Canine oviductal exosomes improve oocyte development via EGFR/MAPK signaling pathway

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Abstract

Oviduct cells produce a favorable environment for the development of gametes by generating multiple growth factors. Particularly, in canine species, immature oocytes undergo serial maturation processes in the oviduct, while the other mammals already possess matured oocytes in ovulatory follicles. However, little is known about the potential effect exhibited by the components released from canine oviduct cells (OCs) for modulating the biological function of oocytes. Recently, exosomes are regarded as promising extracellular vesicles because they represent considerable data for molecular cargo. Therefore, we first investigated the effect of canine oviductal exosomes (OC-Exo) on oocyte development via EGFR/MAPK pathway. Our results showed that OC-Exo labeled with PHK67 are successfully incorporated with cumulus cells and oocytes during IVM. Also, OC-Exo markedly increased the proportion of cumulus-oocyte complexes (COCs) exhibiting cumulus expansion as well as cumulus cell proliferation and maturation rate of oocytes ($P < 0.05$). Furthermore, gene expression patterns related with EGFR/MAPK pathway including *EGFR*, *PKA*, *TACE/ADAM17*, *MAPK1/3*, *MAPK14*, *PTGS2*, *TNFAIP6*, *GDF9*, and *BMP15* were positively modified in COCs cultured with OC-Exo ($P < 0.05$). In addition, OC-Exo significantly up-regulated the protein expression levels of p-EGFR, p-MAPK1/3, GDF9 and BMP15 in COCs ($P < 0.05$). Consequently, the current study provides a model for understanding the roles of OC-Exo as bioactive molecules for canine oocyte maturation via EGFR/MAPK pathway, which would open a new avenue for the application of exosomes to improve assisted reproductive technology in mammals, including humans.

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Introduction

The establishment of in vitro maturation (IVM) of canine oocytes has been limited due to their unique reproductive physiology. Unlike other mammals' in vivo oocyte maturation process, canine oocytes stay in the immature state after ovulation, and they complete the first meiosis in the oviduct within 72 h (Holst & Phemister 1971, Tsutsui 1989). In order to simulate the in vivo environment for oocyte maturation, a variety of studies have introduced the oviductal elements, including synthetic oviductal fluid to the culture medium (Hewitt & England 1998), such as co-culturing canine oviduct cells with oocytes (Bogliolo *et al.* 2002, Vannucchi *et al.* 2006, Lee *et al.* 2017), creating a similar environment by inducing low oxygen tension or presence of 10% bitch serum (Nickson *et al.* 1993, Songsasen *et al.* 2002). However, the oocyte maturation rate is still poor (average 10%) (Luvoni *et al.* 2005) due to the lack of understanding of in vivo reproductive conditions in the bitch (Luvoni *et al.* 2005). Recently, we demonstrated that the canine oviduct cell co-culture system improved the

maturation rate of oocytes in canine and porcine species as well as enhanced the developmental competence of porcine embryos via EGFR/MAPK signaling pathway, an essential mechanism for oocyte development (Lee *et al.* 2017, 2018). Accordingly, we aimed to identify the relationship between canine oviduct cells and oocytes and their specific mechanism that induces in vitro maturation. As various types of growth factors, proteins, and receptors are secreted in the culture medium, the conditioned medium would play an important role in oocyte development (Rieger *et al.* 1995, Maillou *et al.* 2016). Although many soluble factors or extracellular vesicles (EVs) are released into the extracellular space, information about their existences and effects on gametes are still elusive.

Extracellular vesicles, including exosomes, microvesicles, and apoptotic bodies, have been considered as important mediators in cell-to-cell communication (Hoshino *et al.* 2015, Zhang *et al.* 2015). In particular, exosomes are lipid bilayer vesicles with a size of 30–200 nm, and they are released into the extracellular environment by the fusion of

a multi-vesicular body with the cell membrane (El Andaloussi *et al.* 2013, Haney *et al.* 2015). It has been demonstrated that the exosome-transported molecular cargos, such as mRNA, microRNA, DNA, and lipids, could affect the target cells by inducing alterations in their physiological functions and mediating cellular pathways (Valadi *et al.* 2007, Kosaka *et al.* 2010, Quesenberry & Aliotta 2010). Regarding the maternal tracts, including oviductal canal and uterus, they have been shown to regulate gamete and embryo interaction by altering transcript abundance (Al-Dossary & Martin-Deleon 2016, Burns *et al.* 2016, Di Pietro 2016). However, there are only a few studies on the effect of exosomes on gamete/embryos, whereas no study has provided extensive information on the secretion/content of oviductal exosomes and their effect on oocyte maturation. As exosomes have emerged as potential biomarkers to improve reproductive success, the study of oviductal exosomes has the potential for expanding our knowledge of the reproduction biology. Therefore, in the present study, we evaluated the signals from OC-Exo to canine oocytes and investigated for the first time their effects on in vitro maturation of oocytes and cumulus expansion during IVM and the expression of genes/proteins related to EGFR/MAPK signaling pathway.

Materials and methods

Chemicals

All chemicals were obtained from Sigma-Aldrich Co. LLC, unless otherwise stated.

Ethics of animal experiments

In this study, oviduct cells were collected from 1- to 3-year-old mixed breed bitches in the estrus stage. Animal experiments were performed following a standard procedure established by the Committee for Accreditation of Laboratory Animal Care and the Guideline for the Care and Use of Laboratory Animals of Seoul National University (approval number; SNU-140704-1).

