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Female Infertility Is Associated with an Altered Expression Profile of Different Members of the Tachykinin Family in Human Granulosa Cells

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Abstract

Neurokinin B (NKB) and its cognate receptor, NK3R, play a key role in the regulation of reproduction. NKB belongs to the family of tachykinins, which also includes substance P and neurokinin A, both encoded by the by the gene *TAC1*, and hemokinin-1, encoded by the *TAC4* gene. In addition to NK3R, tachykinin effects are mediated by NK1R and NK2R, encoded by the genes *TACR1* and *TACR2*, respectively. The role of these other tachykinins and receptors in the regulation of women infertility is mainly unknown. We have analyzed the expression profile of *TAC1*, *TAC4*, *TACR1*, and *TACR2* in mural granulosa and cumulus cells from women presenting different infertility etiologies, including polycystic ovarian syndrome, advanced maternal age, low ovarian response, and endometriosis. We also studied the expression of *MME*, the gene encoding neprilysin, the most important enzyme involved in tachykinin degradation. Our data show that *TAC1*, *TAC4*, *TACR1*, *TACR2*, and *MME* expression is dysregulated in a different manner depending on the etiology of women infertility. The abnormal expression of these tachykinins and their receptors might be involved in the decreased fertility of these patients, offering a new insight regarding the diagnosis and treatment of women infertility.

Keywords Tachykinins · Polycystic ovarian syndrome · Advanced maternal age · Endometriosis · Human granulosa cells

Introduction

Infertility is a global condition estimated to affect between 8 and 12% of couples of reproductive age around 187 million couples worldwide [1]. Half of these cases have a female origin, including numerous pathologies that can be classified into two wide categories: disorders of the female tract and ovulatory disorders. Disorders of the female tract include endometriosis, tubal damage, and uterine malformations. Ovulatory disorders include hypothalamic pituitary disorders, hyperprolactinemia, ovarian failure, low ovarian response (LOR), polycystic ovary syndrome (PCOS), luteal phase defects, and idiopathic anovulation. Beyond this classification, it is also very important to consider maternal age, which can affect both the ovarian reserve and oocyte quality [2]. This is especially relevant in Western societies, where,

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due to different reasons, parenthood is being increasingly postponed [3].

Assisted reproductive technology (ART) emerged as a tool to overcome this growing issue and give these couples an opportunity to have children. Many advances have been made since first successful in vitro fertilization (IVF), performed more than 40 years ago [2, 4]. However, further research is necessary in order to improve ART results and offer better prospects to infertile patients. In this context, the analysis of the transcripts expressed in follicular cells could provide essential information concerning oocyte genetic constitution and viability [5].

Following a research line developed along the last years, we have focused on investigating tachykinins, a family of peptides deeply involved in fertility regulation. It is now demonstrated that the tachykinin neurokinin B (NKB) and its preferred receptor, the tachykinin NK3 receptor (NK3R), together with kisspeptin and its receptor, KISS1R, play an essential role in the regulation of reproductive functions (5–15). In humans, NKB is encoded by the *TAC3* gene while NK3R is encoded by the *TACR3* gene [6–8]. Kisspeptin is encoded by the *KISS1* gene and its receptor by the *KISS1R* gene [8–13]. They act as key agents in the hypothalamus, regulating the secretion of gonadotropin-releasing hormone and, thus, the hypothalamic-pituitary–gonadal (HPG) axis [8, 9, 12–14]. Beyond their presence in the hypothalamus, the NKB/NK3R and KISS1/KISS1R systems are also expressed in different reproductive organs, as the uterus, the ovary, and the placenta of different mammalian species, including humans [6–8, 15–30]. However, further studies are necessary to clarify their precise role at the peripheral level and how they regulate the reproductive function in these tissues.

Besides NKB and NK3R, the tachykinin family includes other peptides and two additional receptors which are also present in different types of reproductive cells at both central and peripheral levels [7, 16, 20, 24, 25, 31]. In humans, the other known members of the tachykinin family are substance P (SP) and neurokinin A (NKA), both encoded by the gene *TAC1*; hemokinin-1 (HK-1) and the endokinins A, B, C, and D (EKA, EKB, EKC, EKD), generated through alternative splicing and encoded by the different splice variants of the *TAC4* gene, known as α, β, γ, and δ [24, 32]. In addition to NK3R, tachykinin effects are mediated by the specific receptors NK1R and NK2R, encoded by the genes *TACR1* and *TACR2*, respectively. In the case of *TACR1*, two different isoforms have been described, *TACR1-Fl*, encoding the full-length protein, and *TACR1-tr* (*TACR1-T*), encoding a truncated form of the protein which lacked 96 amino acids at the C-terminus [32–35]. NK1R is the preferred receptor for SP and HK-1 while NK2R is the preferred receptor for NKA [24, 25, 32–35]. Nevertheless, the endogenous tachykinins are not very selective and are able to activate all tachykinin receptors [25, 31, 35]. Thus, the rank order of potency for NK1R is SP ≥ HK-1 ≥ NKA > NKB, while it is NKA > NKB > SP ≥ HK-1 for NK2R and NKB > NKA > SP ≥ HK-1 for NK3R [6, 7, 32, 33].

Previous studies have shown that SP, HK-1, NK1R, and NK2R are expressed in human ovarian mural granulosa cells (MGCs) and cumulus cells from healthy oocyte donors [20]. In this study, we intended to widen the knowledge about the expression of these tachykinin family members in granulosa cells from women with different types of infertility. We also analyzed the expression of the enzyme neprilysin, also known as membrane metallo-endopeptidase or neutral endopeptidase (NEP), which is encoded by the *MME* gene [36] and is the main enzyme responsible for degrading tachykinins [16, 37].

Thus, we studied the expression of *TAC1*, *TAC4δ*, *TACR1-T*, *TACR2*, and *MME* in MGCs and CCs from healthy oocyte donors—acting as controls—and patients with different infertility etiologies, including four of the main etiologies that lead patients to require ART treatments: PCOS, endometriosis, LOR, and advanced maternal age (AMA).

