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Effects of oocyte-derived paracrine factors on release of extracellular vesicles by murine mural granulosa cells in vitro

Yuta Matsuno | Natsumi Maruyama | Wataru Fujii | Kunihiko Naito | Koji Sugiura

Laboratory of Applied Genetics, Department of Animal Resource Sciences, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan

Correspondence
Koji Sugiura, Laboratory of Applied Genetics, Department of Animal Resource Sciences, Graduate School of 15 Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan.
Email: aks@mail.ecc.u-tokyo.ac.jp

Present address
Yuta Matsuno, Laboratory of Molecular Reproduction, Research Institute of Agriculture, Tokai University, 9-1-1 Toroku, Higashi-ku, Kumamoto 862-8652, Japan

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Abstract

Both oocytes and extracellular vesicles (EV) have emerged as critical regulators of mammalian follicular development; however, the possible interaction between the oocyte-derived paracrine factor (ODPF) and EV signals has never been examined. Therefore, to explore the possibility of an interaction between oocyte and EV signals, the effects of ODPFs on the biogenesis of EVs as well as the expression levels of transcripts related to EV biogenesis in mural granulosa cells (MGCs) were examined using mice. The results showed that, while oocyte coculture has some effects on the expression levels of transcripts related to EV biogenesis, the number of EV particles present in the conditioned medium were not significantly different between ODPF-treated and non-treated MGCs. Therefore, oocytes have no effects on the EV biogenesis by MGCs, at least with respect to the numbers of EV particles.

KEYWORDS
extracellular vesicles, granulosa cells, oocytes

1 | INTRODUCTION

Development of ovarian follicles requires coordination of multiple extra- and intrafollicular signals in mammals. Oocytes play a fundamental role in this coordination (Emori & Sugiura, 2014; Russell, Gilchrist, Brown, & Thompson, 2016; Su, Sugiura, & Eppig, 2009). For example, oocytes, by producing paracrine factors (oocyte-derived paracrine factors: ODPFs), suppress the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) signals by suppressing expression of LH and FSH receptors in rodents (Elvin, Clark, Wang, Wolfman, & Matzuk, 1999; Eppig, Wigglesworth, Pendola, & Hirao, 1997; McMahon, Hashimoto, Mellon, & Shimasaki, 2008; Otsuka, Moore, Moore, & Shimasaki, 2001; Otsuka, Yamamoto, Yamamoto, Erickson, & Shimasaki, 2001), whereas they promote the epidermal growth factor (EGF) signaling in cumulus cells by augmenting EGF receptor signaling in mice and pigs (Ritter, Sugimura, & Gilchrist, 2015; Su et al., 2010). Moreover, the way cumulus cells respond to the estrogen signal is regulated by ODPFs in rodents (Emori et al., 2013; Otsuka et al., 2005; Sugiura, Su, Li, et al., 2010). Through these abilities to affect the extra- and intrafollicular signals, oocytes are able to determine the rate of follicular development even (Eppig, Wigglesworth, & Pendola, 2002).

In addition to the oocytes, extracellular vesicles (EVs), such as exosomes and microvesicles, are recognized as a novel regulators of ovarian functions (de Avila & da Silveira, 2019; Di Pietro, 2016; da Silveira et al., 2018; Machtinger, Laurent, & Baccarelli, 2016). The presence of EVs in ovarian follicles was first reported in the follicular fluid of mares (da Silveira, Veeramachaneni, Winger, Carnevale, & Bouma, 2012). Since then, EVs have also been detected in bovine (Sohel et al., 2013), porcine (Matsuno et al., 2017), and human

Yuta Matsuno and Natsumi Maruyama contributed equally to this work.

follicular fluid (Diez-Fraile et al., 2014; Santonocito et al., 2014). Therefore, the presence of follicular EVs is likely to be conserved among mammalian species. The follicular EVs affect gene expressions in bovine (Sohel et al., 2013) and equine granulosa cells (da Silveira, Carnevale, Winger, & Bouma, 2014; da Silveira, Winger, Bouma, & Carnevale, 2015), and promote cell proliferation of granulosa cells in cattle (Hung et al., 2017). EVs promote or facilitate cumulus expansion in cattle (Hung, Hong, Christenson, & McGinnis, 2015) and pigs (Matsuno et al., 2017). In addition, EVs affect the developmental competence of oocytes in cattle (da Silveira et al., 2017), and, in humans, the microRNA contents of follicular EVs were implicated as a marker of IVF outcome (Machtinger et al., 2017; Martinez et al., 2018). Therefore, EVs are now considered to be a critical intrafollicular signal regulating follicular development; however, the possible interaction between the EV-and oocyte signals has never been examined.

