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The CC-chemokine receptor 2 is involved in the control of ovarian folliculogenesis and fertility lifespan in mice

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ABSTRACT

The chemokine receptor 2 (CCR2) was first described as a chemotactic factor involved in immune responses, but it also plays an essential function in several biological processes. The chemokine (C–C motif) ligand 2 (CCL2) binds to CCR2 triggering G protein-coupled receptor (GPCR) signaling in leukocytes, including activation of PI3K/Akt/mTOR, a key pathway that is also related to follicular activation and survival. However, the potential role of CCR2 in ovarian follicular physiology remain unexplored. Thus, we investigated the role of CCR2 on follicular growth during adult life and aging. Ovaries and oocytes were collected from wild type (WT) mice at 1.5 months old (mo), and CCR2 expression was observed predominantly in oocytes included in growing follicles, as well as after ovulation. Follicle populations were assessed in WT and CCR2-/- mice at 1.5 mo, and CCR2-/- mice had more primordial and less primary and secondary follicles, while there were no differences in antral follicle numbers. Pro-apoptotic genes *Bax* and *Casp3* were downregulated, while anti-apoptotic *Bcl2* was upregulated in CCR2-/- mice. To further characterize the role of CCR2 in ovarian aging, follicle populations were assessed in WT and CCR2-/- mice at 1.5, 2.5, 6, 10, and 12 mo. A larger ovarian follicular reserve at 1.5–6 mo was observed in CCR2-/- mice. Finally, CCR2-/- aged mice (6–12 mo) ovulated more oocytes than WT mice. Altogether, these data suggest that CCR2 plays an important role in the regulation of murine folliculogenesis, potentially affecting the reproductive lifespan.

1. Introduction

In most mammals, the ovarian follicular reserve is established during embryogenesis and gradually decreases throughout prepubertal and adult life (Monniaux et al., 2014; Goldman et al., 2017; Ye et al., 2019). Regularly, some of the primordial follicles are activated and develop into growing follicles, which are classified as primary, secondary, or antral follicles, according to their morpho-functional aspects (Campos-Junior et al., 2012a; Monniaux et al., 2014). The size of the ovarian reserve, as well as the rates of follicular mobilization and atresia, are the main determinants of the ovarian functional lifespan. A major mechanism responsible for low follicle counts associated with aging is atresia, which is triggered by apoptosis, regulated by activation of pro- or anti-apoptotic genes (Uri-Belapolsky et al., 2014). In that regard, unraveling the mechanism that regulates follicular survival is critical to prevent female infertility (Hsueh et al., 2015; Shen et al., 2019).

The molecular mechanisms that drive follicle activation are still poorly understood. Follicle activation is likely to be regulated by highly controlled pathways to ensure a sustainable balance between quiescence and growth, preventing premature exhaustion of the ovarian reserve (Uri-Belapolsky et al., 2014). Among the potential regulatory mechanisms, a critical role of the PI3K/Akt/mTOR signaling pathway has been proposed (Monniaux et al., 2014; Hsueh et al., 2015; Novella-Maestre et al., 2015; Goldman et al., 2017; Grosbois and Demeestere, 2018; Zhang et al., 2019). The incubation of ovarian tissue with PI3K activators, such as 740Y-P, leads to a substantial primordial follicle activation (Grosbois and Demeestere, 2018).

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Chemokines and chemokine receptors form a complex and diverse system relevant to the physiology of the immune system, including leukocyte activation and cell trafficking (Russo et al., 2010). The production of chemokines also occurs physiologically under tissue homeostasis but may be induced by stress associated with inflammatory stimulus such as infectious diseases and cancer (Russo, 2010). The chemokine (C–C motif) ligand 2 (CCL2) and its receptor chemokine (C–C motif) receptor 2 (CCR2) play important roles in the immune response, such as macrophage chemotaxis, tissue inflammation, tumor invasion, and metastasis (Russo et al., 2010; Castela et al., 2017; Yang et al., 2018). It is well known that CCL2 − CCR2 interaction activates the PI3K/Akt/mTOR signaling pathway in several cell types, including leukocytes (Russo et al., 2010; Yang et al., 2018). Previous studies reported the expression of CCR2 in the theca and perifollicular stroma of human preovulatory follicles (Dahm-Kähler et al., 2009) and in the cumulus-oocyte complexes and follicular wall in feline ovaries (Rojo et al., 2019). It was also over-expressed in the ovaries of obese rats (Ruebel et al., 2016), however, the role of CCR2 in the context of murine ovarian follicular physiology is not fully understood. We hypothesized that CCL2 − CCR2 may be enrolled in follicular mobilization and thus affect fertility lifespan in vivo. To explore this, we evaluated the CCR2 expression in mice ovaries, as well as the impact of CCR2 deficiency on follicular mobilization during adult life and mice aging.