Materials and Methods

Study Population

This study has been registered in ClinicalTrials.gov with the code NCT02877992. It has been approved by the ethics committee of CSIC and Hospital Virgen Macarena (Sevilla, Spain). All recruited subjects signed a written informed consent. Granulosa samples were obtained from preovulatory follicles of Caucasian women, aged 19–45 years, who were undergoing oocyte pick-up after controlled ovarian stimulation (COS). Oocyte pick-up was performed at the clinic IVI-RMA Sevilla (IVI-RMA Global) for Reproductive Care.

Human subjects were classified into two groups. The first one was the control group, composed of 53 healthy oocyte donors. The second group or study group included 125 women. It was divided into four subgroups or categories: PCOS, AMA, LOR, and endometriosis. This is the group of the infertile patients, including 36 patients with PCOS, 44 with AMA, 21 with LOR, and 24 with endometriosis. Sample size was calculated considering an alpha risk of 0.05 and a power of 80% in a two-sided test. All patients underwent IVF treatment in our clinic and oocytes were fertilized through ICSI technique.

A clinical examination of all patients and donors was performed during the first visit to the fertility practice. Blood samples were obtained during early follicular phase of menstrual cycle (day 3 after menses) and after administration of the ovulation inductor. Serum hormone levels were assayed

enzymatically using an automated biochemistry analyzer (cobas e 411; Roche Diagnostics GmbH).

Eligibility Criteria

The control group included women between 18 and 33 years old who had functional ovaries and uterus, an antral follicle count (AFC) ≥ 12, and a normal karyotype. They also underwent a thorough study to exclude subjects with PCOS, mental disorders, hereditary diseases, and carriers of common genetic disorders including cystic fibrosis, fragile X syndrome, and glucose-6-phosphate dehydrogenase (G6PD) deficiency. These women attended the clinic to act as oocyte donors for assisted reproduction treatments and all of them present regular menstruations, no hyperandrogenism, and anti-Müllerian hormone (AMH) levels > 1.1 ng/mL.

The study group includes patients who attended the clinic seeking for fertility treatment. They have been divided into four subgroups of different infertility etiologies: PCOS, AMA, LOR, and endometriosis.

The PCOS subgroup included patients diagnosed with PCOS according to Rotterdam criteria, established in 2003 [38, 39]. According to these criteria, PCOS is diagnosed when two of the following three criteria are fulfilled: (i) menstrual dysfunction (oligo/anovulation), (ii) clinical and/or biochemical hyperandrogenism, and (iii) polycystic ovaries on ultrasound imaging. Patients with other related disorders such as adrenal congenital hyperplasia, thyroid disease, Cushing's syndrome, or androgen-secreting tumors were excluded.

The LOR group included women diagnosed as low or poor responders to COS according to the European Society of Human Reproduction and Embryology (ESHRE) Bologna criteria [40]. Following these criteria, patients are diagnosed as low responders when they have presented two previous episodes of low response after maximal ovarian stimulation (condition sufficient to define a patient as low responder) or when at least two of the following three features are present: (a) advanced maternal age (≥ 40 years) or any risk factor for LOR, (b) a previous LOR (≤ 3 oocytes with conventional stimulation), and (c) an abnormal result in the ovarian reserve test (AFC < 5–7 or anti-Müllerian hormone [AMH] level in blood < 1.1 ng/mL). In our study, the LOR group included patients with AFC < 5, AMH < 1.1 ng/mL, and a number of retrieved oocytes between 1 and 3.

The endometriosis subgroup included patients with infertility primarily associated with endometriosis. This disease was diagnosed through transvaginal ultrasound analysis or laparoscopy according to ESHRE criteria [41].

Finally, the AMA subgroup included patients with 38 years or more, with normal or sufficient pre-stimulation ovarian reserve parameters (AFC ≥ 5 and AMH > 1.1) and whose infertility was primarily linked to the age factor. We have chosen this age limit because it is the most frequently used in scientific literature [42, 43].

The following eligibility criteria were applied to subjects from all groups: body mass index < 28 kg/m^2, nonsmokers, lack of alcohol abuse, lack of diseases such as hydrosalpinx, congenital adrenal hyperplasia, thyroid disease, Cushing's syndrome, androgen-secreting tumors, and lack of use of any drug or medication that could interfere with ovarian folliculogenesis.

Ovarian Stimulation Protocol

Controlled ovarian stimulation for IVF induces a multiple follicular growth that causes a great variation of follicular steroids compared to the physiological levels of a natural cycle. To avoid any impact of these variations, all women included in the study, either fertile or infertile, were subjected to the same standard gonadotropin releasing hormone (GnRH) antagonist protocol for COS. Serum levels of estradiol (E2) and progesterone (P4) were measured following administration of the ovulation inductor.

A combination of two gonadotropins was used to stimulate folliculogenesis in the ovary: recombinant follicle-stimulating hormone (rFSH) (Gonal; Merck Serono) and human menopausal gonadotropin (hMG) (Menopur; Ferring Pharmaceuticals). Depending on AMH level and body mass index (BMI), physicians adjusted gonadotropin daily doses ranging from 150 IU of rFSH + 37.5 IU of hMG to 225 IU of rFSH + 75 IU of hMG. Physicians checked ovarian basal status during either luteal phase of the previous cycle or the first 2 days after menses, using ultrasound scanning. After that, gonadotropin administration began the second day after menses. The GnRH antagonist (Orgalutran; MSD) administration was initiated in a flexible protocol, the fifth or sixth day of stimulation, or when the leading follicle reached a diameter of 14 mm, in a daily dose of 0.25 mg until ovulation induction. Ovulation was induced when at least two follicles reached a diameter of 17 mm, using either 6500 IU of human chorionic gonadotropin (hCG) (Ovitrelle; Merck Serono) or 0.2 mg of the GnRH agonist triptorelin (Ipsen Pharmabiotech). The latter option was chosen in case of risk of ovarian hyperstimulation syndrome. Our previous work has shown that the expression levels of the genes examined are not influenced by the use of hCG or triptorelin for ovulation induction [18, 23]. Gonadotropin doses were adjusted according to patient characteristics and follicular development, which was monitored through periodical ultrasound scans and E2 blood tests.

