ABCC4 is a PGE2 Efflux Transporter in The Ovarian Follicle: A Mediator of Ovulation and A Potential Non-Hormonal Contraceptive Target

Gil Yerushalmi (dratwork@gmail.com)
Assaf Harofeh Medical Center
Bat-el Shuraki
Sheba Medical Center
Yuval Yung
Sheba Medical Center
Ettie Maman
Sheba Medical Center
Micha Baum
Sheba Medical Center
Jon Hennebold
Oregon National Primate Research Center
Eli Adashi
Brown University
Ariel Hourvitz
Assaf Harofeh Medical Center

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Abstract

The role of Prostaglandins (PGs) in the ovulatory process is known. However, the role of the ATP binding cassette subfamily C member 4 (ABCC4), transmembrane PG carrier protein, in ovulation remains unknown. We report herein that ABCC4 expression is significantly upregulated in preovulatory human granulosa cells (GCs). We found that PGE2 efflux in cultured human GCs is mediated by ABCC4 thus regulating its extracellular concentration. The ABCC4 inhibitor Probenecid demonstrated effective blocking of ovulation and affects key ovulatory genes in female mice in vivo. We postulate that the reduction of PGE2 efflux caused by the inhibition of ABCC4 activity in GCs decreases the extracellular concentration of PGE2 and its ovulatory effect. Treatment of female mice with low dose of Probenecid as well as with the PTGS inhibitor Indomethacin or Meloxicam synergistically blocks ovulation. These results support the hypothesis that ABCC4 has an important role in ovulation and might be a potential target for non-hormonal contraception, especially in combination with PGE2 synthesis inhibitors.

These findings may fill the gap in understanding the role of ABCC4 in PGE2 signaling, enhance the understanding of ovulatory disorders, and facilitate the treatment and control of fertility.

Introduction

Prostaglandins (PG) are a group of biologically active compounds that play major roles in human physiology in both health and disease[1]. In the female reproductive system PGs have a role in the ovulatory cascade, fertilization, luteolysis, implantation, and parturition [2-4]. PG biosynthesis is under the control of the prostaglandin synthase (PTGS1 and PTGS2; also commonly known as cyclooxygenase) enzymes [5].

PTGS2 expression and PGE2 biosynthesis are induced by the midcycle luteinizing hormone (LH) surge [6]. Prostaglandin E2 (PGE2) is considered an essential paracrine mediator of the LH surge. The LH surge result in elevation of intrafollicular PGE2, which controls the timing of key ovulatory events [7].

The role of PGs in ovulation became apparent when it was shown that non-selective PTGS inhibitors, indomethacin and aspirin, effectively block ovulation in rats and rabbit. Later it was shown that PTGS inhibition block ovulation in ovine, bovine, and porcine models, as well as humans [6]. Systemic immunoneutralization of PGs blocks follicular rupture in both mice and rabbits [8,9]. PTGS2 null mutant mice exhibit severely impaired ovulation [10].

increased biosynthesis of ovarian PGs consequent to the LH-triggered induction of ovarian PTGS2 activity is essential for the maintenance of a normal ovulatory process (reviewed in [11]).

At physiological pH, PGs are charged anions that diffuse poorly through plasma membranes despite their lipid nature [12]. A carrier-mediated transport mechanism is therefore required for the translocation of PGs across biological membranes.

Three proteins capable of PGE2 transport were previously described, including the PG transporter (PGT), also known as the solute carrier organic anion transporter family member 2A1 (SLCO2A1; mainly a PGE2 influx transporter)[13], ABCC4 (mainly a PGE2 efflux transporter) [14], and SLC22A6 [15].

We have previously identified ovulation-associated genes by comparing the transcriptome of cumulus granulosa cells (CGCs) from immature (compact, germinal vesicle (GV)) cumulus oocyte complexes (COCs) to mature COCs (expanded, pre-ovulatory Metaphase II (MII)) [16].

We speculate that the differentially expressed genes identified (n=1746) serve as regulators of ovulation, cumulus expansion, and/or oocyte maturation.

Review of the resultant transcriptome database revealed ABCC4 to exhibits a 3-fold increase in mature cumulus cells (CCs) compared to immature CCs. SLC22A6, in turn, was not expressed in the library in question. PGT, recently shown by us to be significantly upregulated in the follicle in response to an ovulatory stimulus, was proven to be an important mediator of ovulation by regulating the extracellular concentrations of PGE2 [17]. The above notwithstanding, the transporter that mediates PGE2 efflux in the ovary remains largely unknown.

ABCC4, a functional PGs carrier with high affinity for PGE2 and other PGs [18,19], is a potential PGE2 carrier in the ovary. ABCC4, also known as MRP4 (Multidrug Resistance Protein 4) belongs to the ABCC (ATP Binding Cassette subfamily C) family and serves as an energy-dependent, transmembrane transporter [20]. Earlier work by Furugen et al. has shown that the ABCC4 inhibitors, MK-571 and probenecid, as well as ABCC4 siRNA treatment, reduce the extracellular levels of PGE2 in A549 lung adenocarcinoma derived cells [21].

Worldwide, over 40 million unwanted, pregnancies end in abortion each year [22] thus there is a global need for effective primary contraception, as well as, emergency contraception following unprotected intercourse. PTGS (COX) inhibitors were evaluated as contraceptives in in animal model [23] as well as in human trials but failed to achieve satisfactory results [24-26].

Prompted by the observation that ABCC4 constitutes a highly expressed periovulatory transcript and by its putative role as a prostaglandin efflux transporter, we set out to investigate the physiological role of this key transporter protein in the ovulatory process as well as explore the potential of the combined use of ABCC4 inhibitors with PTGS inhibitors in inhibition of ovulation and possibly contraception (the "combined approach").

Methods

Study design

The objective of this study was to investigate the physiological role of the prostaglandin transporter ABCC4 in the ovulatory process. We used both in vitro and in vivo systems to meet this objective. This

non-randomized laboratory study relied on human tissues and cells as well as on a mouse model. The study was approved by the local Institutional Review Board (IRB) committee of the Chaim Sheba Medical Center at Tel Hashomer (ethical approval #SMC-11-8707 and #SMC-12-9342). All experiments involving mice were conducted in compliance with the principles articulated by of the National Research Council (NRC) and approved by the Institutional Animal Care and Use Committee (IACUC) (approval #919/14/ANIM. The study was carried out in compliance with the ARRIVE guidelines. Mice were eeuthanatized using carbon dioxide in accordance to standard protocol.

Written informed consent was obtained from each patient who provided samples. A total of 27 women were included in the study. All patients were pretreated treated with a Gonadotropin-Releasing Hormone (GnRH) antagonist with an eye toward assuring experimental consistency (see below). The average age of the patients was 32±4 (mean±SD); the average BMI was 21.5±2.3; and the average number of aspirated oocytes was 10±3.

All methods were performed in accordance to the relevant guidelines and regulations.

IVF protocol

Normo-ovulatory young women (< 37 years of age) undergoing IVF because of male factor infertility or pre-implantation genetic diagnosis were selected for this study. Subjects afflicted with BRCA mutations, Fragile X disorder, Endometriosis, or Polycystic Ovary Syndrome (PCOS) were excluded. Ovarian stimulation was carried out as previously described [54,55]. Briefly, a "short antagonist" protocol was used wherein controlled ovarian hyperstimulation with Human Menopausal Gonadotropins (HMG; Menopur®) or recombinant Follicle-Stimulating Hormone)rFSH, either Gonal-F®; Merck Serono or Puregon Pen®; Schering Plough) was initiated 3 days after the onset of menses. The initial gonadotropin dose used was dependent upon age, body mass index, and previous IVF treatment history. Ovarian suppression with a GnRH antagonist (0.25 mg/day, Cetrorelix, Cetrotide®; Serono International, SR) was initiated when the leading follicle was more than 12 mm in diameter. When three or more follicles exceeded18 mm in diameter, 250 µg of human Chorionic Gonadotropin (hCG; Ovitrelle®; Merck Serono) was administered to trigger ovulation. Transvaginal follicular aspiration was performed 36 hours later under ultrasound guidance.

Cumulus granulosa cell collection

After COC retrieval, CGCs of each oocyte were removed with the use of hyaluronidase (SAGE, Trumbull, CT, USA) and a glass denudation pipette (Swemed, Billdal, Sweden). The CGCs were washed in Phosphate-Buffered Saline (PBS) and centrifuged at 5000 × g for 5 minutes at room temperature. The resulting pellets were stored at −80°C until RNA isolation. CGCs of individual oocytes were classified as per the corresponding oocyte maturation stage: CGCs from GV oocytes (CGGV) and CGCs from MII oocytes (CCMII). CGCs obtained from individual oocytes were collected from individual subjects were pooled to generate a single replicate (n=3-4 different subjects). Each experiment was performed at least three times.