2. Materials and methods

2.1. Animals and facilities

Female mice C57BL/6 (wild type - WT, n = 74) and B6.129S4-Ccr2tm1Ifc/J CCR2-/-; (Boring et al., 1997; Russo et al., 2018; n = 68) used in this study were obtained from the Universidade Federal de Minas Gerais and maintained in a positive pressure room isolated from other animals. Cages were filter-topped, and mice had free access to sterilized food and water. The daily light cycle was 12/12 h (light/dark). Animals were inspected daily and, when required, euthanasia was performed using high sedative doses (15.8 mg/kg xylazine and 139.6 mg/kg ketamine). Body and ovarian weight from all animals used in this study were measured, and gonad somatic index (GSI - ovarian mass divided by body weight × 100) also was calculated. The number of puppies per litter and litters from homozygous couples (WT♂ x WT♀ and CCR2-/-♂ x CCR2-/-♀) were recorded during a one-year interval and analyzed. Additionally, the stage of estrous cycle were evaluated by vaginal cytology (Byers et al., 2012) daily, during 2 months, starting at 1.5 mo, and its length was obtained and compared. This study was approved by the Institutional Animal Care and Use Committee of the Universidade Federal University de São João del Rei (Protocol number 27/14 CEUA/FUSJ).

2.2. Experimental design

To immunolocalize CCR2 on ovarian parenchyma, ovaries of WT (n = 3) at 1.5 months old (mo) were collected and processed for immunoperoxidase and immunofluorescence. CCR2 immunolocalization on oocytes was evaluated by immunofluorescence using WT mice (n = 3), at the same age. CCR2 protein detection on ovarian parenchyma was performed by Western Blot, using total protein lysates from WT (n = 4) and CCR2-/- (n = 4) mice.

Ovarian histology was used to address potential differences in follicle population numbers, that were compared in 1.5 mo WT and CCR2-/- mice (n = 6 and 6, respectively). In order to evaluate the follicular mobilization during aging, histological follicle quantification was performed in WT and CCR2-/- mice at the following ages: 1.5, 2.5, 6, 10, and 12 mo (n = 6 per age and group, total n = 60) mo, only ovaries derived from females on proestrus were used, as determined by vaginal cytology (Byers et al., 2012). Oocyte collection was performed in another group of WT and CCR2-/- mice, at the same ages (1.5, 2.5, 6, 10, and 12 mo; n = 6 per age and group, total n = 60). Finally, quantitative Real Time PCR, was performed in WT (n = 4) and CCR2-/- (n = 4) to evaluate the expression of genes related to follicular atresia and growth.

2.3. Immunostaining

Ovaries were collected from WT and CCR2-/- at 1.5 mo, fixed in 4% (wt/vol) paraformaldehyde (PFA; Ref. 30525 − 89-4, Sigma-Aldrich, St. Louis, MO, USA), and embedded in paraffin (Sigma-Aldrich, 327204, St. Louis, MO, USA). Ovarian sections (5 μm) were dewaxed and rehydrated, antigen retrieval was performed in citrate buffer (pH 6.0) for 5 min after boiling in a microwave oven for approximately 10 min. Nonspecific background was blocked for 5 min with Ultra-V-Block (Thermo Scientific, TA-060-UB, Waltham, MA, USA) incubation at room temperature. Tissue sections were then incubated overnight at 4 °C with primary antibody anti − CCR2 antibody [EPR19698] (Abcam, ab222496, Cambridge, UK) at 1:200. Antigens were detected by incubation with Alexa Fluor 488 donkey anti-rabbit IgGA21206 [1:500] (Abcam, Cambridge, UK); for 1 h at room temperature. After nuclear counterstaining using 4′,6-diamidino-2-phenylindole (Sigma-Aldrich; D8417 − 1MG St. Louis, MO, USA), sections were mounted with Mowiol 4–88 solution (Merck; 475904, Frankfurther, Darmstadr, Germany). To evaluate the expression of CCR2 on metaphase II (MII) oocytes, these structures were recovered from WT and CCR2-/- oviducts after gonadotropin stimulation (Campos-Junior et al., 2011). MII oocytes were denuded, fixed in 4% of PFA (Ref. 30525 − 89-4, Sigma-Aldrich, St. Louis, MO, USA), and incubated with primary and secondary antibody as previously mentioned. Negative controls were performed in the absence of primary antibody.