Collection of Human Mural Granulosa Cells and Cumulus Cells

MGCs were collected from the follicular fluids obtained during ultrasound-guided transvaginal oocyte retrieval, which

was performed under intravenous anesthesia 36 h after ovulation induction. After removal of oocyte-cumulus complexes, the remaining follicular aspirates from each patient were pooled, and MGCs were collected using Dynabeads methodology, as described previously [19, 20].

CCs were obtained from the same patients and collected using procedures described elsewhere [19]. After follicular aspiration, the external CCs surrounding the oocyte were removed using cutting needles. CCs closer to the oocyte were removed using a treatment of hyaluronidase (80 IU/mL, K-SIHY, Cook Medical), and cells from corona radiata—closest layer to the oocyte—were removed passing the cumulus multiple times through a very thin glass pipette (Swemed denudation pipette, 0.134–0.145 mm, Vitrolife). CCs were pooled per patient.

RNA Extraction and Real-Time qPCR

Total RNA was extracted from fresh MGCs and CCs using the RNA/protein purification kit (Norgen Biotek Corp., Ontario, Canada) and residual genomic DNA was removed with RNase-free DNase I and RNasin (Promega, Madison, WI). Complementary DNAs (cDNAs) were synthesized using the Transcriptor First Strand cDNA Synthesis kit (Roche, Mannheim, Germany). Samples were then pre-amplificated using the SsoAdvanced PreAmp supermix (Bio-Rad Laboratories, Hercules, CA) following manufacturer's protocol.

Real-time quantitative polymerase chain reaction (RT-qPCR) was used to quantify the expression of *TAC1*, *TAC4* (δ isoform), *TACR1-Tr* (truncated isoform of *TACR1*), *TACR2*, and *MME* in CCs and MGCs using the $2^{-\Delta\Delta CT}$ method, as reported previously [19, 44]. The expression of gremlin 1 (*GREM1*) was also analyzed and used as an internal control for these experiments to asseverate the good quality of MGC and CC samples. According to previously published studies, expression of *GREM1* is related to fertility and its expression levels are lower in infertile patients [45].

qPCR was performed on a Bio-Rad iCycler iQ real-time detection apparatus using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). The parameters of PCR amplification were 10 s at 94 °C, 20 s at 60 °C, and 30 s at 72 °C, for 50 cycles. The sequences of the specific primer pairs designed to amplify each target gene are shown in Supplemental Table S1. Table S1 also shows the primers used to amplify β-actin (*ACTB*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), cyclophilin A (*PPIA*), and succinate dehydrogenase complex, subunit A (*SDHA*) which were chosen as housekeeping genes on the basis of previous studies from other laboratories and ours [18, 28, 46]. The specificity of the PCR reactions was confirmed by melting curve analysis of the products and by size verification of the amplicon in a conventional agarose gel.

A human universal reference total RNA (BD Biosciences Clontech, Palo Alto, CA) was used as a positive control of amplification and three negative controls were run for each assay: no template, no reverse transcriptase, and no RNA in the reverse transcriptase reaction. Each assay was performed in triplicate and the fold change of each target gene expression was expressed relative to the geometric mean mRNA expression of the reference genes in each sample [19, 44].

Statistical Analysis

Results are expressed as mean ± standard deviation and n represents the number of experiments in n different women. Data distribution and homogeneity of variances were analyzed with the Kolmogorov–Smirnov test and Levene test. Non-parametric distributions were observed for all gene expression data, so logarithmic transformations were performed in order to meet the normality assumptions. Statistical differences between these log-transformed values were assessed using the Student t test in the univariate analysis. The relative quantification values are shown in figures without log transformation. When statistical differences were observed, generalized linear models (GLMs) were conducted to control for confounding variables. All models were adjusted by BMI and E2 serum levels after ovulation induction. Association was indicated through β coefficients and p-values after adjusting for BMI and E2. Values of $p < 0.05$ were considered significant. All the statistical analyses were performed using IBM SPSS Statistics software version 24.0.

Results

Clinical Characteristics of Study Subjects

Anthropometric and biochemical parameters of healthy donors and PCOS patients are shown in Supplemental Table S2. All groups of infertile women were significantly older and the PCOS and low responder groups had higher BMI than control women. The serum concentrations of FSH, LH, AMH, and E2 in the early follicular phase of the menstrual cycle fell within the reference range values for each group (Table S2). Thus, the LH/FSH ratio was higher in PCOS and lower in low responders, in comparison with the other groups analyzed. The serum concentrations of E2 measured on the day of administration of the ovulation inductor were similar in controls and PCOS and were lower in the other groups of infertile patients. The serum concentrations of progesterone (P) measured on the day of administration of the ovulation inductor were similar in control and infertile women (Table S2).

Expression of TAC1, TAC4δ, TACR1-T, TACR2, MME, and GREM1 in Healthy Oocyte Donors

In agreement with our previous data [20], the genes *TAC1*, *TAC4δ*, *TACR1*, and *TACR2* were all present in MGCs and CCs of control, healthy women ($n=53$, Figs. 1, 2, 3, and 4). Among the two known isoforms of *TACR1*, only *TACR1-T*, encoding the truncated isoform of the NK1R protein, was expressed [[20], this study]. The *MME* gene, encoding NEP, was also detected in all MGC and CC samples analyzed and its expression was significantly higher in MGCs (~20-fold), compared with CCs from the same donors ($\beta=-1.083$, $p<0.001$, $n=53$, Figs. 1E and 2E).

