanoFil microinjection syringe (NF33FBV-2, World Precision Instruments, Sarasota, USA) under a microscope. The incision was closed in two layers (peritoneal and cutaneous) with 4-0 vicryl suture.

### *Validation of the transfection effect*

Validation of the *in vivo* transfection effect was performed by fluorescence photography, real-time PCR and Western blotting. After 24 hours of *in vivo* transfection with Cy3-labelled control siRNA (Ribobio, Guangzhou China), ovarian tissue was retrieved and imaged using an inverted fluorescence microscope (Carl Zeiss, Germany). Ovarian tissue for real-time PCR and Western blot assays was obtained after 48 hours of transfection with AMHR siRNA. The protocol for real-time PCR is described in the manuscript, and the primers used are listed in **Table 2**. A validation test was independently performed three times.

### *Western blot*

Ovarian tissues were obtained (detailed in the Tissue Preparation section), washed twice with PBS and ground thoroughly on ice. RIPA lysis buffer (Google biotechnology, Wuhan, China) with 2% protease inhibitor (Google biotechnology, Wuhan, China) was used. The tissue was lysed on ice and vibrated 3 times every 10 minutes. Protein concentrations were determined using a BCA Protein Assay Reagent kit (Google biotechnology, Wuhan, China). Samples were

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subjected to 10% SDS-PAGE and transferred onto nitrocellulose membranes using a Bio-Rad electroblot apparatus. Non-specific binding sites were blocked in 5% non-fat dry milk in 0.05% TBS-Tween. The following primary antibodies were used: anti-mouse AMHR2 monoclonal antibody (1:200, Santa Cruz, CA, USA), and anti-mouse  $\beta$ -actin monoclonal antibody (1:800, Google biotechnology, Wuhan, China) at 4°C overnight. The secondary antibody used was rabbit HRP-conjugated anti-goat IgG (1:2000, Google biotechnology, Wuhan, China), and an incubation was performed at 37°C for 1 hour. Protein bands were visualized by enhanced chemiluminescence (Pierce Biotechnology, Inc., Rockford, USA).

### *Murine COH model*

Animals were divided into 4 groups. Group 1 served as the control group (or non-COH group) without any siRNA transfection or ovarian hyperstimulation (n = 10). Group 2 was the Gn-only group with ovarian hyperstimulation but without any siRNA transfection. Group 3 was the control siRNA-transfected group with ovarian hyperstimulation (n = 10). Group 4 was the AMHR2 siRNA-transfected group with ovarian hyperstimulation (n = 10).

Ovarian hyperstimulation treatment began 12 hours after the laparotomy and siRNA transfection mentioned above. PMSG (pregnant mare serum gonadotropin) was injected intraperitoneally (20 IU/d) for 2 consecutive days. HCG (5 IU) was administered to induce ovulation [24].

### *Assessment of the COH effect on each group of mice*

*Plasma proteins in ascites:* Half of the mice from each group were used to detect the plasma protein concentration of ascites. The mice were anesthetized 48 hours after the administration of hCG, and 0.1 ml of 5 mM Evan's blue dye was injected intravenously through the caudal vein [24, 26]. Evan's blue dye binds to plasma proteins and forms leaks, together with the proteins, at sites of high vessel permeability. After 30 minutes, 5 ml of sterile saline were injected intraperitoneally. The fluid was then carefully massaged throughout the abdomen for 1 minute. Then, 1 ml of the fluid was removed and pipetted into a 96-well ELISA plate, and the absorbance was read at 620 nm.

*Ascites volume:* The ascites volume was determined in the remaining mice 48 hours after hCG administration, using the dilution of Evans blue dye. Mice were anesthetized and 2 ml of 0.01% Evans blue in saline was injected intraperitoneally. The fluid was then retrieved after 1 minute of abdominal massage and the concentration of diluted dye was determined at 620 nm. The volume of peritoneal fluid was calculated as follows [24]: Volume [ $\mu$ L] = [(OD 620 nm injected/OD 620 nm retrieved)  $\times$  2000] - 2000.