Collection of canine ovaries and oocytes

Ovaries from domestic bitches were collected during routine ovariohysterectomy at veterinary clinics. All ovaries were used with consent from the owners of these animals. The ovaries were transported to the laboratory in saline at 37°C within 30 min and immediately washed several times with PBS (Invitrogen) and minced with a surgical blade in HEPES-buffered tissue culture medium 199 (TCM-199; Invitrogen) supplemented with 2 mmol/L NaHCO₃, 5 mg/mL BSA, and 1% penicillin–streptomycin. Thereafter, cumulus-oocyte complexes (COCs) were selected for the experiments based on morphological features as follows: (1) dark pigmented homogeneous

cytoplasm, (2) more than two compact multilayers of cumulus cells, and (3) >100 µm in diameter (Lee *et al.* 2017).

Collection and culture of canine oviduct cells from bitches in estrus stage

Canine oviduct cells (OCs) were obtained by flushing method in estrus bitches (Lee *et al.* 2005). For flushing oviduct, a total of eight bitches in the estrus stage without clinical signs of disease were recruited. Briefly, canine oviduct cells (OCs) were obtained from anesthetized females by flushing method. The fimbria of the oviduct was accessed through the bursal slit using a cannula composed of an inverted flanged bulb steel needle mounted on an empty 10 mL syringe without the plunger, and the syringe was inserted into the oviduct to its end. Then, a hypodermic 24-gauge i.v. catheter filled with 10 mL of sterilized normal saline was cannulated into the caudal portion of the oviduct, and OCs were flushed out with normal saline by retrograde flushing with appropriate pressure. All dogs were thoroughly and continuously monitored after the surgery, and we confirmed that they recovered their health after the surgery. The oviduct flushing medium was centrifuged at 1960 g for 2 min and the pellet was collected. The pellets were resuspended in RCMEP medium (K-Stem Cell Research Institute, Seoul, Korea) and centrifugation was performed at 1960 g for 2 min. After discarding the supernatant, the OCs were cultured up to passages 1 to 2 in RCMEP medium with 1 µg/mL of progesterone as previously described by our researches (Lee *et al.* 2019, 2020) and cryopreserved using 10% dimethyl sulfoxide and 90% fetal bovine serum (FBS; Gibco) in liquid nitrogen until further experiments.

Collection of conditioned medium and isolation of oviductal exosomes

First, OCs were seeded on culture dishes at the same volumes of the culture medium containing 10% FBS with 1 µg/mL of progesterone. After culturing for 24 h, the media were exchanged with serum-free medium (containing exosome-depleted FBS; System Biosciences, San Francisco, CA) with 1 µg/mL of progesterone. After an additional 24-h culture, the supernatant was collected and centrifugation was performed (30 min, 2000 g, 4°C). For exosomes extraction, the Total Exosome Isolation Reagent (Life Technologies) was used according to the manufacturer's instructions. Briefly, to remove cell debris, the media were centrifuged at 2000 g for 30 min. Thereafter, the supernatant was transferred to a tube and appropriate volumes of reagents were added. The mixture was incubated at 4°C overnight after vortexing. Then, the mixtures were centrifuged at 10,000 g for 1 h; the pellet was resuspended in PBS and used for further experiments.

Characterization of oviductal exosomes

The OC-Exo were characterized using previously described experimental methods (Lee *et al.* 2019) as follows: (1) transmission electron microscopy (TEM), (2) nanoparticle tracking analysis (NTA), and (3) Western blot analysis.

For TEM, 5 μL of isolated exosomes suspensions were transferred to copper mesh Formvar coated carbon stabilized grids and the suspensions were absorbed in 5 min. Then, the suspensions were wiped out and 5 μL of 1% aqueous uranyl acetate was used to stain OC-Exo for 30 s. Finally, the samples were allowed to air dry for 5 min and analyzed by using a transmission electron microscope (LIBRA 120, Carl Zeiss) at 110 kV. The NTA was performed with a Nanosight LM10 instrument (Nanosight, Malvern, UK) following the manufacturer's protocols to evaluate the concentration, intensity, and size in exosomes. For Western blot analysis, the proteins from exosomes were lysed, then the sample was heated at 95°C for 10 min, and separated by 12% Tris-Tricine SDS-PAGE. The gels were transferred onto polyvinylidene difluoride membranes at 350 mA for 70 min. After transfer, the gels were blocked with the 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 (TTBS) for 1 h at room temperature (RT). The primary antibodies (anti-beta actin (1:2500, ab6276, Abcam) anti-CD9 (1:2500, sc-13118, Santa Cruz Biotechnology Inc) and anti-calnexin (1:2500, ab22595, Abcam) were added and incubated for overnight at 4°C in TTBS. Then, the membranes were washed with TTBS and incubated with the secondary antibody rabbit anti-mouse IgG (1:5000, ab6728, Abcam) for 1 h at RT. After washing the membranes, the proteins were detected using the chemiluminescence reagent (West-Q ECL solution, GenDEPOT). Band density was quantitated by using Fusion Solo software (Vilber Lourmat, France).

