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ORIGINAL RESEARCH

C–C motif chemokine receptor 2 as a novel intermediate in the ovulatory cascade

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ABSTRACT: Expression of immune function genes within follicle cells has been reported in ovaries from many species. Recent work from our laboratory showed a direct effect of the monocyte chemoattractant protein 1/C-C motif chemokine receptor 2 system within the feline cumulus oocyte complex, by increasing the mRNA levels of key genes involved in the ovulatory cascade *in vitro*. Studies were designed to evaluate if C–C motif chemokine receptor 2 acts as a novel mediator of the ovulatory cascade *in vitro*. Therefore, feline cumulus oocyte complexes were cultured in the presence or absence of a highly selective C–C motif chemokine receptor 2 antagonist together with known inducers of cumulus–oocyte expansion and/or oocyte maturation to assess mRNA expression of key genes related to periovulatory events in other species as well as oocyte maturation. Also, the effects of recombinant monocyte chemoattractant protein 1 on spontaneous or gonadotrophin-induced oocyte maturation were assessed. This is an *in vitro* system using isolated cumulus oocyte complexes from feline ovaries. The present study reveals the modulation of several key ovulatory genes by a highly selective C–C motif chemokine receptor 2 antagonist. However, this antagonist was not enough to block the oocyte maturation induced by gonadotropins or amphiregulin. Nonetheless, recombinant monocyte chemoattractant protein 1 had a significant effect on spontaneous oocyte maturation, increasing the percentage of metaphase II stage oocytes in comparison to the control. This is the first study in any species to establish C–C motif chemokine receptor 2 as a mediator of some actions of the mid-cycle gonadotrophin surge.

Key words: C–C motif chemokine receptor 2 / monocyte chemoattractant protein 1 / cumulus oocyte complex / feline / ovulatory cascade genes

Introduction

Shortly before ovulation, the LH surge induces processes critical for fertility, including cumulus–oocyte expansion (C-OE) and resumption of meiosis. While some of the paracrine-acting factors important for these events have been identified, the molecular mechanisms responsible for initiating such complex processes are not fully understood. Thus, understanding the molecular and cellular processes involved in periovulatory events would aid in the diagnosis or treatment of infertility and may also identify novel targets for a non-hormonal form of contraception. Recent work from our laboratory showed a novel direct effect of the monocyte chemoattractant protein 1 (MCP1)/C–C motif chemokine receptor 2 (CCR2) system, by increasing the mRNA levels of key genes [hyaluronan synthase 2 (*HAS2*), amphiregulin (*AREG*), tumor necrosis factor-inducible gene 6 protein (*TNFAIP6*), pentraxin 3 (*PTX3*) and growth differentiation factor-9 (*GDF9*)] involved in the ovulatory cascade *in vitro* (Rojo *et al*., 2019). Expression of immune function genes within follicle cells has been reported in ovaries from many species. Skinner *et al*. showed that the levels of the mRNA of *MCP1, MCP2*, macrophage inflammatory protein 1-beta (MIP-1β) and chemokine C–C motif ligand-5 (*CCL5*) increased in bovine granulosa and/or theca cells during antral follicle development (Skinner *et al*., 2008). Interestingly, different chemokine receptors were also shown to be expressed in the granulosa (*CCR1, CXCR3, CCR5* and *CXCR6*) and theca (*CCR1*) bovine cells, suggesting an autocrine/paracrine role of chemokines in these two cell types. *Mcp1* mRNA levels were shown to be upregulated in the rat preovulatory follicle (Wong *et al*., 2002), and its protein levels were increased in follicular fluid by hCG treatment

of women during IVF cycles (Arici et al., 1997). Also, stromal-derived factor-1 (SDF1, aka CXCL12) is a chemokine that is expressed in ovaries and serves as the ligand to the CXCR4 receptor (Sayasith and Sirois, 2014). Cumulus oocyte complexes (COCs) in mice, cattle and horses express CXCR4, and the hormonal surge that triggers ovulation increases CXCR4 mRNA and protein in equine and bovine follicles (Hernandez-Gonzalez et al., 2006). Recently, our laboratory has demonstrated a direct effect of the chemokine SDF1 within the COC, by increasing the key ovulatory genes *HAS2* and *TNFAIP6,* through its main receptor CXCR4 (Rojo et al., 2018).

The domestic cat (*Felis catus*) serves as a valuable model for studying oocyte biology and also for addressing different infertility syndromes in women due to highly conserved reproductive mechanisms between humans and feline species (Wildt et al., 2010). Remarkably, cat oocytes share several characteristics with human oocytes (Combelles et al., 2002; Comizzoli et al., 2008), including the diameter of the oocyte proper and the germinal vesicle, the time to reach the metaphase II (MII) stage of meiosis in culture, and a nuclear configuration with a small nucleolus and a fibrillar chromatin. In contrast, these morphological features are distinct or lacking in the typical laboratory mouse model. In our laboratory, we have established two different feline culture systems, showing that the culture of feline antral follicles and/or their COCs was a robust and valuable system to study follicular development, steroidogenesis, periovulatory events, as well as follicle and COC biology in general (Rojo et al., 2015; Rojo et al., 2018).

Studies were designed to evaluate if CCR2 acts as a novel mediator of the ovulatory cascade *in vitro*. Therefore, feline COCs were cultured in the presence or absence of a highly selective CCR2 antagonist (RS 504393) together with known inducers of C-OE and/or oocyte maturation [gonadotropins (GNTs), AREG and prostaglandin E2 (PGE2)] to assess mRNA expression of key genes (*HAS2, TNFAIP6, AREG* and *PTX3*) related to periovulatory events in other species and oocyte maturation. Also, the effects of recombinant MCP1 on spontaneous or GNT-induced oocyte maturation were assessed.

Materials and Methods

Animals

Ovaries from adult female ($n = 70$) *F. catus* at different stages of the natural estrous cycle during the breeding season were used. The ovaries were donated following routine spaying procedures conducted at the 'Centro de Salud Animal de la Municipalidad de Merlo' (Prov. de Buenos Aires, Argentina). The excised ovaries were immediately transported to the laboratory in chilled physiological solution.

