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Research Article

Estrous cycle impacts microRNA content in extracellular vesicles that modulate bovine cumulus cell transcripts during in vitro maturation†

Ana Clara Faquineli Cavalcante Mendes de Ávila[1], Alessandra Bridi[1], Gabriella Mamede Andrade[1], Maite del Collado[1], Juliano Rodrigues Sangalli[1], Ricardo Perecin Nociti[1], Wilson Araújo da Silva Junior[2], Alexandre Bastien[3], Claude Robert[3], Flávio Vieira Meirelles[1], Felipe Perecin[1] and Juliano Coelho da Silveira[1,*]

[1]Department of Veterinary Medicine, Faculty of Animal Sciences and Food Engineering, University of São Paulo, Pirassununga, São Paulo, Brazil, [2]Department of Genetics, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, São Paulo, Brazil, and [3]Animal Science Department, Research Center in Reproductive Biology, Institute on Nutrition and Functional Foods, Laval University, Québec, Québec, Canada

***Correspondence:** Department of Veterinary Medicine, University of São Paulo, Av. Duque de Caxias Norte, 225, 13635-900, Pirassununga, São Paulo, Brazil. Tel: +55 (19) 3565-6833. E-mail: julianodasilveira@usp.br

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Abstract

Extracellular vesicles (EVs) are nanoparticles secreted by ovarian follicle cells. Extracellular vesicles are an important form of intercellular communication, since they carry bioactive contents, such as microRNAs (miRNAs), mRNAs, and proteins. MicroRNAs are small noncoding RNA capable of modulating mRNA translation. Thus, EVs can play a role in follicle and oocyte development. However, it is not clear if EV contents vary with the estrous cycle stage. The aim of this study was to investigate the bovine miRNA content in EVs obtained from follicles at different estrous cycle stages, which are associated with different progesterone (P4) levels in the follicular fluid (FF). We collected FF from 3 to 6 mm follicles and evaluated the miRNA profile of the EVs and their effects on cumulus-oocyte complexes during in vitro maturation. We observed that EVs from low P4 group have a higher abundance of miRNAs predicted to modulate pathways, such as MAPK, RNA transport, Hippo, Cell cycle, FoxO, oocyte meiosis, and TGF-beta. Additionally, EVs were taken up by cumulus cells and, thus, affected the RNA global profile 9 h after EV supplementation. Cumulus cells supplemented with EVs from low P4 presented upregulated genes that could modulate biological processes, such as oocyte development, immune responses, and Notch signaling compared with genes of cumulus cells in the EV free media or with EVs from high

P4 follicles. In conclusion, our results demonstrate that EV miRNA contents are distinct in follicles exposed to different estrous cycle stage. Supplementation with EVs impacts gene expression and biological processes in cumulus cells.

Summary Sentence

MicroRNA contents of small EVs obtained from bovine follicular fluid are modified depending on the estrous cycle stage and can modulate the RNA profile of cumulus cells during in vitro oocyte maturation.

Key words: cumulus cells, follicle, microRNA, oocyte maturation, progesterone

Introduction

Oocyte development involves periods wherein the gamete undergoes nuclear, cytoplasmic, and molecular maturation [1]. The competence of the oocyte (i.e., its ability to be fertilized and develop into a blastocyst) depends on these events [1]. Although, in cattle, nuclear maturation rates are over 82% in vitro, only a third of these oocytes are competent enough to reach the blastocyst stage in vitro [2]. The failure to reach the blastocyst stage may be due to inadequate in vitro conditions that fail to mimic the natural follicular environment.

The antral follicle is formed by theca cells, granulosa cells, cumulus cells, and the oocyte, and is filled with follicular fluid (FF) [3]. These cells are in constant communication and are influenced by endocrine and paracrine signals, as well as by physical interactions between follicular cells (FCs) and oocytes [4]. Recently, a study reported that extracellular vesicles (EVs) within the ovarian follicular environment may play a role in follicular development [5]. Granulosa cells and cumulus-oocyte complexes (COCs) are substantial sources of EVs in bovine FF [6]. Extracellular vesicles are nanoparticles with a lipid bilayer and are secreted by cells and found in body fluids [7]. They can be classified as either exosomes or microvesicles, depending on their size, contents, and biosynthesis [8]. Exosomes are small EVs that are between 50 and 150 nm in diameter and are formed by endocytosis which first generates a multivesicular body (MVB). Subsequently, exosomes are secreted upon MVB fusion with the cell membrane. This process of synthesis was first described for reticulocytes [9, 10]. Microvesicles, on the other hand, are large EVs that range between 100 and 1000 nm in diameter and are synthesized upon plasma membrane shedding after cell stimulation [11]. Both types of vesicles carry important biological materials, such as mRNAs, microRNAs (miRNAs), and proteins [12].

Ovarian EVs were first described in the FF of mares; their uptake by granulosa cells was demonstrated in vivo and in vitro [5]. Supplementation of EVs of the FF during in vitro maturation (IVM) improved cumulus expansion and changed transcripts that were associated with this event in bovine cumulus cells [13]. Furthermore, the supplementation of EVs of bovine FF during IVM and the in vitro culture of embryos demonstrated an increase in blastocyst rates and changes in the levels of transcripts and in the global DNA methylation and hydroxymethylation of embryos [14]. Recently, a study using bovine COCs found structures that look like EVs at the end of the transzonal projections (TZP) [15], between the cumulus cells and oocyte, suggesting their relationship with oocyte competence.

MiRNAs are one of the bioactive components of EVs. They are small sequences of noncoding RNAs that act as post-transcriptional regulators [16]. Therefore, they are capable of modulating biological pathways involved in ovarian folliculogenesis [17]. MiRNAs are formed from a primary miRNA (pri-miRNA), which is further processed to the precursor form (pre-miRNA; ~70 nucleotides) inside the nucleus. Subsequently, the pre-miRNA is processed into the mature miRNAs (~20 nucleotides) within the cytoplasm. The mature miRNA can regulate gene translation [18]. The majority of miRNAs in FF are found within the EV [19]. Therefore, EVs play an important role in mediating communication between cells within the follicular environment, and the contents of the EVs may modulate biologic pathways by transferring molecules such as miRNAs.

However, our understanding of the consequences of the different intrafollicular environments and their contents and functions is incomplete. Recently, studies demonstrated that the EVs of different follicular sizes can cause distinct effects in cumulus cell expansion [13] and granulosa cell proliferation [20], and can also differ in terms of concentration and miRNAs contents [21]. We know that the antral follicular environment is dynamic and changes during the estrous cycle [22]. These changes are accompanied with changes in different factors, such as hormones and nutrients, which are found in the FF and can impact oocyte competence [23, 24]. Progesterone (P4) is an important hormone that is associated with different stages of the estrous cycle [25]. Moreover, some studies demonstrated the influence of follicular waves on fertility [26] and the positive effects of high P4 levels during follicular growth in cow fertility compared with that of low P4 levels [26, 27]. Based on these results, our hypothesis is that miRNA contents in the EVs of bovine FF are modulated during the estrous cycle and that the modulation is associated with P4 levels. The aim of this study was to evaluate miRNA contents of the EV in small follicles on different stages of estrous cycle and the effects of these EVs on cumulus cells during IVM.

Materials and methods

Ethics statement

We certify that the proposal "Analysis of a new communication mechanism between female reproductive system cells, oocytes and embryos mediated by extracellular vesicles and their impacts on bovine in vitro production.", utilizing 300 Bovines (300 females), protocol number CEUA 4909010817, under the responsibility of **Juliano Coelho da Silveira** and team; *Ana Clara Faquineli Cavalcante Mendes de Ávila* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Animal Science and Food Engineering - (São Paulo University) (CEUA/FZEA) in the meeting of 10/04/2017.

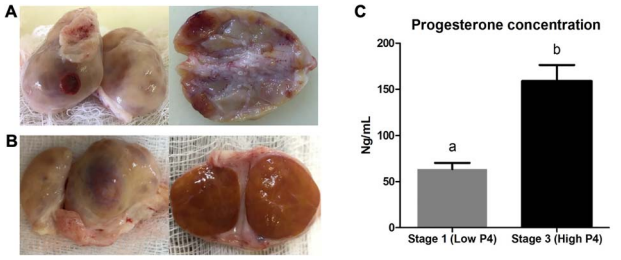

Figure 1. Progesterone concentration in the FF of small follicles obtained from ovaries in different stages of estrous cycle. (A) Ovaries classified as stage 1 of the estrous cycle (low P4 group) based on the appearance of the corpus luteum. (B) Ovaries classified as stage 3 of the estrous cycle (high P4 group) based on the appearance of the corpus luteum. (C) Progesterone concentration (ng/mL) in the FF of ovaries in stage 1/low P4 ($n = 4$ pools) and stage 3/high P4 ($n = 5$ pools) groups classified according to the appearance of the corpus luteum. Different letters indicate $P < 0.05$. Bars represent means and error bars represent standard errors of the means.