In vitro maturation of canine oocytes treated with canine oviductal exosomes

The selected COCs were cultured in a four-well dish for 72 h at 38°C in a humidified atmosphere of 5% CO_2 in IVM medium, containing TCM-199 supplemented with 0.91 mM sodium pyruvate, 0.57 mM cysteine, 10% exosome-depleted FBS, 0.5 $\mu\text{g}/\text{mL}$ follicle-stimulating hormone, and 0.5 $\mu\text{g}/\text{mL}$ luteinizing hormone. In this study, three experimental groups were compared: (1) control, cultured COCs without OC-Exo

treatment; (2) 25 $\mu\text{g}/\text{mL}$, cultured COCs with 25 $\mu\text{g}/\text{mL}$ OC-Exo treatment for 72 h, and (3) 50 $\mu\text{g}/\text{mL}$, cultured COCs with 50 $\mu\text{g}/\text{mL}$ OC-Exo treatment for 72 h. In this experiment, the OC-Exo pellet was resuspended in IVM medium and treated into COCs, while only the IVM medium was treated into the control group. Generally, the OC-Exo adhere very tightly to the surface of the tube after centrifugation; therefore, any small volume of residues in the extraction solution can be completely removed without disturbing the OC-Exo pellet. The IVM system applied in this study is described in Fig. 1A. For IVM experiment for oocytes culture, a total of 310 COCs were used in seven independent replicates and 20–56 COCs were used per repeat (control group: 103, 25 $\mu\text{g}/\text{mL}$ OC-Exo group: 107, 50 $\mu\text{g}/\text{mL}$ OC-Exo group: 100).

Incorporation of oviductal exosomes into cumulus-oocyte complexes using uptake assay

To trace the incorporation of OC-Exo into COCs during IVM, exosomes were labeled with a green fluorescent reagent (PKH67, Green Fluorescent Cell Linker Kit, Sigma Chemical Co.) as per the manual for 24, 48, and 72 h of IVM. In brief, OC-Exo pellets were resuspended in diluent C and PKH67 solution. After incubating for 5 min at RT, the labeling action was stopped by incubating for 1 min with 1 mL of exosome-free FBS. Then, the IVM medium was added and centrifugation was performed at 100,000 g for 30 min at 4°C. After discarding the supernatant, the pellets were resuspended in IVM medium supplemented with 10% exosome-free FBS and stored at -30°C. The COCs were cultured for 72 h, while labeled exosomes were incubated with COCs for 24, 48, and 72 h, respectively. Exosome incorporation by COCs was evaluated under a fluorescence microscope. In total, 48 canine COCs were randomly selected and used in four independent replicates for the incorporation of labeled OC-Exo experiment and four COCs were used per repeat (control group: 16, 25 $\mu\text{g}/\text{mL}$ OC-Exo group: 16, 50 $\mu\text{g}/\text{mL}$ OC-Exo group: 16).

Figure 1 A schematic illustration of the experiment and characterization of oviductal exosomes derived from oviduct cells. (A) A schematic illustration of the experiment with canine oviductal exosomes and cumulus-oocyte complexes. (B) Morphological characterization of exosomes isolated from oviduct cells using transmission electron microscopy; black arrows indicate exosomes (bar: 50 μm). (C) Characterization of exosomes using nanoparticle tracking analysis. (D) Identification of exosomal-specific marker (CD9) and negative calnexin endoplasmic reticulum marker from oviductal exosomes and cell lysates using immunoblotting. Exo, exosomes derived from oviduct cells; Lysates, lysates from oviduct cells.

Assessment of cumulus cell expansion

The degree of cumulus expansion was assessed for 24, 48, and 72 h of IVM in oocytes with cumulus cells after different concentrations of OC-Exo treatment. The scoring system was as follows: no expansion, all layers of cumulus cells were tightly attached to the oocyte; small expansion, the two outer layers showed a little halo around the COCs; expanded, more than three layers of cumulus cells were expanded around the oocyte; and denuded, without any cumulus cells attached. In total, 210 randomly selected canine COCs were used in five independent replicates for evaluating the cumulus expansion experiment and 14 COCs were used per repeat (control group: 70, 25 µg/mL OC-Exo group: 70, 50 µg/mL OC-Exo group: 70).

Assessment of nuclear maturation

After 72 h of IVM, COCs were denuded by gentle pipetting with 0.1% hyaluronidase to evaluate in vitro nuclear maturation rates. An extrusion of first polar body (Metaphase II) from each denuded oocyte was assessed under a stereomicroscope (TE2000-S; Nikon) and immature (without first polar body extrusion) and degenerated oocytes (irregular border and fragmentation of cytoplasm) were also classified. For IVM experiment for oocytes culture, a total of 310 COCs were used in seven independent replicates and 20–56 COCs were used per repeat (control group: 103, 25 µg/mL OC-Exo group: 107, 50 µg/mL OC-Exo group: 100).

Analysis of cumulus cell proliferation rate

We evaluated the cumulus cell proliferation degree using the vibrant MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide) assay. After 72 h of IVM, COCs were denuded with 0.1% hyaluronidase by pipetting and centrifuged at 1975 g for 2 min to collect the cumulus cells. Cumulus cells were cultured at a density of 10,000 cells per well in 96-well plates for 48 h. The media were changed with fresh medium. The MTT solution was added to each well and incubated at 37°C for 4 h. Next, the SDS-HCl solution was added and the plates were incubated at 37°C for 4 h. Then, the absorbance was

determined at 570 nm. In total, 135 randomly selected canine COCs were used in three independent replicates for evaluating proliferation rate of cumulus cells experiment and 15 COCs were used per repeat (control group: 45, 25 µg/mL OC-Exo group: 45, 50 µg/mL OC-Exo group: 45).

Total RNA extraction and cDNA synthesis

Total RNA was extracted from (1) cumulus cells derived from each group of COCs treated with different concentrations of OC-Exo and (2) oocytes derived from each group of COCs treated with different concentrations of OC-Exo using Easy-spin™ Total RNA Extraction Kit (iNtRON Biotechnology Inc., Kyunggi, Korea). We assessed the concentration of isolated RNA by NanoDrop 2000 spectrophotometry (Thermo Fisher Scientific Inc), and cDNA was synthesized using amfiRivert II cDNA Synthesis Premix (GenDEPOT, TX, USA) with the manufacturer's instructions.