COC culture for gene expression

Following the dissection of the antral follicles (0.5–2 mm) from the ovarian tissue under a stereoscopic microscope using 30-gauge needles, they were punctured and their COCs extracted. Isolated healthy COCs ($n = 68$) were randomly divided and cultured, as previously reported (Rojo et al., 2018; Rojo et al., 2019), in minimum essential medium (MEM) containing Hepes (25 mM, Gibco), L-glutamine (2 mM), sodium pyruvate (1 mM, Sigma), penicillin/streptomycin (100 IU/ml-100 mg/ml, Sigma) and 1% fetal bovine serum (FBS)-charcoal/stripped, with known inducers of C-OE and/or oocyte maturation [GNTs, AREG and prostaglandin E2 (PGE2)] in the presence or absence of a highly selective CCR2 antagonist (1 μM; RS 504393, Tocris, Minneapolis, MN, USA) for 3 h. This antagonist was chosen since it is an extremely selective CCR2 chemokine receptor antagonist (IC50: 98 nM and >100 μM for inhibition of human CCR2 and CCR1 receptors, respectively) (Mirzadegan et al., 2000). Mirzadegan et al., (2000) also demonstrated that the RS-504393 compound inhibits MCP-1- and MCP-3-stimulated calcium influx into CCR2-CHL cells (IC50 values as MCP-1 and MCP-3 inhibitors are 35 ± 9 and 160 ± 26 nM, respectively). This antagonist was shown to inhibit MCP-1 chemotaxis (IC50 = 330 nM) and ischemia-reperfusion injury *in vivo* in kidneys (Furuichi et al., 2003). This culture period was chosen since this time interval is when the expression of key genes involved in C-OE (such as *HAS2, TNFAIP6, AREG* and *PTX3*) are induced by GNTs (FSH and LH; 10 and 5 UI/ml, respectively; Merck Serono) in our culture system (Rojo et al., 2018; Rojo et al., 2019). Two different concentrations of AREG (10 and 100 ng/ml; R&D Systems, Minneapolis, MN, USA) and PGE2 (0.5 and 1 μg/ml; Cayman Chemical, Ann Arbor, MI, USA) were tested based on the manufacturer's datasheet, literature and/or previous results (Takahashi et al., 2006; Peluffo et al., 2012, 2014). Serum was included in the media for COC culture because serum factors are required for C-OE to occur (Richards, 2005). COCs were cultured in a 4-well plate (450 μl media, 5–10 COCs/well) for 3 h at 38°C under 5% CO2 in air. At the end of culture, COCs were stored individually at −80°C for subsequent RNA extraction and quantitative RT-PCR (qRT-PCR).

COC culture for oocyte maturation

Healthy COCs ($n = 296$) isolated from antral follicles were randomly divided and cultured using optimal conditions for IVM as previously reported by other authors (Comizzoli et al., 2003). Briefly, COCs were culture in a 4-well plate (450 μl media, 5–10 COCs/well) under mineral oil (at 38°C under 5% CO2 in air) in MEM containing L-glutamine (2 mM, Sigma), sodium pyruvate (1 mM, Sigma), penicillin/streptomycin (100 IU/ml-100 mg/ml, Sigma) and 4 mg/ml bovine serum albumin (BSA) for 28 h under different treatment groups: media alone (Control Group); GNTs (FSH + LH; 75 mIU/ml each); AREG: (10 and 100 ng/ml); PGE2: (0.5 and 1 μg/ml); CCR2 ant (1 μM RS 504393); AREG + CCR2 ant: AREG (10 and 100 ng/ml) + RS 504393); PGE2 + CCR2 ant: PGE2 (0.5 and 1 μg/ml) + RS 504393. At the end of culture, COCs were stripped of cumulus cells and naked oocytes were fixed in 4% paraformaldehyde (PFA) for indirect immunofluorescence (IF) to assess oocyte maturation. The oocytes were then stored in washing buffer (1% BSA, 0.2% powder milk, 0.2% goat serum, 0.2% donkey serum, 0.1% Triton X-100, 0.1 M glycine in PBS) at 4°C until use, as previously described (Peluffo et al., 2010; Rojo et al., 2018).

An additional set of experiments was accomplished to evaluate the effects of recombinant MCP1 on spontaneous or gonadotrophin-induced oocyte maturation. Healthy COCs ($n = 206$) isolated from antral follicles were randomly divided and cultured under different treatment using optimal conditions for IVM as described above. Treatment groups: media alone (Control Group); MCP1: (10 and 100 ng/ml; PHC1011, Life Technologies); GNTs (FSH + LH; 75 mIU/ml each);

GNT+ MCP1: (10 ng/ml and 100 ng/ml). At the end of culture, COCs were stripped of cumulus cells and naked oocytes were fixed in 4% PFA for IF to assess oocyte maturation.

mRNA extraction and gene expression analysis

Total RNA was extracted from individual COCs using Absolutely RNA Nanoprep Kit (Agilent, CA, USA), as previously described (Rojo *et al*., 2018; Rojo *et al*., 2019). In order to obtain DNA-free total RNA, potential DNA contamination was removed by RNase-Free DNase I treatment at 37°C for 15 min (included in the kit). Ten to 100 ng of RNA per sample were eluted in 12 µl of elution buffer (as measured by UV-spectrophotometer, NanoDrop 2000, Thermo Scientific). In order to synthesize single-stranded cDNA from total RNA, the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) was used following the manufacturer's instructions. The reverse transcription was performed for 2 h at 37°C using 10 µl of the RNA extracted from each individual COC, in a 20-µl reaction volume. The kit uses random primers as cDNA priming method and the Multiscribe RT, a recombinant reverse transcriptase obtained from the Moloney Murine Leukemia Virus (MoMuLV). At the end of the reaction, reverse transcriptase was inactivated at 85°C for 5 min. After cDNA synthesis, qPCR for key genes within the ovulatory cascade (such as, *HAS2, TNFAIP6, AREG* and *PTX3*) was conducted as previously described using the same set of primers and TaqMan probes (Rojo *et al*., 2018; Rojo *et al*., 2019). The list of primers and hydrolysis probes (TaqMan, Thermo Scientific), together with the reaction efficiencies and the accession numbers of each target sequence, is listed in Supplementary Table SI. Relative levels of target gene expression were normalized to ribosomal protein *18S* levels.

