Stromal-derived factor 1 directly promotes genes expressed within the ovulatory cascade in feline cumulus oocyte complexes

Julieta L. Rojo¹ · Martina Linari¹ · Kelly A. Young² · Marina C. Peluffo¹

Received: 5 January 2018 / Accepted: 26 February 2018 / Published online: 8 March 2018
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Abstract

Purpose We hypothesized that the chemokine SDF1/CXCR4 system was present in feline cumulus-oocyte complexes (COCs) and that COCs cultured with SDF1 would directly upregulate gene expression in the ovulatory cascade.

Methods Ovaries ($n = 50$) were obtained from adult domestic cats during the breeding season and COCs were recovered from antral follicles. Because IVM media triggers cumulus-oocyte expansion, culture conditions needed to be optimized to study periovulatory genes. After optimization, the effects of 25 ng/ml SDF1 and the CXCR4 inhibitor were examined in a COC culture for 3, 12, and 24 h.

Results MEM-hepes with 1% of charcoal stripped-FBS was the optimized culture medium, assessed by the expansion of COCs at 24 h in the gonadotropin (GNT) group but not in the media with serum alone. The mRNA expression of *HAS2*, *TNFAIP6*, *PTX3*, and *AREG* peaked at 3 h in GNT group as compared to all other groups ($p < 0.05$). COCs cultured with SDF1 showed increased *HAS2* and *TNFAIP6* mRNA expression at 3 h compared to negative controls and to the CXCR4 inhibitor group. CXCR4 and SDF1 immunostaining was present in both cumulus cells and the oocyte.

Conclusions These results demonstrate that GNT stimulation upregulates key periovulatory genes and expansion in feline COCs from antral follicles, and support the use of this culture system to examine molecular processes within the COC. In addition, SDF1 directly promotes key periovulatory genes in feline COCs, suggesting that the SDF1-CXCR4 pathway may extend its function beyond a chemoattractant, and may play a direct role within the COC.

Keywords Chemokines · SDF1 · Ovulatory cascade · CXCR4 · Feline · Cumulus oocyte complex

Introduction

The domestic cat (*Felis catus*) is a species commonly used for the study of oocyte cryopreservation based on its potential to serve as a model species for biomedical research and the conservation of endangered felids [1]. Because highly conserved

reproductive mechanisms between humans and feline species exist, cats serve as good models for addressing infertility syndromes in women, such as asynchronous oocyte cytoplasmic and nuclear maturation, ovarian hypersensitivity, and luteal dysfunction after gonadotropin therapy [1]. Cat oocytes share several characteristics with human oocytes: (1) the diameter of the oocyte proper and the germinal vesicle is equivalent (110 and 45 μm , respectively) in both species; (2) oocytes reach the metaphase II (MII) stage of meiosis after 24 h in culture; and (3) both species have a similar nuclear configuration with a small nucleolus and a fibrillar chromatin [2, 3]. In contrast, these morphological features are distinct or lacking in the typical laboratory mouse model. Moreover, the cat offers a unique and valuable model to study molecular processes within the preovulatory follicle and cumulus-oocyte complexes (COC), as each animal provides between 3 and 7 naturally selected preovulatory follicles, in an “ovulation-ready” state primed during estrus for the copulation-triggered LH stimulus. This stage of the cycle,

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10815-018-1150-4>) contains supplementary material, which is available to authorized users.

✉ Marina C. Peluffo
mpeluffo@cedie.org.ar

¹ Centro de Investigaciones Endocrinológicas “Dr. César Bergadá” (CEDIE), CONICET, FEI, División de Endocrinología Hospital de Niños Ricardo Gutiérrez, Gallo 1330, CABA, C1425EFD Buenos Aires, Argentina

² Department of Biological Sciences, California State University Long Beach, Long Beach, CA 90840, USA

lasting around 7 days, gives a wide window for collecting samples.