EVs are mainly composed of exosomes and microvesicles which are produced through different intracellular machineries: exosomes are formed within the endosomal system as intraluminal vesicles (ILVs) of multivesicular bodies (MVBs) and secreted by the fusion of MVBs with the plasma membrane, whereas microvesicles can form by outward budding of the plasma membrane (Mathieu, Martin-Jaular, Lavieu, & Thery, 2019; van Niel, D'Angelo, & Raposo, 2018). Several molecules involved in the process of exosome biogenesis have been identified. For example, endosomal sorting complex required for transport (ESCRT) machinery (Colombo et al., 2013; Hurley, 2008), syndecan–syntenin–ALIX (Baietti et al., 2012), and/or the ceramide-generating enzyme known as neutral Sphingomyelinase (nSMase; Kajimoto, Okada, Miya, Zhang, & Nakamura, 2013; Trajkovic et al., 2008) are required for the intraluminal budding of endosomal membranes to form MVBs. Then, MVB trafficking to the plasma membrane requires members of the Rab family of small GTPase proteins (Ostrowski et al., 2010), and fusion of MVBs with the plasma membrane to release ILVs as exosomes is mediated by SNARE complexes (Jahn & Scheller, 2006). In contrast to exosome biogenesis, the mechanisms involved in the biogenesis of microvesicles are not well defined but involve some machineries/molecules that are shared in common with those of exosomes, such as the ESCRT machinery (Nabhan, Hu, Oh, Cohen, & Lu, 2012; Wehman, Poggioli, Schweinsberg, Grant, & Nance, 2011) and the Rab family of proteins (Wang et al., 2014).

To explore the possibility of an interaction between oocyte and EV signals, we thought to assess the effects of ODPFs on the production of follicular EVs, since the amount EV production by a cell is known to change in response to different extracellular stimuli (Blanchard et al., 2002; Obata et al., 2018; van der Vlist et al., 2012; Villarroya-Beltri et al., 2016). The majority of follicular EVs are likely to be produced within the follicles rather than the blood supply, since the microRNA contents of the follicular EVs are similar to those of mural granulosa cell (MGC)- and cumulus–oocyte complex (COC)-conditioned culture media in cattle (Andrade, Meirelles, Perecin, & da Silveira, 2017), and the mRNA contents resemble MGCs in pigs (Matsuno et al., 2019). Therefore, we herein evaluate the effects of ODPFs on the biogenesis of EVs by MGCs using primary culture system in mice. The follicular development is critically regulated to provide fertilizable oocytes for the entire duration of female reproductive life span in mammals. The results will provide new insight into the understandings of mechanisms governing the normal female reproductive performance.

2 | MATERIALS AND METHODS

2.1 | Mice

All experiments were conducted using 3-week-old (C57BL/6 x DBA/2) F1 female mice purchased from Sankyo Labo Service Corporation, or produced and raised in the research colonies of investigators at the University of Tokyo. All animal protocols were approved by the Animal Care and Use Committees at the University of Tokyo.

2.2 | Isolation of MGCs and denuded oocytes

Minimum Essential Medium alpha (MEMα; Thermo Fisher Scientific) with Antibiotic Antimycotic Solution (GE Healthcare UK), 0.23 mM pyruvate, and 10 μM of the phosphodiesterase inhibitor, milrinone (Sigma-Aldrich) was used as the basic culture medium. Milrinone was added to the medium to maintain oocytes at the germinal vesicle stage. All cultures were maintained at 37°C in 5% O_2, 5% CO_2, and 90% N_2.

MGCs were isolated from female mice injected with equine chorionic gonadotropin (eCG; GONATROPIN; ASKA Pharmaceutical) 42–48 hr prior to each experiment, as reported previously (Sumitomo et al., 2016). Oocytes were collected from early- to middle-stage antral follicles of 3-week-old female mice that were not treated with eCG (Sumitomo et al., 2016).