IF and oocyte maturation

Like other carnivores, cat oocytes have a dark appearance due to high intracellular concentration of lipids, which makes observance of the germinal vesicle (GV) difficult under the optical microscope. Therefore, IF was performed to assess oocyte maturation. Briefly, oocytes were incubated in primary antibody for 1 h at 37°C, followed by three 10-min washes in wash buffer (1% BSA, 0.2% powder milk, 0.2% goat serum, 0.2% donkey serum, 0.1% Triton X-100, 0.1 M glycine in PBS) and then a 1 h incubation of secondary antibody at room temperature with agitation. Spindle microtubules were labeled with alpha-tubulin clone DM1A (1:1000; Novus Biologicals, Littleton, CO, USA) followed by Alexa 555 donkey anti-mouse IgG (1:500; Invitrogen, Carlsbad, CA, USA); F-actin was probed with Alexa 488-phalloidin (1:50; Invitrogen, Carlsbad, CA, USA); DNA was labeled with Hoechst 33342 (Invitrogen, Carlsbad, CA, USA). Oocytes were mounted on slides with glycerol/PBS solution (1:1), and maturation stage for each oocyte was analyzed and visualized using a fluorescent light microscope.

Statistical analysis

Statistical calculations were performed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA). One-Way ANOVA was used to analyze differences, followed by Newman–Keuls for multiple comparison. Differences were considered significant at $P < 0.05$.

Results

Expression of key genes involved in the ovulatory cascade within the COC blocked by a highly selective CCR2 antagonist

To assess a possible role of the CCR2/MCP1 system as a critical intermediate in the ovulatory cascade, feline COCs were cultured in the presence or absence of a highly selective CCR2 antagonist (RS 504393) together with known inducers of C-OE and/or oocyte maturation (GNT, AREG and PGE2). The level of expression of key genes involved in the ovulatory cascade [*AREG* (Panel a), *HAS2* (Panel b), *TNFAIP6* (Panel c) *and PTX3* (Panel d)] within COC was assessed by qRT-PCR following different treatment conditions (Figs 1–3). The highly selective CCR2 antagonist was able to significantly prevent or interfere with the GNT (Fig. 1) stimulation of *AREG, HAS2, TNFAIP6* and *PTX3* mRNA expression. Similar results were obtained for AREG and PGE2 stimulation (Figs 2 and 3, respectively). However, PGE2 had no effect on PTX3 mRNA levels and the antagonist had no effect on the AREG effect on PTX3 mRNA levels ($P > 0.05$) (Figs 2 and 3, respectively). Two different concentrations of PGE2 (0.5 and 1 µg/ml) and AREG (10 and 100 ng/ml) were used in our culture conditions. Although both concentrations of AREG did significantly stimulate the expression of the evaluated genes, the lower concentration showed more consistent results. In contrast, it was the higher concentration of PGE2 that significantly stimulated the expression of genes involved in the ovulatory cascade.

Oocyte maturation stimulated by GNT or AREG was not affected by CCR2 antagonist

Oocyte maturation was measured as the percentage of oocytes that reached MII stage after 28 h in culture under different treatment conditions (Figs 4 and 5). Interestingly, CCR2 antagonist significantly stimulated oocyte maturation by itself. CCR2 antagonist (RS 504393) did not significantly decrease oocyte maturation triggered by GNTs (Fig. 4a) and AREG (Fig. 4b). However, a trend to decreased oocyte maturation due to RS 504393 was observed after oocyte stimulation with GNTs (Fig. 4a), as well as with the lower concentration of AREG (10 ng/ml; Fig. 4b) ($P > 0.05$). In contrast, PGE2 treatment (Fig. 4c) did not increase the proportion of MII oocytes at either of the two different concentrations tested, under our culture conditions.

Recombinant MCP1 stimulates spontaneous oocyte maturation

When analyzing the effects of recombinant MCP1 on spontaneous and gonadotrophin-induced oocyte maturation (Fig. 5), we observed a significant effect on spontaneous oocyte maturation, increasing the percentage of MII stage oocytes in comparison to the control (media alone, Fig. 5a). In contrast, MCP1 did not significantly affect the percentage of gonadotrophin-induced MII stage oocytes (Fig. 5b).

Discussion

The present study reveals, for the first time in any species, the modulation of several key ovulatory genes by a highly selective CCR2 antagonist. In this regard, we observed that CCR2 antagonist (RS 504393) decreased the mRNA expression level of *AREG, HAS2,*