*Histology and follicular counts of ovaries:* Mice ovaries from every group of mice were fixed in 4% paraformaldehyde, embedded in paraffin, cut into 5- $\mu$ m serial sections and stained with Harris hematoxylin and eosin. As an indicator of recent ovulation, the numbers of corpora lutea were counted and averaged in three serial sections from the largest cross-section through the center of the ovary, as previously described [29-31], under a light microscope (Leica, Germany).

### *Data analysis*

Continuous variables were analyzed for statistical significance with Student's *t* test for two-group comparisons and an ANOVA for multiple-group comparisons. If values were not normally distributed, the Mann-Whitney rank sum test was performed using the SPSS 13.0 software package.

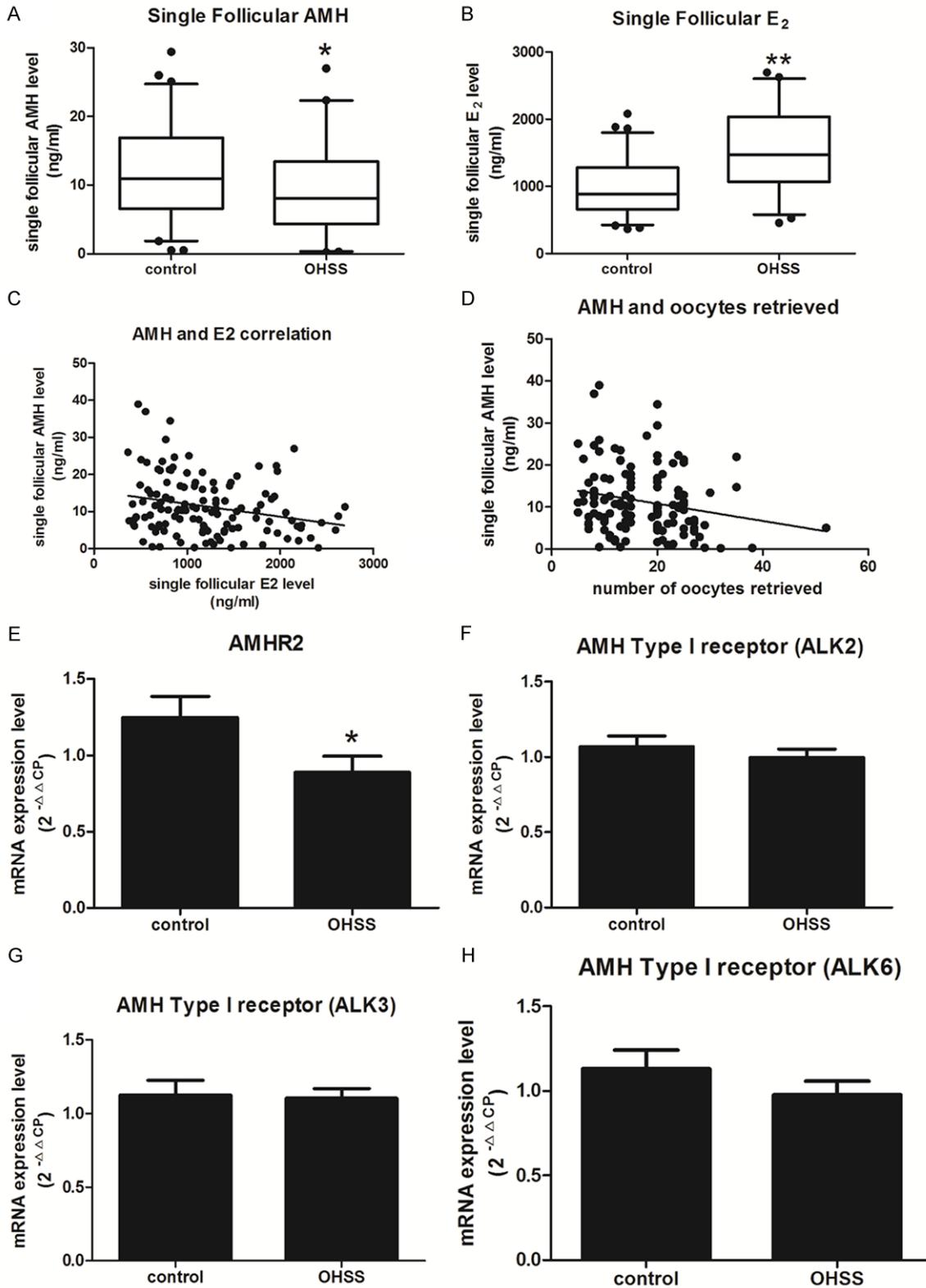
## Results

### *AMH and E<sub>2</sub> concentrations in individual human FF*

A total of 128 FF from 52 OHSS patients and 76 controls were used to measure AMH and E<sub>2</sub> concentrations.

