ABCC4 is a PGE2 efflux transporter in the ovarian follicle: A mediator of ovulation and a potential non-hormonal contraceptive target

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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 in 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.
1 | 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 (PTGS) 1 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 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 Ref. [8]).

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.5 Systemic immunoneutralization of PGs blocks follicular rupture in both mice and rabbits.9,10 PTGS2 null-mutant mice exhibit severely impaired ovulation.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.13

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),14 ABCC4 (mainly a PGE2 efflux transporter),15 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 of prostaglandins as well as cAMP and other compounds.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 However, to date, there is no data regarding ABC4 activity in ovarian follicles except the fact it was shown to be expressed in the ovary.

Worldwide, over 40 million unwanted, pregnancies end in abortion each year22 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,23,24 as well as in human trials but failed to achieve satisfactory results.25–27

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”).

2 | MATERIALS AND METHODS

2.1 | 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 euthanatized using carbon dioxide in accordance with 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 with the relevant guidelines and regulations.

### 2.2 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. 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 Plow) 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 exceeded 18 mm in diameter, 250 μg of human chorionic gonadotropin (hCG; Ovitrelle®; Merck Serono) was administered to trigger ovulation. Transvaginal follicular aspiration was performed 36 h later under ultrasound guidance.

### 2.3 Cumulus granulosa cell collection

After COC retrieval, CGCs of each oocyte were removed with the use of hyaluronidase (SAGE) and a glass denudation pipette (Svedem, Billdal, Sweden). The CGCs were washed in phosphate-buffered Saline (PBS) and centrifuged at 5000xg for 5 min 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.

### 2.4 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). 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 320 g for 5 min 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.

### 2.5 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). 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 960 g 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% CO₂ in air. The cells were cultured for 4 days with daily medium replacement prior to hCG triggering for the indicated times. For gene expression experiments cells were then treated with hCG (1 U) in the absence or presence of probenecid (500 μM) for 3 or 16 h according to the tested gene.

### 2.6 RNA extraction and qPCR

Total RNA was extracted from MGCs or CGCs using a Mini/Micro RNA Isolation I kit (Zymo Research) according to the manufacturer’s instructions. RNA purity and concentration were assessed using a NanoDrop spectrophotometer (NanoDrop 2000C, Thermo Scientific). Total RNA (25 ng) from each sample was used for cDNA synthesis by a high-capacity reverse transcription kit (Applied Biosystems) according to the manufacturer’s instructions in a 10 μL 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 1 μL 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 10 μL. Cycling parameters were 1 cycle at 95°C for 20 s, and 40 cycles each at 95°C for 3 s and at 60°C for 30 s. 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.

2.7 | 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.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

2.8 | IVM protocol

IVM cycles were carried out as previously describe.30 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 150IU/day rFSH for 3 days followed suit. After a second sonographic assessment on Day 6, 10000IU 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 h later.

2.9 | Animals

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

2.10 | Superovulation protocol

25-day-old female mice were initially treated with 10 U of pregnant mare serum gonadotropin (PMSG, Chronoest, Intervet) to stimulate follicular growth. An ovulatory dose of hCG (10 U) (Ovitrelle®, Merck Serono) was administered 48 h later. To elucidate the role of ABCC4 in vivo, probenecid, at different concentrations (200–450 mg/kg), was administrated intra-peritoneally at 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 (2 mg/kg) was administrated intra-peritoneally at the same time as hCG.

Animals were sacrificed 48 h after the initiation of PMSG treatment as well as 9 or 16 h 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.

Treated mice were observed for general health and side effects in accordance with standard guidelines.31

2.11 | 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) containing a protease inhibitor cocktail (Sigma-Aldrich), 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). 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 H2O, 1 mL Tween 20, Sigma-Aldrich) for one hour and afterward 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) and developed using an enhanced chemiluminescence kit (Thermo Scientific).32
2.12 | 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 h with hCG (1 U) 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 s × 3 on ice; CV18 Sonics) and centrifuged at 2560 g for 10 min at 4°C. Supernatants were diluted 1:5 with EIA buffer and subjected to EIA for PGE2 (Cayman Chemical).