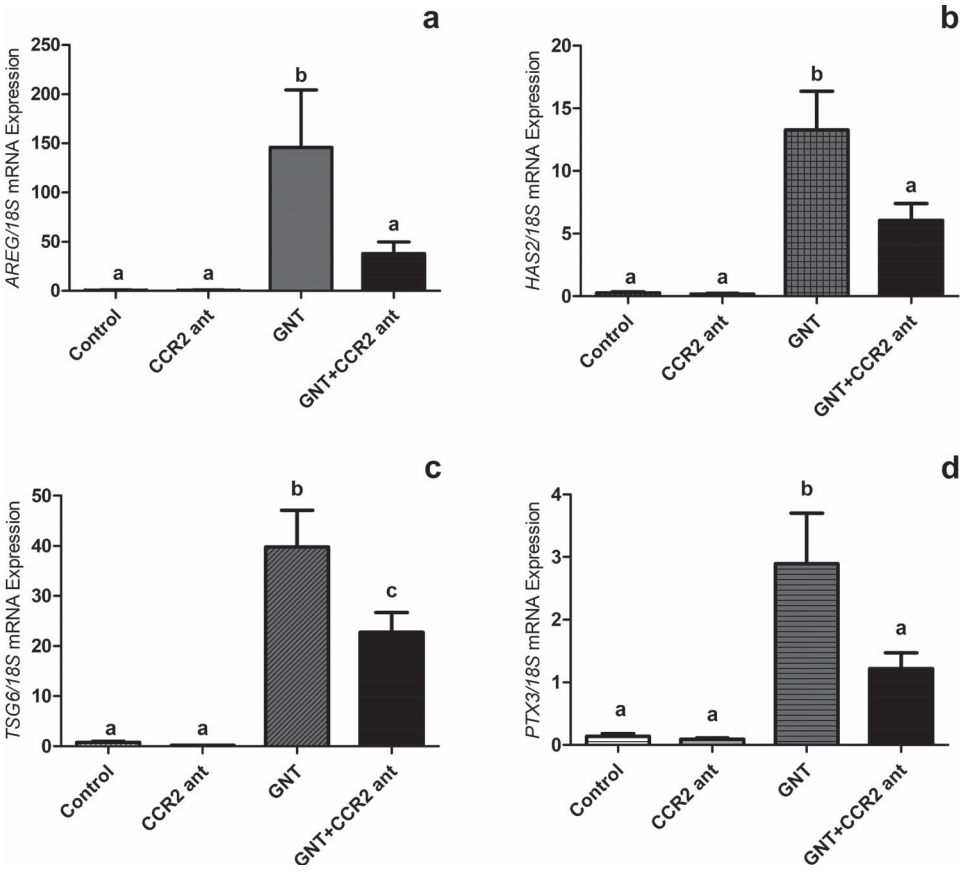

Figure 1 Highly selective C motif chemokine receptor 2 antagonist blocked the gonadotrophin stimulation of mRNA expression levels of key periovulatory genes. Normalized *AREG* (**a**), *HAS2* (**b**), *TNFAIP6* (**c**) and *PTX3* (**d**) mRNA expression levels (mean ± SEM, $n = 5$–6 per group) within the COC after 3 h in culture with GNTs in the presence or absence of a highly selective C–C motif chemokine receptor 2 (CCR2) antagonist (CCR2 ant), assessed by quantitative real-time PCR. *18S* rRNA served as the invariant control for normalization. Different letters represent significant differences between groups (ANOVA; $P < 0.05$). AREG: amphiregulin, HAS2: hyaluronan synthase 2, TNFAIP6: tumor necrosis factor-inducible gene 6 protein, PTX3: pentraxin 3, GNT: gonadotropins, COC: cumulus oocyte complex.

TNFAIP6 and *PTX3* induced by GNTs, AREG or PGE2 within the feline COC. In particular, RS 504393 antagonist interfered with the GNT stimulation of *AREG, HAS2, TNFAIP6* and *PTX3* mRNA expression. Likewise, this antagonist significantly decreased the mRNA levels of *AREG, HAS2* and *TNFAIP6* induced by AREG and PGE2 treatments. However, RS 504393 alone was not enough to block the oocyte maturation (measured as the proportion of MII oocytes) induced by GNTs or AREG. Nonetheless, recombinant MCP1 had a significant effect on spontaneous oocyte maturation (but not gonadotrophin-induced meiosis), increasing the percentage of MII stage oocytes in comparison to the control.

Regarding oocyte maturation, CCR2 antagonist alone significantly increased the proportion of MII stage oocyte in comparison to the control group. It is important to underline that the concentration of the antagonist RS 504393 used to assess oocyte maturation was the same used to study mRNA expression levels, where this compound had no effect by itself but caused a significant reduction of the periovulatory genes induced by GNT, AREG or PGE2. This concentration was carefully chosen based on the ability to block *MCP1* gene stimulation in feline COCs *in vitro,* as previously published (Rojo *et al*., 2019). The RS 504393 compound is a low molecular weight ligand that was shown to block all post receptor events (Mirzadegan *et al*., 2000). It was shown that spiropiperidine compounds (like RS 504393), in addition to inhibiting MCP1 binding and MCP1-driven chemotaxis, are potent inhibitors of MCP1-driven cAMP-mediated gene transcription as well as MCP1-driven calcium influx (Mirzadegan *et al*., 2000). Moreover, these compounds do not act as agonists of MCP1-mediated events (calcium influx, adenylate cyclase inhibition or chemotaxis) even at concentrations 500 times that of their apparent affinities (Mirzadegan *et al*., 2000). For example, RS 504393 was not able to stimulate calcium influx at up to 50 μM (50 times more concentrated than used in the present study). CCR2 blockage by the antagonist within the COC could be affecting other pathways that may be involved in oocyte maturation. Downstream CCR2/MCP1 signaling includes Ca^{2+}, protein kinase C (PKC), mitogen-activated protein kinase (MAPK), and Rho GTPase (Sozzani *et al*., 1991; Yen *et al*., 1997; Ashida *et al*., 2001; Jones *et al*., 2003). Indeed, PGE2 was able to reduce the stimulatory effect of the CCR2 antagonist.

MCP1 has been shown to reduce the accumulation of cAMP following stimulation of adenylate cyclase with forskolin (O'Boyle *et al*.,