Mural granulosa cell collection

Follicular fluid was aspirated from follicles ≥ 17 mm in each subject. The follicular fluid was centrifuged and the pelleted MGCs were re-suspended in PBS (Sigma-Aldrich-Aldrich, St Louis, MO, USA). After allowing the cells to settle by gravity for a few minutes, the top portion of the medium was aspirated and the cells were repeatedly re-suspended until the medium proved clear. The cells were then centrifuged at 1000 rpm for 5 minutes at room temperature and the resulting pellets were stored at −80°C until RNA isolation. Total MGCs from 3 different subjects were pooled to generate a single replicate. Each experiment was performed at least three times.

Mural granulosa cell culture

Each MGCs sample was collected from the aspirated follicular fluid of follicles size ≥ 17 mm from one subject (unless specified otherwise) and re-suspended in PBS (Sigma-Aldrich-Aldrich, St Louis, MO, USA). After allowing the cells to settle by gravity for a few minutes, the top portion of the medium was aspirated and the cells were repeatedly re-suspended until the medium proved clear and then placed on a Percoll® gradient and centrifuged at 3000 RPM for 15 min. The MGCs were collected and washed with PBS, counted and plated in 24-well plates at a density of 100,000 cells/well, and incubated at 37°C in a humidified atmosphere with 5% CO_2 in air. The cells were cultured for 4 days with daily medium replacement prior to hCG triggering for the indicated times.

RNA extraction and qPCR

Total RNA was extracted from MGCs or CGCs using a Mini/Micro RNA Isolation I kit (Zymo Research, CA, USA) according to the manufacturer's instructions. RNA purity and concentration were assessed using a NanoDrop spectrophotometer (NanoDrop 2000C, Thermo Scientific Waltham, MA, USA). Total RNA (25ng) from each sample was used for cDNA synthesis by a high capacity reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions in a 10µl total volume reaction. mRNA levels were analyzed by real-time PCR using the StepOnePlus real-time PCR system (Applied Biosystems). The real-time PCR mix contained 1µl of cDNA, fast SYBR Green Master Mix (Applied Biosystems), and specific primers for ABCC4 or other gene of interest and β-actin (housekeeping gene) in a total volume of 10µl. Cycling parameters were: 1 cycle at 95°C for 20 seconds, and 40 cycles each at 95°C for 3 seconds and at 60°C for 30 seconds. A melting curve analysis was performed at the end of each run to ensure measurements were based on the amplification of the target gene. All samples were run in duplicate. Analysis of the qPCR results was carried out with StepOne software. Relative gene expression was calculated using the delta-delta Ct method. Details of the primers used are shown in Table S1.

RNA sequencing (RNAseq)

Global transcriptome assessments were performed on compact (CCGV) samples obtained from two women during *in vitro* maturation (IVM) and expanded (CCMII) cumulus cells samples obtained from

three women during IVF as described in Yerushalmi et al. 2014 [16]. Briefly, a cDNA library was prepared according to Illumina recommendations (preparing samples for mRNA sequencing; Illumina). Cluster generation and single-end sequencing was carried out using the standard Illumina procedures for the HiSeq 2000 sequencer (Illumina). All sequenced reads were mapped and aligned to the human genome. The number of reads that overlap each of the annotated genes was counted and the differentially expressed transcripts were identified [16].

IVM protocol.

IVM cycles were carried out as previously describe [56]. Briefly, sonographic assessment of the antral follicle count and of endometrial thickness was carried out on day 3 of a spontaneous menstrual cycle. The serum concentrations of estradiol and progesterone were also determined. Treatment with 150 IU/day rFSH for 3 days followed suit. After a second sonographic assessment on Day 6, 10,000 IU hCG (Pregnyl; Organon) was administered when the endometrial thickness was ≥5 mm and the leading follicle was ≥12 mm. Oocyte retrieval was carried out 36 hours later.

Animals

C57BL/6 mice were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA). The mice were maintained under controlled lighting (12 hour light/12 hour dark) conditions with continuous access to food and water.

Superovulation protocol

25-day-old female mice were initially treated with 10 U of Pregnant Mare Serum Gonadotropin (PMSG, Chronogest, Intervet, Israel) to stimulate follicular growth. An ovulatory dose of hCG (10 U) (Ovitrelle®, Merck Serono, Darmstadt, Germany) was administered 48 hours later. To elucidate the role of ABCC4 in vivo, probenecid, at different concentrations (200-450 mg/kg), was administrated intra-peritoneally in the same time as hCG. To evaluate the combined effect of ABCC4 and PTGS inhibitors, probenecid (25 mg/kg) and meloxicam (10 mg/kg) or indomethacin (5 mg/kg) were administrated together intra-peritoneally at the same time as hCG.

Rescue experiments: To examine the specificity of probenecid, and the combined approach (probenecid and meloxicam) effect on ovulation, PGE2 (2mg/kg) was administrated intra-peritoneally in the same time as hCG.

Animals were sacrificed 48 hours after the initiation of PMSG treatment as well as 9 or 16, hours after hCG administration. All mice were sacrificed by CO2 asphyxiation, and the ovaries were removed and either flash frozen in liquid nitrogen, paraformaldehyde-fixed, or punctured in order to collect entrapped oocytes. Blood samples were collected at the time of euthanasia for progesterone measurement and the number of oocytes within the ampullas of each oviduct was recorded.

Western blot

Cells were harvested using 0.5 mL PBS and pelleted. Cell pellets were lysed in TNE buffer (50 mM Tris–HCl, pH 8.0, 250 mM NaCl, 2 mM EDTA, 1% NP-40, Sigma Aldrich St Louis, MO, USA) containing a protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, USA), vortexed, and incubated for 10 min on ice before removal of nuclei and debris by centrifugation. Aliquots of the clarified supernatants were used to determine protein concentration using the Bradford method (Protein Assay Dye Reagent, Bio-Rad, Hercules, CA, USA). Equal amounts (50 µg) of protein were loaded and separated by SDS-polyacrylamide gel electrophoresis (10% acrylamide). Proteins were then transferred onto nitrocellulose membranes. Membranes were blocked in 5% bovine serum albumin (BSA) in TBST (100 mL TBS 10X, 900 mL H_2O, 1 mL Tween 20, Sigma Aldrich St Louis, MO, USA) for one hour and afterwards incubated with a primary antibody against ABCC4 (M4I-10, Abcam, 1:500) or β-actin (housekeeping gene) overnight at 4°C. The membranes were then treated with a goat anti-rat IgG HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA) and developed using an enhanced chemiluminescence kit (Thermo Scientific, Waltham, MA, USA) [57].

PGE2 measurements in cell culture medium and in cells

MGCs were plated in 24-well plates at a density of 200,000 cells/well and cultured as described above for 6 days. The MGCs were then treated for 24 hours with hCG (1U) in the absence or presence of MK-571 (50 µM) or probenecid (500 µM). The concentration of PGE2 in conditioned media was assessed using an enzyme immunoassay kit (Cayman Chemical). For intracellular PGE2 measurements, cells were harvested in PBS, resuspended in 50 µl of sonication buffer (0.1 M phosphate pH 7.4 containing 1 mM EDTA and 10 mM indomethacin), sonicated (7 sec x 3 on ice; CV18 Sonics) and centrifuged at 8000 rpm, for 10 min at 4ºC. Supernatants were diluted 1:5 with EIA buffer and subjected to EIA for PGE2 (Cayman Chemical).

Effect of ABCC4 siRNA on PGE2 levels

MGCs were plated in 6-well plates at a density of 250,000 cells/well and cultured as described above for 2-3 days. The transfection mixture included: (A) 9 µl Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) in 150 µl OptiMEM (Gibco by Life Technologies, Paisley, UK), and (B) 8 µl ABCC4 siRNA or Scramble siRNA (Santa Cruz Biotechnology, Dallas, TX, USA) in 150 µl OptiMEM medium (10 µM final concentration). The transfection mixture was incubated at room temperature for 5 minutes before added to the cells (300 µl) with additional 300 µl of OptiMEM medium. The cells without transfection reagent were covered with 600 µl OptiMEM. After the cells were incubated at 37°C for 4 h, an additional 1.4ml medium (DMED/F12 with 10% FCS media) was added to each well. The medium was changed 24 h later. All the groups, except for control, were cultured for 48 h post transfection and then stimulated with hCG for 24 h. The cells were then harvested and the medium was collected from one duplicate of the group for protein quantification using the Bradford assay. Extracellular PGE2 levels were assessed using a PGE2 Enzyme Immunoassay (EIA) kit. PGE2 levels were analyzed relative to protein levels. The cells in the other duplicate were subjected to RNA lysis buffer, diluted 1:200 for intracellular PGE2 levels and assessed by PGE2 EIA kit and to qPCR to determine ABCC4 mRNA expression levels.