Quantitative real-time PCR

The primers for *EGFR*, *PKA*, *TACE/ADAM17*, *MAPK1*, *MAPK3*, *MAPK14*, *PTGS2*, *HAS2*, *TNFAIP6*, *GDF9*, *BMP15*, and β -actin genes were designed from sequences of canine genes obtained from NCBI; all primer sequences were standardized using a standard curve and are listed in Table 1. Real-time PCR was performed using an ABI 7300 Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. The reaction mixture (20 µL) comprised 2 µL cDNA, 0.4 µL forward and reverse primer, respectively, 10 µL SYBR Green interaction dye (Takara Bio USA Inc.), and 7.2 µL diethyl pyrocarbonate water and the reaction was carried out in a MicroAmp optical 96-well plate. The expression of each target gene was quantified relative to that of the internal control gene (β -actin) using the equation, $R=2^{-(\Delta C_t \text{ target gene in control group} - \Delta C_t \text{ target gene in treatment group})}$ (ΔC_t target gene in treatment group – ΔC_t control gene in treatment group). For real-time PCR, 175 randomly selected canine COCs were used in 12 technical independent replicates for evaluating gene expression levels (control group: 58, 25 µg/mL OC-Exo group: 62, 50 µg/mL OC-Exo group: 55).

Table 1 Sequence-specific primers used for quantification of differentially expressed transcripts for dogs.

Gene	Primer sequences (5' → 3')		GenBank No	Product size (bp)
	Forward	Reverse		
<i>ACTB</i>	GATCTGGCACCACCTTCT	GGGTGTTGAAAGTCTCGAA	XM_005621019.1	121
<i>EGFR</i>	TTGCTGCACTGAGTTGATCC	CCGTCCTGCTTAGGACTCTG	XM_022405164	194
<i>PKA</i>	AGCAGGGCTACATTCAGGTG	TAGATCTGGATGGGCTGGTC	NM_001003032	214
<i>TACE/ADAM17</i>	CTGTGGTGCAAAAGCAGAAA	CTGCCTAATGCCTCCCAATA	XM_005630110	168
<i>MAPK1</i>	ACAGTCTCTGCCCTCCAAGA	GGGTAGATCATCCAGTCCA	NM_001252035	226
<i>MAPK3</i>	TTGTGCTTGGAGCAGAAATG	CTGAGACGTTTGGGCTCTTC	NM_001110800.1	197
<i>MAPK14</i>	TTGGTCAGTGGGATGCATAA	AGTTCATCTTCGGCATCTGG	NM_001003206.1	191
<i>PTGS2</i>	GCCTTACCCAGTTTGTGGAA	AGCCTAAAGCGTTTGGGATA	NM_001003354	163
<i>HAS2</i>	ATCATCCAAAGCCTGTTTGC	TCATCTCCGAGTCCCATC	XM_539153.5	209
<i>TNFAIP6</i>	TACAACCCACATGCAAAGGA	GGGTCATCCTCAAGGTCAAA	XM_533354.6	176
<i>GDF9</i>	CAGAAGGGAGGTCTGTCTGC	CTTGTGCAGTTCCAAGCTGA	NM_001168013	108
<i>BMP15</i>	GACCTTGGCGAATATAGCA	CGGTAAACCACAATGGCTCT	XM_003640274.2	118

All primers showed an efficiency between 90 and 110% and a coefficient value > 0.9.

Immunocytochemistry

To assess the effect of OC-Exo on COCs, immunofluorescence staining was performed. The expression levels of p-EGFR and p-MAPK1/3 were analyzed in COCs and matured oocytes and the expression levels of GDF9 and BMP15 were assessed in matured oocytes from each group. Briefly, all samples were washed in PBS containing 0.2% polyvinyl alcohol and fixed with 4% paraformaldehyde in PBS for 30 min at RT, then washed with PBS. Afterward, the samples were treated with 1% (v/v) Triton X-100 in PBS for 2 h and washed in PBS. Then the samples were incubated in 2% BSA in PBS for 4 h. The samples were incubated with primary antibodies containing p-EGFR (1:200; ab32430; Abcam), p-MAPK1/3 (1:200; ab214362, Abcam), GDF9 (1:200; ab93892, Abcam), and BMP15 (1:200, PA5-34,401, Thermo Fisher Scientific) at 4°C overnight. Then, the samples were washed in 2% BSA/PBS and incubated with goat anti-rabbit polyclonal antibody (1:200; ab6717, Abcam) as a secondary antibody for 3 h at RT. After washing in PBS, the samples were mounted on glass slides. The images of individual COCs and oocytes in each group were captured by a fluorescence microscope (Nikon Corp.). The fluorescence intensities of each protein were quantified by Image J (1.46. version) software (National Institutes of Health, USA). For immunostaining, 180 randomly selected COCs were used in four independent replicates for evaluating protein expression levels on each immunocytochemical analysis and three COCs were used per repeat (control group: 60, 25 µg/mL OC-Exo group: 60, 50 µg/mL OC-Exo group: 60).

Statistical analysis

Data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test using GraphPad Prism 5.0. Values are shown as means $\pm$ S.E.M. The significance level was

$P < 0.05$, and at least three independent replications were performed in all experiments.