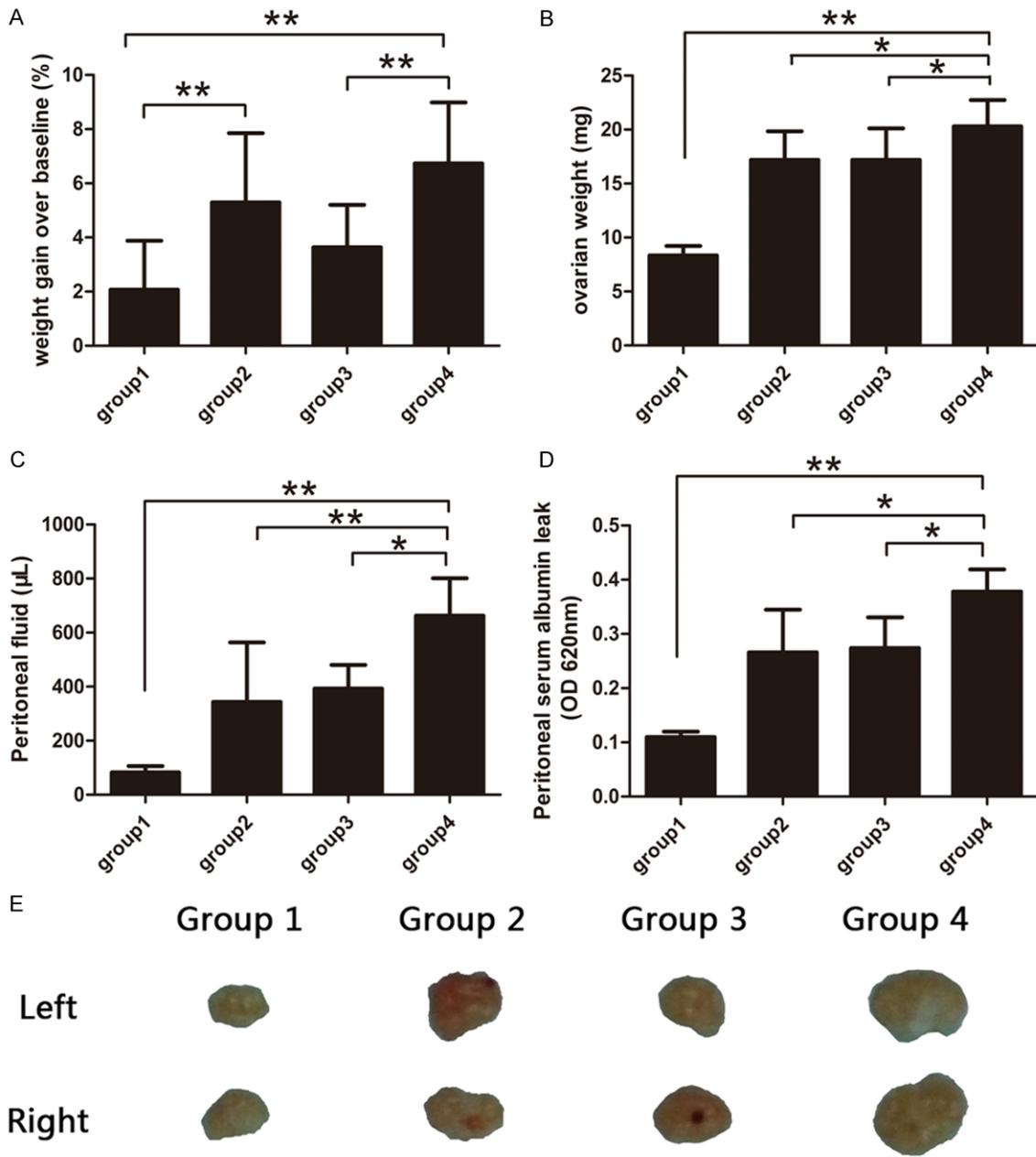
As shown in **Figure 1**, the AMH concentrations in the single FF samples from OHSS patients were significantly lower than those in the control group ( $P < 0.05$ , **Figure 1A**). In accordance with the typical laboratory manifestation of OHSS, the E<sub>2</sub> concentrations in the single FF samples from the OHSS patients were significantly higher than those in the control group ( $P < 0.05$ , **Figure 1B**). A negative correlation was found between the single follicular E<sub>2</sub> level and