In order to study the association of P4 levels in FF with the miRNA contents of EV and the effect of EVs as a supplement in the maturation medium, we designed an experimental model based on the morphology of the corpus luteum. In this model, we used pairs of slaughterhouse ovaries that were in different stages of the estrous cycle and obtained from crossbred Nellore cows (*Bos indicus* background). The ovaries were evaluated based on the appearance of the corpus luteum (using the criteria described by Ireland et al. [28]) and P4 concentration in FF. Accordingly, the ovaries were classified as stage 1 (early corpus luteum, red and recently ovulated, low P4; Figure 1A) or stage 3 (late corpus luteum, tan or orange, high P4; Figure 1B) and used in different analyses. The ovaries were also classified as stage 2 (apex of corpus luteum red or brown) or stage 4 (light yellow to white corpus luteum), in accordance with the criteria described by Ireland et al. [28], but these ovaries were not used in the experiment. Follicles between 3 and 6 mm in diameter were used to collect FF. Extracellular vesicles were isolated by FF using centrifugation and subsequently characterized and used for the supplementation of COCs during IVM. This study was approved by the University of São Paulo Research Ethics Committee (protocol number: 4909010817).

Collection of follicular fluid

Ovaries were collected in pairs and transported to the laboratory in saline solution at 36 °C within 3 h given the proximity of the slaughterhouse to Pirassununga City, in the state of São Paulo. Ovaries were evaluated based on corpus luteum appearance and classified as stage 1 (red, recently ovulated) or stage 3 (tan or orange, vasculature on surface). Ovaries in stage 1 and stage 3 were subsequently referred to as low P4 and high P4, respectively. The pairs of ovaries that were classified as stage 2 and 4 CLs were removed from the experiment. FF was aspired using 18-gauge needle and 10-mL syringe. The FFs were grouped into pools (containing approximately six single ovaries each) of low P4 ($n = 4$ pools) and high P4 ($n = 5$ pools).

Follicular fluid progesterone concentration

The FF from low P4 ($n = 4$ pools) and high P4 ($n = 5$ pools) samples was collected and used to measure P4 concentration using the chemiluminescence assay (ADVIA Centaur-Siemens, Henkestr., Erlangen, Germany). Intra and interassay coefficients of variation were 4.87 and 9.33 ng/mL, respectively. We used FF pools containing similar estradiol/progesterone ratios (data not shown) in order to avoid differences in atresia levels between groups.

Isolation of small extracellular vesicles from follicular fluid

Small EVs were obtained from the bovine FF of low P4 ($n = 4$ pools) and high P4 ($n = 5$ pools) samples. Upon collection, FF was centrifuged at 4 °C in order to remove live cells (300×*g* for 10 min), cellular debris (2000×*g* for 10 min), and large EVs (16 500×*g* for 30 min). The remaining supernatant was maintained at −80 °C until the isolation of small EVs. On the day of use, the FF was filtered through a 0.22-µm sterile syringe filter (PES membrane; KASVI, São José do Pinhais, Paraná, Brazil) in order to remove any remaining large EVs. Finally, this fluid was centrifuged twice at 120 000×*g* for 70 min (Optima XE-90 Ultracentrifuge; rotor 70 Ti; Beckman Coulter, Brea, California, USA) in order to isolate small EVs as previously described [29]. The supernatant was discarded, and the small EV pellets were resuspended in 50 µL of phosphate buffered saline (1× Ca^{2+}/Mg^{2+} free PBS; 137-mM NaCl, 2.7-mM KCl, 10-mM Na_2HPO_4, and 2-mM KH_2PO_4) until further use.

Characterization of small extracellular vesicles

The small EVs isolated from FF were characterized based on their morphology and size using transmission electron microscopy; specific proteins were characterized by western blotting, and particle size and concentration were determined using nanoparticle tracking analysis (NTA).

Transmission electron microscopy

Small EV pellets isolated from 1 mL of FF were diluted in 50 µL of fixing solution (0.1 M cacodylate; 2.5% glutaraldehyde and 4% paraformaldehyde at pH 7.2–7.4) for 2 h at room temperature. Subsequently, the small EVs were diluted in 2 mL of milli-Q water, and the solution was centrifuged once in order to obtain pellets of small EVs (120 000×*g*, 70 min, 4 °C). The pellet was diluted in 20 µL of milli-Q water and placed in a copper grid for 20 min at room temperature in order for it to dry before staining. The grid was inserted into 2% of uranyl acetate and then analyzed using a transmission electron microscope (FEI Tecnai 20; LAB6 emission; 200 kV).

Western blotting analysis

The protein lysate from small EVs and from FCs was obtained using RIPA (radioimmunoprecipitation assay) buffer and proteinase inhibitor cocktail. Laemmli and beta-mercaptoethanol 4× (Bio-rad, Hercules, CA) were added in 10 µL (~15 µg) of protein solution. Denaturation of the proteins in these samples was accomplished by transferring the samples to 95 °C for 5 min. They were then loaded onto SDS-PAGE 10% polyacrylamide gel (456–1033; Mini-PROTEAN TGX; Bio-rad, Hercules, CA). The gel was run at 100 V for 90 min, and then, the proteins were transferred onto a PVDF (Polyvinylidene Difluoride) membrane (1704156; Trans-Blot Turbo; Bio-rad, Hercules, CA). The membrane was washed in 1× Tris buffered saline with Tween-20 (TBST) and maintained in a blocking buffer (5% of bovine serum albumin—BSA—in TBST) at room temperature for 1 h. After that, the membrane was incubated overnight with a primary antibody at 4 °C. The proteins ALIX (ALG-2 interacting protein X) and calnexin were evaluated using a goat polyclonal antibody against the N-terminus peptide of human

origin obtained from ALIX (0.2 µg/mL; SC-49267; Santa Cruz, CA) and a mouse monoclonal antibody against human hepatoma cell line (0.5 µg/mL; SC-23954; Santa Cruz, CA), respectively. After incubation, the membrane was washed three times using 1× TBST for 5 min each and then incubated with secondary antigoat (0.1 µg/mL; SC-2020; Santa Cruz, CA), and antimouse (1:4000; #7076S; Cell Signaling Technology, Danvers, Massachussetts, USA) antibody conjugates in horseradish peroxidase (Supplementary Table S1) for 1 h at room temperature. Finally, the membrane was washed three times using 1× TBST and exposed to a detection solution (170–5060; Clarity Western ECL). The images were obtained and analysis was performed using the ChemiDoc MP Image System (Bio-Rad, Hercules, CA).

Nanoparticle tracking analysis

The small EVs isolated from 100 µL of FF from low P4 and high P4 samples were resuspended in 50 µL of 1× Ca^{2+}/Mg^{2+} free PBS. The particle size and concentrations were measured using Nanosight (NS300; NTA 3.1 Build 3.1.45, Malvern, UK). The dilution factor was 1:500 for 1× Ca^{2+}/Mg^{2+} free PBS. The analysis was performed by capturing five videos of 30 s each, using an sCMOS camera at camera level 15 and under a controlled temperature of 37 °C. A threshold of 5 was considered in this analysis.

Total RNA extraction of small extracellular vesicles and cumulus cells

Total RNA content of small EVs and cumulus cells after 9 h of IVM was extracted using miRNeasy Mini Kit (QIAGEN, Hilden, Germany), in accordance with the manufacturer's instruction. The RNA was treated with DNaseI (Invitrogen, Carlsbad, CA). RNA quality and concentration were analyzed using spectrometry (NanoDrop 2000; Thermo Fisher Scientific, Frederick, Maryland, USA).

Reverse transcription and quantitative RT-PCR analysis of the microRNAs in small extracellular vesicles

The relative expressions of 383 bovine miRNAs in small EVs from FF of low P4 and high P4 samples were evaluated as previously described [30]. The total miRNA was reverse-transcribed using miScript II RT Kit (QIAGEN) according to the manufacturer's instructions. Briefly, we performed 10 µL reactions, containing 100 ng of total RNA, 10× miScript Nucleic mix, nuclease-free water, miScript reverse transcriptase (RT), and 5× miScript HiFlex Buffer, in accordance with the manufacturer's instructions. The reaction was incubated at 37 °C for 60 min followed by 95 °C for 5 min.