2.13 | 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) in 150 μL OptiMEM (Gibco by Life Technologies), and (B) 8 μL ABCC4 siRNA or Scramble siRNA (Santa Cruz Biotechnology) in 150 μL OptiMEM medium (10 μM final concentration). The transfection mixture was incubated at room temperature for 5 min 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 1.4 mL 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.

2.14 | 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) according to the manufacturer's instructions.

2.15 | 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.

2.16 | 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.

2.17 | 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) was used. p-values < .05 were considered statistically significant.

3 | RESULTS

3.1 | 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. 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 \( (p = .0196) \) as compared to 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.\(^3\)\(^3\) We compared the expression levels of \( ABCC4 \) mRNA in MGCs and CGCs by qPCR. MGCs and CGCs were obtained from large preovulatory follicles (>17 mm) during the IVF procedure. As shown in Figure 1A, MGCs expressed significantly higher (2.6-fold) levels of \( ABCC4 \) mRNA relative to CGCs \( (p = .0184) \).

### 3.2 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 h with FSH\(^3\)\(^4\) 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.\(^3\)\(^4\) After hCG stimulation, the cells were harvested 0, 3, 6, 9, 12, 24, and 36 h later. \( ABCC4 \) mRNA

![Image](https://example.com/image.png)

**FIGURE 1** ABCC4 Expression in human GCs. (A) CGCs and MGCs aspirated from preovulatory follicles (>17 mm) during IVF procedures. \( ABCC4 \) was quantified by qPCR and normalized to \( \beta\)-Actin expression. Data represent the mean ± SEM of three independent experiments using \( t \)-test. (B) 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 \( \beta\)-Actin expression. Data represent the mean ± SEM of three independent experiments using ANOVA with Tukey. (C) \( ABCC4 \) protein expression in the hCG-treated cultured MGCs compared with untreated control. The cells were stimulated with vehicle (control) or with 1 U/mL hCG for 9 h and then subjected to Western blot analysis. Protein levels of \( ABCC4 \) expression were determined by Western blotting. \( \beta\)-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 \( \beta\)-Actin level in the same sample. Results are expressed as mean ± SEM of three independent experiments using \( t \)-test.
was observed to be upregulated in hCG-treated cells, peaking at 12 h after hCG treatment \((p < .001, \text{Figure 1B})\), 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 \((p = .0296, \text{Figure 1C})\).

3.3 | 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\(^ {35,36} \) and probenecid.\(^ {35,37} \) FSH-pretreated MGCs (48 h) were incubated with hCG (to induce ABCC4 expression; \(1 \mu M\)) for 24 h in the absence or presence of MK-571 (50 \(\mu M\)) or probenecid (500 \(\mu M\)). 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 (\textit{Figure 2A}). Co-treatment with the ABCC4 blockers probenecid (\textit{Figure 2B}) resulted in a significant decrease \((p < .001)\) of PGE2 levels by 2.7-fold when compared to hCG treatment alone. Similar results were obtained using another ABCC4 blocker, MK-571 (\textit{Figure 2A}). Incubation of MGCs 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 (\textit{Figure 2C,D}). These observations suggest a role of ABCC4 in PGE2 efflux and that cellular mechanisms are in place to keep the intracellular PGE2 levels constant.

3.4 | 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 (\textit{Figure 3}). First, the effect of \textit{ABCC4} siRNA on \textit{ABCC4} mRNA expression was determined. \textit{Figure 3A} shows that \textit{ABCC4} siRNA significantly decreased \textit{ABCC4} mRNA levels 7.75-fold \((p < .001)\) compared to hCG only. Importantly, \textit{ABCC4} mRNA levels in the scrambled siRNA group (negative control) were equivalent to cells treated with hCG only, indicating the specificity of the \textit{ABCC4} siRNA and that siRNA transfection per se does not impact MGC viability or overall gene expression.