Expression of TAC1, TAC4δ, TACR1-T, TACR2, MME, and GREM1 in Patients with Polycystic Ovarian Syndrome

The mRNA expression levels of *TAC1* ($\beta=-0.017$, $p=0.047$) and *TACR1-T* ($\beta=-0.605$, $p=0.031$) were up-regulated in CCs from PCOS patients ($n=36$) while *TAC4δ* levels were down-regulated ($\beta=0.256$, $p=0.004$), in comparison with the control group of oocyte donors ($n=53$, Fig. 1A, B and C). *TACR2* expression was down-regulated in MGCs from PCOS patients ($\beta=0.497$, $p=0.018$) (Fig. 1D). A deeper analysis shows that *TAC1* ($\beta=-1.505$, $p=0.004$), *TAC4δ* ($\beta=0.326$, $p<0.001$), *TACR1-T* ($\beta=-0.701$, $p=0.018$), and *TACR2* ($\beta=-1.096$, $p=0.001$) levels were significantly different in MGCs from oocyte donors, in

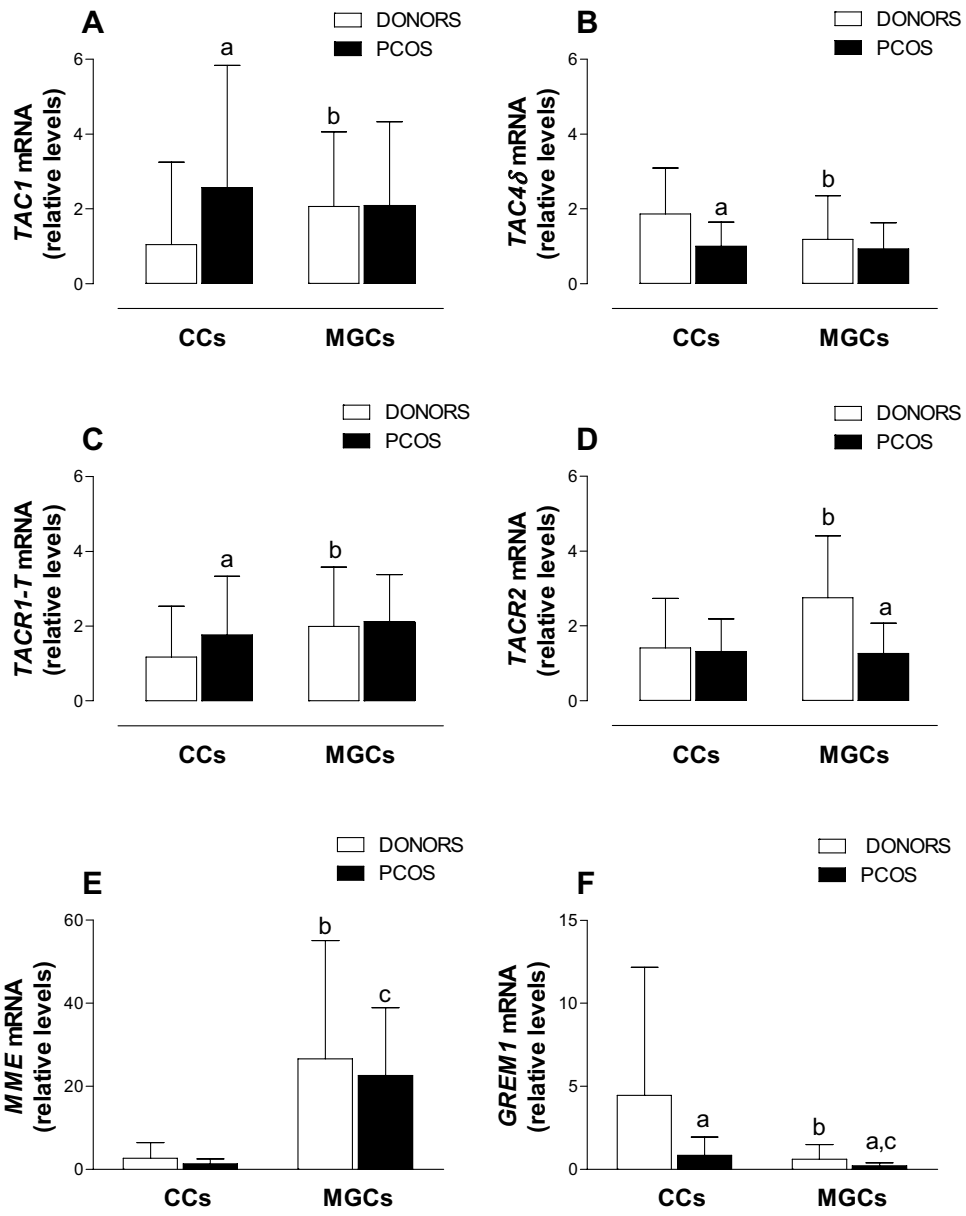

Fig. 1 Expression levels of *TAC1* (**A**), *TAC4δ* (**B**), *TACR1-T* (**C**), *TACR2* (**D**), *MME* (**E**), and *GREM1* (**F**) in human cumulus cells and mural granulosa cells of healthy oocyte donors ($n=53$) and patients with polycystic ovarian syndrome ($n=36$). Results are presented as mean ± standard deviation. Statistically significant differences at $P<0.05$ are represented as **a** between donors and patients, **b** between cumulus and granulosa cells in donors, and **c** between cumulus and granulosa cells in aged patients