For the oocyte coculture experiments, MGCs were resuspended in the basic culture medium supplemented with 5% fetal bovine serum (FBS), and 8.0×10^4 viable cells were plated on individual wells of a 96-well plate (AGC TECHNO GLASS CO., LTD.) pretreated with ECL cell attachment matrix (08-110; Merck). After a 24 hr culture to attach the cells to the bottom of the wells, the medium was changed to the serum-free basic culture medium. Then, oocytes were added to the medium at a density of 2 oocytes/μl. Coculture experiments were conducted for 24 hr.

To collect the MGC-conditioned medium, MGCs were resuspended in the basic culture medium supplemented with 5% FBS, and 8.0×10^5 viable cells were plated on 60 mm tissue-culture dish (AGC TECHNO GLASS Co.) pretreated with ECL cell attachment matrix (Merck). After a 24 hr culture, the medium was changed to 3 ml of the serum-free basic culture medium supplemented with or without recombinant human bone morphogenetic protein 15 (BMP15; 50 ng/ml; R&D Systems), recombinant mouse growth differentiation factor 9 (GDF9; 50 ng/ml; R&D Systems), and recombinant mouse fibroblast

growth factor 8 (FGF8; 50 ng/ml; Sigma-Aldrich). These recombinant proteins were used as substitute for ODPFs in this experiment. The conditioned medium was collected after 48 hr of culture.

2.3 | Isolation of fractions enriched in EVs from MGC-conditioned medium

The MGC-conditioned medium was collected and centrifuged at 2,000 *g* at 4°C for 30 min, followed by 12,000 *g* at 4°C for 45 min, then filtered through a 0.22 μm membrane (Merck) in order to remove cells and debris. After the filtration, the conditioned medium was concentrated using 100 kDa molecular weight cut-off Amicon centrifugal filter units (Merck) to 20 μl. In some experiments, the conditioned medium after the filtration was stored at −80°C until being processed for further analysis.

A fraction enriched in EVs (hereafter "EV-enriched fraction") was isolated from the concentrated conditioned medium samples using a Total Exosome Isolation reagent (from cell culture media; Thermo Fisher Scientific) and centrifugation according to the manufacturer's protocol.

2.4 | Transmission electron microscopy observations of the EV-enriched fraction

Transmission electron microscopy (TEM) observation using the negative stain method was conducted as reported previously (Matsuno et al., 2017). In brief, the EV-enriched fraction isolated from the conditioned medium was resuspended in NaHCa buffer (30 mM HEPES, 100 mM NaCl, 2 mM $CaCl_2$, and pH 7.4) and applied on a 200-mesh copper microgrid covered with a formvar support film that had been pretreated with soft plasma etching equipment (SEDE-AF; Meiwafosis). Then, the microgrid was electron stained with 1% uranium acetate solution for 10 min. Finally, the microgrid was observed with a transmission electron microscope (JEM-1010; JEOL).

2.5 | Western blot analysis

The EV-enriched fraction and MGCs were resuspended in Laemmli buffer (Laemmli, 1970) and immediately boiled for 5 min. Western blot analysis was conducted as reported previously (Matsuno et al., 2019). The primary antibodies used were anti-HSC70 rat antibody (MAB2191; Abnova), anti-ALIX rabbit antibody (ab186429; Abcam), and anti-CYCS mouse antibody (sc-13156; Santa Cruz Biotechnology) as well-known EV markers. The secondary antibodies used were a horseradish peroxidase-conjugated anti-rabbit IgG (AP132P; Merck), anti-rat IgG (81-9520; Thermo Fisher Scientific), and anti-mouse IgG antibodies (115-035-044; Jackson ImmunoResearch). Signals were visualized using an Immunostar LD Kit (FUJIFILM Wako Pure Chemical Corporation) and the C-DiGit Blot Scanner and Image Studio for C-DiGit (LI-COR).

2.6 | Size analysis by nanoparticle tracking

The EV-enriched fraction was resuspended in PBS (-) to analyze the size distribution of particles. Nanoparticle tracking analysis (NTA) was performed with a NanoSight nanoparticle analyzer (NanoSight LM10; Malvern Panalytical) with NTA3.1 software (Build 3.1.46) using the EV-enriched fraction resuspended in PBS (-). Three 30-s videos were recorded for each sample. All postacquisition functions were set at automatic.