Ovulation is a complex, inflammation-like process whereby a fully-developed follicle ruptures in response to the actions of the mid-cycle gonadotropin surge, releasing the COC for passage into the reproductive tract and possible fertilization. Shortly before ovulation, the LH surge induces processes critical for fertility, including cumulus-oocyte expansion (C-OE), resumption of meiosis, and rupture of the follicle wall. C-OE is due to a loss in cell-to-cell contacts and formation of a hyaluronic acid (HA)-rich extracellular matrix, resulting in a large increase in area or expansion of the cumulus granulosa cell layer that surrounds the oocyte [4–6]. The HA-containing matrix is stabilized by several plasma- and follicle-derived components [7] that include inter- α trypsin inhibitor (I α I) and associated heavy chains (HC1–3), proteoglycans such as versican, the tumor necrosis factor alpha-induced protein 6 (TNFAIP6), pentraxin 3 (PTX3), as well as certain members of the a disintegrin and metalloproteinase with thrombospondin-like repeats (ADAMTS) family of proteases [6–10]. Following the gonadotropin surge, hyaluronan synthase 2 (HAS2), a key enzyme required for the synthesis of HA that forms the polymeric backbone of the “expanded” matrix, is induced in cumulus cells [7]. Current data from non-primate species indicate that this process involves a complex interaction of oocyte-, granulosa/cumulus-, and serum-derived factors [7]. While some of the paracrine-acting factors important for C-OE in rodents have been identified, including bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9) homo- and heterodimers [11], epidermal growth factor (EGF)-like ligands (e.g., amphiregulin, AREG; epiregulin, EREG) as well as prostaglandin (PG)E2 [7, 12], the molecular mechanisms responsible for initiating such complex processes are not fully understood. Thus, understanding the molecular and cellular processes involved in C-OE as well as oocyte maturation would aid in the diagnosis or treatment of infertility and may also identify novel targets for a non-hormonal form of contraception.

Chemokines, also known as chemotactic cytokines, are small heparin-binding proteins classified into four families based on the number and location of N-terminal cysteine residues (CC, CXC, C, and CX3C). Many of these chemokines are expressed in ovarian tissue, and chemokine receptors have been identified in ovaries of many species. Chemokine signaling regulates the assembly of the cumulus extracellular matrix and the interaction between PG and chemokine signaling is required for successful fertilization [13, 14]. Skinner et al. showed that mRNA levels of different chemokines (*MCP1*, *MCP2*, *LD78 β* , and *CCL-5*) increased in granulosa and/or theca cells during bovine antral follicle development [15]. Interestingly, chemokine receptors were also expressed in the granulosa (*CCR1*, *CXCR3*, *CCR5*, and *CXCR6*) and theca

(*CCR1*) cells, suggesting an autocrine/paracrine role of chemokines in these two cell types.

Stromal-derived factor-1 (SDF1, aka CXCL12) is a chemokine that is expressed in ovaries and serves as the ligand to the CXCR4 receptor [16]. 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 [17]. While these data suggest that SDF1/CXCR4 signaling may be important in oocyte maturation or CO-E, this has not been examined. Thus, we hypothesized that the SDF1/CXCR4 system would be present in feline COCs and that culture of feline COCs with SDF1 would directly upregulate genes expressed in the ovulatory cascade. Studies were designed to (a) characterize and set up a feline COC culture to study periovulatory events in vitro; (b) evaluate the immunolocalization of CXCR4 and SDF1 with the feline COC; and (c) evaluate the in vitro expression within the COC of key genes expressed in the ovulatory cascade (*HAS2*, *AREG*, *TNFAIP6*, *PTX3*, and *GDF9*) in the presence of SDF1.

Materials and methods

Animals

Ovaries ($n = 50$) at different stages of the natural estrous cycle during the breeding season from adult female *Felis catus* were used. The ovaries were donated from 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 isolation and culture set-up

Based on preliminary results, antral follicles that measured 0.5–2.0 mm were used in the present study. Follicle isolation from the ovary was performed under a dissecting microscope using 30-gauge needles as previously described [18]. Isolated antral follicles were dissected and COCs were prepared for culture. Because media used in feline culture systems examining oocyte maturation can trigger CO-E, a novel culture system was developed where CO-E would only occur with the addition of gonadotropins. The culture environment was optimized by empirically testing different media types (MEM and MEM-Hepes), serum (fetal bovine serum, FBS, and feline serum) at different concentrations (0.5, 1, 2, and 5%), and multiple pre-treatment conditions (heat, oil, and charcoal stripped) in a 4-well plate (500 μ l media, 3–5 COCs/well) for 0, 3, 12, and 24 h. Serum was included in the media for COC culture because serum factors are required for C-OE to occur [7].