Results

Characterization of oviductal exosomes

The OC-Exo from estrus stage were previously identified (Lee *et al.* 2019). Briefly, the OC-Exo exhibited a spherical shape and the mean diameter was 150–200 nm in TEM examination (Fig. 1B). The NTA analysis revealed a single peak for 185–190 nm diameter particles at a concentration of $4.8\text{--}5.8 \times 10^8$ particles/mL in the samples (Fig. 1C). Finally, it was confirmed by Western blot analysis that a specific exosomal marker, CD9, was present in OC-Exo (Fig. 1D). Moreover, in our recent study, CD81 and CD63 (exosomal specific markers) were observed in OC-Exo by Western blot and electrophoresis analysis (Lee *et al.* 2019). Also, calnexin, a non-exosome marker/protein associated with the endoplasmic reticulum, was negative in OC-Exo and positive in cell lysates (Fig. 1D).

Incorporation of oviductal exosomes by cumulus-oocyte complexes

At first, exosomes labeled with PKH67 were observed within the cumulus cells after 24 h of culture during IVM but not in the cytoplasm of oocytes (Fig. 2C and D). After 48 h, the incorporation of exosomes within the cumulus cell and the cytoplasm of oocyte was shown (Fig. 2E and F). In addition, considerable incorporation of labeled exosomes within the oocyte and all layers of the expanded cumulus cells was clearly visible 72 h after the start of IVM, as shown in Fig. 2G and H.

Figure 2 Incorporation of oviductal exosomes (OC-Exo) by oocytes. (A and B) Negative control for ensuring that nonspecific staining of cells from residual dye has not occurred. (C and D) Oocytes observed under visible light and labeled OC-Exo are present within the cumulus cells after 24 h of culture. (E and F) Oocytes observed under visible light and labeled OC-Exo are present within the cumulus cells and in the cytoplasm of oocytes after 48 h of IVM. (G and H) Oocytes observed under visible light and considerable incorporation of labeled OC-Exo are clearly visible within the cumulus cells and in the cytoplasm of oocytes after 72 h of IVM. (I) Measurement of PKH67 fluorescence intensity in each group after the culture period. Data are shown as the means $\pm$ S.E.M. ^{a,b,c} Within a column, values with different superscript letters are significantly different ($P < 0.05$). At least three replications were performed.

To ensure that nonspecific staining of cells from residual PKH67 dye was not occurring, COCs were cultured with the supernatant from the last wash (negative control). As a result, no green fluorescence was observed in the negative control COCs (Fig. 2A and B). These results showed that oviductal exosomes labeled with PKH67 were incorporated into cumulus cells and oocytes in vitro.

Effect of canine oviductal exosomes on cumulus expansion

To determine whether OC-Exo have a physical effect on the COCs, the degree of cumulus expansion from each group was evaluated. After 24, 48, and 72 h of IVM, the degree of cumulus expansion was analyzed in each group of COCs. After 24 h of IVM, there was a significantly increased proportion of small expansion degree in 25 µg/mL OC-Exo group compared with the other groups ($P < 0.05$) (Fig. 3A and B). Furthermore, the expanded degree of COCs was significantly increased in 25 µg/mL OC-Exo-treated group compared to the other groups within 48 h of IVM ($P < 0.05$) (Fig. 3A and B). Finally, the 25 µg/mL OC-Exo group exhibited a significant increase in the rate of expanded COCs compared to the other groups and the proportion of small expansion degree in 25 µg/mL OC-Exo group was also significantly increased compared to the control ($P < 0.05$) (Fig. 3A and B). Assessment of the degree of cumulus expansion confirmed that 25 µg/mL OC-Exo-treated group showed a significant increase in the proportion of COCs exhibiting cumulus expansion ($P < 0.05$) (Fig. 3A and B).

Effect of canine oviductal exosomes on proliferation rate of cumulus cells

As a result, a significant increase in cumulus cell proliferation in exosome-treated group in 570 nm

absorbance was observed. Specifically, significant increased rate of cumulus cell proliferation was observed in 25 and 50 µg/mL oviductal exosome treated group (Absorbance: 0.632 ± 0.006 and 0.606 ± 0.008 , respectively, $P < 0.05$) when compared with the control group (Absorbance: 0.561 ± 0.010 , $P < 0.05$) (Fig. 4A). There were no significant differences in cumulus cell proliferation rate among oviductal exosome treated groups (Fig. 4A).

Effect of canine oviductal exosomes on in vitro maturation of canine oocytes

Based on the extrusion of the first polar body (Metaphase II) from oocytes in each group (Fig. 4D), we analyzed the effects of oviductal exosomes on in vitro maturation of canine oocytes. Canine oocytes with normal morphology were selected for IVM (Fig. 4B). The nuclear maturation rate of oocytes in the 25 µg/mL oviductal exosome-treated group ($22.5 \pm 1.7\%$, $P < 0.05$) were significantly higher than those of control and 50 µg/mL treated groups (9.0 ± 3.3 and $11.2 \pm 3.2\%$, respectively, $P < 0.05$) (Fig. 4C). There were no significant differences in the number of degenerated and immature oocytes among groups.

Relative expression levels of genes relevant to EGFR signaling in cumulus cells and oocytes

The relative mRNA transcript levels of the EGFR/MAPK pathway were analyzed in canine cumulus cells and oocytes from COCs after IVM (Fig. 5). The upstream genes of EGFR pathway, including *EGFR* and *PKA*, were significantly increased in the 25 and 50 µg/mL OC-Exo-treated group compared with the control group ($P < 0.05$) (Fig. 5A and B), and other upstream EGFR signaling genes, *TACE/ADAM17*, were significantly increased in the 25 µg/mL exosome group compared with the other groups ($P < 0.05$) (Fig. 5C). Furthermore,

Figure 3 Effect of canine oviductal exosomes (OC-Exo) on cumulus expansion, proliferation rate of cumulus cells and nuclear maturation rate of oocytes after IVM. The cumulus expansion scoring system was as follows: no expansion, all layers of cumulus cells were tightly attached to the oocyte; small expansion, the two outer layers showed a little halo around the COCs; expanded, more than three layers of cumulus cells were expanded around the oocyte; and denuded, without any cumulus cells attached. (A and B) The degree of cumulus expansion at 24, 48 and 72 h of IVM in control, 25, and 50 µg/mL OC-Exo-treated groups with magnified images of the boxes identified in (A). Bar: 100 µm. Data are shown as the means $\pm$ S.E.M. ^{a,b}Within a column, values with different superscript letters are significantly different ($P < 0.05$).