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**Figure 1.** A. Concentrations of AMH in single FF; B. Concentrations of E<sub>2</sub> in single FF; C. Correlation between FF AMH level and FF E<sub>2</sub> level, Person Correlation coefficient = -0.25, P = 0.004; D. Correlation between FF AMH level and the number of oocytes retrieved, Person Correlation coefficient = -0.21, P = 0.016. E. mRNA expression levels of AMHR2 in GCs; F-H. mRNA expression levels of AMH type I receptors ALK2, ALK3 and ALK6 in GCs. \*P<0.05; \*\*P<0.01. Outliers in A-C are shown as 5<sup>th</sup>/95<sup>th</sup> percentiles.

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**Figure 2.** The parameters of ovarian hyperstimulation. Group 1: the control group; mice were treated with neither siRNA transfection nor PMSG+HCG; Group 2: mice were treated with PMSG+HCG but not with siRNA transfection; Group 3: mice were transfected with negative control siRNA and then treated with PMSG+HCG; Group 4: mice were transfected with AMHR2 siRNA and then treated with PMSG+HCG (n = 10, 10, 10, 10). A. The percentage weight gain in the four groups after COH; B. The mean weight of the bilateral ovaries after COH; C. The volume of peritoneal fluid was determined by Evans blue dye dilution; D. Vascular leakage in the abdominal cavity was analyzed by spectrophotometric measurements of dye concentration in the abdominal fluid after the intravenous injection of Evans Blue (ANOVA, Tukey HSD, \* $P < 0.05$ , \*\* $P < 0.01$ ); E. Photographs of ovaries from mice in each of the four groups after each individual treatment.

the single follicular AMH level (Pearson's correlation coefficient = -0.254,  $P < 0.01$ ) (Figure 1C), and a negative correlation was found between

AMH and the number of oocytes retrieved. (Pearson's correlation coefficient = -0.212,  $P < 0.05$ ) (Figure 1D).