After selecting the best culture media (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% FBS-charcoal/stripped), COCs ($n = 24$) were cultured for 3, 12, and 24 h in the absence (Control) or presence of recombinant human LH + FSH (GNT, 5 and 10 UI/ml, respectively; Merck Serono). Images of the individual COCs at 0, 3, 12, and 24 post-treatment were taken using a digital camera attached to a brightfield microscope to assess the CO-E by changing in COC area. The expression of key genes within the ovulatory cascade (*HAS2*, *TNFAIP6*, *AREG*, *PTX3*, and *GDF9*) were also analyzed at each time point.

Following culture optimization, a new set of experiments was performed to analyze the direct effect of the chemokine SDF1. Two different concentrations of SDF1 were originally tested (5 and 25 ng/ml), based on the manufacturer's datasheet. Because the 25 ng/ml concentration was empirically determined to yield more consistent results, the following experiments were conducted using this concentration. COCs ($n = 48$) were then cultured for different time points (3, 12, and 24 h) in the presence or absence of recombinant human/rhesus macaque/feline CXCL12/SDF1 alpha (25 ng/ml, R&D Systems), with or without a highly selective CXCR4 inhibitor (20 nM, AMD 3100 octahydrochloride, TOCRIS). At the end of culture, COCs were photographed and stored individually at -80°C for subsequent RNA extraction and RT-real time-PCR.

Analyses of gene expression (*HAS2*, *TNFAIP6*, *AREG*, *PTX3*, and *GDF9*)

The RNA extraction for each individual COC was performed using the Absolutely RNA Nanoprep Kit (Agilent) following the manufacturer's instructions. Briefly, each COC was individually homogenized with the Lysis Buffer– β -ME mixture, then treated with 70% ethanol and a fast microcentrifugation. At that time, DNase treatment was carried out followed by washing with buffers containing different salt concentrations together with filtrations steps. The final elution was performed twice, using 10 μl of Elution Buffer for the first elution followed by a second elution with 5 μl of Elution Buffer. The purified RNA was stored at -80°C until used. To synthesize single-stranded cDNA from total RNA, the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used following their instructions. RT was performed for 2 h at 37°C using 10 μl from the RNA extraction from each sample in a 20 μl reaction volume. After cDNA synthesis, real-time PCR for key genes within the ovulatory cascade (*HAS2*, *TNFAIP6*, *AREG*, *PTX3*, and *GDF9*) was conducted as previously described [19]. Primers and probes were designed using gene sequences obtained through the feline genome database (*Felis catus*) accessible at the National Center for Biotechnology

Information (NCBI). Sequences of all primers and probes are listed in Table 1. Relative levels of target gene expression were normalized to *ribosomal protein 18S* levels.

Immunofluorescence and confocal microscopy

To localize both SDF1 and CXCR4 protein levels within the feline COC, at the end of the culture (24 h), a small cohort of COCs from all treatments was randomly selected and fixed in 4% paraformaldehyde and 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 for further indirect immunofluorescence as previously described [20]. Briefly, COCs were incubated with primary antibody (CXCR4 Mouse anti-Feline Clone: 374606, R&D Systems; and rabbit anti-CXCL12/SDF1 alpha, Polyclonal, Novus Biologicals) for 1 h at 37°C followed by three 10 min washes in washing buffer and then a 1-h incubation of secondary antibody at 37°C (secondary antibodies conjugated with Alexa fluor dyes); F-actin was probed with Alexa 488-phalloidin; DNA was labeled with Hoechst 33342 [21]. COCs were mounted on slides with 20 μl of VECTASHIELD® Mounting Medium (Vector Laboratories). COCs were analyzed by confocal microscopy (Olympus FLUOVIEW FV1000 confocal laser scanning microscope) using 2–3 different objectives (PLSAPO 20×0.75 DRY CS UV, UPLFLN 40×1.3 OIL CS UV and PLSAPO 60×1.35 OIL CS UV). Full Z-stack datasets were collected with the $\times 60$ objective for each COC, with images taken every 0.5 μm .

Statistical analysis

Statistical calculations were performed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA). A one or two-way ANOVA analysis was used to analyze differences in gene expression, followed by Newman-Keuls for multiple comparison. Differences were considered significant at $p < 0.05$.