We next tested the effect of treating MGCs with \textit{ABCC4} siRNA on hCG-stimulated extracellular and intracellular levels. \textit{Figure 3B} displays an increase in PGE2 extracellular levels by 1.38-fold \((p = .037)\) in extracellular levels in hCG-treated cells as compared to control. Treatment with \textit{ABCC4} siRNA significantly decreased the PGE2 extracellular levels by 1.36-fold relative to hCG \((p = .017; \text{Figure 3B})\). 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 (\textit{Figure 3C}).

3.5 | 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 (\textit{Figure 4A}), \textit{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 \textit{Figure 4B} and \textit{Table 1}, 100% of the mice receiving hCG with or without vehicle ovulated, releasing an average of 53.7 or 47.8 oocytes, respectively \((p = .99)\). 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%–76% of the mice resulting in the mean release of 9.4 and 4.7 oocytes per animal, respectively \((p < .0001)\). 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, \textit{Figure 4B}).

These results suggest that the inhibition of \textit{ABCC4} blocks oocyte release in mice thereby establishing an essential role of \textit{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 h after hCG administration. PMSG treatment led to the development of numerous preovulatory follicles (\textit{Figure 4C,D}). Removal of the
ovaries from vehicle-treated mice 16 h after the administration of hCG revealed numerous early corpora lutea (Figure 4K). In contrast, the ovaries removed from mice receiving hCG plus probenecid (Figure 4G,H) contained many unruptured preovulatory follicles replete with entrapped oocytes, resembling ovaries obtained from mice treated with PMSG alone. At 40 h after hCG injection, ovaries from hCG-treated mice displayed many corpora lutea composed of luteinized GCs containing lipid droplets in their cytoplasm (Figure 4I,J). By contrast, ovaries collected from hCG- and probenecid-treated mice still contained entrapped oocytes in preovulatory follicles (Figure 4K). 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 2A) and cumulus expansion (Table 2B). 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 h 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. By 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 was no short-term or long-term observable side effects to the mice treated with doses of probenecid up to 450 mg/kg.

3.6 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 h after receiving hCG and vehicle or probenecid. The mRNA levels encoded by known LH/hCG target genes was initially investigated (Figure 5A). The genes in question included those involved in oocyte maturation (Areg, Ereg), progesterone receptor signaling (Pgr), cumulus expansion (Tnfaip6), follicular rupture (Adamts1, Ctsl), gonadotropin signaling (Fshr, Lhcgr), and steroidogenesis [Star and p450Scc (cholesterol side chain cleavage)]. We also investigated the regulation of genes whose products encode proteins involved in PGE2 synthesis and signaling (Figure 5B). Included were those genes critical for PG synthesis (Pla2g4a, Ptgs2, Ptges), PG metabolism (Hpgd), PGE2 signaling (Ptger2, Ptger4) and PG transport (Ptg, Abcc4). Serum progesterone concentration were also measured 24 h 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 Adams1, Ctsl, p450scC, Fshr, and Lhgr was unaffected. The mRNA levels of the PG synthesis and signaling genes Prg2, Ep4, and Pgt (Figure 5B) were also significantly inhibited by probenecid treatment. By contrast, probenecid treatment increased the mRNA levels encoded by the Hpgd, Ep2, and Abcc4 genes. Serum progesterone levels (Figure 5C) were significantly reduced (p = .03) following treatment with 400 mg/kg of probenecid and hCG as compared to hCG alone.
A significant decrease of Ptgs2 and Has2 expression, as well as a non-significant decrease of Areg and increase of Hpgd expression (Figure 6).