For immunoperoxidase, ovaries were collected from WT and CCR2-/- (1.5 mo), fixed in 4% (wt/vol) PFA (Ref. 30525 − 89-4, Sigma-Aldrich, St. Louis, MO, USA), and embedded in paraffin. Ovarian sections (5 μm) were dewaxed and rehydrated, antigen retrieval was performed in citrate buffer (pH 6.0) for 5 min after boiling in a microwave oven for approximately 10 min. Endogenous peroxidase was quenched for 30 min with 3% H2O2 (Sigma-Aldrich; 216763, St. Louis, MO, USA) in phosphate buffer solution (PBS; Ingamed, 216763, Maringá, PR, Brazil). Nonspecific binding was blocked with Ultra-V-Block. Primary antibody anti − CCR2 antibody [EPR19698] (ab222496) at 1:200 was applied, and slides were incubated at 4 °C overnight. Biotinylated anti-rabbit IgG (Abcam, Ab6721, Cambrigde, UK) antibody (1:100) was applied and incubated for 60 min at room temperature. Detection of the signal was performed by incubating in streptavidin (TS-125-HR; Thermo Scientific, Waltham, MA, USA) for 10 min, followed by reaction with peroxidase substrate diaminobenzidine (Sigma-Aldrich; D8001, St. Louis, MO, USA) and counterstaining with hematoxylin (Merck; 105174, Frankfurther, Darmstadr, Germany) at room temperature. Following dehydration, sections were mounted and analyzed. Negative controls were performed in the absence of primary antibody.

To qualitatively evaluate the presence of CCR2 on mice ovaries, western blot analysis was performed using total protein lysates from WT and CCR2-/-. For this evaluation, total protein was isolated from both ovaries of each animal. Protein extraction and western Blot were performed as described by Campos-Junior et al., 2012b. Anti − CCR2 antibody [EPR19698] (Abcam, ab222496, Cambrigde, UK) at 1:200, or Anti-beta Actin antibody (Abcam, ab8227, Cambrigde, UK) at 1:200 were used.

2.4. Histological evaluations and follicle quantification

Ovaries were collected from WT (n = 6) and CCR2-/- (n = 6) adult female mice at 1.5 mo and fixed in PFA (Sigma-Aldrich, St. Louis, MO, USA) solution. Paraffin-embedded ovaries were serially sectioned (5 μm) and stained with hematoxylin and eosin solution. In the third

ovarian section, the number of primordial, primary, secondary, antral, and atretic follicles were counted. Follicles were classified as previously described (Campos-Junior et al., 2012a; Pereira et al., 2020) and follicles (from primordial to antral) showing morphological signs of death such as pyknosis, cellular fragmentation, and disintegration, were classified as atretic. Only follicles containing an oocyte with a visible nucleus were considered to avoid double-counting. Results are shown as the number of counted follicles per ovary. Atresia rate (%) was determined as the number of atretic follicles/total number of follicles*100; and activation rate (%) as the number of growing follicles/total number of follicles*100 (Campos-Junior et al., 2012a). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; Calbiochem, San Diego, CA, USA, Cat. No. QIA33) assay was performed, following the manufacturer's instructions, in paraffin sections (WT and CCR2-/-) to in situ evaluation of follicle apoptosis. The percentage of follicles containing TUNEL positive cells was evaluated in 10 sections per mice in WT (n = 4) and CCR2-/- (n = 4).

2.5. Superovulation and oocyte collection

WT and CCR2-/- female mice at specific ages were primed with gonadotropins as previously described (Campos-Junior et al., 2011). Ovulated cumulus-oocyte complexes were isolated from the oviductal ampullae into PBS 13 h after human chorionic gonadotropin administration. Oocytes were denuded by a brief exposure to 400 IU/mL hyaluronidase (Sigma-Aldrich; H3506, St. Louis, MO, USA) and counted.

2.6. qRT-PCR

Total RNA isolation was performed from WT and CCR2-/- ovaries using RNeasy Mini kit (Qiagen GmbH,74104, Germantown, Maryland, USA), according to the manufacturer's instructions and samples were treated with DNAse I (Qiagen GmbH, 15200 − 50, Germantown, Maryland, USA) and primer sequences are depicted in Supplemental Table 1. The gene expression pattern of the endogenous control (*Gapdh*) was used as calibrators in order to calculate the differences in relative abundance of transcripts between WT and CCR2-/-. Primer efficiency was found as 2 and cycle threshold (Cts) among WT and CCR2-/- samples were similar. Values are shown as n-fold difference. All procedures were performed as previously described by our group (Campos-Junior et al., 2016; Pereira et al., 2020).

2.7. Statistical analyses

Data were tested for normality and homoscedasticity of variances. Parametric data were evaluated by ANOVA and differences between groups compared using the Student *t*-test. Non-parametric endpoints were compared using the Wilcoxon test. All analyses were performed using the GraphPad Prism (Version 5; GraphPad Software, Inc.). Results are shown as mean ± SD. A P value < 0.05 was considered as significant.