Results

Optimized culture with GNT enhances cumulus expansion

A feline in vitro culture to study molecular mechanisms within the COC was established. An optimization of the culture condition was needed since established culture methods focus on feline oocyte maturation and not CO-E [2, 22, 23], and in vitro maturation (IVM) media alone triggers CO-E. Thus, to achieve a culture environment where CO-E would not be triggered unless stimulated by gonadotropins, the media was

Table 1 Q-PCR MGB probe sequences and forward and reverse primers

Gen symbol	Probe (5'-3') 6FAM-Sequence-MGBNFQ*	Primer Forward (5'-3')	Primer Reverse (5'-3')
<i>HAS2</i>	CACGGCTCGATCCAAGTGCC	GAGTCTGGGCTATGCAACAA	TGTACAGCCACTCTCGGAAG
<i>AREG</i>	TCCATGAAGACTCACAGCATGGTTGA	TACTTTGGTGAACGGTGTGG	GACACAAAGGCAGCTAT
<i>TNFAIP6</i>	CCCGTGCTACTGAAGCGTCA	ACGGCTTTGTGGGAAGATAC	GCATCCACAGCAGCATACTT
<i>PTX3</i>	TGCAGGATCCCTCCCTCAGGA	CATGTCCTTGTGGGTAAACG	TTCATCAAAGCCACCACCTA
<i>GDF9</i>	CCCGCTAGAAGACTCGGGCTCC	TGTGAAATGTGTGTGCAACC	CATCCACCTCAATCCATCTG
<i>18S</i>	CAGCAGGCGCGCAAATTACCCA	CGGCTACCACATCCAAGGAA	GGGCCTCGAAAGAGTCCTGT

*For 18S: Probe (5'-3') VIC-Sequence-MGBNFQ

optimized by empirically testing different media types, multiple concentrations of FBS and Feline Serum, with or without different pretreatments (heat, oil or charcoal stripped). MEM-hepes with 1% of charcoal stripped-FBS was determined to be the optimum medium for this culture, as observed by the lack of CO-E at both 0 h (Fig. 1a, c) and 24 h (Fig. 1b) in media lacking gonadotropin, but a positive increase/expansion of the COC at 24 h only in the positive control group (gonadotropin-GNT, Fig. 1d). All COCs analyzed at 3 and 12 h in both groups (Control and GNT) showed no apparent differences between groups (data not shown).

***HAS2*, *TNFAIP6*, *AREG*, and *PTX3* mRNA expression peaks at 3 h after culture with GNT, with no change in *GDF9* mRNA**

To further assess the feline COC culture as a good method to study periovulatory events in vitro, the expression of key genes in the ovulatory cascade (*HAS2*, *TNFAIP6*, *AREG*, *PTX3*, and *GDF9*) was analyzed by real-time PCR (Fig. 2). In order to assess only actively cultured COCs, gene expression was analyzed in the 3, 12, and 24 h groups with and without GNT. The mRNA expression of *HAS2*, *TNFAIP6*, *AREG*, and *PTX3* peaked at 3 h of culture in the GNT group as compared to GNT treatment in the 12 and 24 h groups and, as compared to controls at all time points ($p < 0.05$); however, *GDF9* mRNA expression did not change, regardless of GNT treatment or time ($p > 0.05$; Fig. 2).

CXCR4 and SDF1 proteins were immunolocalized to cumulus cells and oocytes

To visualize the CXCR4/SDF1 within the feline COC, a small cohort of COCs was randomly selected and fixed at the end of the culture (24 h). All samples exhibited the same immunostaining pattern for both proteins (CXCR4 and SDF1), independently from treatment, showing immunolocalization within the cumulus as well as the oocyte. A representative picture of the immunofluorescence for CXCR4 (panel A) and SDF1 (panel B) within the feline COC is shown in Fig. 3.

***HAS2* and *TNFAIP6* mRNA expression peaks at 3 h after culture with SDF1, with no change in *AREG*, *PTX3*, or *GDF9* mRNA**

COCs cultured with 25 ng/ml SDF1 showed increased *HAS2* and *TNFAIP6* mRNA expression at 3 h as compared to negative controls and COCs treated with the CXCR4 inhibitor. In contrast, no changes were observed in *HAS2* and *TNFAIP6* at other time points, and no changes in *AREG*, *PTX3*, and *GDF9* expression were noted between SDF1-treated groups and negative controls, at any time point (Fig. 4) (Online Resources 1 and 2).