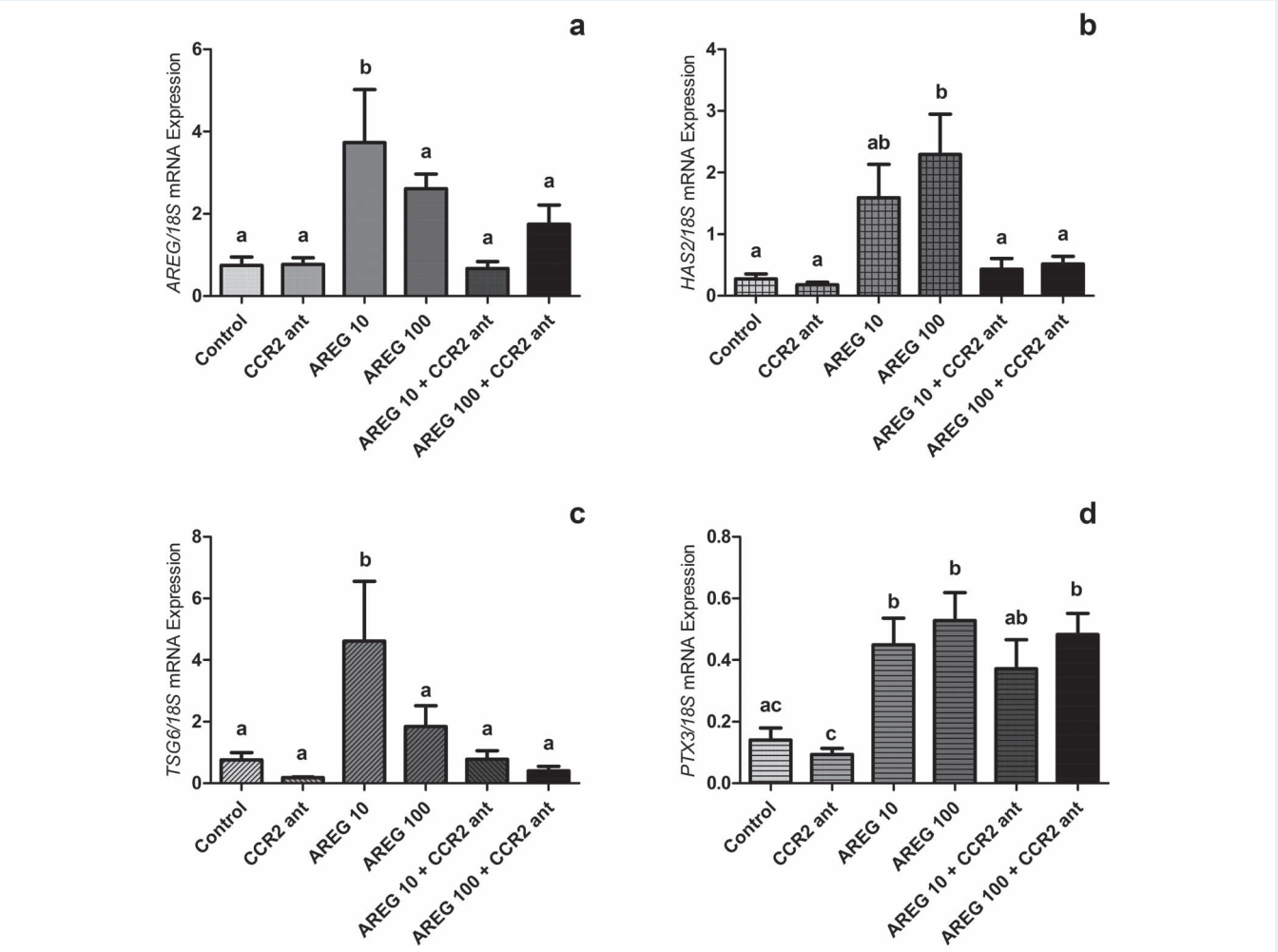

Figure 2 Highly selective CCR2 antagonist significantly blocked the AREG stimulation of mRNA expression levels of key periovulatory genes. Normalized *AREG* (**a**), *HAS2* (**b**), *TNFAIP6* (**c**) and *PTX3* (**d**) mRNA expression levels (mean ± SEM, $n = 5$–6 per group) within the cumulus oocyte complexes COC after 3 h in culture with AREG in the presence or absence of a highly selective CCR2 antagonist (CCR2 ant) assessed by quantitative real-time PCR. *18S* rRNA served as the invariant control for normalization. Different letters represent significant differences between groups (ANOVA; $P < 0.05$).

2007). Thus, the CCR2 antagonist may allow cAMP to accumulate within the oocyte and/or cumulus cells to levels that are stimulatory to meiosis. cAMP plays a critical role in the control of oocyte maturation, as a high level of cAMP maintains oocyte arrest at the first meiotic prophase. However, *Chen et al.* suggested that a transient increase in oocyte cAMP can induce meiotic resumption by providing AMP, which activates AMP-activated protein kinase, inducing oocytes to overcome meiotic inhibition and re-enter meiosis. (Chen *et al.*, 2009). It has also been suggested that meiotic resumption requires high cAMP levels in the granulosa cells and low or decreasing levels in the oocyte and that such opposing levels of cAMP may result from the selective expression and regulation of these phosphodiesterases in the two compartments of the COC (Tsafriri *et al.*, 1996). Also, since we previously reported that CCR2 and MCP1 immunoreactivity was observed in both the oocyte and cumulus cells of the feline COC (Rojo *et al.*, 2019), differential effects on both cell types cannot be discarded. Thus, further studies are needed to elucidate the involvement of CCR2 antagonist in cAMP stimulation or inhibition within the COC together with oocyte maturation.

Surprisingly, RS 504393 did not provoke a significant effect on the proportion of MII oocytes stimulated by GNT or AREG. In agreement with these results, CCR2 antagonist outcomes using the optimal culture condition for studying C-OE (media containing charcoal-stripped FBS without oil) showed no significant effect on the proportion of MII oocytes stimulated by the GNTs and AREG (data not shown). One explanation for these intriguing results could be that the CCR2/MCP1 pathway is important for C-OE (thus affecting periovulatory genes), but redundant (dispensable) for oocyte maturation. Nevertheless, in the present study, we also demonstrated that recombinant MCP1 had a significant effect on spontaneous oocyte maturation, increasing the percentage of MII stage oocytes in comparison to the control (media alone). MCP1, besides stimulating chemotaxis, also inhibits adenylate cyclase and stimulates calcium influx. Both processes could be responsible for the resumption of meiosis. It is well established that calcium is