### 3.7 The effect of combined ABCC4 and PTGS blockade on ovulation in vivo

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

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 3A). The PTGS inhibitor indomethacin (1–5 mg/kg) also resulted in partial inhibition of ovulation to 25.2±3.6–37.5±5.8
TABLE 2 Effect of probenecid on oocyte maturation and cumulus expansion.

| Treatment                  | Mature Oocytes (MII) | Immature Oocytes (GV) |
|----------------------------|---------------------|-----------------------|
| hCG (n = 5)                | 100% (93/93)        | 0% (0/93)             |
| hCG + Probenecid 300 mg/kg (n = 3) | 17% (10/59)        | 83% (49/59)          |
| hCG + Probenecid 400 mg/kg (n = 3) | 0% (0/88)          | 100% (88/88)          |

| Treatment                  | Expanded cumulus    | Compact cumulus       |
|----------------------------|---------------------|-----------------------|
| hCG (n = 5)                | 100% (213/213)      | 0% (0/213)           |
| hCG + Probenecid 300 mg/kg (n = 3) | 17.5% (14/80)     | 82.5% (66/80)        |
| hCG + Probenecid 400 mg/kg (n = 3) | 2.2% (2/88)       | 97.7% (86/88)        |
| hCG + Probenecid 450 mg/kg (n = 2) | 0% (0/22)          | 100% (22/22)         |

Note: Immature 25-day-old female mice were superovulated with 10 U of PMSG for 48 h, followed by the injection of 10 U hCG or hCG with 300, 400, and 450 mg/kg probenecid at the same time. Mice were sacrificed 16 h after hCG administration and the ovulated oocytes in the oviducts and the remaining oocytes in the ovaries were examined in terms of (A) oocyte maturation and (B) cumulus expansion. The number of oocytes is represented as the percentage of oocytes in the subgroup relative to the total group. The oocytes were obtained from the oviduct (300 mg/kg probenecid) or from the ovary by punctures (400/450 mg/kg probenecid). Results were compared using ANOVA with Tukey. The p value shown for each treatment represents the post hoc analysis comparing the specific treatment to the hCG group.

Oocytes (Table 3A). 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 3B). 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 7A). 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, Figure 7B). Total inhibition of ovulation was achieved with a dose of 200 mg/kg of probenecid plus 40 mg/kg of meloxicam.

4 | 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 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. It was shown that ABCC4 expression is significantly increased in MGC in vivo following hCG stimulation (5.6-fold, p < 0.0001). These observations suggest a role for MGCs in facilitating the known effects of prostaglandins following the LH surge.

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 h 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. It was also found that the transcription factor FOS facilitated hCG induction of both ABCC4 and SLCO2A1. 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 (Figure 4A). However, this difference between species, might also be due to a dilutional effect of multiple cell types, because in the mouse, 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. 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 (Figure 2 and Figure 3). These results are similar to those reported for other cell types that demonstrated that chemical inhibitors of ABCC4, MK-571
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and probenecid, decreased the extracellular PGE2 while intracellular PGE2 remained at constant levels.20,21,51 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.

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. The addition of PGE2 to probenecid negated its inhibitory effect on ovulation and strengthened the role of ABCC4 in the ovulatory cascade.

Earlier ABCC4 knockout studies that did not focus on female fertility and ovulation reported normal fertility.52 A more recent a ABCC4 null mice study that focused on the effect on male gametes reported a small reduction in litter size despite normal adult male gametes.53 This might be attributed to reduced ovulatory efficiency. These observations demonstrate that PGE2, although an important component in the ovulatory cascade, is not the only critical component of ovulation and other factors may compensate for the loss of ABCC4 function. 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.9,10,54 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.55 PGE2 induces progesterone production in the rat and mouse ovaries56,57 and inhibiting PG signaling reduce progesterone production. In primates, PG inhibition does not compromise ovarian progesterone production.54,58 We found that inhibition of ABCC4 activity

![Figure 6](image-url)
TABLE 3 Effect of combined ABCC4 and PTGS blockade on ovulation in vivo.