Mouse ovary RNA isolation

Mouse ovaries were removed immediately after the mice were sacrificed, dissected from the surrounding fallopian tubes and fat tissue, and flash frozen in liquid nitrogen. Frozen ovaries were crushed by mortar and pestle and the RNA was purified using the Micro RNA Isolation I kit (Zymo Research, CA, USA) according to the manufacturer's instructions.

Mouse ovarian morphology

Fixed ovaries (4% formalin) were embedded in paraffin, sectioned, mounted on slides, and stained with hematoxylin/eosin. Mouse ovarian morphology was assessed by examining 4-μm serial histological sections.

Measurement of progesterone concentrations

Blood samples for hormone assays in female mice were obtained at the time of euthanasia by cardiac puncture. Sera were separated from whole blood and frozen until the time of analysis. Progesterone concentrations were measured in duplicate by the American Medical Laboratories in Herzliya, Israel.

Statistics

Each experiment was carried out at least three times. Data were expressed as mean ± standard error of the mean (SEM) and evaluated with Student's t-test with a two-tailed distribution, with two samples equaling variance, or with ANOVA for more than two variances using the post hoc Tukey test, assuming equal variances, or the Games–Howell test for unequal variances. When appropriate, Kruskal–Wallis non-parametric comparison test was used. For all statistical analysis, SPSS 22 software (IBM, Armonk, NY, USA) was used. P values < 0.05 were considered statistically significant.

Results

ABCC4 expression in human granulosa cells in vivo

Illumina-based RNA-sequencing (RNAseq) was carried out on RNA extracted from compact (CCGV) and expanded (CCMII) cumulus cells and sequence counts were used to assess gene expression [16]. As revealed in Fig. 1A, the *in vivo* expression of *ABCC4* transcripts in CGCs of expanded post-ovulatory MII COCs after *in vitro* fertilization (IVF) was 2.74-fold higher (p=0.0196) as compared with CGCs of compact GV COCs obtained during *in vitro* maturation (IVM).

In humans, during follicular antrum formation, GCs produced two distinct lineages; MGCs that line the follicular wall and CGCs that surround the oocyte [27]. We compared the expression levels of *ABCC4* mRNA in MGCs and CGCs by qPCR. MGCs and CGCs were obtained from large preovulatory follicles (>17mm) during the IVF procedure. As shown in Fig. 1B, MGCs expressed significantly higher (2.6-fold) levels of *ABCC4* mRNA relative to CGCs (p=0.0184).

ABCC4 expression in human mural granulosa cells in vitro

To confirm the effect of LHCGR (luteinizing hormone/choriogonadotropin receptor) activation on the expression of *ABCC4* transcripts in GCs, primary MGCs were cultured for 4 days and subsequently pretreated for 48 hours with FSH [28] were then incubated in the absence or presence of hCG. These cells provided an *in vitro* model of non-luteinized human mural granulosa cell culture to study the effects of LH/hCG signaling [28]. After hCG stimulation, the cells were harvested 0, 3, 6, 9, 12, 24 and 36 h later. *ABCC4* mRNA was observed to be upregulated in hCG-treated cells, peaking at 12 h after hCG treatment ($p<0.001$, Fig. 1C) and returning to basal levels at 24 and 36 h post-hCG treatment. Similar changes in the ABCC4 protein levels were observed by Western blot, with a 1.5-fold induction following hCG treatment ($p=0.0296$, Fig. 1D).

Assessment of ABCC4-mediated efflux of PGE2 in cultured human mural GCs

To investigate the hypothesis that ABCC4 mediates efflux of PGE2 we used two known ABCC4 chemical inhibitors; MK-571 [29,30], and probenecid [29,31]. FSH-pretreated MGCs (48 hours) were incubated with hCG (to induce ABCC4 expression; 1 U/ml) for 24 hours in the absence or presence of MK-571 (50 μM) or probenecid (500 μM). The medium was collected and analyzed by a PGE2 specific enzyme immunoassay (EIA). Treatment with hCG produced a 5.3-fold increase in the concentration of PGE2 in the conditioned medium (Fig. 2A). Co-treatment with the ABCC4 blockers probenecid (Figure 2A) resulted in a significant decrease ($p<0.001$) of PGE2 levels by 2.7-fold when compared to hCG treatment alone. Similar results were obtained using another ABCC4 blocker, MK-571 (Figure 2C). Incubation of MCGs with probenecid or MK-571 in the absence of hCG did not have a significant effect on basal PGE2 levels. Moreover, intracellular PGE2 levels remain unchanged (Figure 2B, 2D). These observations suggest a role of ABCC4 in PGE2 efflux and that cellular mechanisms are in place to keep the intracellular PGE2 levels constant.

Assessment of the ABCC4-mediated PGE2 efflux in cultured human mural GCs using ABCC4 siRNA.

To confirm the role ABCC4 may play in regulating PGE2 efflux, a more specific approach was utilized, namely ABCC4 knockdown in MGCs through the use of siRNA (Fig. 3). First, the effect of *ABCC4* siRNA on *ABCC4* mRNA expression was determined. Fig. 3A shows that ABCC4 siRNA significantly decreased *ABCC4* mRNA levels 7.75-fold ($p<0.001$) compared to hCG only. Importantly, *ABCC4* mRNA levels in the scrambled siRNA group (negative control) were equivalent to cells treated with hCG only, indicating the specificity of the *ABCC4* siRNA and that siRNA transfection per se does not impact MCG viability or overall gene expression.

We next tested the effect of treating MGCs with *ABCC4* siRNA on hCG-stimulated extracellular and intracellular levels. Fig. 3B displays an increase in PGE2 extracellular levels by 1.38-fold ($p=0.037$) in extracellular levels in hCG-treated cells as compared to control. Treatment with *ABCC4* siRNA significantly decreased the PGE2 extracellular levels by 1.36-fold relative to hCG ($p=0.017$; Figure 3B). As was the case with the chemical ABCC4 inhibitors, the intracellular levels of PGE2 following siRNA and hCG treatment did not differ significantly from the hCG-treated controls (Figure 3C).

The effect of ABCC4 blockade on ovulation in vivo

To evaluate the physiological role of ABCC4 in the ovulatory process, we tested the effect of probenecid on ovulation in mice undergoing a PMSG/hCG superovulation protocol. As shown (Figure 4A), *ABCC4* transcripts were expressed in the mouse ovary throughout the periovulatory interval, with no significant differences in mRNA levels between the different stages. We then assessed the effect of different doses of probenecid on ovulation. As shown in Fig. 4B and Table S2, 100% of the mice receiving hCG with or without vehicle ovulated, releasing an average of 53.7 or 47.8 oocytes, respectively (p=0.99). The percentage of mice that ovulated and the number of oocytes ovulated was decreased in probenecid-treated mice in a dose-dependent manner, with a significant decrease from the concentration of 350 mg/kg probenecid. Concurrent injection of hCG and 350 or 400 mg/kg of probenecid prevented ovulation in 72% to 76% of the mice resulting in the mean release of 9.4 and 4.7 oocytes per animal, respectively. Treatment with 350 or 400 mg/kg of probenecid represents a significant 82.5% and 91.2% reduction in ovulation efficiency, respectively, compared to animals receiving hCG alone. Administration of 450 mg/kg of probenecid effectively blocked ovulation in 80% of the mice, while the remaining 20% had ovulated on average one oocyte per mouse, which represents a 99.6% reduction in ovulatory efficiency. In order to assess the specificity of probenecid effect on ovulation we used probenecid (350 mg/kg) in conjunction with PGE2 (2 µg/kg). The addition of PGE2 to the inhibitors abolished most of the inhibitory effect of ovulation by probenecid (rescue effect, Fig. 4B).

These results suggest that the inhibition of ABCC4 blocks oocyte release in mice thereby establishing an essential role of ABCC4 in ovulation.

To further validate the ovulation-blocking activity of probenecid, we performed a histological examination of ovaries removed from vehicle- or probenecid (400 mg/kg)-treated mice at 16 and 40 hours after hCG administration. PMSG treatment led to the development of numerous preovulatory follicles (Fig. 4, C and D). Removal of the ovaries from vehicle-treated mice 16 hours after the administration of hCG revealed numerous early corpora lutea (Fig. 4, E and F). In contrast, the ovaries removed from mice receiving hCG plus probenecid (Fig. 4, G and H) contained many unruptured preovulatory follicles replete with entrapped oocytes, resembling ovaries obtained from mice treated with PMSG alone. At 40 hours after hCG injection, ovaries from hCG-treated mice displayed many corpora lutea composed of luteinized GCs containing lipid droplets in their cytoplasm (Fig. 4, I and J). In contrast, ovaries collected from hCG- and probenecid-treated mice still contained entrapped oocytes in preovulatory follicles (Fig. 4, K). These histological results further demonstrate that treatment of mice with an ABCC4 inhibitor prevents ovulation.