3. Results

3.1. CCR2 is expressed by oocytes included in growing follicles and ovulated oocytes

CCR2 was observed by immunofluorescence in the oocytes (Fig. 1A) from WT adult mice, and was not observed in the negative controls (Fig. 1B). The immunoperoxidase also confirmed the expression of CCR2 by WT oocytes at diverse developmental stages. CCR2 was observed on growing oocytes included in primary, secondary, antral, or atretic follicles (Fig. 1C–E). Interestingly, on dormant oocytes, included in primordial follicles, CCR2 was not observed (Fig. 1C). There was no staining for CCR2 on negative controls (Fig. 1F) as well as in CCR2-/- ovarian samples (Fig. 1G-H).

We also evaluated the expression of CCR2 in MII oocytes recovered from WT and CCR2-/- mice oviducts after in vivo superovulation protocol. Immunofluorescence confirmed the expression of CCR2 in MII oocytes (Fig. 1I). Positive staining for CCR2 was observed neither on the negative controls (Fig. 1J) nor in CCR2-/- (Fig. 1K).

3.2. CCR2 deficiency attenuates follicular activation and atresia

Body and ovarian weight, as well as GSI, were similar ($p > 0.05$) between WT and CCR2-/- mice (Sup. Fig. 1A–C). Western blot analyses confirmed the presence of CCR2 protein on WT ovaries; on the other hand, this protein was not detected on CCR2-/- ovaries. To validate these analyses, B-actin was detected in both WT and CCR2-/- (Sup. Fig. 1D, E). There was no difference ($p > 0.05$) in the number of puppies per litter between homozygous WT and CCR2-/- couples (Sup. Fig. 2 A). However, CCR2-/- delivered less ($p < 0.05$) litters in a one-year interval (Sup. Fig. 2B), and showed longer estrous cycles ($p < 0.05$; Sup. Fig. 2C), compared to WT.

Histological analyses from a middle section of WT and CCR2-/- ovaries indicated that both had a similar size (Fig. 2A,B). However, the follicle counts showed that adult (1.5 mo) CCR2-/- mice had more primordial follicles than WT mice (Fig. 2C). CCR2-/- mice also showed less primary and secondary follicles (Fig. 2D), while there was no difference in the number of antral follicles when compared to WT mice (Fig. 2D). Follicular activation and atresia rates were also quantified as previously described (Campos-Junior et al., 2012a). The results indicated lower follicular activation and atresia in CCR2-/- mice when compared to WT mice (Fig. 2E,F).

3.3. CCR2 deficiency alters follicular atresia and the expression of genes related to follicle survival

Morphologically, CCR2-/- mice showed ($p < 0.05$) a reduced number of atretic follicles compared to WT (Fig. 3A). These findings were confirmed by the TUNEL assay (Fig. 3B-C), where follicles containing TUNEL positive cells were less frequently ($p < 0.05$) observed in CCR2-/- mice at 1.5 mo (Fig. 3D). By real-time PCR, we found a pro-survival balance of genes in ovaries of CCR2-/- mice. Pro-apoptotic related genes *Bax* and *Casp3* were downregulated in ovaries of CCR2-/- mice when compared to WT (Fig. 3E), while the anti-apoptotic gene *Bcl2* was upregulated (Fig. 3E). Finally, there is no difference in the expression of *Fshr* (a follicle growth marker) on CCR2-/- mice when compared to WT mice (Fig. 3E).

3.4. CCR2 deficiency prolongs ovarian lifespan

Despite the observation that CCR2 deficiency attenuates follicular activation at 1.5 mo when compared to WT mice, we further characterized the ovarian lifespan during mice aging (1.5, 2.5, 6, 10, and 12 mo). Based on primordial follicle counts, we found a greater ovarian follicular reserve in CCR2-/- mice at 1.5, 2.5, and 6 mo, but not at 10 or 12 mo, when compared to the WT mice (Fig. 4A). Finally, more oocytes ($p < 0.05$) were harvested after super stimulation of aged CCR2-/- mice (6–12 mo), compared to the WT (Fig. 4B).