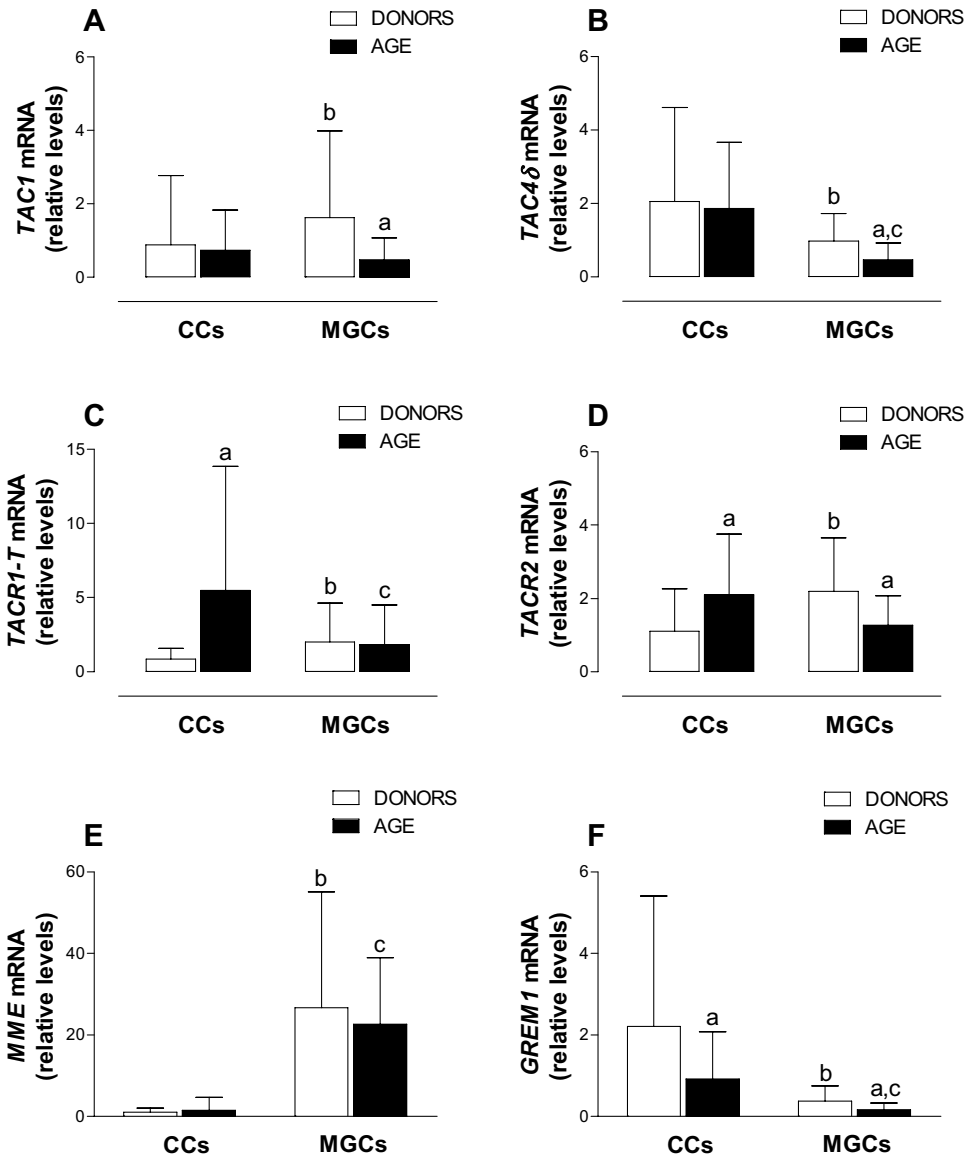

Fig. 2 Expression levels of *TAC1* (**A**), *TAC4δ* (**B**), *TACR1-T* (**C**), *TACR2* (**D**), *MME* (**E**), and *GREM1* (**F**) in human cumulus cells and mural granulosa cells of healthy oocyte donors ($n=53$) and women of advanced maternal age (≥ 38 years, $n=44$). Results are presented as mean ± standard deviation. Statistically significant differences at $P<0.05$ are represented as **a** between donors and patients, **b** between cumulus and granulosa cells in donors, and **c** between cumulus and granulosa cells in aged patients

comparison with CCs from the same women. These differences were not observed in PCOS women.

MME expression levels were similar in PCOS patients and donors ($\beta=-0.034$, $p=0.904$ for MGCs and $\beta=-0.375$, $p=0.269$ for CCs) and were significantly higher (~20-fold) in MGCs than in CCs from both studied groups ($\beta=-1.083$, $p<0.001$ and $\beta=-1.104$, $p=0.009$, respectively) (Fig. 1E). *GREM1* levels were lower in the group of PCOS patients, both in CCs ($\beta=0.695$, $p<0.001$) and MGCs ($\beta=1.058$, $p<0.001$) (Fig. 1F).

Expression of TAC1, TAC4δ, TACR1-T, TACR2, MME, and GREM1 in Patients with Advanced Maternal Age

The expression of *TAC1* ($\beta=1.378$, $p=0.044$), *TAC4δ* ($\beta=0.697$, $p<0.001$), and *TACR2* ($\beta=0.585$, $p=0.013$) was down-regulated in MGCs from the group of patients with AMA ($n=44$), in comparison with mRNA levels in the control group ($n=53$, Fig. 2A and B). *TACR1-T* and *TACR2* levels were up-regulated in CCs from aged patients ($\beta=-0.867$, $p=0.003$ and $\beta=-1.203$, $p=0.001$, respectively) (Fig. 2C and D).

Similarly to the PCOS group, *MME* mRNA levels were much lower (~20-fold) in CCs than in MGCs from both groups (donors: $\beta=-1.281$, $p<0.001$; PCOS: $\beta=-1.370$, $p<0.001$), but there were no significant differences in its expression between donors and aged patients (CCs: $\beta=-0.043$, $p=0.867$; MGCs: $\beta=0.018$, $p=0.929$) (Fig. 2E). *GREM1* mRNA expression levels were lower in aged patients, in both types of granulosa cells (CCs: $\beta=0.549$, $p<0.001$; MGCs: $\beta=1.085$, $p<0.001$) (Fig. 2F).