We also determined the effect of *in vivo* treatment with probenecid on oocyte maturation (table S3A) and cumulus expansion (table S3B). Oocytes were collected from control mice (treated with hCG and PMSG) as well as from mice treated with different doses of probenecid (300, 400 and 450 mg/kg), which were co-injected with hCG. The ovaries were removed 16 hours after hCG injection. The results show that oocytes from the hCG-treated group underwent germinal vesicle breakdown (GVBD) and progressed to

the metaphase II stage of meiosis and cumulus expansion. In contrast, oocytes from the probenecid-treated group (400 mg/kg) were all immature (GV stage) and a compact cumulus cell layer surrounded 97.7% of the oocytes. From the 450 mg/kg probenecid-treated group all oocytes examined possessed a compact cumulus.

There were no short term or long term observable side effects to the mice treated with doses of probenecid up to 450 mg/kg.

Inhibition of ABCC4 affects the levels of mRNA encoded by genes that regulate ovulation and corpus luteum function

We next examined the effect of probenecid on genes involved in ovulation and luteal formation in ovaries obtained from mice undergoing a superovulation protocol with or without 400 mg/kg of probenecid. Animals were sacrificed 3 hours after receiving hCG and vehicle or probenecid. The mRNA levels encoded by known LH/hCG target genes was initially investigated (Fig. 5A). The genes in question included those involved in oocyte maturation (*Areg, Ereg*) [32,33], progesterone receptor signaling (*Pgr*) [34], cumulus expansion (*Tnfaip6*) [35], follicular rupture (Adamts1, Ctsl) [36], gonadotropin signaling (*Fshr, Lhcgr*) [37], and steroidogenesis [(*Star, Cyp11a1*) [38] and p450Scc (cholesterol side cleavage chain)] [39]. We also investigated the regulation of genes whose products encode proteins involved in PGE2 synthesis and signaling (Fig. 5B). Included were those genes critical for PG synthesis (*Pla2g4a, Ptgs2, Ptges*), PG metabolism (*Hpgd*), PGE2 signaling (*Ptger2, Ptger4*) and PG transport (*pgt, Abcc4*) [40]. Serum progesterone concentration were also measured 24 hours after hCG administration to assess corpus luteum function. As shown in Figure 5A, treatment with the ABCC4 inhibitor probenecid significantly inhibited the expression of *Areg, Ereg, Tnfaip6, Pr and Star*, whereas the expression of *Adamts1, Ctsl, p450scc, Fshr and Lhcgr* was unaffected. The mRNA levels of the PG synthesis and signaling genes *ptgs2, Ep4 and Pgt* (Fig. 5B) were also significantly inhibited by probenecid treatment. In contrast, probenecid treatment increased the mRNA levels encoded by the *Hpgd, Ep2 and Abcc4* genes. Serum progesterone levels (Fig. 5C) were significantly reduced ($p=0.03$) following treatment with 400 mg/kg of probenecid and hCG as compared with hCG alone.

The effect of combined ABCC4 and PTGS2 blockade on ovulation in vivo

PTGS inhibitors were shown to partially inhibit ovulation in animal models as well as in human studies (see introduction).

Based on the hypothesis that concurrent inhibition of prostaglandin synthesis (blocking PTGS2) and transport (blocking ABCC4 transporter) may synergistically work to inhibit ovulation, we examined the effect of using combined low dose of ABCC4 and PTGS inhibitors (the "combined approach") on ovulation in mice undergoing a PMSG/hCG superovulation protocol.

As shown here, using a low dose of probenecid (25-200 mg/kg) in mice undergoing PMSG/hCG superovulation protocol resulted in partial inhibition of ovulation, to 36.9-48.8 oocytes (versus the mean

of 56.7 in controls, Table S4A). The PTGS inhibitor indomethacin (1-5 mg/kg) also resulted in partial inhibition of ovulation to 25.2±3.6-37.5±5.8 oocytes (Table S4A). When mice were treated with both probenecid (25 mg/kg) and indomethacin (5 mg/kg) concurrently, we observed significant inhibition of ovulation efficiency to 4.6±1.4 oocytes (with 8% ovulation rate, Table S4B). Thus, at the tested doses, each inhibitor alone had no significant effect or only a partial effect, while the "combined approach" resulted in almost complete blockade of ovulation (Figure 6A). We used the PTGS inhibitor meloxicam (10 mg/kg) in combination with probenecid (25 mg/kg) and w/wo PGE2 (2 µg/kg). The addition of PGE2 to the inhibitors abolished most of the inhibition of ovulation by meloxicam and probenecid (rescue effect, Fig. 6B). Total inhibition of ovulation was achieved with a dose of 200 mg/kg of probenecid plus 40 mg/kg of meloxicam.

Discussion

Ovulation, a complex and highly regulated process, is central to mammalian female reproduction. It is initiated by the midcycle LH surge, which leads to the differential expression of a large number of ovarian genes. Thus, the identification of the genes involved in ovulation is of obvious importance. In an earlier report, an RNAseq approach was used to systematically isolate genes with an ovulation-selective pattern of expression [16] in human CGCs wherein *ABCC4* was found to be significantly upregulated. Subsequent analysis of *ABCC4* expression in MGCs and CGCs obtained from preovulatory follicles revealed that *ABCC4* mRNA levels are significantly higher in MGCs. These observation suggest a role for MGCs in facilitating the known effects of prostaglandins following the LH surge [41].

ABCC4 expression during the ovulatory interval was also tested *in vitro*. The *in vitro* model comprised of four days incubated primary human MGCs followed by hCG stimulation. The stimulation of cultured MGCs with hCG caused a significant increase in ABCC4 mRNA and protein expression, peaking between 9 and 12 hours after hCG stimulation. Altogether, these results establish ABCC4 as a new LH-induced ovulatory gene in humans. ABCC4 induction by hCG was also recently shown in an *in vitro* model [42]. It was also found that the transcription factor FOS facilitated hCG induction of both ABCC4 and SLCO2A1 [43]. The regulation of ABCC4 gene expression may be species-specific since its mRNA levels did not change in the ovaries of mice undergoing a superovulation protocol (Fig. 4A). However, this difference between species, might also be due to a dilutional effect of multiple cell types, because in the mice, *ABCC4* mRNA was tested in the whole ovary and not in isolated GCs.

Little attention has been paid to the mechanisms by which PGs cross biological membranes in granulosa cells, until our previous study, which showed that PGT is required to PGE2 influx during the ovulatory process [17]. In the current study we used probenecid and MK-571, chemical inhibitors of ABCC4 as well as *ABCC4* knockdown siRNA in an in vitro assay and tested their effect on the extracellular and intracellular levels of PGE2 in response to hCG. Our results show that probenecid and MK-571, as well as *ABCC4* siRNA knockdown of ABCC4 expression, significantly decreased the extracellular PGE2 levels as compared to hCG treatment alone. Rescue experiments using PGE2 abolished the inhibitory effect on

ovulation of probenecid alone as well as probenecid combined with NSAIDs supporting the specific role of ABCC4 in ovulation.

Interestingly, the ABCC4 chemical inhibitors and siRNA experiments were without effect on the intracellular PGE2 concentrations which remained constant under all conditions tested (Fig. 2 and Fig. 3). These results are similar to those reported for other cell types that demonstrated that chemical inhibitors of ABCC4, MK-571 and probenecid, decreased the extracellular PGE2 while intracellular PGE2 remained at constant levels [20,21,44]. These findings, suggest that there is an intracellular system that maintains constant PGE2 levels, potentially through either increasing the intracellular degradation of PGE2 and/or restraining PGE2 synthesis. One possible mechanism whereby intracellular PGE2 levels are regulated includes a reduction in PG synthesis and influx in parallel with a concomitant increase in degradation. *Ptgs2* (PGE2 synthesis) and *Pgt* (PGE2 influx) mRNA levels were significantly inhibited by probenecid treatment, whereas *Hpgd* (responsible for PGE2 metabolism) mRNA and *ABCC4* levels were increased in the ovaries of probenecid treated mice. These results are similar to those reported for other cell types that demonstrated that chemical inhibitors of ABCC4, MK-571 and probenecid, decreased the extracellular PGE2 while intracellular PGE2 remained at constant levels [20,21,44].