2.7 | Quantitative reverse transcription (RT) polymerase chain reaction (PCR) analysis

Real-time PCR analysis was conducted as reported previously (Matsuno et al., 2016). In brief, total RNA was extracted from MGCs using a ReliaPrep™ Cell Miniprep System (Promega KK), and reverse transcribed using a ReverTraAce qPCR Master Mix with gDNA Remover (Toyobo, Osaka, Japan). Real-time PCR reactions were performed using a THUNDERBIRD qPCR Mix (Toyobo) and an Applied Biosystems StepOnePlus Real-Time PCR system (Thermo Fisher Scientific). The transcript levels were normalized to the levels of a house-keeping gene, ribosomal protein L19 (RPL19), using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Dissociation-curve analyses were performed at the end of the analyses to avoid false-positive signals. The PCR primers used are shown in Table 1. The PCR primer set used to amplify *Rpl19* was reported previously (Sugiura et al., 2007). Only one product of the appropriate size was identified with agarose gel electrophoresis for each set of primers.

2.8 | Statistics

All experiments were repeated at least three times. Statistical analyses were conducted using Microsoft Excel software (Microsoft). Student's *t* test was used for paired comparisons. A $p < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Detection of EVs in conditioned medium of MGCs

The EV-enriched fractions were isolated from the MGC-conditioned medium using a commercially available exosome isolation reagent, and were analyzed by transmission electron microscopy (TEM) observation, western blotting analysis, and nanoparticle tracking analysis (NTA) to characterize and demonstrate the purity of the samples. TEM using the negative stain method demonstrated that round-shaped vesicles of about 100 nm in diameter were present in the fraction isolated from the conditioned medium (Figure 1a). NTA revealed the presence of EVs with a particle diameter ranging from 40 to 200 nm in the EV-enriched fraction (Figure 1c). The western blotting analysis revealed that two well-known markers for EVs, programmed

TABLE 1 Sequence of PRC primers used for real-time PCR

Gene symbol	Accession number	Forward (5′ to 3′)	Reverse (5′ to 3′)
Chmp2a	NM_001360730	CCGAGCCATGAGAGAACTGG	GCACCAGGTCTTTTGCCATG
Chmp4b	NM_029362	GAAGGCTGCCCACGACAA	GCTCATCCTCGTCGAACTCTTC
Chmp5	NM_029814	AGATGAGAGAGGGTCCTGCT	AGGTTGTCTCGCTGTTGCTC
Hgs	NM_008244	GGTGGTCCAGGACACATACC	AACTGTACTCTGCACCGATGG
Pdcd6ip	NM_001164677	ATTGCAGCAGAGCAGAACCT	TGTCCACAGTAGGCTCTCGA
Rab27a	NM_001301230	TCACCACAGTGGGCATTGAT	CGTGTCCCATAACTGCAGGT
Rab7	NM_009005	AAGGTCATCATCCTGGGGGA	TTGGTCAGAAAGTCCGCTCC
Sdc1	NM_011519	ATGGCTCTGGGGATGACTC	CACACGTCCTTCCAAGTGG
Sdcbp	NM_016807	GGTCCTCGGGGCAGAAAATA	CAGCAGAAGCATCCACCAGA
Smpd2	NM_009213	TTGCTCCGAAGCACTCCAG	GGGATGTCCCAGCAGTTGAG
Smpd3	NM_021491	CAATGGGTGCAGCTTCGATG	TTGGTCCTGAGGTGTGCTTC
Smpd4	NM_029945	GCGGTCAGAAACTCTCCTCC	TGGAGACAGTGAGTCGGTAGT
Snap23	NM_001177792	GCTGAGGGAACTAGTCAGCG	CAGCTGAACTTCCTCTGGGG
Stam	NM_011484	CAGCTGAACTTCCTCTGGGG	TTTGGTAGCCACAGTCCCAG
Tsg101	NM_001348089	ACCGTCCGTCAAACTGTCAA	GCTCGATAACGCACTGGGAT
Vamp7	NM_011515	TCAAGTGTTTTGGCTGCACA	ACCTTTCTCCACGTTGAGCA