4. Discussion

The ovarian follicle reserve and the dynamics of follicular mobilization, growth, and atresia are key components of female fertility, as well as important predictors of the success of artificial reproductive technologies. In this study, we investigated the role of the CC-chemokine receptor 2 (CCR2) in ovarian physiology. The main findings were that CCR2 was expressed in oocytes included in all classes of growing follicles, as well as in MII oocytes, but not in primordial follicles. For the first time, we showed that the lack of CCR2 resulted in reduced follicle activation but also reduced follicle atresia, and thus prevented the early

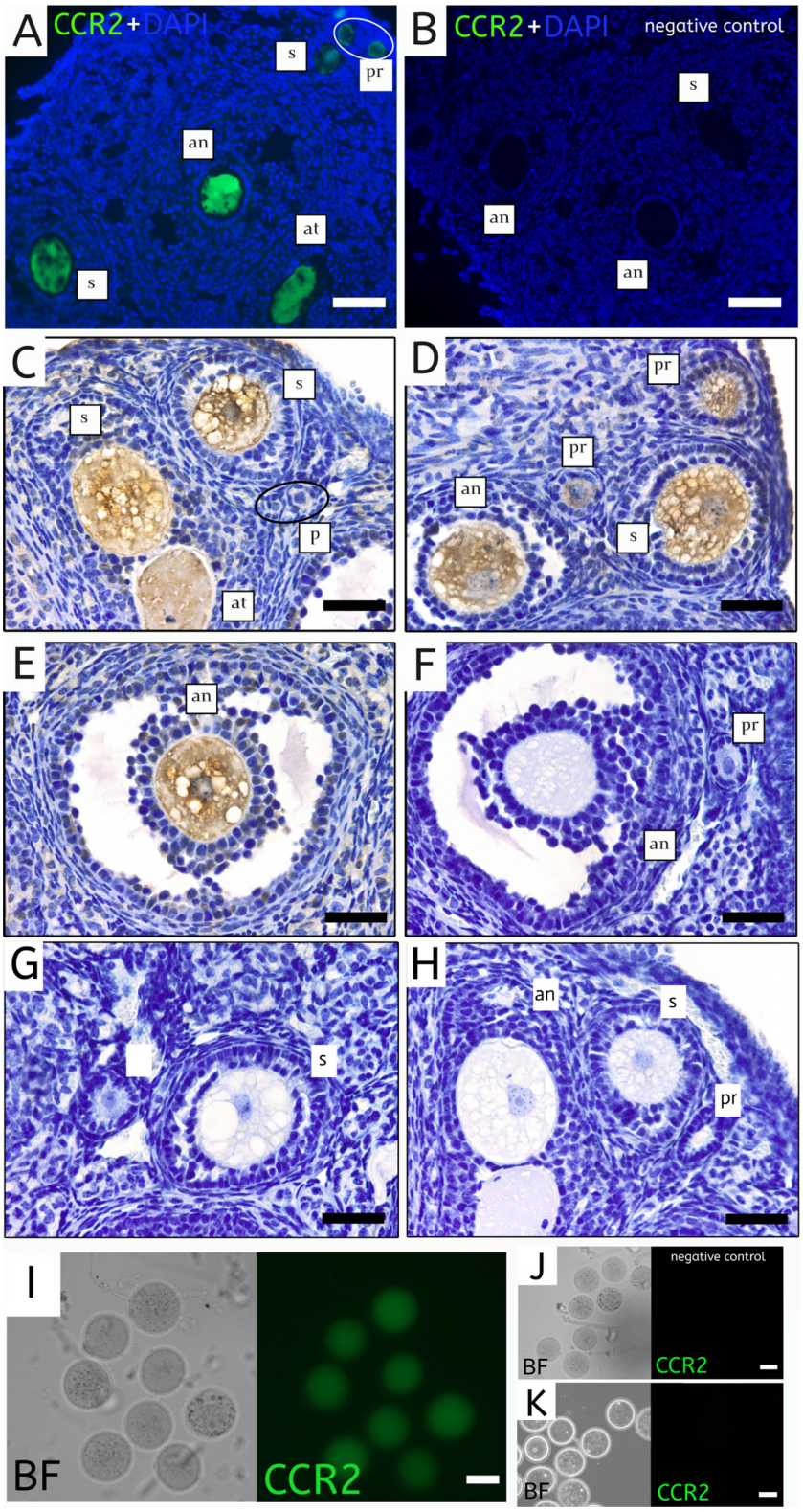

Fig. 1. Chemokine receptor 2 (CCR2) immunolocalization on wild type (WT) and CCR2-/- mice ovaries and oocytes. (**A**) CCR2 was immunolocalized in the oocyte of adult WT ovaries, while (**B**) no staining was observed in negative controls. In a more descriptive analysis using immunoperoxidase, CCR2 (**C–E**) was observed only in growing follicles: Primary (pr), Secondary (s), Antral (an), and Atretic (at); on the other hand, (**C**) CCR2 was not immunolocalized in Primordial (p) follicles. (**F**) No staining was observed on WT negative control samples, as well as on (**G**) CCR2-/- and (**H**) its negative control. (**I**) The expression of CCR2 was also observed in ovulated MII oocytes, but not in (**J**) negative control, or in (**K**) CCR2-/-. Bars A and B = 150 μm; C and D = 100 μm; E - H = 50 μm; I - K = 60 μm.

depletion of ovarian follicle reserves associated with aging. These results confirmed our hypothesis that CCR2 participates in the mechanisms that control the balance between follicle mobilization and atresia.