Quantitative RT-PCR of small EVs was performed using miScript SYBR Green PCR Kit (QIAGEN). The total volume of the reaction mixture was 6 µL, and it contained 2× Power SYBR Green PCR Master Mix (Applied Biosystems, Frederick, Maryland, USA), 10× miScript Universal Primer, nuclease-free water, 0.2 ng of cDNA, and 1 µL of specific forward primer, which was designed based on bovine mature miRNA sequences and according to mirBase database (http://www.mirbase.org). Amplifications were performed using QuantStudio 6 Flex (Applied Biosystems) equipment. The reactions were exposed to 95 °C for 15 min, followed by 45 cycles for 15 s at 94 °C, 30 s at 55 °C, and 30 s at 70 °C. This was followed by melting curve according to the manufacturer's instructions. We considered the miRNA present when cycle threshold (CT) was less than 37 cycles in at least three biological repetitions with adequate melting curves; CT was normalized using the geometric mean of miR-99b obtained in duplicate and using the method described by da Silveira et al. [14]. Data analyses were performed to evaluate the miRNAs that were described as common, exclusive as well as differently abundant in the low P4 and high P4 samples. Only differently abundant miRNAs ($P < 0.05$) were analyzed using mirpath v.3 software from DIANA TOOLS. Since this software uses human data, we used bovine miRNAs that were present with homology greater than 90% in human sequences. The homology was determined using miRbase, for bioinformatics analysis.

Small extracellular vesicle labeling and co-incubation with cumulus-oocyte-complexes

For tracking experiments, the small EVs isolated from 1 mL (mean of $5.93 \times 10^{11} \pm 2.48 \times 10^{11}$ particles/mL) of FF independent of estrous cycle stage were labeled using a fluorescent dye PKH26 (red; Sigma-Aldrich, Taufkirchen, Germany) that labels lipid membranes, in according with the manufacturer's instructions. Briefly, we added 250 µL of diluent C in the solution containing small EVs. Next, we added 250 µL of diluent C and 1 µL of dye in order to stain small EVs. The same protocol was used for the negative control group (using 1× Ca^{2+}/Mg^{2+} free PBS alone, without small EVs). These solutions were incubated at 37 °C for 15 min, followed by the addition of 1 mL of TCM199 + 1% BSA for 1 min in order to inactivate the unbound dye. Finally, we centrifuged the solution twice at 100 000×*g* for 30 min at 4 °C, to form pellets and wash the stained small EVs. The small EVs and the negative control pellets were resuspended in 1 mL of maturation medium, which was used for the supplementation of COCs during IVM.

In order to analyze the interaction between COCs and EV, we used live cell imaging during IVM. Fifty semidenuded COCs were obtained from 3 to 6 mm follicles and co-incubated with either maturation medium containing labeled EVs or negative control. Both the maturation medium and the negative control contained Sir-actin (0.1 µM; Cytoskeleton, Inc, Denver, Colorado, USA) to label the actin filaments. The COCs were placed in a custom dish (modified from Bioptechs #190310-35) containing 60 wells of diameter 280 µm and under mineral oil. The dish was maintained in the microscope chamber at 38.5 °C and a humidified atmosphere with 5% of CO_2. We performed time-lapses in series of 20 and at 10 s intervals using a confocal microscope (Zeiss LSM 700 confocal live cell system; Carl Zeiss, Germany) from 1 to 9 h of the IVM, at 40×/1.2NA objective in Immersol W. Each time point corresponded to five z-stacked slice, with an interval of 1 µm each.

Supplementation of small extracellular vesicles during in vitro maturation

During IVM, the effect of small EVs in bovine COCs was evaluated after supplementation. For this, small EVs isolated from 200 µL of either low or high P4 FF were resuspended in 200 µL of IVM medium. Good quality COCs obtained from 3 to 6 mm follicles (n = 805) were washed with TCM 199 medium (GIBCO), buffered with HEPES (20 mM), and supplemented with sodium pyruvate (0.2 mM) and gentamycin sulfate (50 µg/mL). Groups of 20–25 COCs were distributed in 100 µL drops of the maturation medium containing TCM 199 (GIBCO) buffered with sodium bicarbonate (25 mM). The maturation medium was also supplemented with sodium pyruvate (0.2 mM), gentamycin

sulfate (50 µg/mL), follicle stimulating hormone (0.5 µg/mL, Folltropin-V, Bioniche Animal Health Canada, Belleville, Canada), and human Chorionic Gonadotropin (5 U/mL, Vetecor, Hertape Calier).

These COCs were maintained during 9 and 24 h in a maturation medium that was supplemented with either: (1) 10% EV-depleted fetal calf serum (EVs free-FCS; Control), (2) 10% of EV free-FCS supplemented with small EVs from low P4 samples (EVs–low P4), or (3) 10% of EVs free-FCS supplemented with small EVs from high P4 samples (EVs–high P4). The in vitro maturation dishes contained mineral oil were maintained at 38.5 °C and in an atmosphere with 5% CO_2. After 9 and 24 h of maturation, 20–25 COCs per group were collected, washed using 1× Ca^{2+}/Mg^{2+} free PBS, and completely denuded by pipetting. Cumulus cells collected after 9 h of IVM of COCs were centrifuged twice at 300×*g* for 5 min, and resultant the pellet was immediately placed in liquid nitrogen. These samples were maintained at −80 °C until RNA extraction and RNA sequencing. After 24 h of IVM, the presence of first polar body was confirmed with the aim of evaluating the maturation rate. A total of six replicates were performed.

RNA library preparation and sequencing

The libraries containing three pools of cumulus cells per group (each pool containing two replicates) supplemented with either EVs free-FCS, EVs-low P4, or EVs-high P4 during 9 h of IVM were prepared using truseq stranded mRNA sample prep kit, and the sequencing was performed in 1 lane of Hiseq 2500 V4 (2 × 100 pb). Data were processed in the Laboratory of Molecular Genetics and Bioinformatics (LGMB) in the Medical School of Ribeirão Preto, University of São Paulo (FMRP-USP). Quality of the reads was evaluated using the FASTQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Reads were filtered using the software, TRIMMOMATIC [31] and the following parameters "threads 24 LEADING: 22 TRAILING: 22 SLIDINGWINDOW: 4: 26 MINLEN: 36 AVGQUAL: 24"; considering the minimum phred quality at the start/end of the read to be 22, medium phred quality to be 22, and minimum read size to be 36 bps. Samples that passed the quality screening were then aligned with the *Bos taurus* genome (assembly ARS-UCD1.2), using the STAR software [32] and default parameters. A final report of the alignment quality was created using the MULTIQC software [33]. Analysis of differential gene expression was performed using DESEQ2 [34] R package considering Benjamini–Hochberg method ("BH") for adjusted *P* values <0.10, and the log2 fold change greater than 0.5 or less than −0.5. Furthermore, since we have three groups, the genes in a group were classified as up or down regulated based on if they were always up or downregulated in all possible contrasts. For the enrichment analysis, we used the R software package ClusterProfiler [35] with the compareCluster function and the flag "enrichGO" for the biological process. In order to assist in the interpretation of the results, principal component analysis (PCA), volcanoplots, and Heatmaps were constructed using R.

Statistical analysis

Analysis of FF P4 concentration, particle size, and concentration, and the relative expression of miRNAs in small EVs obtained from low and high P4 samples were performed using Student *t*-test with a significance level of 5%. All data followed the criteria of normality. The analyses were performed using the JMP software from SAS. The images of confocal microscopy were analyzed qualitatively, in comparison to the negative control group.

Results

Progesterone concentrations in small follicles indicate different stages of estrous cycle

The FF from small follicles was collected from pairs of ovaries classified as either low P4 or high P4 of the estrous cycle and was used to determine follicular P4 concentration (Figure 1A and B). The results confirmed the difference in P4 concentrations of FF obtained at different stages of estrous cycle (Figure 1C). The means of the P4 were statistically different between low P4 (63.62 ± 6.79 ng/mL) and high P4 (158.8 ± 17.47 ng/mL) groups. Thus, different phases of estrous cycle, based on corpus luteum appearance, could be confirmed using FF P4 levels.