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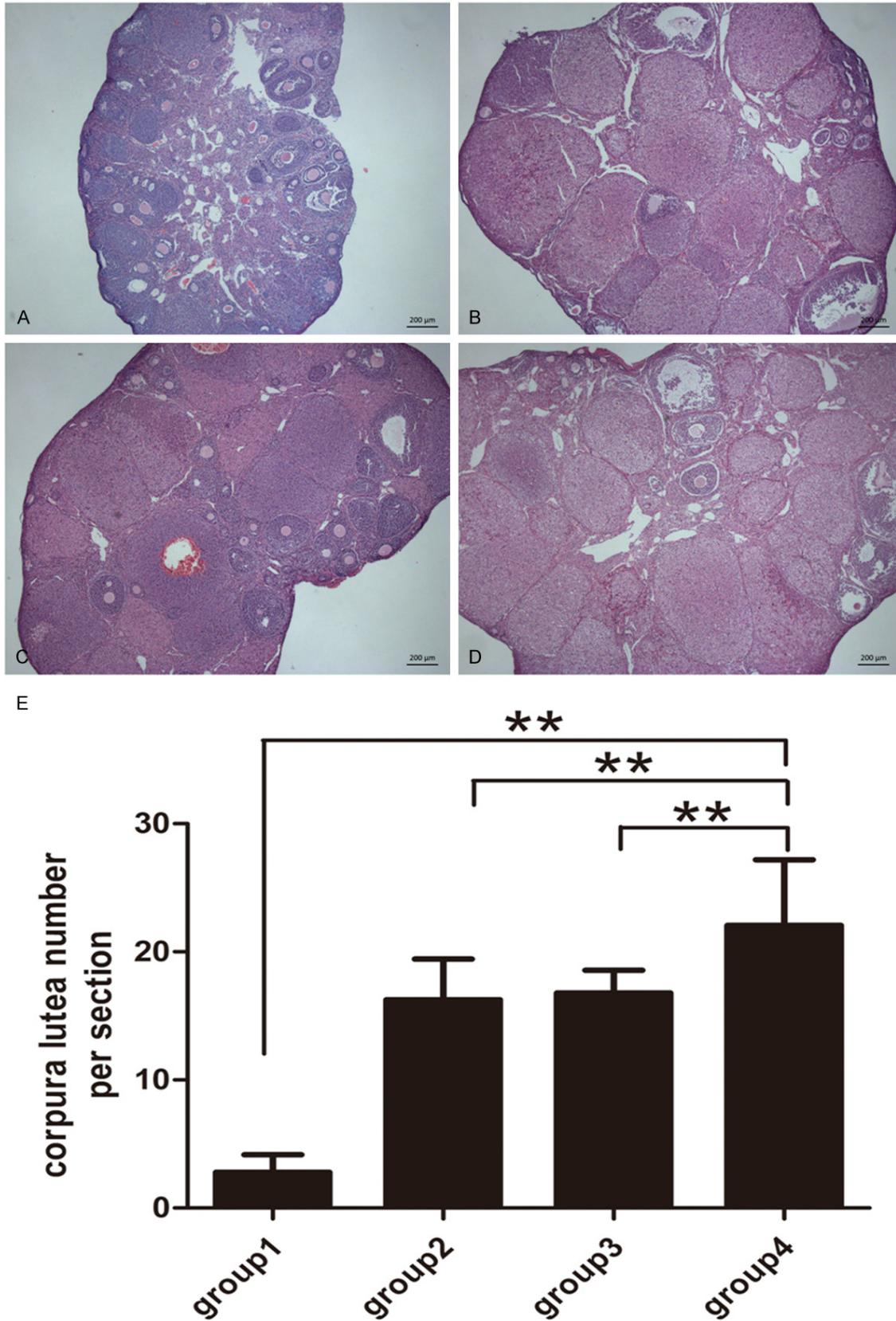
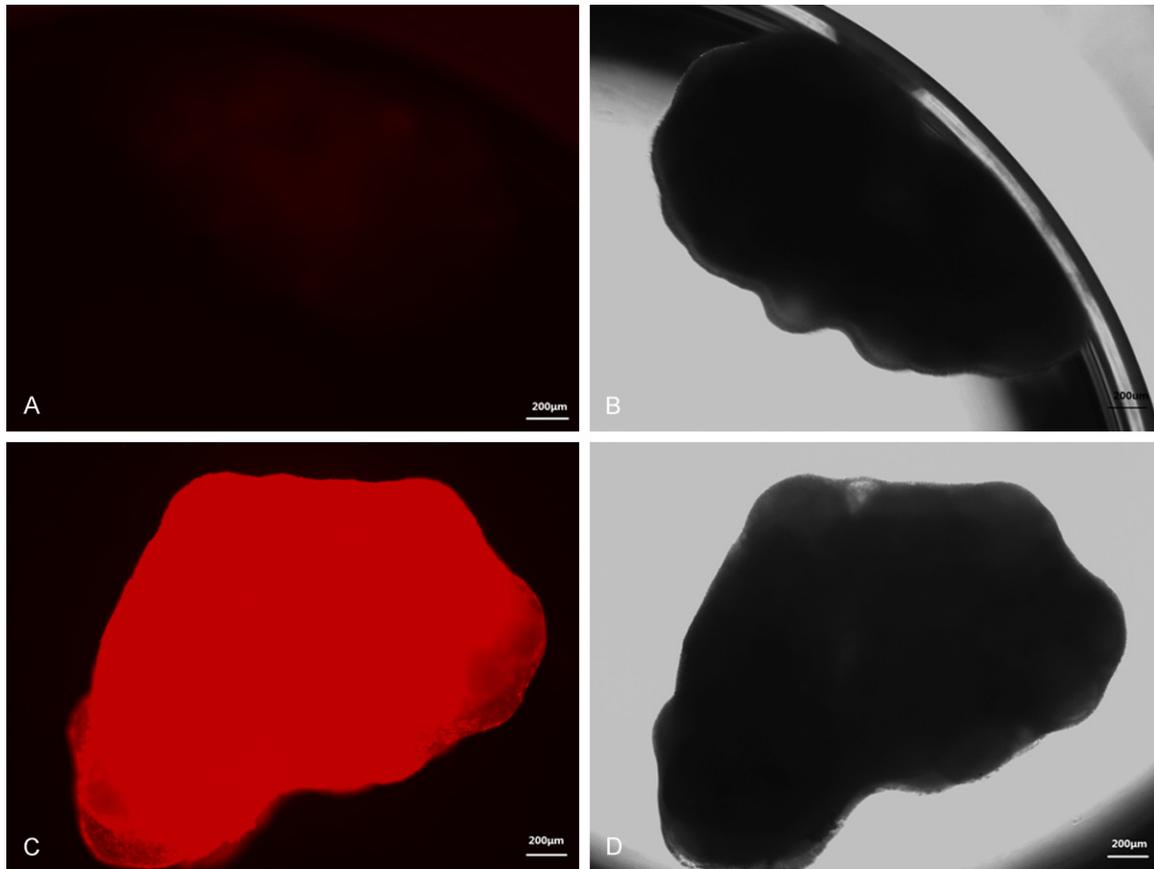