Since increased intrafollicular PGE2 levels and PGE2 signaling is required for ovulation, we postulate that ABCC4 serves as a key efflux transporter that allows for movement of PGE2 from its intracellular site of synthesis to its extracellular site of action. To investigate the role PGE2 efflux plays in ovulation, experiments were performed in mice using the ABCC4 inhibitor probenecid. PMSG-primed/hCG-triggered immature female mice were concurrently treated with different concentrations of probenecid or vehicle at the time of hCG administration. We found that treatment with probenecid markedly decreased ovulation efficiency in a dose-dependent manner. The maximal inhibition was achieved at a dose of 450 mg/kg with 99.6% reduction of the average number of ovulated oocytes per mouse.

It is interesting to note that ABCC4 null mice displayed a small reduction in litter size [45] which might be attributed to reduced ovulatory efficiency. A tissue-specific conditional knockout of ABCC4 might overcome the limitations of constitutive knockout models and provide a clearer fertility phenotype.

Ovulation and luteinization of the mature follicle are essential processes for successful reproduction. The process includes the reinitiation of meiosis and cytoplasmic maturation in the enclosed oocyte, cumulus expansion, follicular rupture and luteinization.

By looking into these ovulatory functions, we can further understand the mechanism of the inhibition of ovulation by probenecid. Histological evaluation of ovaries obtained from mice treated with probenecid revealed entrapped oocytes, indicating that the follicles did not rupture, as previously shown in several species that inhibition of PG signaling or synthesis prevented follicular rupture [8,9,46]. Luteinization and subsequent progesterone production can occur in the absence of ovulation, and vice-versa, indicating that luteinization and ovulation can be viewed as independent processes [47]. PGE2 induces progesterone production in the rat and mouse ovaries [48,49] and inhibiting PG signaling reduce progesterone production.

In primates, PG inhibition does not compromise ovarian progesterone production [46,50]. We found that inhibition of ABCC4 activity inhibits progesterone synthesis in mice as described for other PG signaling inhibitors in non-primate model [17,51].

Molecular analysis of ABCC4 inhibition by 400 mg/kg probenecid on ovarian gene expression revealed dysregulation of genes involved in oocyte maturation, cumulus expansion, steroidogenesis genes as well as PGE2 synthesis, metabolism, signaling and transport. Nevertheless, there were several genes that were not affected by ABCC4 inhibition and for some of these genes, a tendency that did not reach significant difference was observed (*Adamts1*, *p450scc* and *Lhcgr*). It was shown that AREG and EREG signaling affect ovulation pathway through PGE2, independently from LH signaling [52].The results observed in this study corroborate these assertions.

One might speculate that these genes are not regulated at the mRNA levels, are not the limiting factors in this process, or perhaps, these genes change their expression pattern at different times, and not necessarily at the 3h time point we chose to examine. To summarize, we found that the inhibition of ABCC4 has critical effect on the entire ovulatory process.

In the past 30 years, numerous studies evaluated ovulation inhibition with cyclooxygenase inhibitors in women. However, the inhibition of ovulation was only partly efficient (reviewed in [51]). One of the challenges in the field of contraception is emergency contraception also known as "the day after pill". One study investigated whether a PTGS2 inhibitor could enhance the ability of the progestin levonorgestrel to reduce rates of ovulation in a model of emergency contraception with promising preliminary results but it was not further pursued [53].

Our study of combined inhibition of prostaglandin synthesis and transport (the "combined approach") using low dose of PTGS2 and ABCC4 inhibitor achieved over 90% inhibition of ovulation in a super-ovulation protocol. Total inhibition was achieved using a higher dose, though still much lower than using only probenecid. Further mammal and human studies are needed to support our results but is can be postulated that in a mono-ovulatory cycle, the efficacy of the multi-drug approach would be effective enough for emergency contraception and perhaps suitable for monthly non-hormonal contraception.

We observed no short- or long-term side effects of probenecid treatment in mouse experiments. Probenecid is used in human clinical setting (for several indications) for many years and is considered safe even in the pregnant and pediatric population.

The doses of probenecid to be used in clinical studies and in equivalence with mouse studies is currently unknown. Based on our study in the mouse, we believe that the doses needed for blocking ovulation, will be safe and efficient. We suggest that the doses that will be used will be derived from the doses of probenecid used in other clinical settings. Regarding the PTGS2 inhibitors, there are several studies in the literature using accepted doses of PTGS inhibitors as mentioned, those studies show partial effect in inhibiting ovulation (~50-80%) [51]. Based on the synergistic effect shown in this study (Fig. 6), we believe

that the combined approach will enable us to use lower doses while achieving better efficacy, with no side effects.

In summary, our studies reveal ABCC4 to serve as an essential component of the ovulatory process. We demonstrated that ABCC4 functions as a PGE2 efflux transporter. Probenecid, an ABCC4 inhibitor, effectively blocked ovulation in mice in a dose-dependent manner. Specific effects of probenecid administration *in vivo* on ovulatory processes included alterations in cumulus expansion, oocyte maturation, follicular rupture, and luteinization. All these findings mark ABCC4 as a novel ovulatory gene, responsible for the efflux transport of PGE2. Combining a low dose of Probenecid and Indomethacin synergistically blocked ovulation and suggested a potential new non-hormonal contraceptive. Better understanding of the precise mechanism of ABCC4 and PGT-mediated modulation of PGE2 levels during ovulation may provide effective treatments for some types of infertility as well as contraception. This study demonstrates that the modulation of ABCC4 activity may provide an opportunity to pharmacologically control ovulation.

Declarations

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Author contributions:

G.M.Y., B.S., Y.Y., E.M., M.B., J.D.H., E.Y.A., and A.H. contributed to the study design and analysis, the interpretation of the data, and the writing of the manuscript. B. S. and Y.Y. were involved in sample preparation and in vivo and in vitro experimental work. All the authors have contributed to data analysis and to finalizing the manuscript.

Competing interests:

A provisional patent application has been filed by A.H., Y.Y. and G.M.Y. (application no. 62/103,091): prostaglandin transporter inhibitors for inhibiting ovulation. All other authors have no competing interests to declare.