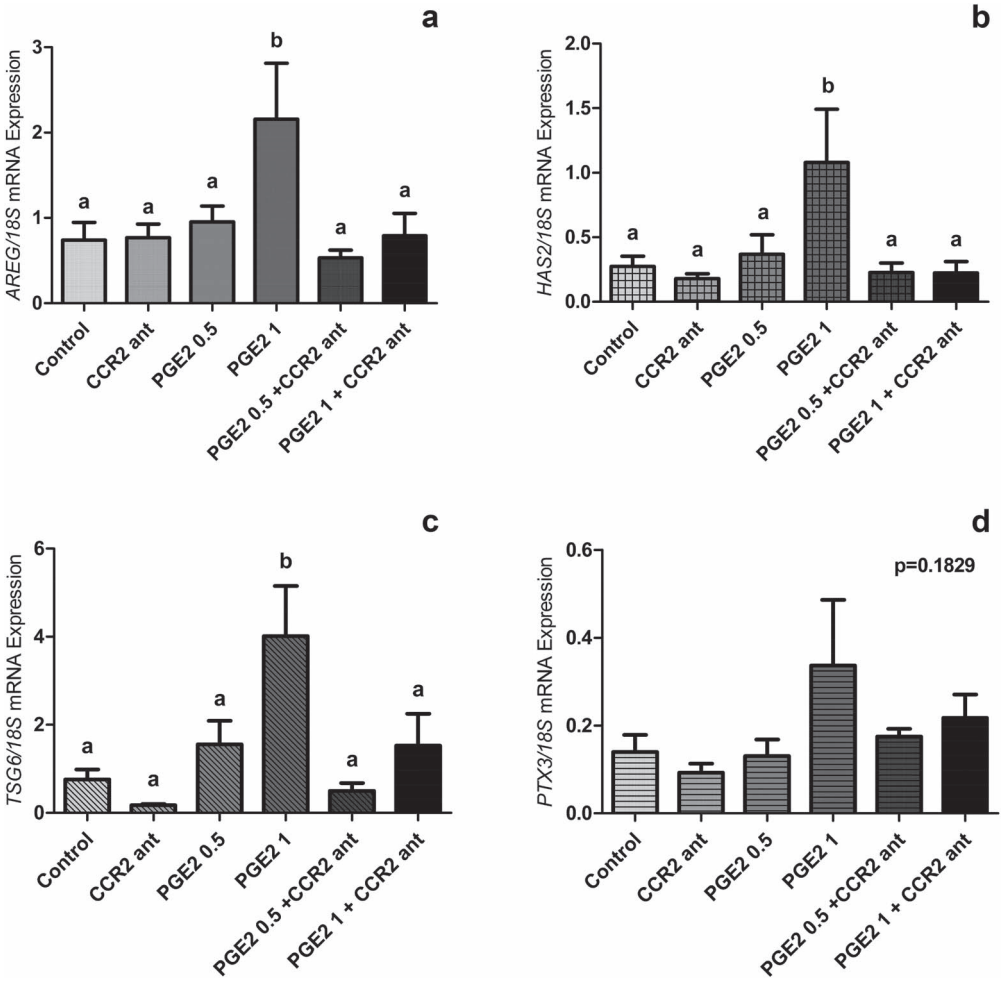

Figure 3 Highly selective CCR2 antagonist significantly blocked the prostaglandin E2 stimulation of mRNA expression levels of key periovulatory genes. Normalized *AREG* (**a**), *HAS2* (**b**), *TNFAIP6* (**c**) and *PTX3* (**d**) mRNA expression levels (mean ± SEM, $n = 5–6$ per group) within the COC after 3 h in culture with prostaglandin E2 (PGE2) in the presence or absence of a highly selective CCR2 antagonist assessed by quantitative real-time PCR. *18S* rRNA served as the invariant control for normalization. Different letters represent significant differences between groups (ANOVA; $P < 0.05$).

involved in the control of oocyte maturation (Mattioli *et al*., 1998; Mattioli and Barboni, 2000; Tosti, 2006). Intracellular Ca^{2+} modifications have been recorded in the oocyte after exposure to LH (Zuelke and Keith, 1991) and the addition of a putative inhibitor of phosphoinositide turnover, neomycin, prevents GV breakdown in the bovine and porcine oocyte (Homa, 1995). On the other hand, spontaneous meiosis resumption does not occur in the absence of intracellular calcium elevation *in vitro* (Carroll *et al*., 1994). cAMP within the oocyte has been shown to play a critical role in maintaining oocyte meiotic arrest, where a decrease in its levels is associated with the resumption of meiosis (Schultz *et al*., 1983; Dekel *et al*., 1984). On the other hand, MCP1 was not able to significantly affect the percentage of gonadotrophin-induced MII stage oocytes, suggesting that the CCR2/MCP1 pathway may be downstream of GNTs within the ovulatory cascade.

An increasing number of studies support a direct role for chemokines in regulating events necessary for C-OE and oocyte maturation. Essentially, rodent studies suggest that chemokine signaling regulates the assembly of the cumulus extracellular matrix and thus fertilization (Tamba *et al*., 2008). Also, it was reported that PGE2 actions, critical for C-OE and fertility, executed through the PGE2 receptor subtype 2 (PTGER2) were mediated by certain chemokines in rodents (Yodoi *et al*., 2009). Additionally, we have previously shown a direct effect of different chemokines within the feline COC. For instance, SDF1 significantly increased the expression of key ovulatory genes *HAS2* and *TNFAIP6*, through its main receptor CXCR4, within the COC (Rojo *et al*., 2018). In addition, we have demonstrated the expression and a novel direct effect of the MCP1/CCR2 system within the feline COC, augmenting the mRNA levels of several key ovulatory genes (*HAS2, AREG, TNFAIP6, PTX3* and *GDF9*) *in vitro* (Rojo *et al*., 2019).

The localization of CCR2 in non-immune cells within the feline ovary (Linari *et al*., 2014, 2016), together with the fact that the highly selective CCR2 antagonist interfered with the expression of *AREG, HAS2, TNFAIP6* and *PTX3* induced by GNTs within the COC, supports a role of CCR2 receptor as a novel mediator of the ovulatory cascade.