Figure 4 (A) Effect of oviductal exosomes (OC-Exo) on the proliferation rate of cumulus cells in control, 25, and 50 µg/mL OC-Exo-treated groups. (B) Morphology of canine oocytes selected for IVM and (C) effect of different concentration of oviductal exosomes (OC-Exo) on nuclear maturation of canine oocytes. Metaphase II indicates oocyte maturation. (D) First polar body extrusion in control, 25, and 50 µg/mL exosome-treated group. Data are shown as the means ± S.E.M. ^{a,b}Within a column, values with different superscript letters are significantly different ($P < 0.05$). Bar: 50 µm.

Figure 5 The mRNA expression levels of EGFR signaling pathway-related genes in cumulus cells and oocyte-maturation-related genes in oocytes derived from cumulus-oocyte complexes in control, 25, and 50 µg/mL OC-Exo-treated groups. (A, B and C) Expression of upstream genes related with EGFR/MAPK signaling pathway in cumulus cells derived from each group. (D, E and F) Expression of downstream genes related with EGFR/MAPK signaling pathway in cumulus cells derived from each group. (G, H and I) Expression of downstream genes related with cumulus EGFR/MAPK signaling pathway and cumulus expansion in cumulus cells derived from each group. (J and K) Expression of genes related with oocyte maturation in oocyte derived from each group. EGFR, epidermal growth factor receptor; PKA, protein kinase A; TACE/ADAM17, tumor necrosis factor alpha-converting enzyme and a disintegrin and metalloprotease 17; MAPK1, mitogen-activated protein kinase 1; MAPK3, mitogen-activated protein kinase 3; MAPK14, mitogen-activated protein kinase 14; PTGS2, prostaglandin-endoperoxide synthase 2; HAS2, hyaluronan synthase 2; TNFAIP6, tumor necrosis factor alpha induced protein 6; GDF9, growth differentiation factor 9; BMP15, bone morphogenetic protein 15. Data are shown as the means ± S.E.M. ^{a,b,c}Within a column, values with different superscript letters are significantly different ($P < 0.05$). At least six replications were performed.

the downstream genes of the EGFR signaling pathway, such as *MAPK1*, *MAPK3*, and *MAPK14*, were also significantly up-regulated in OC-Exo-treated groups compared to those of the control ($P < 0.05$) (Fig. 5D, E and F). Particularly, the expression level of *MAPK3* was significantly up-regulated in 25 µg/mL OC-Exo group compared to those of 50 µg/mL group ($P < 0.05$) (Fig. 5E). Moreover, 25 µg/mL exosome group showed a significant increase in the cumulus expansion-related genes (*PTGS2* and *TNFAIP6*) compared with the control group ($P < 0.05$) (Fig. 5G and I). In addition, the 25 µg/mL exosome group showed a significant increase in the mRNA expression of the oocyte-maturation-related genes such as *GDF9* and *BMP15* compared to the other groups ($P < 0.05$) (Fig. 5J and K).

Relative fluorescence intensity of p-EGFR, p-MAPK1/3, GDF9, and BMP15 in canine cumulus-oocyte complexes and oocytes

For evaluating the effect of OC-Exo on the relative protein levels of the EGFR/MAPK signaling pathway, immunocytochemistry analysis was performed in each group of COCs and oocytes. The fluorescence intensity of p-EGFR in COCs in the 25 and 50 µg/mL OC-Exo-treated group was significantly increased compared with those of control ($P < 0.05$) (Fig. 6A and B). Moreover, the protein expression level of p-MAPK1/3 in COCs was significantly up-regulated in exosome-treated groups; the 25 µg/mL OC-Exo group showed markedly increased p-MAPK1/3 levels compared with those of 50 µg/mL OC-Exo group ($P < 0.05$) (Fig. 7A and B). The p-EGFR and p-MAPK1/3 were also detected within the oocyte (Fig. 8A), and their expression level was significantly up-regulated in 25 µg/mL OC-Exo-treated

group compared to those of other groups ($P < 0.05$) (Fig. 8A and B). Moreover, a significant increase in the fluorescence intensities of GDF9 and BMP15 was observed in 25 µg/mL OC-Exo group compared to the control ($P < 0.05$) (Fig. 8A and B).

Discussion

In the present study, for the first time, we demonstrated the effect of oviductal exosomes on canine oocyte maturation during IVM and the gene/protein expression levels relevant to EGFR/MAPK pathway. Based on our result, we propose that canine oviductal exosomes can be incorporated into COCs and have beneficial effects on canine oocyte development and the physiological function of cumulus cells as well as enhance the gene/protein expression related with EGFR/MAPK pathway.