Characterization of small extracellular vesicles obtained from follicular fluid

The small EVs obtained from FF were characterized in order to verify the quality of the isolation protocol. Initially, we evaluated the morphology and size of EVs using transmission electron microscopy (Figure 2A). The images showed consistent morphology and diameter of about 50–150 nm. Next, we analyzed the presence of proteins such as ALIX and Calnexin in the small EVs and FCs using western blotting (Figure 2B). ALIX is an endosome pathway marker and was detected in small EVs and FCs. Calnexin is an endoplasmic reticulum marker and was not found in small EVs, but was present in FCs, consistent with the reports by Lötvall et al. [36]. Finally, we determined particles size and concentration using NTA between small EVs isolated from low P4 and high P4 FF (Figure 2C). There was no difference in size (mean size of low P4: 113.8 ± 4.70 nm; mean size of high P4: 123.62 ± 3.40 nm) or particle concentration (mean concentration of low P4: $5.2 \times 10^{11} \pm 1.03 \times 10^{11}$ particles/mL; mean concentration of high P4: $6.52 \times 10^{11} \pm 1.28 \times 10^{11}$ particles/mL) between the EV groups. These results demonstrate the efficiency of the protocol of isolation of small EVs since their morphology and size are equivalent to EVs, as well as the pellet enrichment in ALIX and in the absence of cell contamination based on the absence of calnexin. Additionally, there was no difference in particle size and concentration between small EVs from the low and high P4 EVs groups.

MicroRNA contents of small extracellular vesicles are modulated by the estrous cycle

In order to investigate the miRNAs contents of small EVs isolated from FF that was collected from low P4 (n = 4 pools) and high P4 (n = 5 pools) groups, we performed the analysis of 383 bovine miRNAs. The results identified a total of 357 miRNAs in both groups. Of these, 293 miRNAs were commonly detected in low and high P4 groups and a total of 161 miRNAs were upregulated in low P4 compared with high P4 group ($P < 0.05$; Figure 3A). Additionally, 37 miRNAs were exclusively detected in the low P4 group and 27 miRNAs were detected exclusively in the high P4 group (Supplementary Table S2). These results showed that the miRNAs content in small EVs obtained from FF are being modified in accordance to the FF P4 levels. This suggests that miRNAs contents of small EVs could be modulating biologic pathways in response to different follicular environments.

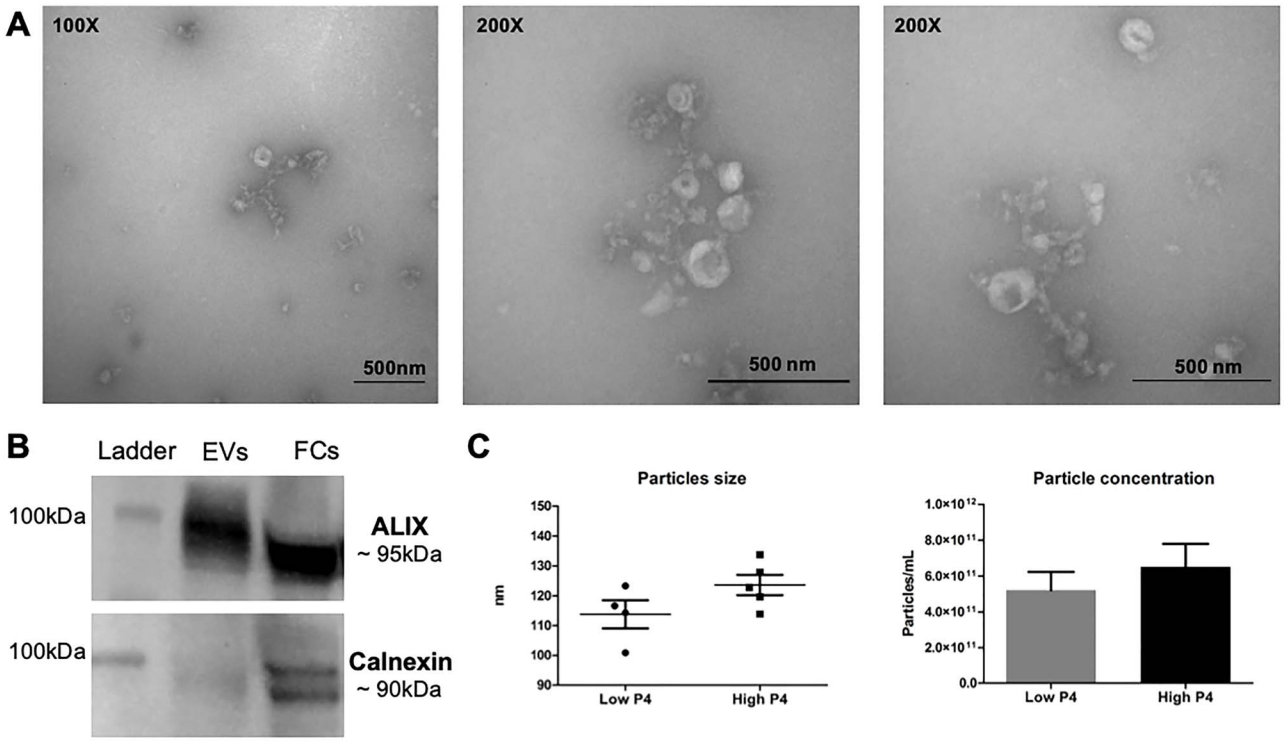

Figure 2. Characterization of small EVs isolated from FF. (A) Transmission electron microscopy showing small EVs, around 50–150 nm in diameter and their cup-shaped appearance (scale bar 500 nm). (B) Western blotting analysis of proteins ALIX and Calnexin in small EVs and FCs. We detected the presence of ALIX, an endosome pathway marker, in EVs and FCs. The protein calnexin, an endoplasmic reticulum marker, was only detected in FCs, thus confirming the absence of cell contamination in EVs. (C) Analysis of the particle size (nm) and concentration (particles/mL) of small EVs that were isolated from follicles exposed to either low P4 or high P4 using NTA. Bars represent means and error bars represent standard errors of the means.

Bioinformatics analyses of microRNAs present in small extracellular vesicles that are associated with different stages of the estrous cycle indicate the modulation of reproduction associated pathways

In order to determine the predicted biological functions of miRNAs carried out by small EVs in FF obtained from different P4 levels, we performed bioinformatics analysis of the upregulated miRNAs using mirPath v.3 software in DIANA TOOLS considering the *P*-value and homology to be similar to human sequences (Supplementary Table S3). Since the 161 miRNAs were more abundant in small EVs obtained from low P4 groups compared those obtained from high P4, we used only the top 100 miRNAs to perform bioinformatics analysis. These miRNAs were predicted to be involved in the modulation of 22 physiologically relevant pathways (Figure 3B). Some of the reproductive physiology-related pathways are MAPK signaling pathway (179 genes), endocytosis (170 genes), RNA transport (127 genes), Hippo signaling pathway (115 genes), cell cycle (110 genes), FoxO signaling pathway (108 genes), oocyte meiosis (90 genes), P4-mediated oocyte maturation (71 genes), and TGF-signaling pathway (65 genes). Interestingly, we identified a significant number of pathways that were modulated by miRNAs were present in higher abundance in small EVs obtained from FF in low P4. These pathways are involved in cell proliferation, follicular development, and oocyte maturation.

Small extracellular vesicles are taken up by cumulus cells during in vitro maturation

In order to evaluate the mechanism of communication between small EVs and COCs, the small EVs were labeled with a membrane dye and supplemented during IVM. Live cell images were captured during the 9 h of IVM. The results demonstrated that the uptake of small EVs by cumulus cells occurs from the first hour of IVM. The time-lapse images showed that labeled small EVs are taken up by cumulus cells and move toward the zona pellucida (ZP) over time (Figure 4; Supplementary Movie S1). We designed negative controls (PKH26 labeled PBS) and did not see any dye staining in cumulus cells (Supplementary Figure S1). In conclusion, we confirmed that the uptake of small EVs by cumulus cells occurs from the first hour of the IVM of COC.