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Figures

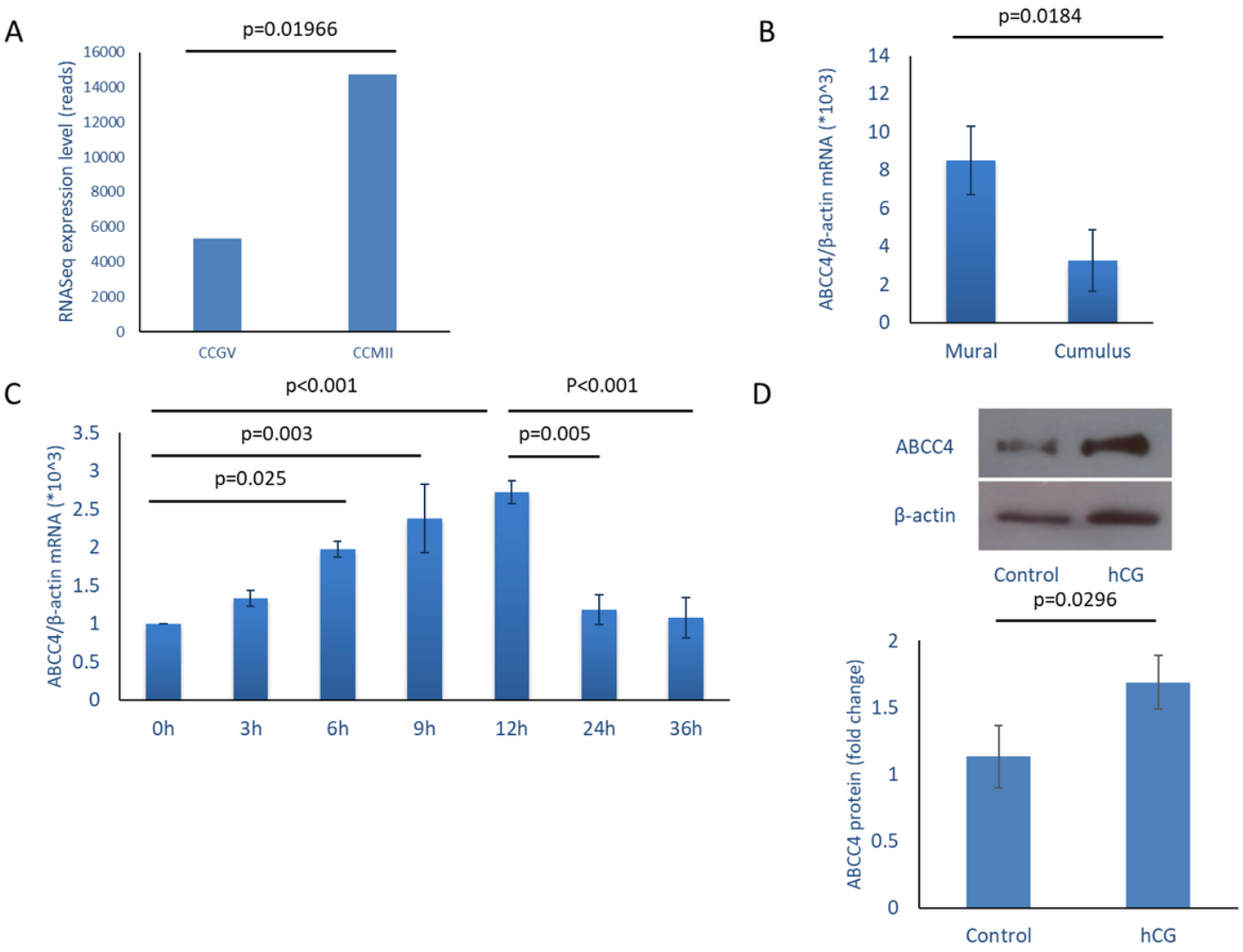

Figure 1

ABCC4 expression in human GCs. (A-B) Expression of ABCC4 in human GCs in vivo. (A) RNAseq expression (reads) of ABCC4 in the CGCs surrounding a GV oocyte after IVM treatment and in expanded CGCs surrounding MII oocytes. Global transcriptome sequencing was performed on CCMII samples obtained from three women and CCGV samples obtained from two women as described previously (15) (B) CGCs and MGCs aspirated from preovulatory follicles (>17 mm) during IVF procedures. ABCC4 was quantified by qPCR and normalized to β-actin expression. Data represent the mean ± SEM of three independent experiments. (C) In vitro expression of ABCC4 in MGCs. MGCs aspirated during IVF procedures were initially cultured for 4 days with daily medium replacement and then exposed to hCG for the indicated time. ABCC4 was quantified by qPCR and normalized to β-actin expression. Data represent the mean ± SEM of three independent experiments. (D) ABCC4 protein expression in the hCG-treated cultured MGCs compared to untreated control. The cells were stimulated with vehicle (control) or with 1U/ml hCG for 9 hours and then subjected to western blot analysis. Protein levels of ABCC4 expression were determined by western blotting. β-actin was used as control. The image is a representative of three independent experiments. Protein levels of ABCC4 expression were analyzed by image studio lite (LI-COR

Biosciences) and calculated relative to the β-actin level in the same sample. Results are expressed as mean ±SEM of three independent experiments.

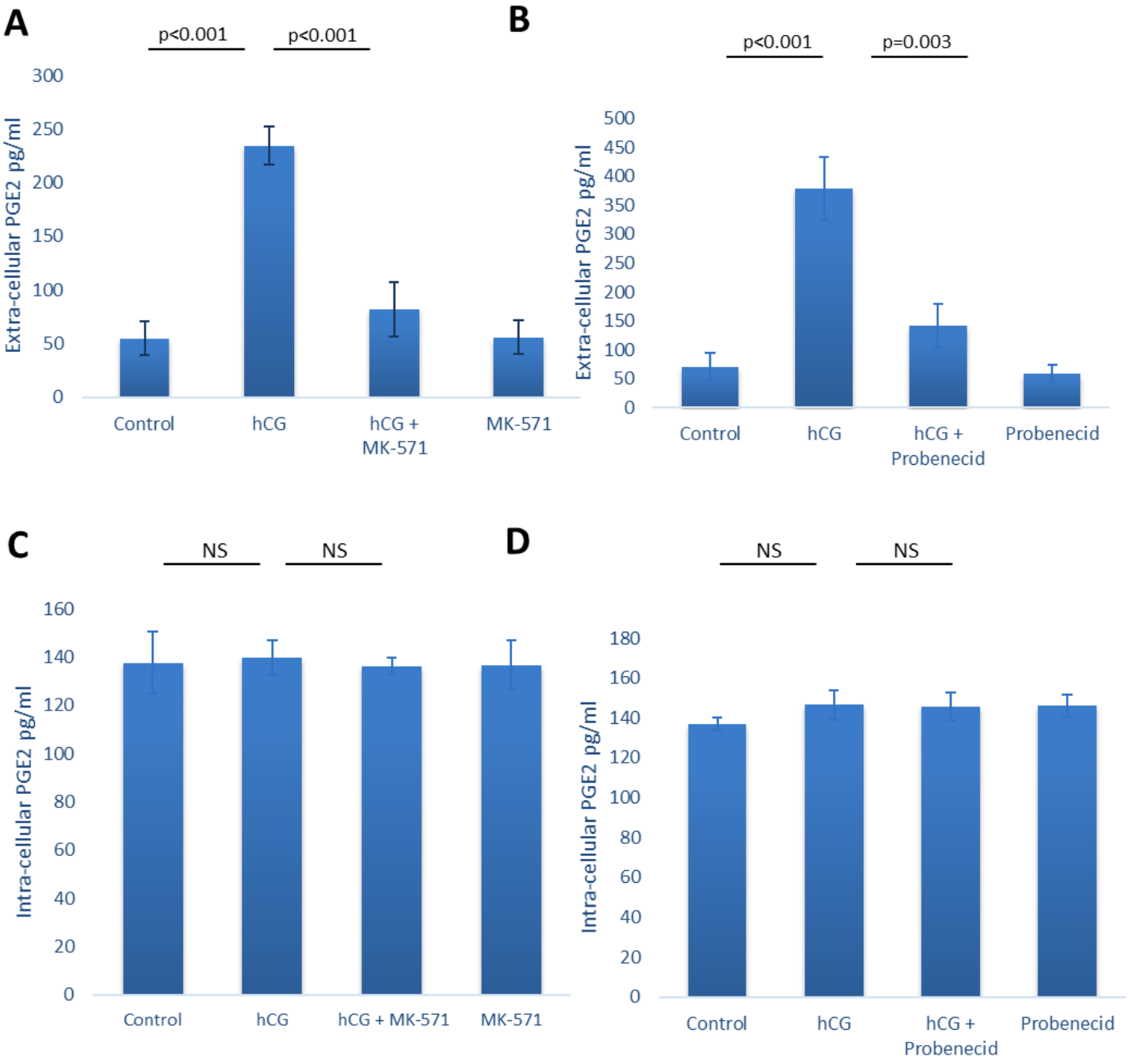

Figure 2

The role of ABCC4 in the regulation of extra and intra cellular PGE2 levels in MGCs – ABCC4 inhibitors studies MGCs aspirated from preovulatory follicles (>17 mm) during IVF procedures, were cultured for 3-4 days. The cells were either stimulated with 1U/ml hCG in the presence of 50 μM MK-571 or 500μM probenecid for 24 hours. PGE2 levels in the culture media (A, C) and intracellular (B, D) were measured by PGE2 EIA kit. Each sample included pooled cells from 2 to 4 women. The results are expressed as mean ±SEM of four independent experiments.