|                     | Control | Dose (mg/kg) | 0  | 25  | 50  | 100 | 200 | 1  | 5 |
|---------------------|---------|--------------|----|-----|-----|-----|-----|----|----|
| Number of mice that ovulate | 10/10 (100%) | 7/7 (100%) | 8/9 (89.9%) | 4/4 (100%) | 11/11 (100%) | 11/11 (100%) | 48.8 ± 7.5 (p = 97%) | 37.5 ± 5.8 (p = 31%) |
| Number of ova/mouse (mean ± SEM) | 54.5 ± 5.0 | 42.1 ± 7.3 (p = 94%) | 47.2 ± 7.3 (p = 94%) | 49.8 ± 6.1 (p = 99%) | 48.8 ± 7.5 (p = 97%) | 37.5 ± 5.8 (p = 31%) |

Note: (A) Immature 25-day-old female mice were treated with 10 U PMSG for 48 h, followed by injection of 10 U hCG, or hCG plus low dose probenecid or indomethacin. Intrapituitary injection of probenecid or indomethacin was carried out at the same time as hCG administration. Doses of probenecid were tested, including 25, 50, 100, and 200 mg/kg. Doses of indomethacin were 1 and 5 mg/kg. (B) Immature 25-day-old female mice were treated with 10 U PMSG for 48 h, followed by injection of 10 U hCG, or hCG plus low dose probenecid together with indomethacin (5 mg/kg). Different doses of probenecid were tested, including 25, 50, 100, and 200 mg/kg. Mouse were sacrificed 16 h after hCG administration and the number of oocytes in the oviducts was recorded. Number of oocytes is represented as mean ± SEM. Results were compared using ANOVA with Tukey. The p-value shown for each treatment is the post hoc analysis comparing the specific treatment to the hCG group.

Inhibits progesterone synthesis in mice as described for other PG signaling inhibitors in non-primate model.8,17

Molecular analysis of ABCC4 inhibition by 400 mg/kg probenecid in the mouse in vivo model, 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 ABC4 inhibition and for some of these genes, a tendency that did not reach significant difference was observed (Adams1, p450scc, and Lhcr). It was shown that AREG and EREG signaling affect ovulation pathway through PGE2, independently of LH signaling.59 Also, in PTGS2 KO mice it was shown that AREG and EREG levels are significantly reduced, linking prostaglandins as a messenger to LH signaling.60 The results observed in our study corroborate these assertions. We also analyzed several ovariary gene expression in vitro in primary mural granulosa cell culture (Figure 6) and observed similar results albeit not as significant as seen in vivo.

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 3 h time point we chose to examine. The effect of ABCC4 inhibition on ovulation can also be attributed to in inhibition of ABCC4 mediated cAMP efflux. However, oocyte is attributed to the increase in cAMP intracellular concentration (not the extracellular concentration).61 Also, we observed that the addition of PGE2 to the inhibitors abolished most of the inhibition of ovulation (Figure 7B). 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 in8). One of the challenges in the field of contraception is emergency contraception also known as “the day after pill.” One study investigated whether a PTGS 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.62
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.

High dose of PTGS inhibitors were shown to efficiently block ovulation in rats. However, several human studies using accepted doses of PTGS inhibitors show partial effect in inhibiting ovulation (~50% to 80%). Moreover, in human use, lower doses are required in order to keep side effects to minimum. Therefore, our goal was to use low dose of PTGS inhibitors combined with ABCC4 inhibition to try to find a synergistic effect with ABCC4 inhibitors (Figure 7) 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.

**AUTHOR CONTRIBUTIONS**
Gil M. Yerushalmi, Bat-el Shuraki, Yuval Yung, Ettie Maman, Micha Baum, Jon D. Hennebold, Elia Y. Adashi, and Ariel Hourvitz contributed to the study design and analysis, the interpretation of the data, and the writing of the manuscript. Bat-el Shuraki and Yuval Yung 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.

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**DISCLOSURES**
A provisional patent application has been filed by A.H., Y.Y., and G.M.Y. (application no. 62/103091): prostaglandin transporter inhibitors for inhibiting ovulation. All other authors have no competing interests to declare.
DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available in the methods and supplementary material of this article.

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SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.

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