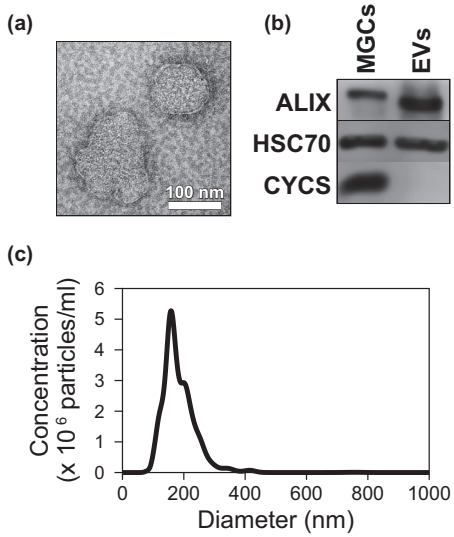

FIGURE 1 Detection and characterization of EVs in MGC-conditioned medium. (a) Representative photograph of vesicles in the EV-enriched fraction isolated from MGC-conditioned medium observed by TEM with the negative-staining method. The scale bars indicate 100 nm. (b) Western blotting analysis for ALIX, HSC70, and CYCS. (c) NTA for size distribution of particles in the EV-enriched fraction. NTA was repeated on three independent samples, and histogram of NTA is shown as an average of the three repeats. EVs, the EV-enriched fraction; MGCs, mural granulosa cells; NTA, nanoparticle tracking analysis; TEM, transmission electron microscopy

cell death 6 interacting protein (PDCD6IP, also known as ALIX), and heat shock protein 70 (HSC70) were present in both MGCs and the EV-enriched fraction (Figure 1b). In contrast, cytochrome C (CYCS) was only detected in MGCs but not in the EV-enriched fraction (Figure 1b). This indicates that the EV-enriched fraction was free of contaminants such as apoptotic bodies or cell debris.

3.2 | Oocyte effects on the levels of transcripts related to EV biogenesis in MGCs in vitro

To assess the effects of ODPFs on the expression levels of transcripts encoding proteins involved in EV biogenesis in MGCs, MGCs were cultured with or without oocytes, and the expression levels of transcripts involved in ESCRT machinery (Figure 2a; Colombo et al., 2013), the syndecan–syntenin–ALIX (Figure 2b; Baietti et al., 2012), nSMase (Figure 2c; Trajkovic et al., 2008), the Rab family of small GTPase proteins (Figure 2d; Ostrowski et al., 2010), and SNARE complexes (Figure 2e; Jahn & Scheller, 2006) were examined with RT-qPCR. As shown in Figure 2, the levels of the *Sdcbp* transcript encoding the syndecan-binding protein (also known as Syntenin-1) were significantly suppressed with oocyte coculture (Figure 2d). Similarly, the transcript levels of charged multivesicular body protein 5 (*Chmp5*) and sphingomyelin phosphodiesterase 2, neutral (*Smpd2*) tended to be lower in MGCs cultured with oocytes (p = .051 and .064, respectively; Figure 2aB). The *Smpd3* transcript was barely detectable regardless of the presence of oocytes (Figure 2b). Other transcripts examined were not significantly affected by the presence of oocytes, at least under the present culture conditions.

3.3 | Effect of ODFPs on the release of EVS by MGCs in vitro

We next examined the effects of ODPFs on the release of EVs by MGCs. For this experiment, we used recombinant proteins for ODPFs (i.e., BMP15, GDF9, and FGF8; Emori & Sugiura, 2014; Russell et al., 2016; Su et al., 2009), instead of the natural ODFPs (i.e., oocyte coculture), to avoid the possibility of detecting oocyte-derived EVs in the conditioned medium. Moreover, it was not practical to collect