Discussion

The current study demonstrates that feline COCs from antral follicles can be reliably cultured and can respond to a gonadotropin stimulus, as evidenced by general COC expansion as well as elevated mRNA levels of key periovulatory genes (*HAS2*, *AREG*, *TNFAIP6*, and *PTX3*). These genes play key roles in rodents during oocyte maturation and CO-E [7], and *HAS2*, *AREG*, and *TNFAIP6* mRNA peak within the preovulatory follicle 12 h after an in vivo bolus of hCG in primates [24]. The mRNA expression of *HAS2*, *TNFAIP6*, *AREG*, and *PTX3* peaked at 3 h of culture in the presence of gonadotropins, suggesting that this time point is ideal for studying periovulatory genes using this feline COC culture system. The rapid response obtained in the peak expression of these genes in the COC culture is likely due to direct application of the stimulus to the COC, whereas in vivo application occurs indirectly through growth factors secreted in a paracrine manner through the follicle [25]. In rodents, LH-dependent intrafollicular expression of the EGF-like family members, such as AREG, EREG, and BTC mediate or synergize with the gonadotropin surge to promote periovulatory events including re-initiation of meiosis (germinal vesicle breakdown, GVBD) in both oocytes and C-OE [25]. Indeed, EGF-related factors mediate gonadotrophin action through the induction of steroid and prostaglandin production [26, 27]. In addition, AREG is induced in the follicular fluid of rhesus macaques

Fig. 1 The optimum culture environment was empirically determined based on C-OE at 24 h. Images of COCs were taken prior to (a, c) and at the end of culture (24 h; b, d). MEM-HEPES with 1% of charcoal stripped-FBS was the optimum medium for this culture, as observed by the increase/expansion of the COC at 24 h in the presence of GNT (d) compared to the negative control (b) at the same time point. Bar represents 300 μ m

preovulatory follicles by an ovulatory stimulus, and AREG enhances primate oocyte in vitro maturation [21]. Although

GDF9 has also been reported to be an important paracrine-acting factor important for C-OE in rodents [11], no

Fig. 2 The relative mRNA expression of a *HAS2*, b *TNFAIP6*, c *PTX3*, and d *AREG* peaked at 3 h of culture in the GNT group as compared to GNT treatment in the 12 and 24 h groups and as compared to controls at all time points ($p < 0.05$). In contrast, e *GDF9* mRNA expression did not change, regardless of GNT treatment or time ($p > 0.05$). Bars represent the real-time PCR relative-quantitation of mRNA levels normalized to *18S* (mean $\pm$ SEM; $n = 4$ /time point). Different letters above the columns indicate a significant difference ($p < 0.05$) between the time points

Fig. 3 Representative confocal microscopy images ($\times 60$) of feline COCs after immunofluorescence staining. Blue = Hoechst, Green = F-actin, Red = CXCR4 (a) or SDF1 (b). No staining was observed in the negative control (inserts). Immunostaining for the chemokine receptor CXCR4 and its principal ligand SDF1 was observed in the oocyte and the cumulus cells. Bar represents 50 μm

significant differences were observed in its mRNA expression levels in the presence of gonadotropins in our culture system. However, GDF9 action may be restricted to only preantral follicles and early stages of follicle maturation in feline follicles since GDF9 receptor (BMP RII) is mainly abundant in primordial and primary follicles [28], unlike the antral follicle used in the current study.

Limited published studies support a novel role for chemokines in regulating events necessary for C-OE and oocyte maturation. Chiefly, PGE2 actions mediated through the PGE2 receptor subtype 2 (PTGER2), which are critical for C-OE and fertility in rodents [29], are mediated through certain chemokines. Rodent studies suggest that chemokine signaling regulates the assembly of the cumulus extracellular matrix and thus fertilization [13]. SDF1 and CXCR4 are both expressed

Fig. 4 COCs cultured for 3 h with 25 ng/ml SDF1 showed increased **a** *HAS2* and **b** *TNFAIP6* mRNA expression at 3 h as compared to negative controls and COCs treated with the CXCR4 inhibitor. In contrast, no changes were observed in **c** *PTX3*, **d** *AREG*, and **e** *GDF9* expression. Bars represent the real-time PCR relative-quantitation of mRNA levels normalized to *18S* (mean $\pm$ SEM; $n = 4$ /time point). Different letters above the columns indicate a significant difference ($p < 0.05$) between the time points