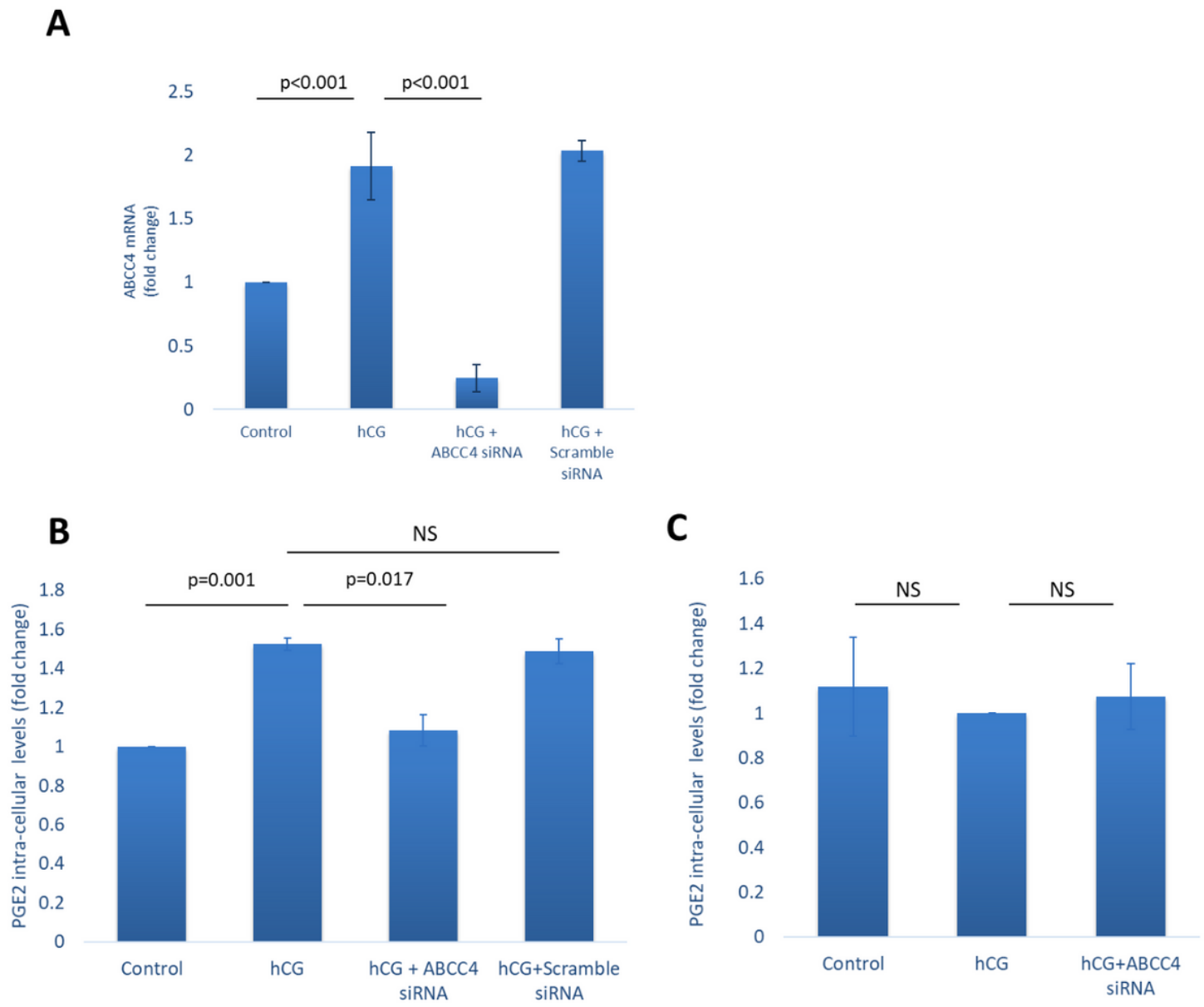

Figure 3

The role of ABCC4 in the regulation of PGE2 extra and intra cellular levels in MGCs – siRNA studies. MGCs aspirated from preovulatory follicles (>17 mm) during IVF procedures, were cultured for 2-3 days. The cells were transfected with either ABCC4 siRNA or Scramble (control) siRNA. 48 hours post transfection, the two groups of transfected cells were stimulated with 1U/ml hCG, whereas two not-transfected groups were stimulated either with 1U/ml hCG or none. (A) The cells were subjected to total RNA extraction. ABCC4 mRNA expression was assessed by qPCR and calculated relative to the β-actin level in the same sample. The results are expressed as mean ±SEM of three independent experiments. PGE2 levels in the culture media (B) and intracellular (C) were measured by PGE2 EIA kit. The results are expressed as mean ±SEM of three to seven independent experiments. Each culture included pooled cells from 2 to 4 women.

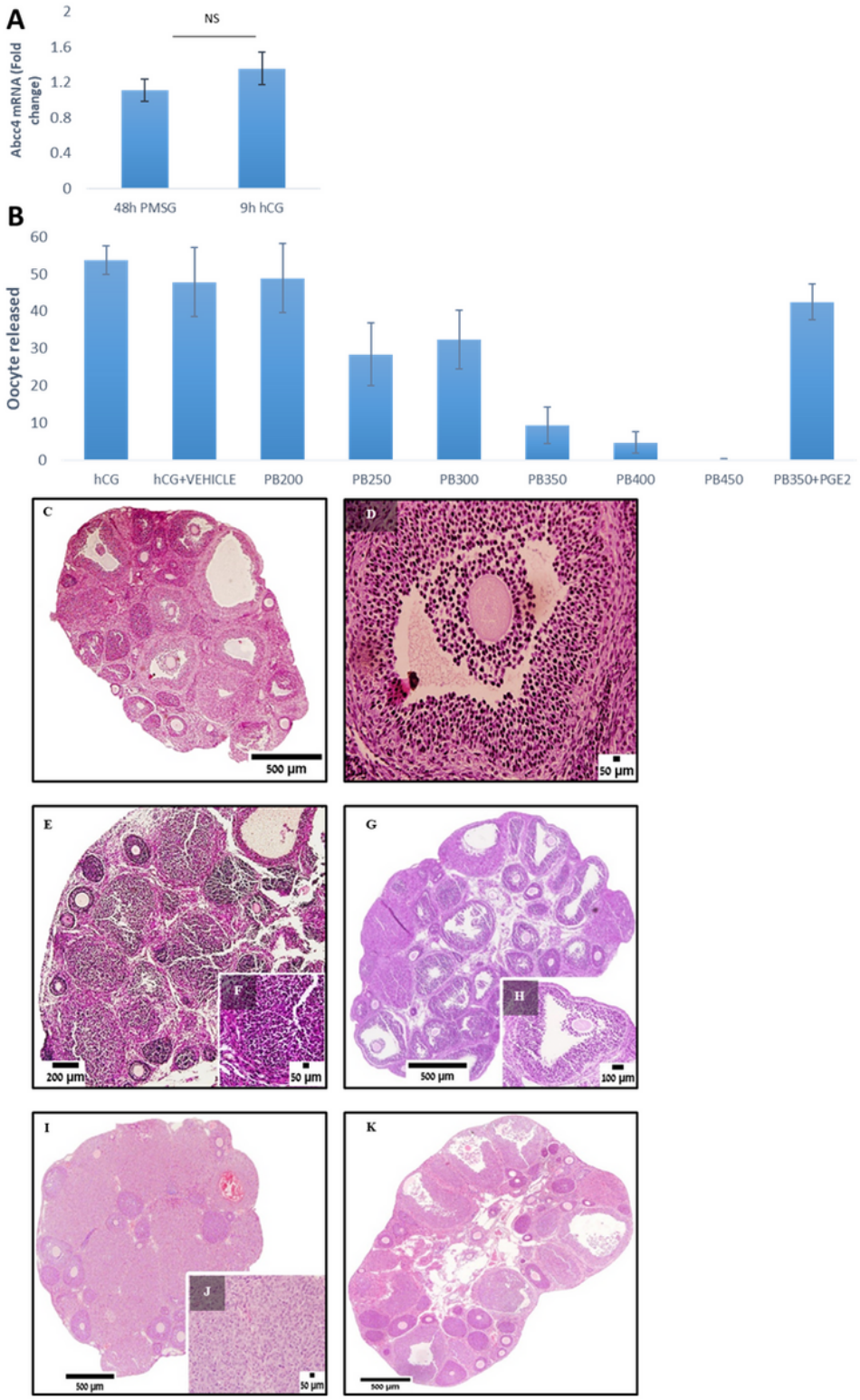

Figure 4

ABCC4 mRNA expression pattern in mice ovaries and the effect of probenecid on ovulation in mice undergoing a superovulation protocol. (A) ABCC4 mRNA expression pattern in mice ovaries during the preovulatory period. Immature 25-day old female mice were injected with saline or 10U PMSG to stimulate follicle growth, and 48 hours later with an ovulatory dose of 10U hCG to induce ovulation. The mice were sacrificed 48 hours after PMSG and 9 hours after hCG treatment. The ovaries were collected

and the total ovarian RNA were extracted. ABCC4 mRNA levels were assessed by qPCR and expressed relative to β-actin mRNA in each sample. The results are expressed as mean ±SEM of four independent experiments. (B) The effect of probenecid on ovulation in mice undergoing a superovulation protocol. mice were injected with saline or 10U PMSG to stimulate follicle growth, and 48 hours later with 10U hCG with Probenecid (PB, different doses) and w/wo PGE2 (2µg/kg). Mice were sacrificed 16 hours after hCG administration and the number of oocytes in the oviducts was recorded. Number of oocytes is represented as mean±SEM. (C-K) Ovarian histology of probenecid-treated and control mice. Ovaries collected from mice super-ovulated with 10U PMSG for 48h, followed by 10U hCG or 10U hCG and 400 mg/kg probenecid for 16h and 40h. The ovaries were fixed in 4% formaldehyde. Paraffin-embedded sections were serially sectioned and stained with hematoxylin and eosin and examined by light microscopy. Representative images of at least three independent experiments are displayed: (C) 48h after treatment with PMSG. (D) Higher magnification of a portion of the PMSG-primed ovary at the 48-hour time point. (E) 16h after the administration of hCG. (F) Higher magnification of one postovulatory follicle forming an early corpus luteum. (G) 16h after the administration of hCG + probenecid. (H) Higher magnification of the time point 16h after the administration of hCG + probenecid. (I) 40h after the administration of hCG. (J) Higher magnification of postovulatory follicular cells 40h after the administration of hCG. (K) 40h after the administration of hCG + probenecid.