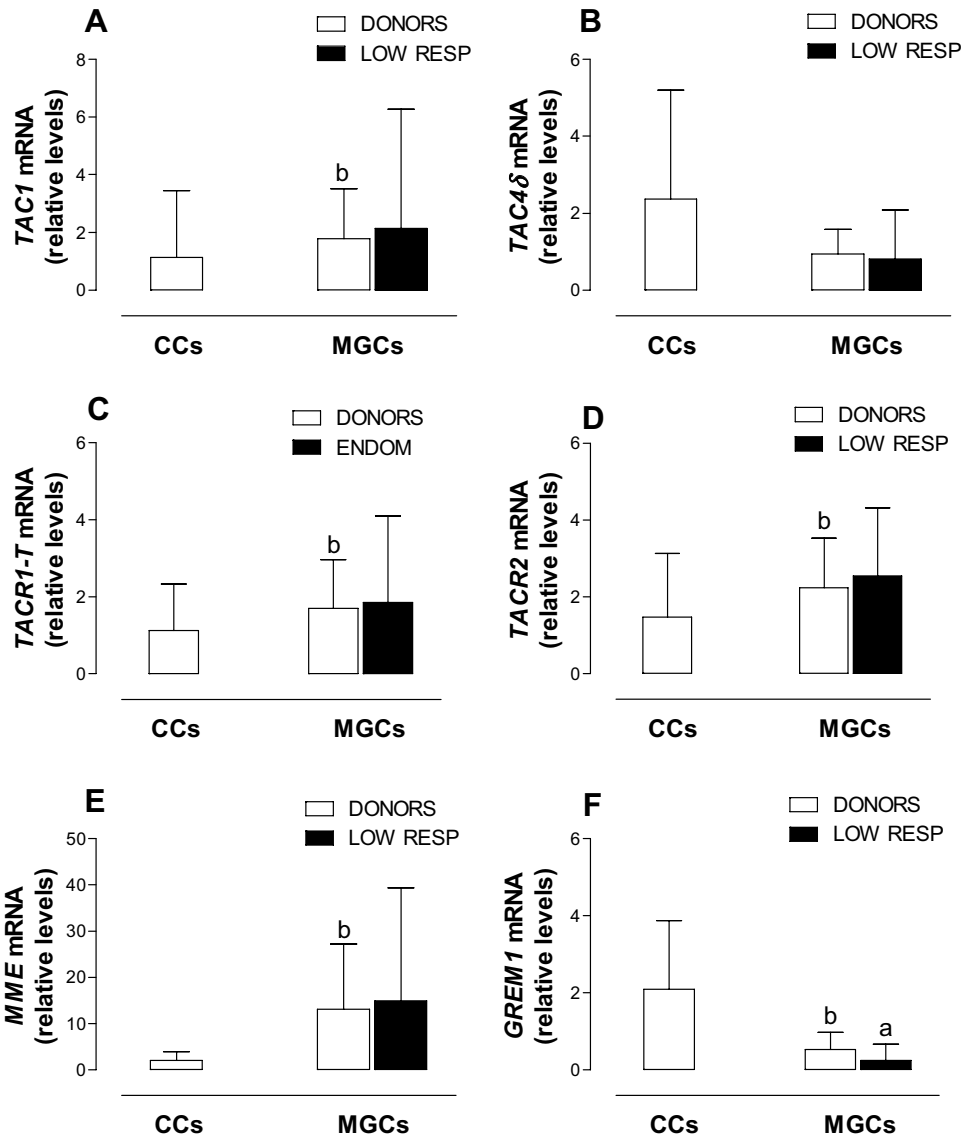

Fig. 3 Expression levels of *TAC1* (**A**), *TAC4δ* (**B**), *TACR1-T* (**C**), *TACR2* (**D**), *MME* (**E**), and *GREM1* (**F**) in human mural granulosa cells of healthy oocyte donors ($n=53$) and patients with low ovarian response ($n=21$). Results are presented as mean ± standard deviation. Statistically significant differences between donors and patients at $P<0.05$ are represented as **a** between donors and patients and **b** between cumulus and granulosa cells in donors

Expression of TAC1, TAC4δ, TACR1-T, TACR2, MME, and GREM1 in Patients with Low Ovarian Response

Due to the small number of CCs obtained from patients with LOR, the expression of the target genes could only be analyzed in MGCs from LOR patients. In this case, we did not find statistically significant variations in the mRNA expression levels of *TAC1* ($\beta=1.172$, $p=0.346$), *TAC4δ* ($\beta=0.181$, $p=0.430$), *TACR1-T* ($\beta=-0.244$, $p=0.431$), *TACR2* ($\beta=-0.117$, $p=0.540$), *or MME* ($\beta=0.074$, $p=0.821$) between LOR patients ($n=21$) and donors ($n=53$, Fig. 3A–E).

In the case of the control gene *GREM1*, its expression was significantly lower in MGCs from LOR patients than in the donor group ($\beta=0.433$, $p=0.049$) (Fig. 3F).

Expression of TAC1, TAC4δ, TACR1-T, TACR2, MME, and GREM1 in Patients with Endometriosis

In order to avoid the analysis of damaged MGCs from patients with endometriosis, in this study we only included CCs from this type of patients. MGCs are isolated from the follicular fluid, which, in the case of endometriosis patients, contains big aggregates of granulosa cells and other cell types like blood cells. Since it is not possible to correctly isolate MGCs in this context, they have been excluded from the analysis.

The results showed that *TAC4δ* ($\beta=0.052$, $p<001$), *TACR2* ($\beta=0.080$, $p<0.001$), and MME ($\beta=0.043$, $p=0.007$) were overexpressed in CCs from endometriosis patients ($n=24$), in comparison with the control group ($n=53$, Fig. 4B, D, and