Figure 3. Ovarian histological study and corpora lutea counts. Histologic sections were prepared from the ovaries of each treatment group 48 hours after hCG treatment and stained with H&E. A-D. Ovarian sections with the largest

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diameters from groups 1-4, respectively. E. The average number of corpora lutea per section from each group was determined (ANOVA, Tukey HSD,  $**P<0.01$ ).



**Figure 4.** Validation of *in vivo* siRNA transfection by Cy3-labelled siRNA and fluorescent photography. Photos in the left panel were taken under fluorescence. Those in the right panel were taken under visible light. A, B. Negative control siRNA-transfected ovary; C, D. Cy3-labelled siRNA-transfected ovary.

### *Expression of AMH and AMHR2 in human GCs*

We measured the mRNA expression levels of AMH and its receptors in human GCs. Thirty-one OHSS patients (OHSS group) and thirty-two control patients (control group) were included in this part of the study.

AMH mRNA levels were barely detectable in both the OHSS group and the control group. The AMHR2 mRNA levels were significantly (0.71-fold) lower in the GCs of the OHSS group compared with those of the control group ( $P<0.05$ , **Figure 1E**). However, the mRNA levels of AMH type I receptors (ALK2, ALK3, and ALK6) were similar to those of the control group ( $P>0.05$ , **Figure 1F-H**).

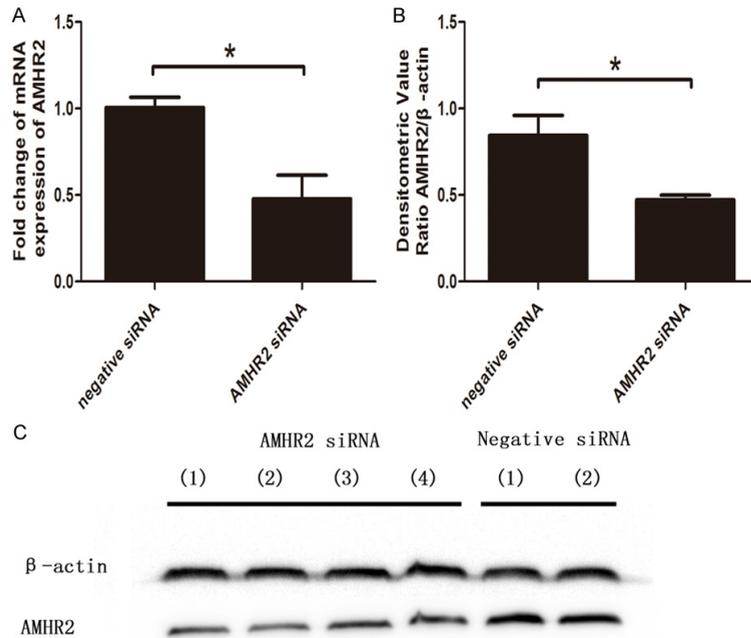
### *Down-regulation of ovarian AMHR2 gene expression in a murine model of induced ovarian hyperstimulation*