Previous studies have demonstrated that the PI3K/Akt/mTOR signaling pathway leads to primordial follicle activation (Novella-Maestre et al., 2015; Goldman et al., 2017; Grosbois and Demeestere, 2018). As well, the CCL2 ligand activates CCR2 triggering this pathway in several cell types (Yang et al., 2018), it was reasonable to hypothesize that CCR2 could be part of the mechanism controlling follicular activation. To confirm this, we first immunolocalized CCR2 in the ovary. The presence of CCR2 in all classes of growing follicles suggests that the expression of CCR2 could be a requirement for follicle activation and growth. Interestingly, CCR2 was found in the oocytes, rather than in the granulosa. It is well known that there is cross-talk between the oocyte

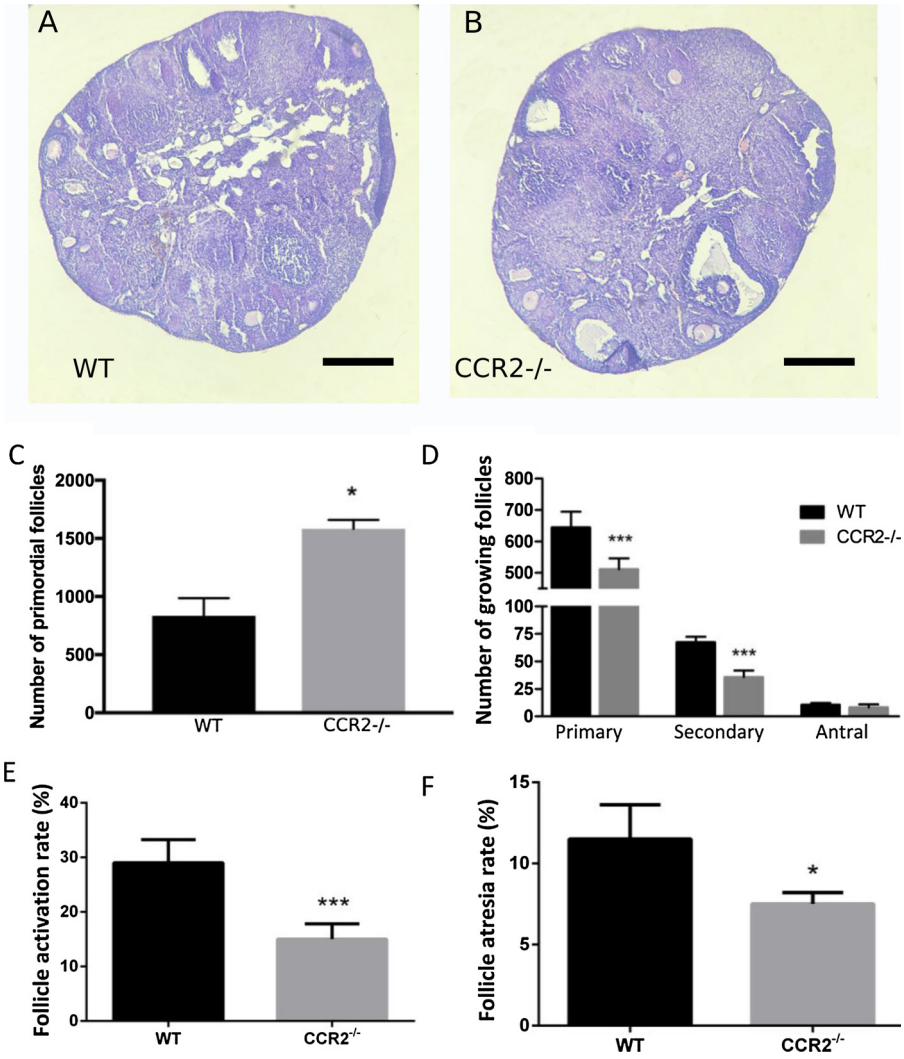

Fig. 2. The effects of CCR2 deficiency on adult mouse ovaries. A middle section of (**A**) WT and (**B**) CCR2-/- ovaries, showed CCR2 ablation does not affect ovarian size. (**C**) In CCR2-/- ovaries was observed a greater number of primordial follicles ($p < 0.05$), however numbers of (**D**) primary and secondary were lower in CCR2-/- compared to WT ($p < 0.05$), and the number of antral follicles was similar ($p > 0.05$). (**E, F**) Rates of follicle activation and atresia were lower in CCR2-/- ($p < 0.05$). Bars = 4000 μm.