Global RNA profile indicates different modulation of the transcripts in cumulus cells after supplementation with small extracellular vesicles from different estrous cycle stage during in vitro maturation

In order to study the effects of small EVs during IVM, cumulus cells were analyzed for 9 h after IVM. We used RNAseq technique to perform a global analysis of cumulus cells, with the aim of evaluating the RNA profile and biological processes that are modulated in these cells when exposed to different supplementations. The RNA profile demonstrated that the groups of cumulus cells are distinct depending on different supplementation, as shown by the PCA (Figure 5A). Additionally, we evaluated differently expressed genes (DEGs) between contrasts. The results demonstrated that COCs exposed to maturation media supplemented with EVs free-FCS or EVs from low or high P4 group for 9 h are able to change gene expression in cumulus cells, as shown in the volcano plot. Briefly, in contrast to the cells supplemented with EVs-low P4 and EVs-high P4, we demonstrated that 63 genes are upregulated in the EVs-low P4

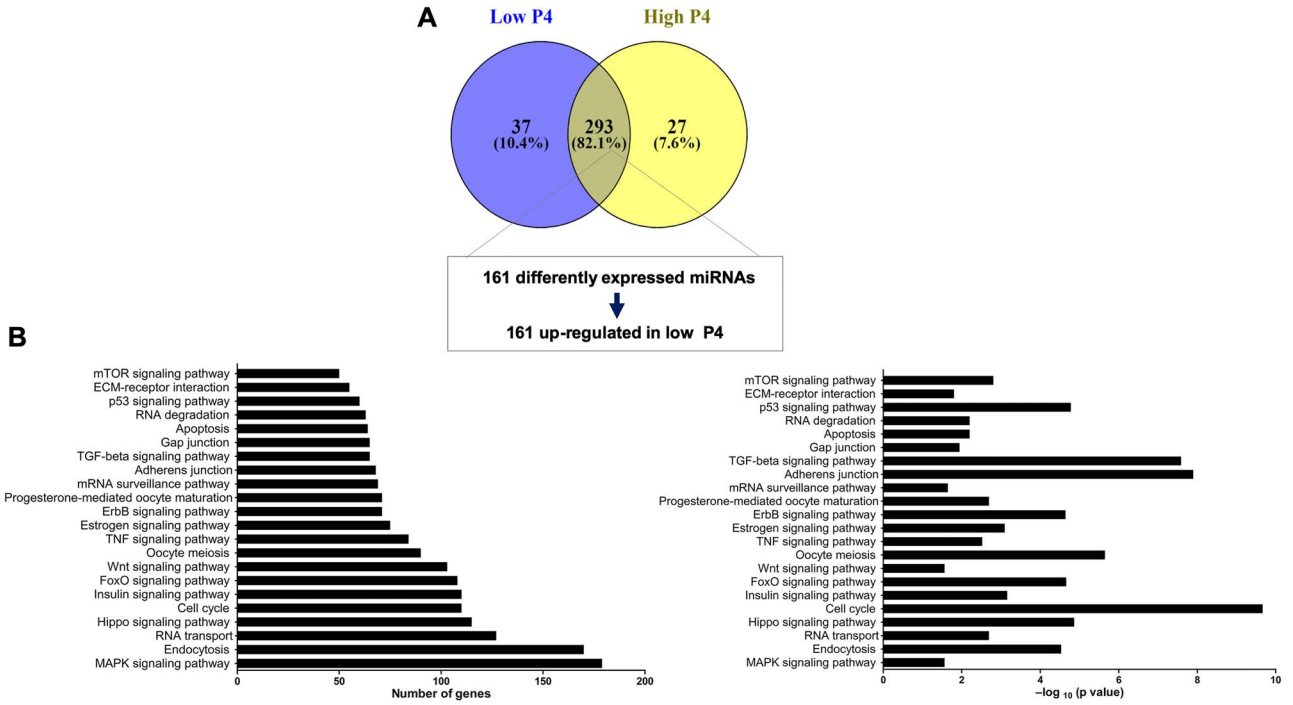

Figure 3. Profile of the 383 miRNAs in small EVs that are associated with different progesterone levels. (A) Venn diagram representing the profile of miRNAs in small EVs obtained from the FF of low and high P4 group. We detected a total 357 miRNAs, of which 293 were common between the groups. The differences in relative expression levels between the groups revealed a total 161 miRNAs that were upregulated in low P4 group compared with high P4 group ($P < 0.05$). (B) Predicted pathways regulated by top 100 miRNAs that were upregulated in the low P4 group. Graphs represent the number of genes (bottom left) and transformed ($-\log_{10}$) *P*-value (bottom right) from the selected pathways that were predicted to be regulated by these miRNAs.

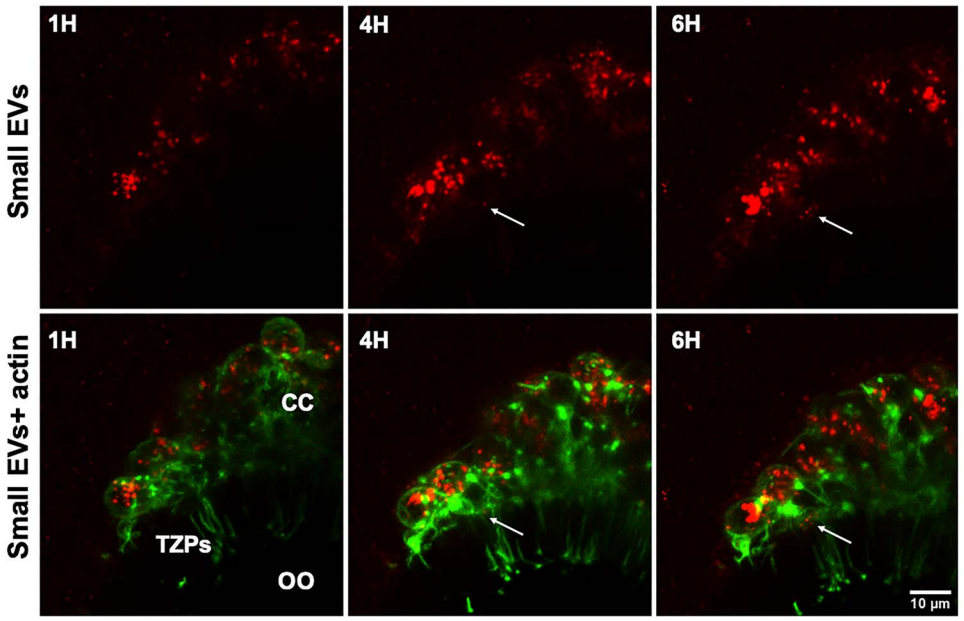

Figure 4. Live cell imaging of small EVs and COCs during IVM. Time lapse of the IVM supplemented with labeled small EVs (red). Images demonstrate that the small EVs are taken up by cumulus cells since the first hour of IVM and the proximity of the EVs with the ZP over time (white arrows). COCs were labeled with Sir-Actin (actin; green). Images were obtained at 1, 4, and 6 h (H) of IVM using a 40× objective in water. OO, oocyte; TZP, transzonal projections; CC, cumulus cells.