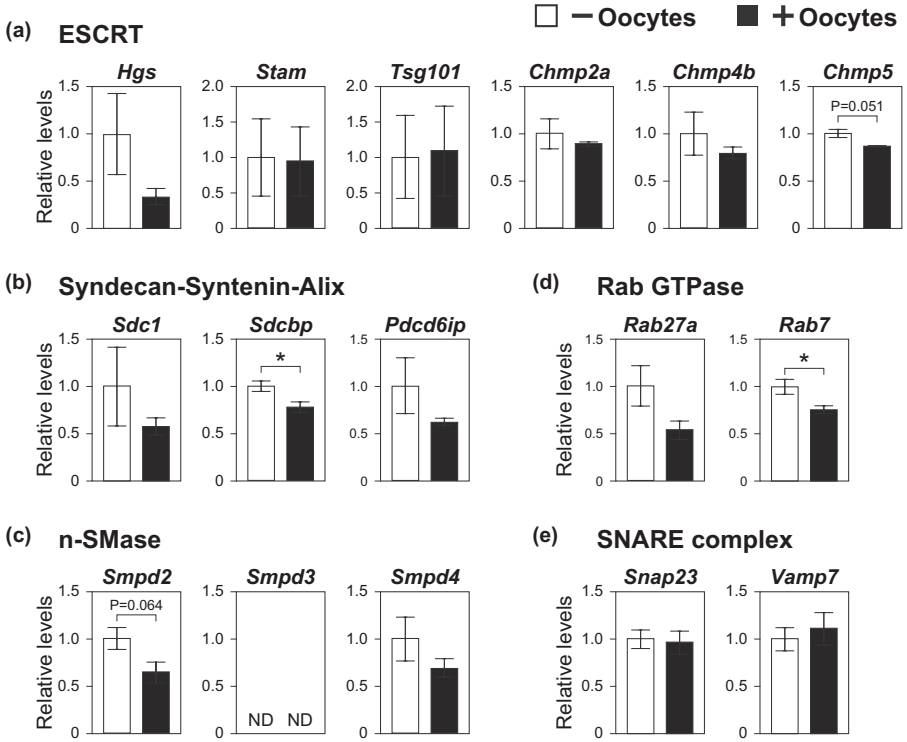

FIGURE 2 Effects of oocyte-derived paracrine factors on the levels of transcripts related to extracellular vesicle biogenesis in mural granulosa cells (MGCs). MGCs were cultured with or without oocytes (2 oocytes/μl), and the expression levels of transcripts involved in ESCRT machinery (a), syndecan–syntenin–ALIX (b), nSMase (c), the Rab family of small GTPase proteins (d), and SNARE complexes (e) were examined. An asterisk denotes significant differences between the indicated groups ($p < .05$)

sufficient numbers of oocytes (6,000 oocytes per experiment) for this experiment. EV-enriched fractions were isolated from the conditioned medium of MGCs treated with or without recombinant ODPFs, and the numbers of EV particles were assessed by NTA (Figure 3). The results showed that the particle numbers of EVs were not significantly different between the control and ODPF-treated groups (Figure 3).

4 | DISCUSSION

The present study was conducted to assess the possibility that oocytes regulate EV production by MGCs in mice. The results showed that MGCs indeed produce EVs during culture. While the oocyte co-culture exhibited some effects on the transcript levels of proteins involved in EV biogenesis in MGCs, the particle numbers of EVs present in the conditioned medium were not significantly different between the ODPF-treated and non-treated groups. These results suggest that oocytes have no effects on the EV biogenesis by MGCs, at least in terms of the numbers of EV particles.

There are several examples of the regulation of EV production by extracellular stimuli in cell systems other than ovarian follicles. For example, in the immune system, the activation of T-cell receptor signaling results in a large increase in EV production by T cells (Blanchard et al., 2002). In addition, stimulating T cells with interferon signals promotes ISGylation (a ubiquitin-like modification of proteins, in which proteins are conjugated with ISG15) of the MVB protein, TSG101. This results in the lysosomal degradation of MVBs, and thereby in the reduction in EV production by the interferon-treated T cells (Villarroya-Beltri et al., 2016). Moreover, adipocyte-derived adiponectin promotes EV production by T-cadherin expressing cells, which in turn promotes cellular protection and cell survival by stimulating ceramide efflux from the cells (Obata et al., 2018). On the other hand, the present results showed that the production of EV particles by MGCs is not affected by oocytes, at least under the present culture conditions. However, since it was not practical to collect the required number of oocytes for the experiments (6,000 oocytes per experiment), recombinant proteins (i.e., BMP15, GDF9, and FGF8) were used as ODPFs to assess the effect of ODPFs on the EV production by MGCs. Therefore, it is possible that other oocyte-derived factors, such as BMP6 (Sugiura, Su, & Eppig, 2010) and TGFB2 (Schmid, Cox, van der Putten, McMaster, & Bilbe, 1994), as well as metabolites produced by oocytes may have some effects on EV production by MGCs.