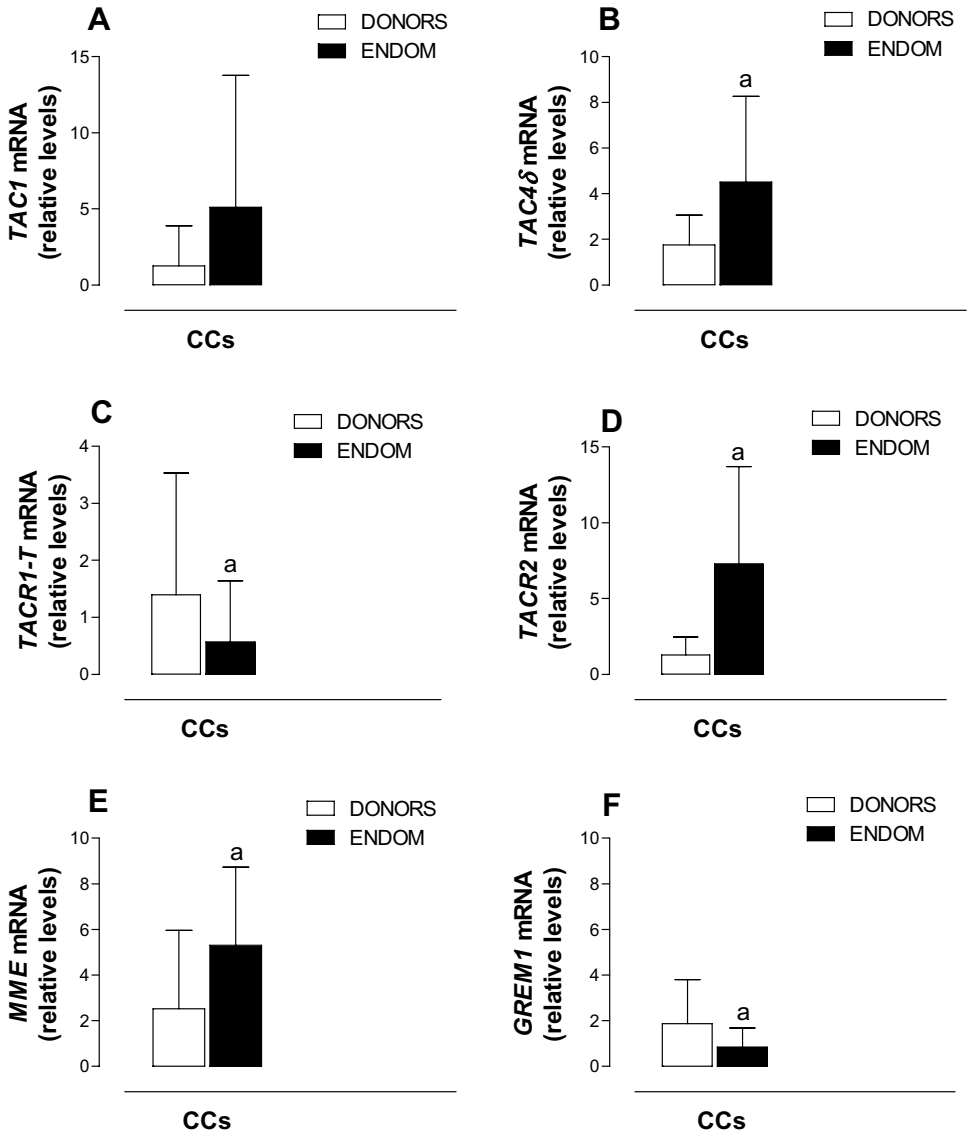

Fig. 4 Expression levels of *TAC1* (**A**), *TAC4δ* (**B**), *TACR1-T* (**C**), *TACR2* (**D**), *MME* (**E**), and *GREM1* (**F**) in human cumulus cells of healthy oocyte donors ($n=53$) and patients with endometriosis ($n=24$). Results are presented as mean ± standard deviation. Statistically significant differences between donors and patients at $P<0.05$ are represented as (**a**)

E). *TAC1* mRNA levels were ~ fivefold higher in the endometriosis group, but they showed great individual variations in these patients, with values between 0 and 35, and, as a consequence, expression differences between the endometriosis and the donor groups did not reach statistical significance (Fig. 4A). The expression levels of *TACR1-T* were lower in the endometriosis group than in oocyte donors ($\beta=-0.032$, $p=0.019$) (Fig. 4C).

The mRNA expression level of *GREM1* was significantly lower in the endometriosis group, compared to oocyte donors ($\beta=0.514$, $p=<0.001$) (Fig. 4F).

Discussion

NKB and its preferred receptor, NK3R, play a key role in the regulation of human reproduction [8–16, 31, 47]. However, the effect of NKB may be mediated by activation of the other known tachykinin receptors, NK1R and NK2R, and the NK3R may be activated by the tachykinins SP, NKA, or HK-1 [6, 7, 32, 33]. Moreover, the effects of NKB in human MGCs involve activation of tachykinin receptors other than NK3R [19]. We previously found

that *TAC3* and *TACR3* mRNA levels are dysregulated in CCs and MGCs from infertile women [18, 23]. The present finding shows that the expression of *TAC1*, *TAC4δ*, *TACR1-T*, and *TACR2* in MGCs and CCs from infertile women is also altered, in a selective manner, depending on infertility etiology. This suggests that the whole tachykinin family acts in the ovary to regulate reproductive function.

The effects of tachykinins on reproduction might include a direct action at peripheral reproductive tissues (8,9,18–21,29,33,49,50). Within the ovary, *TAC1*, *TAC3*, *TAC4*, *TACR1*, *TACR2*, and *TACR3* are constitutively expressed in human granulosa cells, where they may play essential roles regulating fertility [19, 20, 28, 48]. According to a study performed in the ascidian *Cyona intestinalis*, tachykinins could be involved in the stimulation of oocyte growth [49]. Similar results have been obtained in healthy women, where NKB regulates ovarian follicle growth and ovulation [14]. Other studies based on the culture of granulosa and luteal cells of bovine and porcine origin suggest a role for SP in the regulation of steroid hormone secretion [50]. Nevertheless, the precise function of tachykinins in the ovary remains mostly unknown.