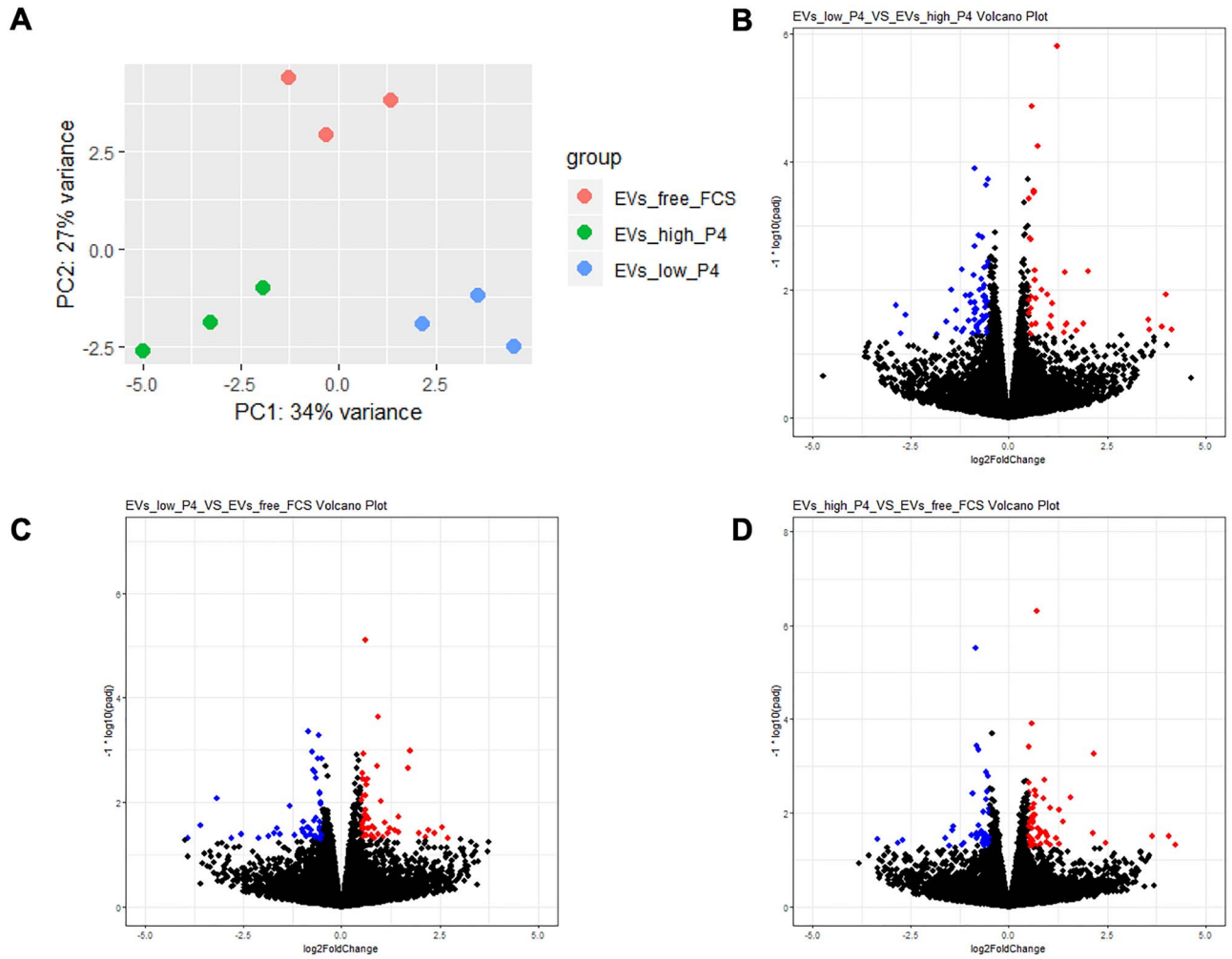

Figure 5. PCA and volcano plot representing the RNA profile of cumulus cells supplemented with EVs free-FCS, small EVs from low P4 (EVs-low P4), and small EVs from high P4 (EVs-high P4) group for 9 h of IVM. (A) PCA representing the variation in RNAs in cumulus cells supplemented with EVs free-FCS ($n = 3$, red), EVs-low P4 ($n = 3$, blue), and EVs-high P4 ($n = 3$, green). Volcano plot representing variation in DEGs between the following: (B) EVs-low P4 and EVs-high P4 groups, (C) EVs-low P4 and EVs free-FCS, and (D) EVs-high P4 and EVs free-FCS. Red dots indicate the DEGs that were upregulated in the first group, while blue dots represent the DEGs that were upregulated in the second group. DEGs were considered to be upregulated when *P*-adjusted was <0.1 and log2 fold change >0.5.

group and 76 are upregulated in the EVs-high P4 group (Figure 5B). When we compared cumulus cells supplemented with EVs-low P4 and EVs free-FCS, we found that 77 genes are upregulated in EVs-low P4, while 75 genes are upregulated in EVs free-FCS group (Figure 5C). Then, upon comparing the cumulus cells supplemented with EVs-high P4 group and EVs free-FCS, we detected 69 genes that were upregulated in EVs-high P4 group and 64 genes that were upregulated in EVs free-FCS group (Figure 5D). Differently expressed genes are listed in Supplementary Table S4. Thus, these results demonstrate that supplementation with small EVs of different follicular origins (under low or high P4) is capable of impacting RNA profiles in cumulus cell in only 9 h (Figure 6).

The functional enrichment analysis of genes that were either always upregulated or always downregulated in the cumulus cells compared with all contrasts, demonstrated that biological processes are modulated differently in these cells depending on supplementation. A large number of biological processes are upregulated in EVs-low P4 group, whereas a large number of biological processes in EVs free-FCS and EVs-high P4 supplemented cells are downregulated. Briefly, genes related to the regulation of reproductive processes such as oocyte differentiation and development and ovulation cycle, cellular process involved in the reproduction of multicellular organisms, and sexual reproduction are upregulated in cumulus cells supplemented with EVs-low P4 group and downregulated when supplemented with EVs free-FCS group. Similarly, regulation of the immune system processes and inflammatory responses are upregulated in cells supplemented with EVs-low P4 and downregulated in those supplemented with the EVs free-FCS group. Especially, genes involved in cell activation and immune response are upregulated in the EVs-low P4 group and downregulated in the EVs-high P4 and in EVs free-FCS groups. Genes that are involved in biological processes such as regulation of signaling receptor activity and Notch signaling pathway are upregulated in small EVs-low P4 and downregulated in small EVs-high P4. Finally, cumulus cells supplemented with small EVs-high P4 presented presented downregulated genes that modulate biological processes involved in cellular and immune responses and

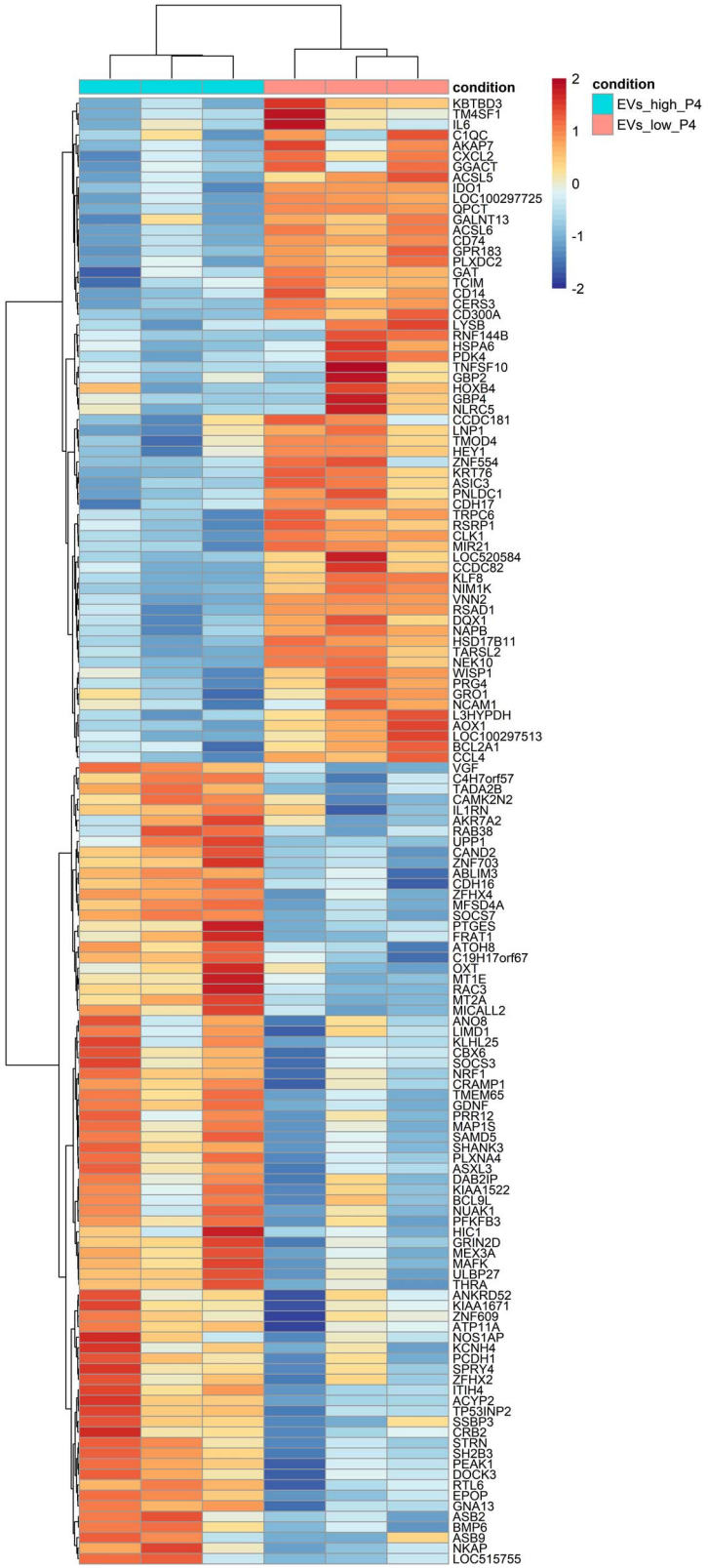

Figure 6. Heatmap representing the variations in 139 DEGs between cumulus cells supplemented with small EVs from low P4 (EVs-low P4) or high P4 groups (EVs-high P4). Color scales vary from red (upregulated genes) to blue (downregulated genes). DEGs were considered to be either upregulated or downregulated when the *P*-adjusted was <0.1 and log2 fold change >0.5.

Table 1. Biological processes that were upregulated in cumulus cells after supplementation with EVs-low P4 for 9 h of IVM.