Till date, the potential physiological characteristics of oviduct cells have been investigated in the field of reproduction (No *et al.* 2018, Budna-Tukan *et al.* 2019, Ferraz *et al.* 2019) because serial reproductive processes, including gamete maturation, fertilization, and embryo development, occur within the oviductal canal. The maternal tract, including oviductal canal and uterus, has been shown to regulate gamete and embryo interaction by altering transcript abundance (Al-Dossary & Martin-Deleon 2016, Burns *et al.* 2016, Di Pietro 2016). In particular, oviduct in canine species has peculiar properties that enhance oocyte development and prolong the survival of the germ cells (Luvoni *et al.* 2005). In our previous study, the co-culture of oocytes with canine oviduct cells had markedly improved the oocyte and embryo development by stimulating the EGFR signaling pathway (Lee *et al.* 2019). However, the indirect positive effects of oviduct cells on the improvement of

Figure 6 Association between oviductal exosomes (OC-Exo) and cumulus-oocyte complexes (COCs) in the EGFR signaling pathway. (A) Immunocytochemical analysis of phospho-EGFR (p-EGFR, green) in COCs and their nuclear staining (DAPI, blue) in control, 25, and 50 µg/mL OC-Exo-treated groups. (B) The fluorescence intensity of p-EGFR in each group of COCs. Data are shown as the means $\pm$ S.E.M. ^{a,b} Within a column, values with different superscript letters are significantly different ($P < 0.05$). At least five replications were performed. Bar: 50 µm.

Figure 7 (A) Effect of oviductal exosomes (OC-Exo) on the expression of phospho-MAPK1/3 (p-MAPK1/3, green) protein in cumulus-oocyte complexes (COCs) and their nuclear staining (DAPI, blue) in control, 25, and 50 μ g/mL OC-Exo-treated groups. (B) The fluorescence intensity for p-MAPK1/3 in each group of COCs. Data are shown as the means $\pm$ S.E.M. ^{a,b,c} Within a column, values with different superscript letters are significantly different ($P < 0.05$). At least five replications were performed. Bar: 50 μ m.

oocyte competence are yet to be established. As in vivo immature canine oocytes indirectly interact with oviduct for maturation, it is conceivable that the secretome of oviduct cells would play a key role in canine oocyte development. Among numerous secretomes, exosomes have been considered as the important mediators in cell communication through the transfer of their molecular cargoes (protein, mRNA, miRNA) (Hoshino *et al.* 2015, Zhang *et al.* 2015). In our recent study, we demonstrated the relationship between canine oviduct cell-derived exosomes and cumulus cells in the EGFR/MAPK pathway and showed that oviductal exosomes improve the physiological state of cumulus cells as well as gene/

protein expression levels related with EGFR/MAPK signaling in cumulus cells (Lee *et al.* 2019). However, to the best of our knowledge, still, none of the research has provided the effect of exosomes on canine oocyte development at a molecular fashion. Therefore, in the present study, we investigated their effects on in vitro maturation of oocytes and cumulus expansion during IVM and the expression of genes/proteins related to EGFR/MAPK pathway.

To investigate whether canine oocytes could take up OC-Exo, PKH67-labelled OC-Exo were co-incubated with oocytes during IVM. According to our results, the intensity of labeled OC-Exo appeared in the surrounding

Figure 8 The effect of oviductal exosomes (OC-Exo) on protein expression of the EGFR/MAPK signaling pathway and oocyte maturation in oocytes derived from control, 25, and 50 μ g/mL OC-Exo-treated groups. (A) Immunocytochemical analysis of phospho-EGFR (p-EGFR), phospho-MAPK1/3 (p-MAPK1/3), GDF9, and BMP15 in oocytes derived from control, 25 and 50 μ g/mL OC-Exo-treated groups. (B) The fluorescence intensity for p-EGFR in each group of COCs. Data are shown as the means $\pm$ S.E.M. ^{a,b,c} Within a column, values with different superscript letters are significantly different ($P < 0.05$). At least five replications were performed. Bar: 50 μ m.

cumulus cells after 24 h of IVM and the OC-Exo was gradually increased within the cytoplasm of oocytes after 48 h and 72 h of IVM. Thus, we demonstrated that cumulus cells play a role in the paracrine mechanism as a messenger between oocytes and OC-Exo, considering that cumulus cells are incorporated within exosomes before oocyte uptake during IVM. As oocyte maturation in other mammals, such as cow, horse, and pig, occurs in the ovarian follicle in vivo, recent studies focused on the relationship between ovarian follicular EVs/exosomes and gamete maturation (Hung *et al.* 2015, Rodrigues *et al.* 2019). Meanwhile, the canine oviduct cells have potential physiological characteristics for oocyte maturation compared with other mammals. It has been identified that oviduct-derived EVs cargo has a set of proteins with essential roles between gamete and oviduct interactions (Machtinger *et al.* 2016, Alminana *et al.* 2017). In the present study, we demonstrated for the first time that OC-Exo could enhance the COCs development and markedly increase the maturation rate in canine COCs compared with the other groups. Therefore, the results support our hypothesis that oviductal exosomes play potential roles in the release of various proteins into the oviduct milieu, which are incorporated into the oocytes, allowing successful oocyte maturation.

In previous studies, EGF-like peptides influenced cumulus cell expansion as well as gene expression in pig and rat (Sekiguchi *et al.* 2004, Prochazka *et al.* 2012). Also, it has been proved that EGF can enhance oocyte maturation factor including MAPK, p34^{cdc2} and cyclin B (Pereira *et al.* 2019) and that it exerted a positive effect on the progression of MII phase of oocyte in dog (Hatoya *et al.* 2009, Song *et al.* 2011). In addition, another report implicated exosomes in the gene expression of cultured granulosa cells (da Silveira *et al.* 2014), which suggested that exposure to the exosomes is closely related with the gene expression change. In the present study, we showed that OC-Exo supported cumulus expansion and genes associated with cumulus expansion and oocyte maturation. Therefore, we speculated that OC-Exo could mimic the physiological changes in canine COCs, which can be seen in intact canine COCs undergoing development in vivo.