Although the present results suggest that the number of EV particles produced by MGCs is not affected by oocytes, this may not necessarily mean that the signals of oocytes and follicular EVs are independent. EVs contain many biologically active molecules, such as proteins, lipids, metabolites, and nucleic acids including DNA, mRNA, and non-coding RNAs (Kalluri & LeBleu, 2020; Sedgwick & D'Souza-Schorey, 2018; van Niel et al., 2018). Although they may not be completely identical, the properties of these EV components reflect the phenotypic and physiological state of their originating

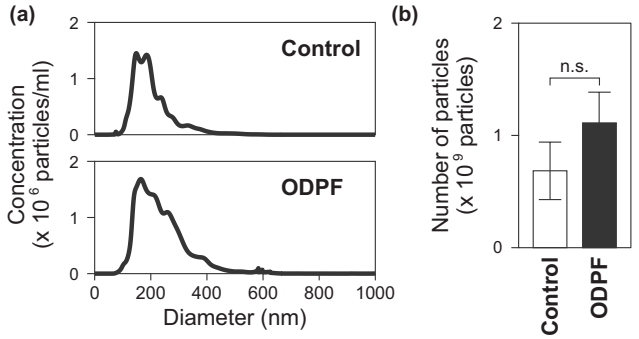

FIGURE 3 Effects of oocyte-derived paracrine factors (ODPFs) on the release of extracellular vesicles (EVs) by mural granulosa cells (MGCs) in vitro. MGCs were cultured with or without recombinant BMP15 (50 ng/ml), GDF9 (50 ng/ml), and FGF8 (50 ng/ml), and the numbers of EV particles were assessed by nanoparticle tracking analysis (NTA). (a) Histograms of NTA for the EV-enriched fraction of MGCs treated with (ODPF) or without (Control) ODPFs. NTA was repeated on three independent samples, and the histogram of NTA is shown as an average of the three repeats. (b) Total number of EV particles measured by NTA. n.s., not significant

cells to some extent. In fact, our previous study showed that the mRNA content in EVs isolated from porcine follicular fluid was significantly correlated with that of MGCs (Matsuno et al., 2019). It is well accepted that oocytes regulate expression of mRNA (Emori et al., 2013) and miRNA (Sumitomo et al., 2016), as well as metabolic activities (Eppig, Pendola, Wigglesworth, & Pendola, 2005; Su et al., 2008; Sugiura et al., 2007) in follicular granulosa cells, such as MGCs and cumulus cells. Thus, it is possible that the EVs produced by MGCs stimulated with ODFPs contain components with different properties than the EVs produced by non-stimulated MGCs, and therefore, the two types of EVs may have different effects on the target cells.

The other possibility of an interaction between oocyte and EV signals would exist at the point of cellular uptake of EVs. EVs are taken up by recipient cells through several different mechanisms, such as simple fusion with the plasma membrane, endocytotic pathways mediated by clathrin or caveolae, phagocytosis, and micropinocytosis (Kalluri & LeBleu, 2020; Sedgwick & D'Souza-Schorey, 2018; van Niel et al., 2018). The physiological targets of follicular EVs are yet to be determined, but previous studies have shown that follicular EVs are taken up by MGCs and cumulus cells in several mammalian species, such as horses, cattle, and pigs (da Silveira et al., 2012; Hung et al., 2015; Matsuno et al., 2017; Sohel et al., 2013). The mechanism by which these follicular cells take up EVs is not known; however, it has been reported that MGCs or cumulus cells are capable of taking up extracellular substances thorough several endocytotic pathways (Shimada, Hernandez-Gonzalez, Gonzalez-Robanya, & Richards, 2006). In addition, our previous transcriptomic analysis showed that the expression levels of transcripts related to endocytosis in cumulus cells are affected by oocytes in vivo and in vitro (Emori et al., 2013; Su et al., 2008). Therefore, it is possible that oocytes may interact with EV signals by affecting the EV uptake of recipient cells through the regulation of endocytotic pathways. The mechanism by which each cell type in follicles incorporates EVs, and the question of whether the pathways are regulated by oocytes will require further investigation.

Both ODPFs and EVs are now considered as critical regulators of follicular development. Therefore, understanding the precise interaction between the ODPFs and EV signals will bring new insights into the regulatory mechanisms of follicular development. This will be a critical step for the future development or improvement of animal reproductive techniques for production of fertilizable oocytes.

5 | CONCLUSION

In summary, the present results suggest that oocyte signals have no effect on the production of EVs by MGCs in mice, at least with regard to the number of EV particles. Further studies are warranted to investigate whether oocytes affect the EV components produced by MGCs, and whether oocytes regulate uptake of EVs by recipient cells.

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ORCID

Yuta Matsuno https://orcid.org/0000-0001-9927-1756
Koji Sugiura https://orcid.org/0000-0001-8945-3353

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