Biological process	*P*-adjusted	Genes
Regulation of reproductive process (GO:2000241)	4.1×10^{-04}	*GDF9/PTGS2/ZP2/ZP3*
Oocyte differentiation (GO:0009994)	8.65×10^{-08}	*LGR5/GDF9/PTGS2/ZP3*
Oocyte development (GO:0048599)	2.6×10^{-04}	*GDF9/PTGS2/ZP3*
Ovulation cycle (GO:0042698)	9.9×10^{-09}	
Cellular process involved in reproduction in multicellular organism (GO:0022412)	4.7×10^{-04}	*LGR5/GDF9/CYP26B1/PTGS2/ZP3*
Sexual reproduction (GO:0019953)	1.0×10^{-03}	*LGR5/GDF9/CYP26B1/PTGS2/PPP1R1B/ZP2/ZP3*
Regulation of immune system process (GO:0002682)	2.86×10^{-08}	*IGFBP2/FCER1G/A2M/CYP26B1/LRRC24/ADORA2B/ZP3/IL6/CD14/GPR183/TCIM/ARG1*
Inflammatory response (GO:0006954)	2.8×10^{-04}	*FCER1G/A2M/CYP26B1/PTGS2/ADORA2B/ZP3/IL6*
Cell activation (GO:0001775)	1.71×10^{-08}	*IGFBP2/FCER1G/CYP26B1/ADORA2B/ZP2/ZP3/IL6/GPR183/TCIM/ARG1*
Immune response (GO:0006955)	2.1×10^{-04}	*FCER1G/A2M/LRRC24/ADORA2B/ZP3/IL6/CD14/GPR183/TCIM/ARG1*
Regulation of signaling receptor activity (GO:0010469)	1.7×10^{-03}	*GDF9/CARTPT/ZP3/IL6/CXCL2/GRO1/CCL4*
Notch signaling pathway (GO:0007219)	3.3×10^{-02}	*HEY1/TCIM*

Genes were considered if they were always upregulated compared to the genes of cells supplemented with EVs free-FCS and EVs-high P4 groups.

Table 2. Biological processes that were downregulated in cumulus cells after supplementation with EVs-high P4 for 9 h of IVM.

Biological process	*P*-adjusted	Genes
Regulation of signaling receptor activity (GO:0010469)	$5.3E^{-04}$	*CXCL8/IL1B/IL6/CXCL2/GRO1/CCL4/CXCL5*
Cell activation (GO:0001775)	$1.3E^{-03}$	*CXCL8/GPR183/FGG/IL6/TCIM/CXCL5*
Notch signaling pathway (GO:0007219)	$1.6E^{-03}$	*TMEM100/HEY1/TCIM*
Immune response (GO:0006955)	$5.2E^{-03}$	*CD36/IL1B/GPR183/IL6/CD14/TCIM/CXCL5*

Genes were considered if they were always downregulated compared to the genes of cells that were supplemented with EVs free-FCS and EVs-low P4 groups.

cell homeostasis, differentiation and chemotaxis (Tables 1 and 2; Supplementary Figure S2 and Supplementary Table S5). Therefore, as a consequence of the distinct supplementations and different RNA profiles of cumulus cell, biological processes are differently modulated in cumulus cells.

Supplementation of small extracellular vesicles during in vitro maturation did not affect maturation rate

Based on the uptake of small EV by cumulus cells, we evaluated the effects of supplementation with EVs free-FCS, EVs-low P4, or EVs-high P4 on maturation rate during IVM. We did not observe any difference in maturation rates between groups after 24 h of IVM (67.8 ± 4.9; 75.2 ± 4.9; $68.6 \pm 5.5\%$, respectively).

Discussion

Extracellular vesicles secreted by FCs are involved in different biological functions related to follicle development and oocyte maturation [5, 13, 14, 20]. However, EV functions reflect the follicular environment, which can modulate different molecular and phenotypic changes within the cumulus [13] and granulosa cells [20]. Hung et al. [13] demonstrated that EVs from small follicles (3–6 mm) compared with large follicles (>9 mm) are capable of inducing cumulus expansion. Additionally, it was described that follicles of small (3–5 mm), medium (6–9 mm), and large (>9 mm) size can differ in terms of EVs concentration as well as miRNAs contents [21]. In addition, EVs from small follicles were much more capable of inducing granulosa cell proliferation, compared with EVs from medium and large follicles [20]. These effects can be related to the ability of cells to uptake EVs, as well as to EVs concentration and miRNAs contents [21]. The present study evaluated the impact of different follicular environments on EV contents and IVM of COCs. To our knowledge, this is the first study that characterizes EVs from follicles of the same size that are exposed to distinct estrous cycle stages and its associated P4 levels, as well as the first study that performs functional experiments to evaluate the effects of these vesicles on IVM of COC.

The bovine estrous cycle comprises of follicular waves [37] that are controlled by variations in hormones and their receptors, thus affecting the follicle microenvironment [22]. Therefore, follicles go through different phases that impact their morphological and biochemical characteristics [22]. The FF is composed of hormones as P4, estradiol, insulin, IGFs, and other factors secreted by the oocyte and cumulus cells, as well as nutrients and metabolites [23, 24]. Another study, that used the same method of classification of estrous cycle stages as the present study, demonstrated that the composition of nutrients, such as carbohydrates and amino acids, changes in the FF in different stages of estrous cycle [24]. Therefore, the concentration of many factors can change according to the changes in the physiology of the follicles during different estrous cycle stages [38]. The stages of estrous cycle can be estimated by the appearance of the corpus luteum with an accuracy of 85% [28]. These conditions are associated with hormonal changes during the estrous cycle such as changes in the P4 concentration in the plasma [39] and FF [25, 38]. Considering that pairs of ovaries act as a single unit [22], P4 concentration in FF could be a good indicator of estrous cycle stage. Based on that and differences in P4 concentration, we

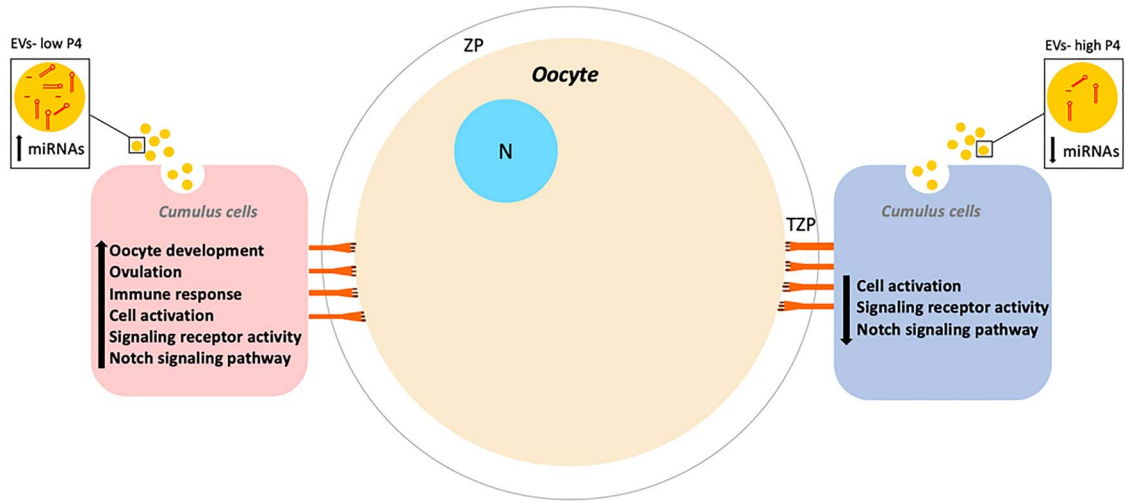

Figure 7. Hypothetical model. Small EVs from different estrous cycle stages carry distinct miRNAs and impact biological processes in cumulus cells. N, nucleus; ZP, zona pellucida; TZP, transzonal projections (orange).

classified pairs of ovaries according to the appearance of the corpus luteum in early and late stages of the estrous cycle. We classified the ovaries as belonging to either stage 1 or 3 according Ireland et al. [28]. The results demonstrated that these stages were associated with different P4 levels in the FF of small follicles, creating groups of follicles that had either low or high P4 levels. Our results of P4 levels are in accordance with the concentrations observed in a previous study performed by Ireland et al. [25].

Progesterone production in the ovary is initiated by a steroidogenesis processes that occurs in the theca and granulosa cells [40], as well as in the luteal cells [41]. Importantly, the corpus luteum that is formed after ovulation is a central source of P4, and circulating P4 concentration increases progressively during the estrous cycle [42], and high levels of P4 are produced during diestrus or pregnancy [22]. Previous studies have demonstrated that progestin concentrations in the FF increase more during stage 3 than during stage 1 of the estrous cycle in follicles with 3–9 mm diameter [25]. Additionally, circulating progestin concentration increases as well and is correlated with the concentration of FF [25]. Progesterone has been associated with important functions in mammalian ovaries, as reported by Peluso [43]. The role of P4 in FF was initially associated with oocyte maturation due to the description of steroid changes in FF, which are characterized by the fall of estradiol concentration and an increase of P4 concentration in preovulatory follicles, shortly before ovulation [44]. P4 receptors are present in granulosa, cumulus, and oocyte cells and are related to cell cycle and oocyte maturation [45–47]. Some studies demonstrated that P4 has an antiapoptotic function in granulosa cells [48, 49]. Moreover, the inhibition of P4 synthesis by COCs during IVM negatively impacts oocyte maturation as well as the in vitro development of blastocysts [45, 50]. Additionally, in vivo studies demonstrated that follicles growing with high P4 levels were related to greater fertility in dairy cows [26, 51] and beef cows compared those growing with low P4 levels [27]. Therefore, these observations show that, in addition to being associated with estrous cycle stages, P4 plays an important role in the follicular environment and can affect follicular contents and oocyte competence.