in the activating mammalian ovarian follicle [30]. In the present study, immunolocalization of the receptor CXCR4 and its chemokine ligand SDF1 was observed within the oocyte as well as the cumulus cells of the feline COC from antral follicles. These observations, especially the CXCR4 receptor protein expression within the COC, suggest that chemokine action in the ovary may extend beyond its role as a chemoattractant, including a possible direct effect on promoting C-OE and/or oocyte maturation, as the LH surge that triggers ovulation increases *CXCR4* mRNA and protein in ovarian follicles in other species [17]. Interestingly, the addition of recombinant SDF1 to the feline COC culture increased *HAS2* and *TNFAIP6* mRNA expression at 3 h, as observed for GNT treatment, in comparison to negative controls. Furthermore, this increase was blocked by the addition of the specific CXCR4 inhibitor, demonstrating that the effect of the recombinant SDF1 was direct and through its main receptor, CXCR4. A remarkably similar mRNA pattern after a bolus of hCG in primates [24] was also noted for *HAS2* and *TNFAIP6*, two critical factors that are induced to produce extracellular matrix during CO-E in rodents [7]. Thus, key components of this process are temporally expressed and may play a critical and rapid role in C-OE and oocyte quality [31]. In contrast, no changes in *AREG*, *PTX3*, and *GDF9* mRNA expression were noted in the current study between SDF1 and control treated groups, at any time point. These results indicate that SDF1 may play a role in only some, but not all, of the events trigger by the ovulatory stimulus. Based on the stimulation of *HAS2* and *TNFAIP6* mRNA levels by recombinant SDF1, this chemokine may be involved in regulating the CO-E process. However, no apparent changes in COC area were observed in the presence of SDF1 after 24 h in culture. Thus, further studies are warranted to investigate the molecular features of C-OE and chemokines in felines.

The development of models, such as the one presented in the current study, can assist be used enhance our knowledge of oocyte quality. Nevertheless, as any model does, the feline COC in vitro culture system presented here has its limitations and may not completely recapitulate in vivo follicular dynamics. Future comparisons with in vivo oocytes can help to further our understanding of peri-ovulatory events within the COC. In contrast to models that emphasize the oocyte maturation state, the model presented here focuses on CO-E since it was reportedly the most reliable index of oocyte maturation in cats [32]. Further investigations are warranted to elucidate the oocyte maturation state using this feline culture system. In addition to the potential to expand knowledge of feline reproduction, the use of this model system has the advantage of providing an excellent surrogate for understanding events involving human COCs that are necessary for fertility. Despite these limitations, these types of animal culture systems, with defined in vitro characteristics, can contribute to

our understanding of parameters necessary for basic oocyte function and quality.

In summary, these data indicate, for the first time, that culture of feline COCs from antral follicles is a robust and valuable system to study periovulatory events such as CO-E as well as oocyte and cumulus cell biology in general. Using this culture system, we demonstrated the immunolocalization of the chemokine SDF1 and its receptor CXCR4 within the oocyte and the cumulus cells of the feline COC. And more notably, these results demonstrated a novel direct effect of the chemokine SDF1 within the COC, by increasing the key ovulatory genes *HAS2* and *TNFAIP6*, through its main receptor CXCR4. Together, these data suggest that chemokine action in the ovary extends beyond its role as a chemoattractant, including a possible direct effect of the SDF1-CXCR4 pathway on promoting C-OE and/or oocyte maturation. Understanding the molecular and cellular processes involved in C-OE as well as oocyte maturation would aid in the diagnosis or treatment of infertility and may also help identifying novel targets for a non-hormonal form of contraception. Finally, since ovaries are a reservoir of competent GV-stage oocytes which represent a potential source for female fertility preservation [33], using an in vitro culture model to study feline ovarian function may prove to be important for the conservation of endangered felids.

Acknowledgements We are grateful to Olga Bustamante from the “Centro de Sanidad Animal de la Municipalidad de Merlo” (Provincia de Buenos Aires) for the donation of the feline ovaries. Recombinant human FSH and LH (Merck Serono) were generously donated for this project. A special thanks to German La Iacona for his technical assistance with the confocal microscope.

Funding This study was supported PRESTAMO BID PICT 2014 N° 666 (MCP), Small Faculty Grants Program CNSM CSULB (KAY), the CSULB Professors Around the World Award (KAY), and by the Fogarty International Center, of the National Institutes of Health under Award R01TW009163 (MCP).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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