and granulosa cells (Liu, 2007) and that the oocyte controls follicle development through the production of paracrine factors such as growth differentiation factor 9 (GDF9) and bone morphogenetic protein (BMP15) (Sugiura et al., 2010). Our findings are coherent with the active role of the oocyte in primordial follicle activation, supporting the idea of the oocyte as "the conductor" of the transition from primordial to primary follicles (van den Hurk and Zhao, 2005). The oocyte-specific expression of CCR2 observed in the current study differs from that observed in previous reports, in which this receptor was observed in human and feline ovarian somatic cells (Dahm-Kähler et al., 2009; Rojo et al., 2019). However, in these studies different antibodies and experimental models were used, and comparisons among these findings requires caution.

Our results, using the CCR2 knockout mice, supported the hypothesized importance of CCR2 in the control of follicle activation. The lower follicular activation in CCR2-/- mice resulted in lower primary and secondary follicle populations, and thus in a greater number of primordial follicles. In this regard, the effects of the CCR2 deficiency seem to be the opposite of those observed in the lack of the anti-Mullerian hormone (AMH), another TGF-β family member involved in the control of follicle activation. The AMH is also expressed only in growing follicles, and its deficiency in mice does not impair fertility but leads to precocious depletion of primordial follicle reserve (Durlinger et al., 2002). It is noteworthy that the lack of CCR2 did not compromise ovarian follicular reserve formation, somatic development, ovarian size, or ovulatory capacity, suggesting that its action was through very specific pathways and time windows. On the other hand, the maintenance of CCR2 expression in MII oocytes suggests that this receptor may play roles in the reproductive process other than controlling follicular development.

Interestingly, reduced follicular activation in CCR2-/- mice, and consequent reduction in the populations of primary and secondary follicles, did not affect the number of antral follicles. It was previously demonstrated that mechanisms balancing activation and atresia resulted in a similar number of antral follicles among mice strains with different primordial follicle reserves (Campos-Junior et al., 2012a). In the current study, we demonstrate that CCR2 acts in this mechanism. The lower follicular activation in CCR2-/- mice was balanced by reduced follicular atresia, triggered by an upregulation of anti-apoptotic (*Bcl2*) and a downregulation of pro-apoptotic (*Bax*, *Casp3*) genes and confirmed by a reduced percentage of follicles containing TUNEL positive cells. The result was a similar antral follicle population when compared to the WT mice, thus preserving the fertility of the CCR2-/- mice. Coherently, there was no effect of CCR2 deficiency on *Fshr*