Previous research suggested that when exosomes imparted their genetic material to recipient cells by releasing vesicles, they possessed an EGFR ligand, AREG (Higginbotham *et al.* 2011, Taverna *et al.* 2017). In addition, based on our previous research on the physiological properties of OC-Exo on EGFR/MAPK pathway (Lee *et al.* 2019), it is possible that OC-Exo improved the gene expression levels of canine COCs via the EGFR/MAPK signaling pathway.

Early evidence substantiates that the participation of the EGFR signaling pathway in oocyte maturation and cumulus expansion originated from in vitro studies. A research demonstrated that the EGFR signaling pathway

in COCs is critical for oocyte meiotic resumption as well as provided evidence of how this network influences the oocyte developmental capacity (Park *et al.* 2004). To activate the EGFR signaling pathway, production of EGF-like peptides is necessary and these proteins stimulate protein kinase A (PKA) in follicular cells, which is required for cumulus expansion (Fan *et al.* 2010, Prochazka *et al.* 2012). In our recent studies, we showed that oviduct cells stimulated with progesterone successfully increased the production of AREG (Lee *et al.* 2019) and that the exosomes derived from oviduct cells activated the EGFR/MAPK signaling pathway in canine cumulus cells in vitro (Lee *et al.* 2019). The released EGF-like peptides are tethered to follicular cells and then cleaved at the ectodomain by the TACE/ADAM17 (Dong *et al.* 1999, Sahin *et al.* 2004), which is required for the activation of EGFR and its downstream targets in cumulus cells. The EGFR signaling activation promotes the MAPK1/3 pathway in cumulus cells (Oda *et al.* 2005), which is fundamental in the mediation of serial gene expression related to oocyte maturation (Shimada *et al.* 2016). The gene transcription of EGFR-MAPK1/3 is responsible for the MAPK14 (p38MAPK) induction in cumulus cells, which up-regulates and maintains the overall EGF network for inducing oocyte maturation and cumulus expansion as key downstream effectors (Park *et al.* 2004, Downs & Chen 2008). Thus, it is evident that once the EGFR/MAPK signaling is activated, the downstream pathway related to cumulus expansion is enhanced (Yamashita *et al.* 2011). Therefore, in the current study, the expression levels of genes relevant to EGFR/MAPK pathway in matured oocytes and cumulus cells were measured to ascertain whether characteristics of canine COCs were changed by exosomes derived from oviduct cells. Based on our results, we suggested that exposure of OC-Exo to oocytes during IVM significantly increased expression levels of the upstream and downstream EGFR/MAPK pathway-related genes in the resultant COCs. Taken together, our results support the hypothesis that OC-Exo would act on canine COCs during oocyte maturation to improve the competence of oocytes and enhance the gene expression levels via EGFR/MAPK pathway.

For the maturation of oocytes, not only EGFR/MAPK signaling activation in cumulus cells is required but also the cues of oocytes are essential to promote oocyte development (Diaz *et al.* 2006, 2007). The GDF9 and BMP15 have been described as important factors for oocyte maturation (Dong *et al.* 1996, Galloway *et al.* 2002). A recent study suggested that the participation of oocyte-secreted factors, GDF9 and BMP15, is required for obtaining an AREG-responsive state in porcine COCs (Sugimura *et al.* 2015). A study proved that mouse oocytes are closely involved in the expression of EGFR in cumulus cells via GDF9 and BMP15 activation (Su *et al.* 2010) and another study suggested that a cross-talk between EGFR-MAPK1/3 and GDF9/BMP15 existed

Figure 9 A schematic illustration of the relation between canine oviductal exosomes and cumulus-oocyte complexes via the EGFR/MAPK signaling pathway. Exosomes form by inward budding of the endosomal membranes of oviduct cells. After the exosomes are released into the extracellular space by fusion with the plasma membrane of oviduct cells, their interaction with cumulus cells induces rapid activation and internalization of EGFR and endocytosis. The alteration in EGFR on cumulus cells via oviductal exosomes significantly activates the EGFR/MAPK signaling pathway in cumulus cells, and it would subsequently improve oocyte maturation.

for oocyte maturation (Sasseville *et al.* 2010). Likewise, we proposed that the increased transcript levels of the EGFR pathway induced by OC-Exo might activate the response to oocyte-secreted factors. Based on our results, we suggested that an up-regulated expression of genes in cumulus cells and oocytes via OC-Exo markedly improved the protein levels of GDF9, BMP15, p-EGFR, and p-MAPK1/3 in oocytes and p-EGFR and p-MAPK1/3 in cumulus cells, which substantiate that signals derived from exosomes can reach beyond the oocyte and modulate their gene expression during IVM of oocytes. Such insights might represent a potential IVM system that mimics the signaling events occurring in vivo.

In conclusion, for the first time, we have demonstrated the effect of oviductal exosomes on in vitro maturation of canine oocytes and the alteration of gene/protein levels relevant to the EGFR/MAPK signaling within COCs (Fig. 9). The alteration of EGFR on cumulus cells via oviductal exosomes significantly activated the EGFR/MAPK signaling pathway in cumulus cells and subsequently improved oocyte maturation, which would clarify the role of oviductal exosomes on oocyte development, fertilization, as well as embryo implantation in the field of reproductive biology in the future.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S H L conceived the study, carried out the experiments, then performed the statistical analysis and drafted the manuscript. H J O and B C L assisted with designing the experiment and statistical analysis of the data. M J K assisted with implementation of the experiments and manuscript drafting. All co-authors revised the manuscript. B C L supervised the research and supplied the funding. All authors read, revised, and approved the final manuscript.

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