Although we evaluated small EVs obtained from the fluids of small follicles that were in different stages of the estrous cycle, it is important to consider that the use of different biotechniques enables the oocytes to produce embryos and pregnancies independent of estrous cycle stages. However, the intensive use of assisted reproductive techniques in bovine and humans can affect embryo quality and the health of individuals born. Considering various changes that occur in the in vitro environment, as disturbed gene expression and epigenetics [52], oocyte meiosis [53], disturbs in cumulus cell transcription [54] and metabolism [55, 56], and thus effects in embryo quality [2]. This study used small EVs from FF, which are physiologically in contact with the oocyte, as a tool to modulate oocyte maturation in an attempt to modulate the in vitro environment similar to in vivo environment.

Follicular fluid samples from small follicles exposed to low and high P4 levels were collected and used to isolate small EVs. We did not find any differences in the particle size and concentration of small EVs obtained from low and high P4 group. Thus, we investigated if the follicular environments exposed to different P4 levels can modulate EVs miRNAs contents. The results demonstrated that miRNAs contents were distinct between the groups. Similarly, other studies showed that contents of small EVs miRNAs vary depending on the follicular origins. The profiles of different miRNAs were described in females of different ages [5, 57, 58], associated with reproductive diseases [59] and with stages of follicle development [20, 21]. However, no study has characterized EVs obtained from follicles that have been exposed to different stages of estrous cycle and associated with different P4 levels. A recent study demonstrated that bovine oviductal EV composition is influenced by hormonal changes in stages 1–4 of the estrous cycle [60], and a study in sheep showed that P4 regulates the miRNAs contents in EVs obtained from the uterine lumen [61].

The profile of bovine miRNAs in small EVs was evaluated with the aim of correlating the miRNA contents with the predicted biological pathways that are modulated by them. The results demonstrated differences in miRNA cargo between small EVs obtained from low and high P4 groups. We identified the presence of 357 miRNAs in both groups. Importantly, 161 miRNAs were upregulated in the low P4 level group compared with the high P4 levels group ($P < 0.05$). Therefore, this result suggests that small EVs that have distinct origins can differently modulate biological pathways in recipient cells. Potential biological pathways that are modulated by the upregulated

miRNAs in the low P4 group were evaluated using bioinformatics analyses. We found pathways relevant to reproduction, which were involved in follicle development and oocyte maturation. Pathways such as MAPK, Hippo, cell cycle, FoxO, and TGF-beta are involved in biological processes, such as proliferation, cell differentiation, and apoptosis. These pathways are important for follicular development [3, 62–65]. RNA transport, oocyte meiosis, and P4-mediated oocyte maturation pathways are also modulated by miRNAs that are upregulated in EVs-low P4 and these processes that can, in turn, impact oocyte competence [45, 66]. After evaluating the contents of small EV miRNA and the pathways that were predicted to be regulated by it, we evaluated the uptake of EV by COCs during IVM. We demonstrated that the uptake of small EVs by cumulus cells starts after 1 h of IVM, using live cell imaging. The uptake of EVs by cumulus cells was demonstrated for the first time by Hung et al. [13]. Thus, these results suggest that small EVs from the FF can enter cumulus cells and modulate these cells, thus impacting the oocyte.

Since the contents of the miRNA of small EVs were different in low and high P4 groups and the uptake of these vesicles by cumulus cells was demonstrated, we investigated the effects of the supplementation of cumulus cells with small EV during IVM. The results demonstrated that the maturation rate did not change in response to supplementation. However, global analysis using RNAseq demonstrated that the RNA profile is distinct between the cumulus cells supplemented with EVs free-FCS and with small EVs from low and high P4 group. The analyses of DEGs demonstrated that a significant number of RNA are different between the groups, and there are differences in the cumulus cells supplemented with small EVs of different follicular origin (low or high P4 levels). Importantly, functional enrichment analysis indicated that DEGs in the cumulus cells differently modulate biological processes depending on supplementations. In general, the results demonstrate that cumulus cells supplemented with EVs-low P4 group present a large number of genes that are upregulated and cells supplemented with EVs free-FCS and EVs-high P4 groups present a large number of genes that are downregulated when all contrasts were compared. Thus, when we compared cells supplemented with EVs-low P4 and EVs free-FCS, we found that processes involved in reproduction and immune responses were differently modulated by two groups. Moreover, we found that processes as cell activation, immune responses, regulation of signaling receptor activity, and Notch signaling pathway were upregulated in the EVs-low P4 group comparing to that in the EVs-high P4 group. Interestingly, Notch signaling pathway plays an important role in follicular development [67, 68] and was included in the top functions associated with oocyte competence [23]. Additionally, this pathway can be downregulated by hormonal changes, such as higher P4 levels during mid-pregnancy, which suppresses primordial follicle formation in the ovary of the fetal mouse [69]. According to our results, follicles exposed to high P4 levels can also affect their small EVs, which are responsible for the downregulation of the Notch signaling pathway in cumulus cells. From these results, we showed that a different supplementation for only 9 h during IVM is sufficient to modulate cumulus cells. We summarized the results using a hypothetical model in Figure 7.

Together, the results of miRNA profiles in small EVs and RNA profile variation in cumulus cells after different supplementation during IVM can be an explanation for the effect of follicular origin on the fertility of COCs, which was reported in previous studies [26, 27, 51]. This study was able to show that the miRNA of small EVs is an important tool for intercellular cross-talk and is affected by estrous cycle stage. Pathways such as MAPK, RNA transport, Hippo, Cell cycle, FoxO, oocyte meiosis, P4-mediated oocyte maturation, and TGF-beta, that were predicted to be modulated by the upregulated miRNAs in small EVs obtained from the low P4 samples, are involved in processes such as follicle and oocyte development. Consequently, cumulus cells supplemented with EVs-low P4 had upregulated genes that can modulate biological processes related with follicular and oocyte development, as well as with immune and inflammatory responses associated with cumulus cell expansion and ovulation [70]. These results suggest that EVs-low P4 resulted in a greater response in cumulus cells. These responses were consistent with changes in the predicted pathways that are modulated by miRNAs. The changes in these pathways can be associated with the upregulation of the cell activation process in cumulus cells. Moreover, it is possible that miRNAs within EVs-low P4 act by repressing RNA translation in cumulus cells [71], thus justifying the large number of upregulated genes and biological processes in these cumulus cells. On the contrary, miRNAs in EVs obtained from high P4, and cumulus cells supplemented with these EVs contained genes downregulated. Therefore, small EVs with different follicular origins generated cumulus cells with distinct functional characteristics. These cells have the potential of affecting the oocyte, since cumulus cells send RNA molecules to the ooplasm [72] and the variation in the gene abundance of cumulus cells is associated with the developmental competence of oocytes [54].

In conclusion, the results showed for the first time that miRNA contents of small EVs are modified depending on the estrous cycle stage and are associated with follicular P4 concentration. Small EVs are clearly taken up by the cumulus cells during IVM. Additionally, small EVs obtained from low and high P4 FF are capable of inducing a distinct RNA profile in the cumulus cells within only 9 h of IVM. Small EVs obtained from low P4 were associated with a large number of upregulated genes that modulate biological processes involved in reproduction and immune responses. Moreover, small EVs obtained from low and high P4 follicles differently modulate the Notch signaling pathway in cumulus cells. Finally, this study generated cumulus cells with distinct functional characteristics depending on supplementation with small EVs. These cumulus cells have the potential of impacting oocyte developmental competence.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Author contributions

A.C.F.C.M. and J.C.S. designed the study. A.C.F.C.M., A.B., G.M.A., M.C., and J.R.S. collected the samples and performed the experiments. R.P.N. and W.A.S.J. performed RNA sequencing analyses. A.B. and C.R. collaborated with live cell images. A.C.F.C.M., J.C.S., and F.P. analyzed the data and wrote the manuscript. F.V.M. contributed expertise and reagents. All authors discussed the study, edited and reviewed the manuscript.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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