To our knowledge, this is the first study showing an alteration of the expression of *TAC1*, *TAC4*, *TACR1*, and *TACR2 i*n MGCs and CCs from infertile women. This and our previous results with *TAC3* and *TACR3* show that women infertility is associated with a dysregulation of the tachykinin system in ovarian granulosa cells. Furthermore, the tachykinin expression profile varies depending on the studied infertility etiology. In the case of PCOS patients, *TAC1* and *TACR1-T* were up-regulated and *TAC4δ* was down-regulated in CCs, while *TACR2* was down-regulated in MGCs. In the case of the aged patients, however, *TAC1, TAC4δ*, and *TACR2* were down-regulated in MGCS, while *TACR1-T* and *TACR2* were up-regulated in CCs. The group of LOR patients was the one with fewer variations in tachykinin expression and we failed to find significant variations in the mRNA levels of *TAC1*, *TAC4δ*, *TACR1-T*, *or TACR2* between LOR patients and donors. Finally, in the endometriosis group, *TAC1*, *TAC4δ*, and *TACR2* were up-regulated in CCs while *TACR1-T* was down-regulated. The profound differences in the expression of the tachykinin family members in MGCs and CCs of infertile patients and the dependence on the type of infertility suggest that these genes might be involved in the pathophysiology beneath PCOS, AMA, LOR, and endometriosis. These are clearly differentiated in their clinical (40–45) and biochemical (Table S2) characteristics, but the mechanism through which they lead to infertility remains unclear. The unique genetic profile of tachykinins in each etiology provides a molecular basis to differentiate these disorders and might improve our diagnostic capabilities in the field of ART and infertility, being particularly useful in the diagnosis and treatment of women with unexplained infertility.

Regarding PCOS, this endocrinopathy affects approximately 5–15% of reproductive-aged women and is one of the most common causes of female subfertility [18]. The pathogenesis of this syndrome is strongly associated with dysfunction of granulosa cells [18] whose differentiation in MGCs and CCs is essential for coordination of follicular development, oocyte maturation, and ovulation [51]. In this context, if we consider the expression of the tachykinin and kisspeptin systems in healthy donors, the transition of MGCs to CCs is accompanied by (a) an increase in the expression of *KISS1R* [18, 19]; (b) an increase in the expression of *TAC3* and *TACR3* [18, 19]; (c) a decrease in the expression of *TAC1*, *TACR1-T*, and *TACR2* (this study); and (d) an increase in the expression of *TAC4δ* (this study). All these changes did not occur in the transition of MGCs to CCs in PCOS patients, where the mRNA levels of these genes, with the exception of *TAC3*, were similar in MGCs and CCs [[18, 19], this study]. Thus, it seems that CCs of PCOS women remain in an immature, MGC-like state, with the consequent failures in follicle maturation and ovulation characteristics of PCOS. This lack of normal maturation of MGCs could be signaled to the hypothalamus, contributing to the maintenance of high circulating levels of kisspeptin, LH, and AMH, as observed in PCOS [[52–54], this study]. Further studies are needed to determine whether the dysregulation of tachykinins and their receptors is a cause or a consequence of the alteration of granulosa cells in PCOS.

Advanced maternal age is clearly associated with a decrease in both ovarian reserve and oocyte competence. In our study, the mean age of the AMA group was significantly higher than that of the LOR group, but both groups included women with ages ≥ 38 years old. However, AMA and LOR patients were clearly differentiated in their biochemical and clinical parameters (see Table S2): (a) in contrast to LOR, patients of the AMA group showed a normal or sufficient ovarian reserve [55]; (b) FSH levels were significantly higher and the FSH/LH ratio significantly lower in the LOR than in the AMA group; and (c) E2 levels after ovulation induction were significantly lower in the LOR than in the AMA group. This indicates a marked hyporesponse to gonadotropins in the LOR group. The differences in the mRNA levels of tachykinins and tachykinins receptors between the AMA and LOR groups add support to our finding that the expression profile of these genes in ovarian granulosa cells varied depending on the infertility etiology.

Endometriosis is an estrogen-dependent chronic disease that causes infertility and pelvic pain with different degrees of severity. It has been shown that this condition has a harmful impact on ovarian physiology, especially when endometriotic lesions are located at this level. The *MME* gene did not show expression differences between healthy oocyte donors and patients with PCOS, AMA, or LOR. However, there were important differences between CCs and MGCs,

with a much higher expression in MGCs, which evidences a higher restriction of tachykinin effects in these cells. In the case of endometriosis patients, *MME* was significantly up-regulated in CCs, suggesting a possible relationship with the etiopathogenesis of endometriosis. This is reinforced by the observation that *TAC1*, *TAC4δ*, *TACR1-T*, and *TACR2* expression was also dysregulated in endometriosis patients. In this line, endometriosis is associated with an inflammatory response [56], tachykinins are important inflammatory mediators [6], and there is increasing evidence suggesting that tachykinins might play a role in the development of endometriotic lesions [57].

The fact that tachykinins show altered genetic profiles in infertility opens a new area of therapeutic options, as antagonists of tachykinin receptors could be useful for the treatment of different types of infertility. In fact, NK3R antagonists have been suggested as potentially useful for treatment of PCOS, a disorder for which there is actually no specific treatment [53, 54]. NK3R antagonists are also being assayed for treatment of menopausal hot flashes and have shown good efficacy and tolerability in phase 2 trials [57]. Completion of phase 3 clinical trials is required to validate the therapeutic use of NK3R antagonists to treat infertility and other reproduction-related disorders.

In summary, the different members of the tachykinin family could mediate, at least in part, the effects of the NKB/NK3R system in granulosa cells, as NKB also acts on NK1R and NK2R and all tachykinins are able to activate NK3R. This study shows that *TAC1*, *TAC4δ*, *TACR1-T*, and *TACR2* expression is altered in ovarian granulosa cells from women presenting different infertility etiologies, including PCOS, AMA, and endometriosis. A dysregulated expression of tachykinins and their receptors might thus be involved in the decreased fertility of these patients. In fact, the genetic expression profile differs depending on the etiology, highlighting the diverse genetic background of each type of infertility, which are still far from being understood. These insights could become the first step to unveil new molecular mechanisms underneath infertility and design new treatment options in the future.

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Data Availability Data is available for transparency.

Code Availability Not applicable.

Declarations

Ethics Approval Approved by the ethics committee of CSIC and Hospital Virgen Macarena (Sevilla, Spain).

Consent to Participate All recruited subjects signed a written informed consent.

Consent for Publication All recruited subjects have consented for publication.

Conflict of Interest The authors declare no competing interests.

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