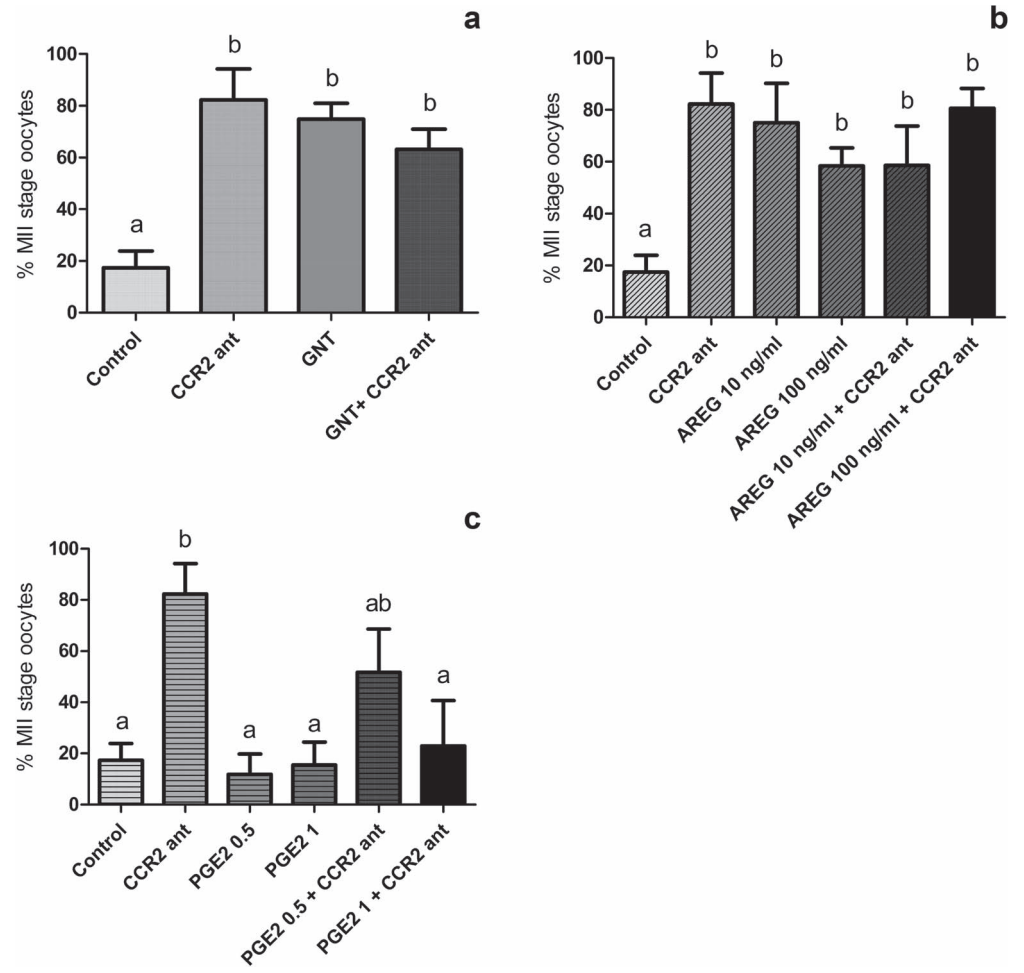

Figure 4 Highly selective CCR2 antagonist was not able to reduce the proportion of metaphase II oocytes stimulated by GNT and AREG. Percentage (mean ± SEM, $n = 4–6$ experiments) of metaphase II (MII) stage oocytes after 28 h in culture with GNTs (**a**), AREG (**b**) and PGE2 (**c**) in the presence or absence of a highly selective CCR2 antagonist ($n = 21–31$ COC per group). Different letters represent significant differences between groups (ANOVA; $P < 0.05$).

In this regard, we had previously observed that LH stimuli significantly increased the *CCR2* mRNA levels within the COC from antral feline follicles *in vitro* (Rojo *et al*., 2019). In women, physiologic levels of MCP1 are transiently elevated in the periovulatory follicular fluid and ovarian stromal cells during the ovulatory process (Dahm-Kahler *et al*., 2009) to produce a normal ovulatory event. Likewise, MCP1 was also shown to be upregulated in the rat preovulatory follicle (Wong *et al*., 2002). Besides being part of a larger and complex ovulatory process (e.g. macrophage infiltration), this increase in MCP1 during the periovulatory interval might be triggering a direct mechanism within the preovulatory follicle, such as in C-OE and/or oocyte maturation. In this study, we showed for the first time that inhibition of its main receptor CCR2 within the COC prevents or interferes with the GNT stimulation of several key ovulatory genes.

Stimulation of the MCP1/CCR2 system by LH directly or via ovarian steroids may be a reinforcement of the ovulatory cascade. The early events of ovulation involve significant changes in ovarian steroid synthesis, as well. Within a few hours after the LH/hCG stimulus, estradiol synthesis declines sharply, whereas progesterone (P4) production increases dramatically (Espey and Lipner, 1994). According to some studies, these steroids can either induce or inhibit chemokine expression, probably depending on the cell type and its environment. For example, in human endometrial endothelial cell cultures from patients with endometriosis, it was observed that MCP1 expression was induced by P4 and estradiol (E2) (Luk *et al*., 2010). Upregulation of MCP1 by E2, P4 and hCG in human decidual stromal cells in early gestation was also reported (He *et al*., 2007). In contrast, another report showed that E2 and P4 inhibit MCP1 expression in human endometrial cells *in vitro* (Arici *et al*., 1999). In macrophages, on the other hand, E2 caused inhibition of the MCP1 expression induced by hydrogen peroxide, while P4 enhanced its expression (Huang *et al*., 2008). Moreover, interactions between MCP1/CCR2 and steroids have been reported in other systems. For example, it was reported that estrogen promotes progression of hormone-dependent breast cancer through the MCP1-CCR2 axis by upregulation of the transcription factor Twist, via phosphoinositide 3-kinases/AKT/nuclear factor-κB

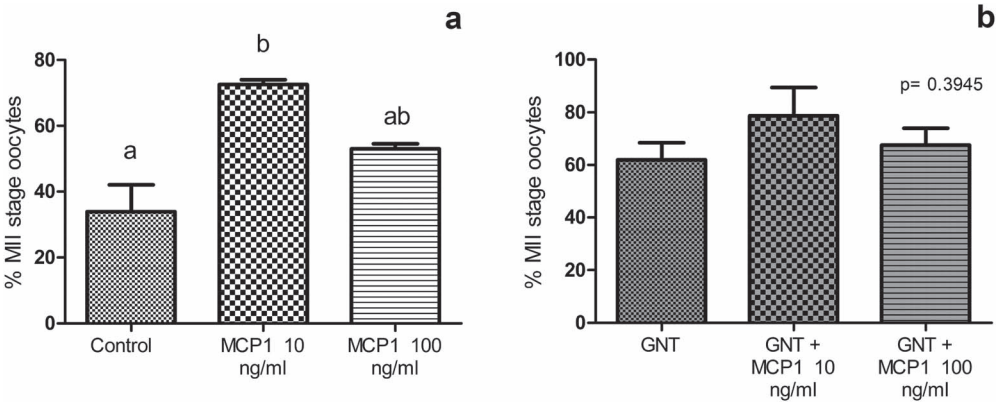

Figure 5 Recombinant monocyte chemoattractant protein 1 had a significant effect on spontaneous oocyte maturation but not on GNT-induced meiosis. Percentage (mean ± SEM, $n = 3$–4 experiments) of MII stage oocytes after 28 h in culture without GNTs (control, **a**) or with GNT (**b**) in the presence or absence of recombinant monocyte chemoattractant protein 1 (MCP1) (10 or 100 ng/ml; $n = 31$–42 COC per group). Different letters represent significant differences between groups (ANOVA; $P < 0.05$).

signaling (Han *et al*., 2018). In addition, cholesterol has also been shown to play an important role in regulating chemokine receptor function and dimerization (Legler *et al*., 2017; Gahbauer *et al*., 2018), since steroids influence the spatial organization of G protein-coupled receptors (GPCRs) within the membrane bilayer, consequently tuning chemokine receptor signaling.