To identify whether the attenuation of the AMH signaling pathway plays an important role in the development of OHSS, we established a murine model of ovarian AMHR2 gene knockdown by *in vivo* siRNA transfection.

We tested the weights of the ovaries, the number of corpus lutea, generalized edema (measured by weight gain), the formation of ascites and the elevated vascular permeability in our murine model.

As shown in **Figures 2B, 2E** and **3**, ovaries from the AMHR2 siRNA treatment group weighed

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**Figure 5.** Validation of the transfection effect by real-time PCR and Western blotting. Three samples were used for each PCR assay, and each sample was measured in triplicate. The expression levels of AMHR2 mRNA in the negative transfected group and in the AMHR2 siRNA transfected group are shown (Figure 2A). In the Western blot, four samples from the AMHR siRNA group and two from the control group were used. The AMHR2 protein levels were calculated as the ratio of their densitometric values to that of the  $\beta$ -actin reference gene (Figure 2B). The densitometric values for the  $\beta$ -actin reference protein were comparable among all samples. \* $P < 0.05$ .

more than those from mice in the negative siRNA treatment group ( $P = 0.027$ ), the Gn-only group ( $P = 0.027$ ) and the non-COH group ( $P = 0.001$ ), and had more corpus lutea per section ( $P = 0.002, 0.006, 0.001$ , respectively).

Mice transfected with AMHR2 siRNA showed a nearly two-fold increase in weight after COH compared to those transfected with the negative control siRNA, manifesting more severe generalized edema ( $P = 0.01$ ) (Figure 2A). The AMHR2 siRNA treatment also led to a significant increase in the volume of peritoneal fluid ( $P = 0.031$ ) (Figure 2C) and increased peritoneal vascular leakage ( $P = 0.031$ ) (Figure 2D).

The transfection efficiency of the siRNA is described in Figures 4 and 5.

### Discussion

The present study proposed a novel perspective on, and possible explanation for, the relationship between AMH and OHSS. We explored

the concentrations of AMH and  $E_2$  in the FF of single follicles as well as the gene expression levels of genes in the AMH-signaling pathway. According to the observed characteristics of the AMH signaling pathway in OHSS patients, we established a murine model using *in vivo* AMHR2 siRNA transfection to establish the relationship between a deficient AMH-signaling pathway and OHSS.

Follicular fluid is a vital and dynamic element of the ovarian follicle that is in close proximity to developing oocytes. Its accumulation in the growing follicle begins at the pre-antral stage with the diffusion of proteins in the blood, through thecal capillaries, and of secretions from GCs, theca cells and oocytes [32]. Thus, knowledge of the proteins present in follicular fluid could provide insight into the follicular microenvironment.

The protein content of follicular fluid reflects changes in the secretory processes of GCs and theca cells and, more directly, their metabolic states during follicular growth. We observed that AMH concentrations in the follicular fluid of women with OHSS are significantly lower than those from women in the control group. In contrast, the  $E_2$  levels in individual follicular fluid samples from OHSS patients were significantly higher than those from women in the control group. We also found a negative correlation between AMH and  $E_2$  levels in follicular fluid and a negative correlation between the FF AMH level and the number of oocytes retrieved. These findings are in accordance with the recently established theory that AMH inhibits FSH-induced adenylyl cyclase activation, aromatase expression, and estradiol production in human granulosa-lutein cells [18]. The amount of AMH secreted by GCs in OHSS patients was less than in the control group, which weakened the inhibitory effect of AMH; as a result, the synthesis of  $E_2$  by single follicles in OHSS patients was significantly high-

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er than in the control group. The weakening of the inhibitory effect of AMH led to decreased suppression of the FSH responsiveness of follicle FSHR, resulting in larger numbers of growing follicles and a larger number of oocytes retrieved.