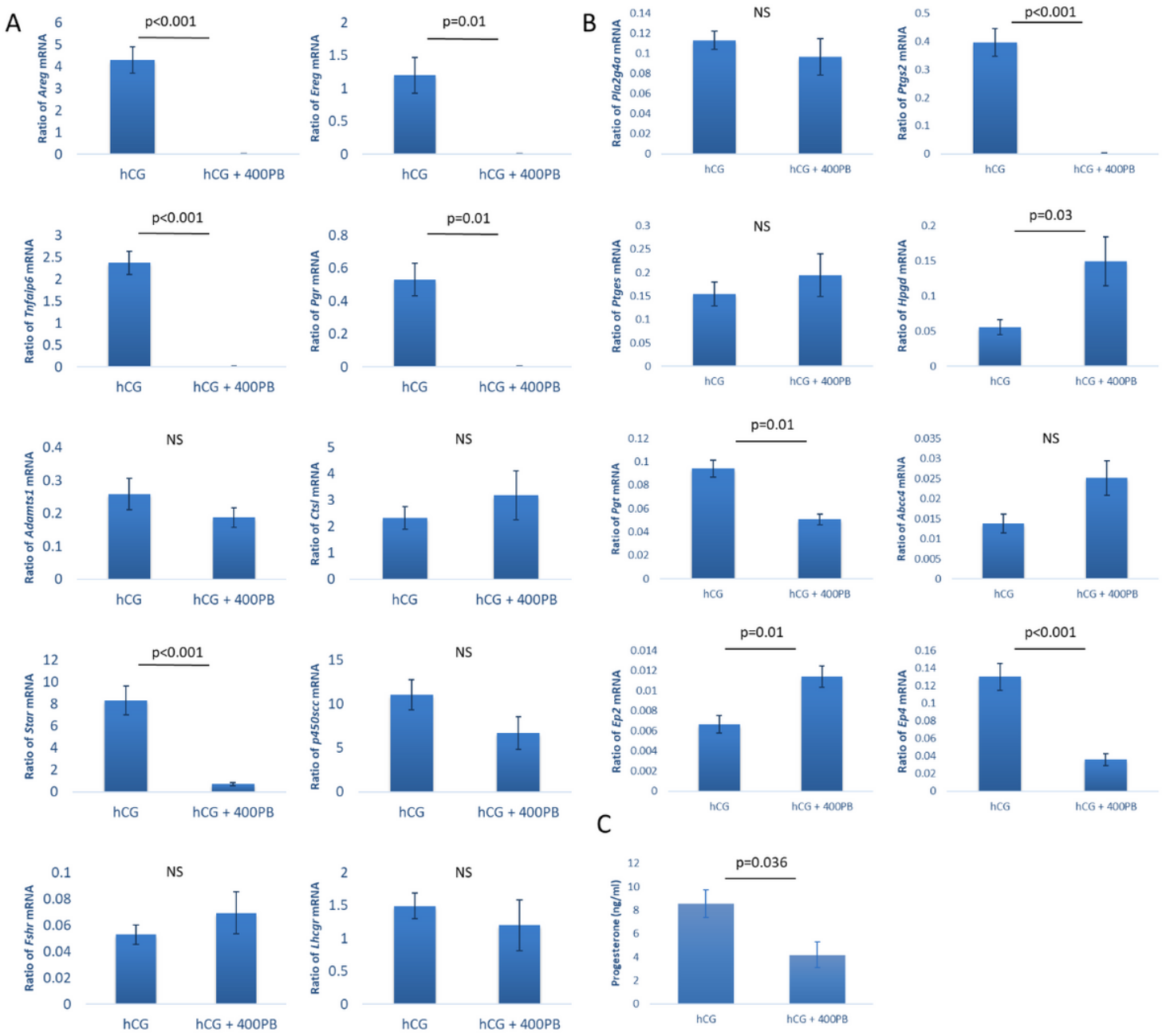

Figure 5

The effect of ABCC4 inhibition by probenecid on the expression of LH/hCG target genes and serum progesterone concentration. Immature 25-day-old female mice were superovulated with 10U PMSG for 48h, followed by injection of 10U hCG with or without the 400 mg/kg probenecid. Animals were sacrificed 3 hours after hCG administration. The ovaries were collected and the RNA were extracted. mRNA levels of each gene were assessed by qPCR and expressed relative to β-actin mRNA in each sample. The results are expressed as mean ±SEM of five to seven independent experiments. (A) Expression of LH/hCG target genes: Areg, Ereg, Tnfaip6, Pr, Adamts1, Ctsl, Star, P450Scc, Fshr and Lhcgr. (B) Expression of PGs synthesis, metabolism, receptors and transporters genes: Pla2g4a, Ptgs2, Ptges, Hpgd, Ep2, Ep4, Pgt and Abcc4. (C) Serum progesterone concentration in hCG treated ovaries compare to hCG and probenecid

treated ovaries. For progesterone concentration, the animals were sacrificed 24 hours after hCG administration. The results are expressed as mean ±SEM of four to seven mice per group.

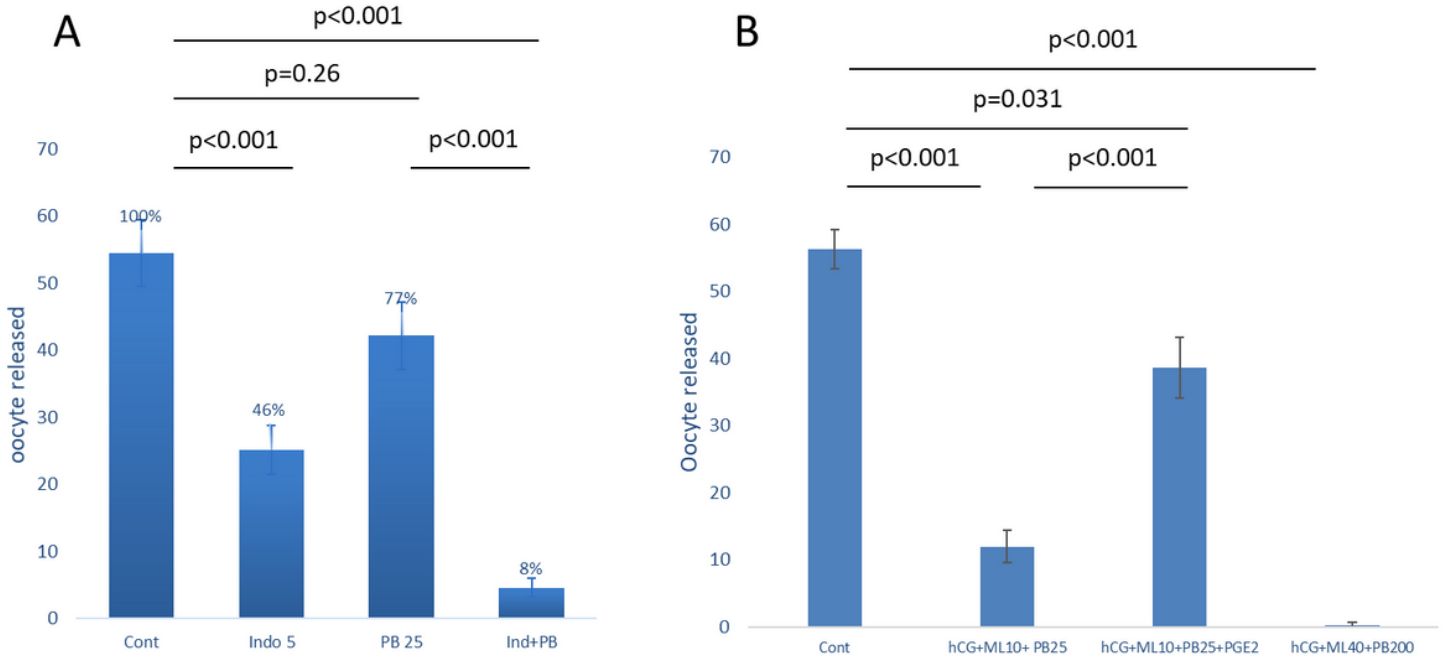

Figure 6

Inhibition of ovulation in vivo by a combination of probenecid and indomethacin or probenecid and meloxicam and the rescue effect of PGE2. 25-day old C57BL/6 female mice were injected with 10U of PMSG and 48 h later (A) with an ovulatory dose of 10U hCG w/wo Probenecid (PB, 25 mg/kg) w/wo Indomethacin (Indo, 5mg/kg). (B) With an ovulatory dose of 10U hCG with Probenecid (PB, 25 mg/kg) plus Meloxicam (ML, 10mg/kg) and w/wo PGE2 (2µg/kg) and combination of Probenecid (200 mg/kg) plus Meloxicam (40mg/kg). Mice were sacrificed 16 hours after the administration of hCG and the number of oocytes within the ampullas of both oviducts was recorded.

Supplementary Files

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- RoleofABCCsuppTablev2.docx