Park *et al*. demonstrated that LH stimulation induced the transient and sequential expression of the epidermal growth factor (EGF) family members AREG, epiregulin and betacellulin and that these EGF-related growth factors were paracrine mediators that propagate the LH signal throughout the follicle to promote periovulatory events in both oocytes and COC (Park *et al*., 2004). Indeed, EGF-related factors mediate gonadotrophin action through the induction of steroid and prostaglandin production (Jamnongjit and Hammes, 2005; Shimada *et al*., 2006). Interestingly, when we inhibited the CCR2 receptor in the COC, we significantly repressed the stimulation of *AREG, HAS2* and *TNFAIP6* mRNA levels triggered by AREG and PGE2. Consequently, these data suggest that the MCP1/CCR2 system may be downstream the EGF-related factors and PGE2 in the ovulatory cascade. Supporting this idea, studies on MCP1 stimulation by PGE2 (Nakayama *et al*., 2006; Sun *et al*., 2007) or EGF-like ligands (Zhu *et al*., 2007; Liu *et al*., 2008) have been reported in other tissues. Furthermore, both signaling pathways are able to activate the PKC and MAPKs (Samoto *et al*., 2003; Rabinovitz *et al*., 2004; Ghayor *et al*., 2005; Frijns *et al*., 2010), which will then induce the activation and translocation of nuclear factor (NF)κb to the nucleus. Moreover, it has been shown that induced expression of MCP1 is strongly dependent on activation of the transcription factor NFκb (Ueda *et al*., 1994, 1997). As critical mediators of these processes, a positive feedback loop between them is very likely and therefore they would create an amplified effect. In agreement with this idea, as mentioned above, we have previously demonstrated a direct effect of the recombinant MCP1 in the CCR2/MCP1 system within the feline COC, characterized by increased mRNA levels of periovulatory genes, including *AREG* (Rojo *et al*., 2019). Supplementary Fig. 1 illustrates a scheme proposing possible signaling and cross talk within the cumulus cells during the ovulatory cascade.

In addition to the role as receptors for their own specific ligands, receptor tyrosine kinases (RTKs; such as EGFR) can also serve as signal transducers for a variety of extracellular stimuli. For instance, EGFR was identified as an essential signaling effector of GPCRs in rat fibroblasts (Daub *et al*., 1996), and the cross talk between GPCRs and EGFR can be generalized to several GPCRs and diverse cell types (Eguchi *et al*., 1998). Moreover, it was shown that EGFR transactivation is obligatory for protein synthesis stimulation by GPCRs (Voisin *et al*., 2002). Also, both types of receptors—GPCRs (such as CCR2) and RTKs—share common signaling intermediates in the pathway leading to activation of the extracellular signal-regulated kinase (subfamily of MAPK (Luttrell *et al*., 1999). It was reported that GPCR signaling can activate the phosphatidylinositol-3-kinase (PI3K) γ isoform directly (Vecchione *et al*., 2005) or indirectly through RTKs (Voisin *et al*., 2002; Baudhuin *et al*., 2004). For example, Fougerat *et al*. demonstrated a direct link between MCP1/CCR2 activation and the PI3K/PKB pathway in smooth muscle cell migration (Fougerat *et al*., 2012), providing evidence that the γ isoform of PI3K activated is specifically involved in this MCP1/CCR2 signaling pathway. Although an increasing number of studies show the cross communication between GPCRs and EGFR, the biological significance of this cross talk within the ovarian follicles remains unknown and further studies are warranted.

The highly selective CCR2 antagonist was able to significantly prevent or interfere with the GNT stimulation of *PTX3* mRNA expression within the feline COC; however, it was not able to inhibit the stimulation of *PTX3* mRNA levels induced by AREG. Therefore, this may indicate that *PTX3* stimulation by AREG is independent of the CCR2/MCP1 pathway. PTX3 plays a key role in organization of the cumulus extracellular matrix and in *in vivo* fertilization (Salustri *et al*., 2004). Notably, targeted disruption of *PTX3* gene results in viable mice with impaired C-OE and infertility (Varani *et al*., 2002). The matrix is synthesized by cumulus cells a few hours before ovulation and the combined action of gonadotropins and soluble oocyte factors is required for triggering this process in mice (Buccione *et al*., 1990; Salustri *et al*., 1990). One possible explanation why RS 504393 was able to significantly prevent or interfere with the AREG stimulation of

AREG, HAS2 and *TNFAIP6* mRNA levels but not *PTX3*, might be the fact that CCR2/MCP1 acts by modulating *PTX3* indirectly through the induction/stimulation of AREG. PTX3 was shown to be expressed by cumulus cells before ovulation, playing a role in cumulus matrix stability (Salustri *et al*., 2004), which may be a later event in the matrix formation, while HAS2 and TNFAIP6 play an earlier role.

It is worthy to emphasize that chemokines, like MCP1, are commonly elevated in disorders with a state of chronic low-grade inflammation; including polycystic ovary syndrome (PCOS), obesity, metabolic syndrome and type 2 diabetes. PCOS is the most common endocrinopathy of women; however, its etiology remains unknown. This disorder is characterized by reproductive abnormalities including hyperandrogenemia, menstrual disorders, polycystic ovary morphology and infertility (King, 2006; Barthelmess and Naz, 2014). Interestingly, it was reported that genetic variation in the *MCP1*-gene promoter is associated with PCOS risk by affecting transcriptional activity, leading to an increased expression level of *MCP1* (Li *et al*., 2015). Thus, in PCOS patients a likely high and endless activation of the chemokine receptor CCR2 present in the different ovarian cell types may alter its physiological role and it could explain the alteration of follicle function. Possible roles for this chemokine in the ovary may extend beyond its capacity to serve as a chemoattractant since mRNA high levels of expression and immunostaining of CCR2 (the main receptor of MCP1) were observed in several non-immune cell types in the feline ovaries during the natural estrous cycle (Linari *et al*., 2014, 2016; Rojo *et al*., 2019). In fact, taking into account our previous study showing a direct effect of the MCP1/CCR2 system within the COC, together with our current data, additional studies are warranted to further assess the physiological processes triggered by the CCR2/MCP1 system in the ovary and potentially overcome the disruption of ovarian function that occurs in several metabolic disorders, such as PCOS.

In summary, our results demonstrate that the GNT, AREG and PGE2 stimulation of periovulatory gene mRNA levels occurs, at least in part, through the CCR2/MCP1 pathway, therefore proposing the CCR2 receptor as a novel mediator of the ovulatory cascade. Furthermore, this is the first study in any species demonstrating a stimulatory effect of MCP1 on meiosis. A better understanding of this novel role of CCR2 within the COC could eventually aid in the diagnosis or treatment of infertility related to metabolic disorders. Alternatively, this might help in the identification of novel targets for a non-hormonal form of contraception.

Supplementary Data

Supplementary data are available at *Molecular Human Reproduction* online.

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Authors' roles

J.P.J. contributed with design and performance of the research, analyzed data and contributed with the writing of the manuscript; M.U. and E.D. contributed with design and performance of the research; G.J. contributed with design and performance of the research together with analyses of data; M.C.P. designed and performed research, analyzed data and wrote/corrected the manuscript. All the authors read and reviewed the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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