Many previous studies have associated high serum AMH levels with the incidence of OHSS during the ART procedure [33-35]. However, we observed a negative association between the AMH concentrations of single follicles and the incidence of OHSS. This result might be attributed to a cumulative effect in the peripheral blood because OHSS patients usually have more total follicles, which could lead to the high levels of AMH we found in sera from these patients.

We also studied the expression of AMH and its receptors in the granulosa cells of OHSS patients compared with patients in the control group. AMH signals through two types of serine/threonine kinase receptors called type I and type II receptors [36, 37]. The type II receptor of AMH (AMHR2) is highly specific and plays a major role in the AMH signaling pathway, while the identity of AMHR1 is unclear. The most widely accepted AMH type I receptor candidates include ALK2, ALK3 and ALK6, which are also type I receptors of bone morphogenetic proteins (BMPs) [36]. Dimerization of AMHR2 with type I receptors initiates AMH signaling via SMAD1, -5, and -8 [36, 38]. AMH acts through the type II AMH receptor (AMHR2) to inhibit FSH-induced adenylyl cyclase activation, aromatase expression, and  $E_2$  production in human granulosa-lutein cells [18]. We found that the expression of AMHR2 in the granulosa cells of OHSS patients on the day of ovum selection is significantly lower than that of the control group. This expression pattern of AMHR2 in OHSS patients is thought to result in the attenuation of the AMH-signaling pathway. Consequently, the inhibitory effect of AMH on follicular growth is weakened, resulting in the hypersensitivity of growing follicles to exogenous gonadotropins. All participants in our study experienced normal ovulation and regular menstruation. The attenuation of the AMH signaling pathway appears to have little influence on natural folliculogenesis. The excessively high doses of exogenous gonadotropins involved in the COH procedure may have an

amplifying effect, leaving certain patients more sensitive to FSH.

Based on our results in OHSS patients, we established a murine model of controlled ovarian hyperstimulation using AMHR2-down-regulated mice to demonstrate the potential role of the AMH signaling pathway in the progress of OHSS. The administration of PMSG and hCG forms a classical model of ovarian hyperstimulation and superovulation, high doses of which can result in increased ovarian weights, vascular permeability, and expression of VEGF and its receptors [39-41]. We compared the weight gain and ovarian weights and the magnitude of peritoneal vessel permeability among treatment groups by weighing mice and their ovaries, quantifying protein leakage into the peritoneal cavity and measuring the accumulation of ascites. In accordance with our hypothesis, mice transfected with AMHR2 siRNA exhibited heightened sensitivity to exogenous gonadotropin stimulation and a more obvious tendency toward OHSS. This tendency is supported by the increased numbers of corpora lutea, higher percentage of body weight gained, increased weights of ovaries and increased vascular permeability (as determined by Evans Blue leakage) in the treatment group compared to the control group, all of which mimic the clinical manifestations of human OHSS. These phenomena could be explained by the theory that the AMH signaling pathway inhibits ovarian responsiveness to FSH. After the down-regulation of AMHR2 by siRNA transfection, the efficiency of AMH signaling pathway decreases, which can lead to relative increases in ovarian response and a higher risk of OHSS.

In conclusion, we explored the potential contribution of the AMH signaling pathway in OHSS by investigating clinical human samples and simulating clinical findings in a murine model. The present findings suggest that an attenuated AMH signaling pathway (involving lower concentrations of AMH in single follicular fluid and the lower expression of AMHR2 in GCs) plays an important role in the progress of OHSS. As a result of its attenuation, the inhibitory effect of the AMH signaling pathway on folliculogenesis is lessened, and the balance between promoting and inhibiting follicle development is disrupted. Consequently, patients with an attenuated AMH signaling pathway are more sensitive

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to excessive exogenous FSH, and their ovaries are more easily hyperstimulated. Further research is necessary to clarify the mechanisms of OHSS.

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### Disclosure of conflict of interest

None.

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