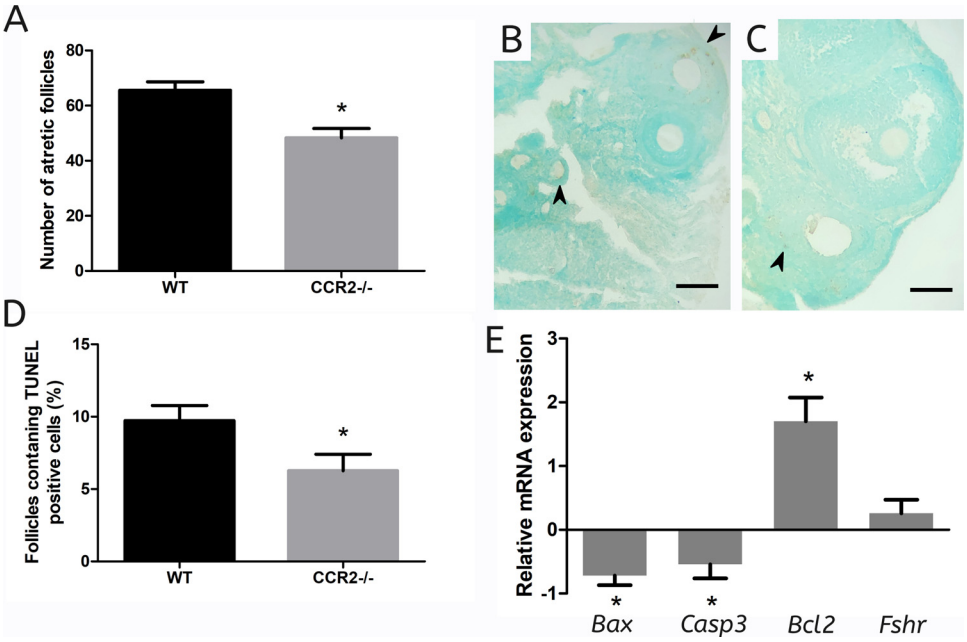

Fig. 3. The effects of CCR2 deficiency on follicular atresia and expression of genes related to follicle survival and growth in adult mice. (**A**) CCR2-/- mice showed a reduced ($p < 0.05$) number of atretic follicles compared to WT. TUNEL staining revealed that (**B**) WT presented more apoptotic cells (arrowheads) compared to (**C**) CCR2-/-, and this data was quantitatively demonstrated, once in (**D**) CCR2-/- ovaries a reduced ($p < 0.05$) percentage of follicles containing TUNEL positive cells was observed. (**E**) Apoptotic markers *Bax* and *Caspase3* were downregulated in CCR2-/- ovaries ($p < 0.05$), while the anti-apoptotic gene *Bcl2* was downregulated ($p < 0.05$). There was no difference in the expression of *Fshr* between CCR2-/- and WT ovaries ($p > 0.05$).

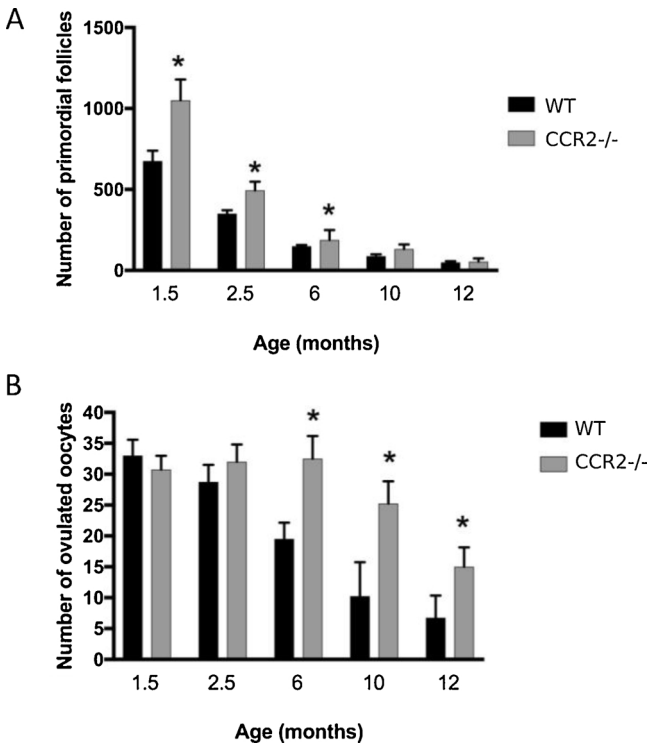

Fig. 4. The effects of CCR2 deficiency on ovarian aging. (**A**) CCR2-/- animals showed a larger primordial follicle reserve from 1.5 to 6 mo compared to WT ($p < 0.05$), however, this difference was attenuated after 10 mo. Additionally, (**B**) from 6 to 12 mo, CCR2-/- animals ovulated more MII oocytes compared to WT ($p < 0.05$) after exogenous stimulation.

expression, which has a key role in supporting antral follicle development (Hillier, 2001).

Our results show that the lack of CCR2 in mice delayed the age-related depletion of ovarian primordial follicle reserve, preserving the capacity to respond to exogenous FSH hyperstimulation in older mice. This is a very exciting finding, leading to the possible use of CCL2 or CCR2 modulators (anti−CCL2 antibodies or CCR2 antagonists) as a strategy to increase the female reproductive lifespan, particularly in those conditions associated with precocious depletion of the ovarian follicle reserve, such as with oncologic treatments. It was shown, for example, that some drugs used in chemotherapy, such as cyclophosphamide (Cy), causes ovarian depletion by activating the PI3K signaling pathway and thus causing massive follicle activation, but this effect could be counterbalanced by drugs or factors that downregulate activation (Kalich-Philosoph et al., 2013; Sonigo et al., 2019). However, potential side effects due to the participation of CCR2 in other several biological processes, particularly related to the immune system (Yang et al., 2018) must be considered. It is also to be determined if, and to which extent, the maintenance of a greater ovarian reserve associated with CCR2 deficiency also delays the progressive adverse effects of aging on the follicular environment and oocyte quality (Krisher, 2019). To the best of our knowledge, the present study is the first suggesting that, in mice, CCR2 plays an important role in ovarian folliculogenesis, potentially affecting the reproductive lifespan.

Author contributions

PHACJ designed and supervised the experiments, and prepared the manuscript. AGAS and LAACP performed and analyzed experiments. RCR and JHMV contributed to the analysis of experimental findings, discussion of data, and revised the final manuscript.

Declaration of Competing Interest

The authors declare that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jri.2020.103174.

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